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Coronavirus Disease 2019: The Public Health Challenge and Our Preparedness

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ABSTRACT

Coronavirus disease 2019 (Covid-19) is the most dreadful among all types of corona viruses so far, whose first wave is currently invading the world, and countries are struggling to fight for a low morbidity and mortality, aiming to reduce the overall tension on their health-care as well their nationals. Nevertheless, the control of the epidemic, with regard to less mortality and morbidity as found in some Asian countries, including China, South Korea and others has been perhaps accomplished by certain important factors including immediate response to the calamity under instructions of WHO as well as its directions to the international community via strict social distancing, quarantines and global lock downs. In this communication, a literature based analysis has been made using the Internet, Pub Med and other data bases to explore the status of the pandemic between January and June 2020 in order to have an idea of the steps various countries have taken for combating the corona virus disease. It has been observed that the principle of over-reaction all over the world was preferred, than under reacting, showing positive results. The summary is, we have to be prepared for such catastrophic events at all times and for a huge population, its necessary for their respective governments to allocate funds for such crisis management without the stigma of corruption, dishonesty, procrastination and irresponsibility. One thing is clear, we have to save ourselves, no matter the tall claims, as in case of health care in any developing country, it's always the out of pocket expenditure which comes first, for any health related emergency.

INTRODUCTION

Coronavirus Disease 19 is the most dreadful among all types of Corona viruses so far, whose first wave is currently invading the world, and several countries are struggling to fight for a low morbidity and mortality, aiming to reduce the overall tension on their health-care as well their nationals. Since the coronavirus disease 2019 outbreak began in late months of 2019, spreading to different parts of the world, early information showed that the USA ranked first, and the UK second; South Korea was ranked ninth, and China 51st; most African countries were at the bottom of the ranking. Things look different now with more deaths in the US and UK, where the governments have not been, able to provide adequate responses to the pandemic, with errors and flaws infecting the already burdened health systems globally.

Literature review suggests that since Co-vid 19 outbreak and despite implementation of tough measures such as strict lockdowns, the success in containing the disease were marked by varied results. According to the latest statistics with >10.2 million cases reported till date, the top 10 countries affected are USA being ranked first followed by Brazil, India and UK being ranked fourth and fifth respectively, Italy at ninth and Iran at tenth position, (Ritchie et al., 2020).

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Looking to the severity of the sudden worldwide gripping of the population by the less known virus, the World Health Organization (WHO) has done its job quite well, in issuing the preparedness instructions, (Thorp 2020). Though, losing over five hundred thousand people and afflicting over a million is heart-breaking, destroying the world's economy, starving millions worldwide is indeed a paradox in the 21st century. The curves are not showing signs of flattening for now, but will certainly disappear sooner or later, with a determined effort of all of us. The present paper describes the current scenario of world preparedness focusing on certain countries which have shown positive responses to the pandemic.

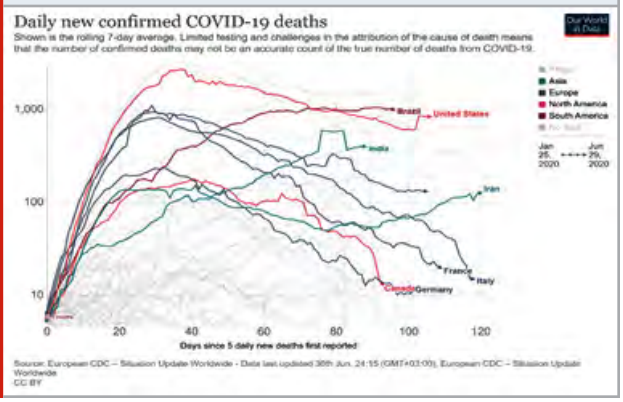
METHODOLOGY

In the present study a literature based analysis has been made using the Internet resources, Pub Med and other data bases to explore the status of the corona virus pandemic of late, between January and June 2020 in order to have an idea of the steps various countries have taken for the preparation of combating the corona virus disease. The compiling of literature is an attempt to short list vital precautionary steps under taken in the back ground of the world wide recommendations proposed by the World Health Organization (WHO) in its hurried strategies to face boldly the worlds most unprecedented onslaught of the viral epidemic killing more than half a million and affecting over a million people all over the globe, (JHU 2020).

While the situation in some of the developed economies namely United States and UK is grim, other nations Germany, France, Italy and Canada have been able to control the pandemic quite successfully, after the initial exponential rise, as depicted in Fig.1, (Ritchie et al., 2020). Comparing it with the developing economies such as Brazil and India, continuous elevation in the graph is visible as steady increase among the Co-Vid positive cases is witnessed. Universally, the commonest medical strategy for countering the Co-Vid 19 challenge is trace, test, isolate and quarantine, since it will take quite a while to prepare vaccination and anti-viral medicine to treat the disease. Countries like China, Taiwan, South Korea, Singapore have provided rapid, effective and often innovative responses by means of strictly implementing the basics with full public support, the other middle east countries like Saudi Arabia, UAE have been able to quite control well in time not only implementing the WHO recommended protocol but also because of their earlier experiences with Middle East respiratory Syndrome in 2012,(Dalgish 2020).

Countries like India and Brazil suffer from different socio-economic challenges among many fronts making it more difficult to counter the Co-vid 19. Despite these, the principle of over-reaction all over the world was preferred than under-reacting, which brought some better positive results for at least India, which otherwise would have been graver, (WHO,2020 b, Miraj and Miraj,2020).

Figure 1: Showing the country wise control of the Pandemic -19 deaths



Source European CDC Ref. Ritchie H.,Ortiz-Ospina E., Beltekian D., Mathieu E.,Hasell J.,Macdonald B. Giattino C and Rose M (2020) Statistics and Research Coronavirus Pandemic (COVID-19).

Recently, the example of New Zealand, is an eye opener, it first went into lockdown on March 25, setting up a new four-stage alert system and going in at level four, where most businesses were shut, schools closed and people told to stay at home. This resulted in controlling the pandemic much better, of late by early June, all of New Zealand moved to level one, the lowest of a four-tier alert system. Under the new rules after moving into level one, all schools and workplaces can remain open.

In the face of this global health crisis and its struggle, yet another example is of Saudi Arabia the kingdom is bringing back traditional policy approaches. The dual qualities of firmness and determination have been the motto of the policy makers. In this spirit, the government acted decisively as the coronavirus spread to implement comprehensive and unprecedented precautionary measures that were largely applauded. The kingdom started by quarantining an entire city and later imposed local lockdowns and a strict nationwide curfew that included the two holy cities of Mecca and Medina.

Due to the corona virus pandemic, only "very limited numbers" of people will be allowed to perform the annual Hajj pilgrimage that traditionally draws around 2 million Muslims from around the world. The annual pilgrimage is to take place in July August this year. The Saudi government said its decision to drastically limit the number of pilgrims was aimed at preserving global public health due to the lack of a vaccine for the virus or a cure for those infected, as well as the risks associated with large gatherings of people.

Alleged rumour spreaders, curfew violators and opportunist suppliers in the kingdom have been prosecuted. There has been no shortages of any kind and the execution of well - planned strategies have yielded results. Violators of these measures have been denounced as citizens, rally behind the slogan, "we are all responsible,"(Farook 2020).

Meanwhile, the health response to corona pandemic by India has probably gone as well as can be expected, given the generally not so good state of India's infrastructure for delivering health care. Different states have responded with varying levels of effectiveness, reflecting not just their resource levels but also their institutional capacities. The crisis has highlighted the importance of state and city governments in the delivery of public services. This certainly does have challenges, but is also something as basic as getting the message out that healthcare providers should be protected rather than being ostracised.

The timing of lock down in India has been controversial, lockdown when infection levels are low, but in such a manner that produces intensified infection among the forcibly disrupted millions of informal workers. Then, when the infection has been properly incubated and intensified, through some combination of incompetence and callousness, allow millions to be at greater risk. In this way, the actual policy, as enacted on the ground, maximises both the economic catastrophe, and the ferocity of the epidemic. For countries around the world, there is a difficult choice to be made between two broad policy options: Containment, possibly via lockdown; or, alternatively, allowing the infection to spread, in the hope of achieving "herd immunity." There are costs involved in both strategies, (Singh 2020 IE 2020).

India's 'cluster-containment' strategy means focusing on early detection of cases. In India, Kerala a developed and highly educated state flattened the infection curve via the creation of a contagion route map. Similarly, Odisha's susceptibility to natural disaster gave it an advantage in crisis preparedness. The state with experiences of fighting the natural calamities like the cyclones, hurricanes and tornadoes has developed a one year contingency plan for the corona virus combat, using the Zero Causality Advanced Planning and Community Control formula, which has been highly successful.

The Odisha government fought its battle against the Covid-19 pandemic by using high-end analytic tools, AI-based questionnaire and WhatsApp chatbot, among other technologies, to flatten the curve. Even before the entire country bore the Covid-19 brunt; Odisha became the first state to enforce lockdown and incentivise self-declaration. It incentivised the quarantine scheme and offered INR 15,000 to people coming from abroad and who declared their travel history on the portal.

The government of Odisha received an overwhelming response from the travellers largely because of the incentive. The state deployed all its resources to ensure travellers complete the quarantine period. The call centre team called them up thrice a day for thorough monitoring of their quarantine. Incentivisation literally helped them to keep travellers inside quarantine centres. Odisha has always been a pioneer in managing disasters through cutting edge technology, (Dass 2020).

In a large country like India, thus different responses have been noted some with responsibility, swift decision making and its implementation with corruption free systems ably supported by well aware and educated public as seen in these states which have shown different positive results. Recently, the WHO praised India for its tough and timely efforts to control the spread of coronavirus. There has been no community transmission of coronavirus since the country went into lockdown, and the growth factor of cases has declined by 40%, according to recent press briefings from the Indian Union Health Ministry.

A good sign which has emerged globally is of our awareness to frequent hand -washing, social distancing, use of masks, gloves and avoiding spitting, a menace of pre-lock down era, showing that pandemics have always been the paradigmatic example of diseases, that show how our health is interlinked on a large scale. This impulse is laudable and, indeed, useful. It has helped, for example, ensuring that during this pandemic an enormous number of people worldwide have followed guidance and orders to physically distance with relatively little demonstrable social disturbance.The people by large have learned important lessons of living, believing a modified Cannons Law: of fright and fight and survive.

The summary is, we have to be ourselves prepared for such catastrophic events at all times though its necessary for the governments to come forward and allocate special funds for such crisis management without the stigma of corruption, dishonesty, procrastination and irresponsibility. One thing is clear, we have to save ourselves, no matter the tall claims, as in case of health care in any developing country, it's always the out of pocket expenditure which comes first for any emergency.

As world experts struggle to deal with the new coronavirus and its aftermath, they will have to confront the fact that the global economy doesn't work as they thought it did. For developing economies, the economic impact from the corona pandemic is multiple. Even for the affluent class, residential lockdown, travel bans, loss of jobs have resulted in a greater social vacuum. In this hour of crisis, the worst may be yet to be seen and therefore it would be wise to be pragmatic and start afresh where ever possible. Meticulous planning taking into confidence the real experts of public health, epidemiologists, physicians, scientists and academicians with corporate industries, a plan for the future is the need of the hour, rather than a team of sycophants which may lead to disaster.

Hope of humanity also now lies in the concentrated efforts of all of us, leading from the front, especially countries like US, Canada, UK, China, Russia, Europe and India who can make the difference in tackling this calamity. We also will have to develop the same attitude of being determinant, strong and confident of combating the pandemics, no matter what the system is prevailing.

If the various nations of the world do their job well in areas of public health, hygiene, sanitation, environmental

awareness, cleanliness, rest assured, the epidemics will not enter in the first place and if they do so, we will as a strong public health entity through that of public- private –partnership, should be ready to support the existing systems of health care by our own sustained combined efforts. Beyond the health system, social determinants of health, including adequate housing, safe drinking water and food, social security, and protection from violence, are also the central elements of the right to health and must be protected under international law as interconnected rights, rightly suggested by WHO (2020c) and Puras et al (2020).

I wonder is this a Gods way to tell us, that the time has come to limit the nuclear weapons and invest more on healthcare facilities for a better and a healthier world.

I wish these exorbitant atomic weapons could kill the virus !

Conflict of interest: The author has no conflict of interest

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An Updated Review on the Properties of Graphene Nano Filled Composites and Their Applications in Dentistry

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ABSTRACT

Researchers looking for innovative nanomaterials to be used in dentistry have recently been attracted to graphene nanofillers which demonstrate exceptional biological, chemical and mechanical characteristics over other nanofillers. Even though literature contains data for implementing graphene in different areas of medicine like utilization of their tissue regenerative potentials and antibacterial activities; only small numbers of studies are focused on their applicability in dental nanocomposites. This literature review aims to summarize current information and the latest progress made relating to graphene properties, synthesis and use as fillers for dental nanocomposites. Reports included in this review showed that graphene as well as its derivatives have recently turned into promising tools to be used in the biomedical field. These nano-size-materials exhibit great versatility and peculiar properties like huge surface area and impressive thermal stability, electrical conductivity and mechanical strength. Moreover, these nanomaterials can be incorporated into other materials for improvement in their bioactivity and optimization of their mechanical, physical and chemical characteristics. Accordingly, this indicate the potential utility of graphene for several different dental applications. Thus, it is highly recommended that more studies must be conducted for investigating the advantages and the utility of graphene nanomaterials in dental applications. This review would give valuable data toward new material fabrication and practical application of graphene nanofilled composite.

KEY WORDS: GRAPHENE, NANOFILLER, COMPOSITE, NANOCOMPOSITE, DENTISTRY.

INTRODUCTION

Graphene is one of the allotropes of elemental carbon. It is a one-atom-thick, sp2 hybridised, two-dimensional sheet of carbon atoms arranged in a honeycomb lattice, with 0.142 nm carbon-carbon bond length and with individual graphene sheets of 0.34 nm thickness, (Papageorgiou et al., 2017). Since (Novoselov et al., 2004) succeeded in identifying single-layer graphene at Manchester University in 2004, graphene has attracted enormous scientific attention in recent years, due to its unique electrical, thermal, and mechanical properties. In particular, it has many interesting characteristics, including unprecedented mechanical properties: modulus of elasticity about one TPa; tensile strength of 130GPa which can be considered as the strongest material ever discovered, remarkable thermal conductivity (5300 Wm-l.K-1), (Jun Han et al., 2013, Erol et al., 2018), and high electron mobility at room temperature (250,000 cm2/Vs)(Novoselov et al., 2012, Zhao et al., 2017). However, the properties of graphene depend on the perfection of

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its structure, which is significantly influenced by the methods of its synthesis (Skoda et al., 2014, Erol et al., 2018).

Graphene Synthesis And Production Methods: The Graphene nanofillers are produced with lightweight, high performance, low cost, and have a wide range in biomedical applications,(Zhang et al., 2012, Erol et al., 2018). However, large scale production of graphene has been a challenge although much research has been done to produce high quality sheets of graphene (Amiri et al., 2018). Hence, it has created more research and understanding of the graphene nanofilled composites. Enhancement of various properties like physical and mechanical will increase the suitability of graphene nanofilled polymers in many technological fields, (Zhang et al., 2012, Wang et al., 2016). Consequently, graphene can be synthesised by employing various methods such as; deposition of chemical vapor, growth of the epitaxial, exfoliation of compounds intercalated with graphite, and methods that include the oxidation and reduction of chemicals, (Lee et al., 2017, Lin et al., 2018).

In addition, high quality graphene can be produced through the micromechanical cleavage method that is more reliable and effective. However, the technique experiences a draw back since it produces very low yields and, thus, cannot be applied for large scale production of graphene. (Novoselov et al., 2004) Moreover, graphite precursors are the most suitable polymer for the extraction of graphene since they are considered to be relatively cheaper than other polymers. Consequently, the thorough analysis of different techniques of graphene synthesis is highly applicable to the current world of science (Verdejo et al., 2011, Lee et al., 2017).

Exfoliation: It refers to the process of separating layered materials into single sheets in order to make them thin due to the high demand of very thin nanomaterials. Thin graphene nanofillers are very beneficial since they have enhanced electrical conductivity, thermal stability, and reduced gas permeability than bulk materials(Rao et al., 2009). Moreover, graphite oxidation is a good approach of separating bulk materials into single layers and involves various techniques such as; stirring, shaking, and ultra-sonication. The process of oxidation is normally done in water by a sonication process since the polar functional groups are present in water. However, the exfoliation process can be done in an organic solvent which is much easier than in water because of the hydrophobic nature of sheets. Also, the thermal exfoliation process is also employed in exfoliation and produces single layer graphene in gaseous media in a relatively shorter time as compared to graphite oxidation, (Dimiev and Tour, 2014).

When the exfoliation process is done under heat, it leads to the production of gases from the functional groups which increase pressure thereby leading to disruption of molecular forces of association which exist between the layers hence causing separation. Moreover, the strategy is employed in large scale production since it produces high quality graphene with a perfect structure. This method, however, has limitations since it produces multilayered sheets because it allows the restacking of graphene sheets even after de-intercalation.(Verdejo et al., 2011, Shang et al., 2019).

Chemical Vapor Deposition: Chemical Vapor Deposition (CVD) process is normally used to produce high quality graphene which is achieved by using substrates of transitional metals like copper and nickel, which are used as a catalyst (Seah et al., 2014). This process is cheaper compared to others and requires the addition of the polycrystalline nickel films to a mixture of methane and hydrogen at a very high temperature. Once methane gas decomposes, it produces carbon atoms that react with nickel and solidifies in the presence of argon which decreases its solubility, and removes carbon atoms, and precipitates on the nickel film leading to the formation of graphene films. In addition, copper can alternatively be used as a catalyst in the formation of single layered graphene (Zhang et al., 2013). Moreover, another method used to produce single layered graphene is the Plasma-enhanced chemical vapour deposition which is a promising method, and it is likely to be used in large scale production. The process is flexible of various substrates, and it is achieved at a very low temperature and pressure which allows the generation of reactive species in the plasma. However, the process needs to be modified to control the graphene thickness, (Chan et al., 2013, Lin et al., 2018).

Chemical Oxidation-Reduction Methods: The reduction of graphite oxide is currently considered the most reliable route of producing graphene in considerably large quantities (Dreyer et al., 2010). The method involves the employment of oxidizing agents, and even strong mineral acids for the oxidation of graphite through the treatment with potassium permanganate and sulfuric acid as per hummer's method (Hummers and Offeman, 1958). As opposed to pristine oxide, the graphite oxide oxygenated heavily with its carbon atoms at the basal plane decorated with groups such as hydroxyl and epoxide. Moreover, its edge atoms are also decorated with carboxyl and carbonyl groups, and, thus, making graphite oxide extremely hydrophilic. The presence of the functional groups within the graphite oxide decreases the forces between the planes as the interaction of faces between polymers and the graphite oxide reduces, and thereby leading to the state of separation of the graphite oxide element, (Potts et al., 2011).

However, the chemical treatment during oxidation openly yields defects of the structures such as; multiple and single vacancies, defects of dislocation, chemical groups accessory and the defect type known as Stone- Wales. Subsequently, the structural defects affect the mechanical functionality of graphene in a great way. In addition, these defects of the structure lead to the interruption of the electronic framework of graphene and, thus, change its conductivity effect. Also, the graphite oxide that is insulated can be transformed into conductive graphene by the employment of procedures such as chemical

reduction with low temperatures and thermal annealing with high temperatures, (Verdejo et al., 2011).

Moreover, there are two reducing methods employed which include the chemical reduction process which involves the use of reducing agents of chemical nature in the graphite oxide chemical reactions (Park et al., 2009) and the thermal reducing process which is normally conducted by the intense heating of a vacuum or inactive environment to temperatures of the range between one thousand and fifty to two thousand degrees Celsius per minute, (Schniepp et al., 2006). Besides, there are various chemical reducing agents that are employed on graphite oxide reduction including hydroquinone, metal hydrides, hydro iodide, and p-phenylene diamine among others, (Galpaya et al., 2013, Singh et al., 2012).

Other Methods: There are other mechanisms that can be employed in the synthesis of graphene such as the conversion of carbon nanotubes to graphene nanoribbons by the unzipping process which includes the use of a mixture of potassium permanganate and concentrated sulfuric acid (Rafiee et al., 2010, Shukla et al., 2016). The unzipping procedure is possible since carbon nanotubes are normally considered as a roll of graphene sheets, and thus the process of unzipping them will reduce the thickness and increase the quality and demand of the sheets. However, the technique has a challenge of defecting sites which will hamper the properties of graphene, (Wei et al., 2013, Lee et al., 2017).

Preparation Of Graphene-Nanocomposites: The dispersion of nanofillers is a very critical step in the preparation of polymer nanocomposite. The properties of a whole matrix are equally improved by a good dispersed state that ensures a maximum reinforced surface area that affects other polymer chains. There are certain strategies that have been used in the preparation of graphene polymer nanocomposites and they include the process of melting, solvent processing, and in situ polymerization (Galpaya et al., 2013, Papageorgiou et al., 2017).

Solvent Processing: The graphite-oxide is scrubbed into single sheets of graphene oxide by the chemical processing or treatment using heat as previously mentioned. Moreover, the solvent process involves the partial oxygenation of graphene layers that allow dispersion in polar solvent, which is a preferred method of preparation of graphene composites through the process of solvent blending technique (Verdejo et al., 2011, Sharma et al., 2018). In addition, the procedure involves three primary steps that include; the dispersion by ultra-sonication in a preferable solvent, followed by the addition of polymers and finally the removal of the solvent through evaporation or distillation. The solvent processing technique is typically simple and, thus, more of graphene polymers are developed using it. However, the procedure has its disadvantage such as the permanent adsorption of organic solvents on the graphite oxide (Galpaya et al., 2013, Ahmad et al., 2018).

In situ polymerization: This is a procedure that involves the use of the chemically modified graphene which are combined with monomers in the existence of a selected solvent, thereby allowing polymerization response by regulating both temperature and time parameters. The chemically modified graphene (CMG) contains minute molecules that are employed in covalent bonding or during grafting of polymer chains via atom polymerization by transferring of radicals (Lee et al., 2010, Ahmad et al., 2018, Sreenivasulu et al., 2018). A good example of the in situ polymerization is epoxy, polymethylmethacrylate (PMMA) and polydimethylsiloxane (PDMS) foams (Chhetri et al., 2018, Zhang et al., 2018, Yang et al., 2018). However, despite the findings generated regarding the technique, there is still need for more research to be done on curing reaction so as to enhance the understanding and discovering of the consequence of nanofillers in the morphology of polymer matrix and its properties and curing reaction (Verdejo et al., 2011, Sharma et al., 2018).

Studies have shown that the polymerization reaction rate of PDMS decreases by the addition of graphene which has been exfoliated by thermal treatment, and leads to robust relations between the matrix of polymer and the incorporated particles that facilitate stress transfer (Verdejo et al., 2009). Moreover, the in-situ polymerization method also leads to a homogenous and an exceptional dispersion. The limitation of this strategy is that it is accompanied by increased viscosity that prevents loading and manipulation fraction (Papageorgiou et al., 2017, Varenik et al., 2017).

Melt process: This process is the most useful technique employed commercially as compared to in situ polymerization and solvent process, in particular for thermoplastic composites (Galpaya et al., 2013). The melt-process involves direct incorporation of the CMGs into the selected polymer which has been melted by the use of an extruder (twin screw) and the regulation of speed, time, and temperature (Verdejo et al., 2011). Moreover, for exfoliated graphene nanofillers that are prepared thermally, most available strategies are developed by using various polymers like polycarbonate, isotactic polypropylene, and polystyrene-co-acrylonitrile, (Kim and Macosko, 2009, Ajayan and Tour, 2007). The limitation of this method is the inadequate dispersion at a high level of fillers loading and increased viscosity, in addition to that graphene sheets are prone to rolling or folding during the blending procedure leading to a lower dispersion degree which consequently, resulted in poor mechanical properties, (An et al., 2010, Ji et al., 2016, Sreenivasulu et al., 2018).

Mechanical and physical properties of graphene-polymer-nanocomposites: Properties of Polymer-Nanocomposites are classified into mechanical and physical properties. There are variations between the mechanical features of the chemically exfoliated graphene and the thermally exfoliated graphene (Jang and Zhamu, 2008). The mechanical properties of nanocomposite include features such as raise in modulus as a loading fraction function.

Moreover, the graphite nanoplatelets have seen to reduce crack propagation in epoxy polymer. Thermally exfoliated graphene has been shown to have a larger interfacial area and aspect ratio. Subsequently, one of the limitations of thermal exfoliation is causing defects and wrinkles on the single layered sheets rather it has been seen to increase mechanical reinforcement,(Galpaya et al., 2013).

In addition, the wrinkles increase surface roughness which enhances the interlocking and mechanical properties of the graphene nanocomposite. Chemically and thermally exfoliated graphene has been seen to increase tensile modulus. Ultimately, it is important to note that, the tensile strength, toughness of the fracture, and the modulus of elasticity increase significantly in graphene based nanocomposite. Moreover, it is also interesting to note that plasticity decreases as compared to other pure polymers. The mechanical characteristics' improvement in graphene polymer nanocomposites is due to various key factors such as the fillers have a high surface area, homogenous scattering experienced within the matrix of the polymer and due to the binding strength between the polymer matrix and graphene nanofillers, (Mogharabi and Faramarzi, 2015).

Thermal Properties: Polymers are sensitive and are degradable at low temperatures, and considered as a limitation since they cannot be used at high temperatures. This degradation behavior of polymers is due to three parameters that include: the onset temperature which starts to degrade them, the degradation temperature, where maximum degradation takes place and the rate of degradation. Moreover, thermal stability has been seen to be increased in graphene that is exfoliated both thermally and chemically based nanocomposites. The increased area of the surface and excellent dispersion in graphene nanocomposites have been demonstrated as a contributing factors of thermal stability. Besides, thermally exfoliated graphene based nanocomposites have also been shown to have a radical raise in the glass-transition-temperature, (Singh et al., 2012, Galpaya et al., 2013, Ji et al., 2016).

Graphene nanofillers have been reposed to reduce gas permeability of polymer based nanocomposites, whereas reduction of gas permeability and water vapour permeation are a great enhancement to increase potential applications in various fields. Moreover, the elevated surface area of graphene is a factor, which attributes to the improvement of both physical and mechanical properties which make the graphene based nanocomposite very useful for the application in biomedical research and development (Mogharabi and Faramarzi, 2015, Sharma et al., 2018).

Biocompatibility of Graphene-Based Nanocomposite: There is recent research and interest geared towards the understanding and exploitation of graphene nanocomposite because of its significant contribution to the use of graphene in the field of biomedical research. These applications include important techniques such as bio imaging, bio sensing, drug delivery, cancer therapy, and antibacterial materials, (Pinto et al., 2013, Kuila et al., 2012). Moreover, graphene materials have a high surface area that absorbs many molecules that yielding a negative health concern in human and environmental ecosystem. In microbiology research, the graphene based composites has been shown to be of great significance because of its antimicrobial effect to suppress the growth of E coli and different types of bacteria, (Lim et al., 2012, Some et al., 2012, Panda et al., 2018) and also common oral pathogens such as S. mutans, F. nucleatum and P. gingivalis , (He et al., 2015), hence showing promising results of production of antibacterial composites (Pinto et al., 2013). In genetic engineering, the graphene nanocomposite has shown promising results because of its advantageous interaction with the nucleic acids, (Wang et al., 2014). This biocompatibility will be of use in diagnostic purposes and also in development of recombinant DNA vaccines, (Yang et al., 2010, Kuila et al., 2012, Erol et al., 2018).

The graphene based nanocomposite applications in tissue engineering have been reported, though faced with limitations of toxicity related to fibroblasts, epithelial cells of the lungs among others. The toxicity levels are attributed to reactive oxygen species which are known to induce oxidative stress (Pinto et al., 2013). However, this cytotoxicity can be related to the use of graphene oxide in graphene based nanocomposite since studies on graphene oxide showed that it is cytotoxic to cells (Sasidharan et al., 2011), while exfoliated graphene from liquid media does not have cytotoxic effects which emphasizes the importance of graphene, not its derivatives in fabrication graphene based nanocomposite since it will be more useful especially in biomedical applications. In the research findings from (Fan et al., 2010), it showed conclusive evidence that graphene based nanocomposites have good biological safety since it displayed almost non cytotoxic effects. In addition, research conducted recently showed that the toxicological and biological activity of a material was dependent on size, quality, state, and oxygen threshold, (Singh et al., 2012).

In a study conducted by Olteanu et al., (2015), they assessed the cytotoxic effects of three graphene based materials: graphite oxide (GO), thermally reduced graphene oxide (TRGO) and N-doped graphene (N–Gr), on stem cells obtained from the human dental follicle. The study was conducted in vitro and they evaluated cell viability, damage of the cell membrane, mitochondria properties and its effect on cytoskeleton. They also evaluated the oxidative stress induced by the graphene derivatives. From this study, it was shown that GO had the lowest cytotoxic effects compared to the other two. It further showed that GO induced oxidative stress without causing damage to the cell membrane which is a very important property in drug studies since it will not affect the host's cell membranes. The biocompatibility evidence on graphene based nanocomposite determined

that it can be very useful in biomedical applications and development, (Erol et al., 2018).

Graphene In Dental Nanocomposites: In research by Nuvoli et al., (2013), Bis-GMA/TEGDA was the polymer system selected for the experiment, instead of the commonly used Bis-GMA/TEGDMA polymer system. TEGDA is a copolymer of TEGDMA, with similar structures, and TEGDA was selected for the experiment because it is one of the best graphene exfoliation media. During the research, in the experimental section, the dispersion of graphene in TEGDA with graphene nanofiller loadings were acquired by distributing graphite flakes in the TEGDA and ultrasonicating for enabling graphite flakes exfoliation find then centrifuging for letting the un-exfoliated graphite flakes precipitating and recovering liquid-phase that contain graphene. Additionally, it was diluted with appropriate amounts of liquid monomer to obtain the required concentration (0.003, 0011, 0.053 and 0.27 wt.%) and the actual graphene content was checked using UV -Vis and Raman spectra. Thereafter, the right amount of Bis-GMA was applied to obtain 1:1 w/w mixture. Also, 1 wt.% of Benzoyl peroxide was added and the mix homogenized and further poured into silicon molds for polymerization at 80 degrees for 24 hours.

The results of this study showed that this synthesis technique reduced the phenomena of re-aggregation at the time of recovery of graphene nano- filler during solid state. As graphene concentration increases, a rougher surface was observed with the SEM results. Also, the increase of graphene content in the polymer relates to better mechanical reinforcement of the polymer in a proportional manner. Also, due to the presence of graphene in low amounts, the modulus of elasticity and hardness of the surface increased significantly in comparison to the absence of graphene, but glass transition temperature in nano-filled composites was independent of the filler content. The significant result of the study was that the graphene obtained by direct exfoliation is a good and safe alternative to carbon oxidized forms, due to the non-involvement of chemical manipulation in the synthesis.

The research done by Sava et al., (2015) is based on the efficiency and viability of the graphene- silver nano-particles used for the purpose of dental restoration. The aim of the research was to analysis an experimental composite material's mechanical properties, prepared both with and without, graphene-silver nano-particles. The materials used in the composition were monomer mixture with BIS-GMA/TEGDMA, as a matrix, in addition to hydroxyapatite along with silica and bioglasses in addition to graphene-silver nano-particles (0, 5, 10 wt.°/c) forming the reinforcing components in the mixture. During the experimentation section of the study, a total of 10 different composite samples were prepared from each group for each test. Tests for flexural strength of the specimens, diametrical tensile strength, and compressive strength test were performed, as was modulus of elasticity test.

The result revealed that when 5- 10 wt.% of graphene-silver nanoparticles, mixed with hydroxyapatite, silica, and bio-glass, was added to unfilled monomer mixtures of BIS-GMA and TEGDM A, then compressive strength, modulus of elasticity, surface hardness, and flexural strength increased significantly. Thus, the research empirically measures the effects regarding the mechanical properties when graphene-silver nanoparticles were added, and thus, it acts as nanofiller reinforcing material for resin-based dental composite. Recently, Lee et al., (2018a) found when 0.5% of graphene oxide nanosheet (nGO) was added to PMMA, the Vickers micro hardness and resistance to 3-point flexural bending were significantly increased in comparison to control specimens. Furthermore, (Alamgir et al., 2018) added GO and TiO2 to PMMA for reinforcement. The researchers synthesized two types of nanocomposites (PMMA / GO + TiO2 and PMMA / GO).

The structural, mechanical and thermal characteristics of these nanocomposites were then studied. Microstructural and thermal properties demonstrated by these nanocomposites were quite different from pure PMMA. They also demonstrated higher resistance to localized deformation. In comparison to pure PMMA and PMMA/GO specimens, the PMMA/GO+TiO2 nanocomposites exhibited greater surface hardness and modulus of elasticity. Impact of addition of graphene nanosheets (GNS) on physical, chemical, mechanical and bioactive characteristics of two different dental cement specimens namely EndocemZr (ECZ) and Biodentine (BIO) was investigated by (Dubey et al., 2017). Different proportions (1,3,5 and 4 wt%) of GNS was added to the cement specimens. It is noteworthy that GNS addition made cement setting quicker and improved the hardness without changing the pH of the two types of cements. However, when GNS was added to ECZ, it resulted in considerable decrease in adhesion strength.

Researchers have also tried to add graphene derived nanomaterials to glass ionomers (GI) for the purpose of reinforcement. Several different clinical applications have made use of GI demonstrating ideal coefficient of linear thermal expansion, dynamic fluoride discharge and capability to attach chemically with the intact tooth structure. Still, the utility of this substance continues to be challenged by unsatisfactory physio mechanical characteristics even though considerable progress has been made relating to GI constituents added with different filler types like hydroxyapatite powders, metallic powders and fibers (Baig and Fleming, 2015). Substantial improvement in physio mechanical characteristics of GIs has been achieved by combining graphene with glass ionomer synthesized from poly (acrylic acid) (Malik et al., 2018).

A GICs/FG composite matrix can be formed by the mechanical blending of glass ionomer with fluoride graphene synthesized from the hydrothermal reaction of graphene oxide. In this way, antibacterial, tribological and mechanical characteristics of glass ionomer can be considerably improved (Sun et al., 2018). It has been

reported that increment in proportion of FG in glass ionomer results in increment in antibacterial activity, reduction in micro cracks and pores and reduction in vulnerability to microbial attack and erosion. Moreover, surface characteristics and degree of conversion has been reported to be improved with reinforcing resin polymer matrices containing graphene gold nanoparticles as fillers. It can, therefore, be considered as a good approach for improving the physicochemical characteristics of dental nanocomposites (Sarosi et al., 2016).

The development of antibiofilm adhesives has become a necessity because of the existence of micorgaps between tooth healthy tissues and dental restoration resulting in microbial invasion (Ferracane and Hilton, 2016). Considering the antibacterial characteristics of graphene, utilization of GFNs as antibiofilm filler and antimicrobial filler for dental adhesives has been suggested by (Bregnocchi et al., 2017). GFNs modified dental adhesives were shown to cause inhibition of growth and adhesion of S. mutans with no effect on adhesion properties and mechanical efficiency. In another study, (Lee et al., 2018b) have investigated the biological and mechanical characteristics of orthodontic bonding adhesive (Transbond XT (LV) enriched in GO and bioactive glass mixture (BAG) in a proportions of 1,3 and 5% (BAG+GO). In comparison to LV, improved microhardness has been demonstrated by the adhesive containing 3% and 5% by wt. of GAG+GO. However, the shear bond strengths of all specimens were found to be statistically indifferent. Also, anti-demineralization and antibacterial action of the BAG+GO group were found to be superior.

CONCLUSION

Owing to their impressive structural and other characteristics, graphene as well as its derivatives have recently turned into promising tools to be used in the biomedical field. These nano-size-materials exhibit great versatility and peculiar properties like huge surface area and impressive thermal stability, electrical conductivity and mechanical strength. Moreover, these nanomaterials can be incorporated into other materials for improvement in their bioactivity and optimization of their mechanical, physical and chemical characteristics. Reports included in this review indicate the great utility of graphene for several different dental applications. Accordingly, it is highly recommended that more studies must be conducted for investigating the advantages and the utility of this nanomaterials in dental applications.

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A Mini Review on the Beneficial Source of Saudi Medicinal Plants for the Human Health Sector

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ABSTRACT

Medicinal plants also known as herbal plants are oftenly used in traditional medicine to cure the human diseases. These plants have been used for thousands of years as medicine. The medicinal plants are naturally derived from an indeed resources of vegetable origin. Herb is used for bark, flower, leaf, seed, stigma, stem and non-woody plant. Herbal plants have a significant potential source of therapeutics, and these plants have attained the commanding role in the health care system. In Saudi Arabia, flora has adjacent resemblance with African, Iranoturanian and Mediterranean countries. The prevalence rate of herbal plants in Saudi Arabia is in between 8-76%. Saudi Arabia also have the historical tradition of using herbal medicine in daily life. From the natural source, numerous medications were extracted from the plants. Black seeds, costus, fenugreek, garlic, ginger, henna, miswak and pomegranates are the list of prophetic medicines worked effectively for treating humans. The Saudi medicinal plants are positioned at various regions in kingdom. However, information of Saudi medicinal plants was stored in the Saudi Herbal Plants information system. This review was majorly focused on Leguminosae, Compositae, Labiatae and Euphorbiaceae herbal plant species which are used for the human treatment. This review concludes the available herbal medicines in Saudi Arabia and herbal medicines contain wide range of active pharmacological components have therapeutic effects. Lots of research shows that herbal medicines are used for the human treatment. Saudi Arabia has a great history in treating the diseases with herbal medicines.

KEY WORDS: MEDICINAL PLANTS, HERBS, LEGUMINOSAE, COMPOSITAE, LABIATAE AND EUPHORBIACEAE.

INTRODUCTION

Human therapies depend upon plants and its supplementation, known as the conventional complementary treatments in the health issues. Plant compounds play a perfect role in managing human diseases. Plants not only deliver food and fiber but also useful as medicine in treating social conditions. Before 5000 years, plants were used as medicines through various sources such as antineoplastic, antibiotics, cardioprotective and analgesics. Natural compound products were used for the infections. The term medicinal plant is defined as numerous kinds of plants applied in herbarium for the medicinal purposes and for its use. The medicinal plant can be used for therapeutic purpose and use for drugs. Randomly, 70% of the world population uses medicinal plants to cure human diseases,

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(Brima and health, 2017, Anand et al., 2019, Zielinska-Blizniewska et al., 2019).

The word medicine is derived from Latin word indicates "arsmedicina", specifies as art of healing. Modern medicine is replaced with recent medical methodologies applied for treating the human diseases. Plants and herbs supply enormous chemicals towards their metabolic activities and to guard them from diseases (Mosihuzzaman, 2012).The stochastic estimation of 30,000 plant species was connected towards medicine globally and approximately, 15,000 plants were used for the preparation of drugs. Plant harbors the known and unknown chemical molecules which are applied in the pharmaceuticals (Manoharachary and Nagaraju, 2016). World Health Organization (WHO) defines herbal plants as used in treatment, diagnosis, or prevention of illness and to maintain the human health (Organization, 2019).

Herb is used for the reference for bark, flower, leaf, seed, stigma, stem and non-woody plant. Herbal plants have a significant potential source of therapeutics, and these plants have attained the commanding role in the health care system. Almost, two-third of the global population depends on herbal medicine for primary health care issues because of its cultural acceptability. Plant-derived drugs were discovered with traditional and therapeutics studies (Schulz et al., 2001, Oladeji, 2016). Herbal usage is widely involved in major global countries with complementary-alternative medicines (CAMs) (Ekor, 2014). Saudi Arabia also have the historical tradition of using herbal medicine in daily life. From the natural source, numerous medications were extracted from the plants. Black seeds, costus, fenugreek, garlic, ginger, henna, miswak and pomegranates are the list of prophetic medicines worked effectively for treating humans (Aati et al., 2019). The purported benefits of herbal medicines were applied for natural healing, inadequate side-effects, affordable, strength in the immune system, maintenance of hormones and metabolisms (Mohammadi et al., 2020).

However, Alghamdi et al (Alghamdi et al., 2018) studies confirm high usage of herbal plants in Saudi Arabia for human treatment. Arabian Peninsula covers Saudi Arabia, UAE, Bahrain, Yemen, Oman and Kuwait, which is located in the south-west Asian region. Saudia epitomizes the 2.2 Million of the longest part in the Arabian Peninsula (Schulz and Whitney, 1986). In Saudi Arabia, flora has adjacent resemblance with African, Iranoturanian and Mediterranean countries (Alfarhan, 1999). The prevalence rate of herbal plants in Saudi Arabia is in between 8-76% (Alrowais and Alyousefi, 2017). Saudi plants play a vital role in a portion of eco-system, sustaining and maintaining ecological balance and stability.

The practice of medicines from the plants was processed earlier in 3000BC (Pharaohs), 400BC (Greeks), 37BC

Table 1. Available of Plant species from Tabuk Region in Saudi Arabia for herbal medicine (Ali et al., 2017)

Family/Species	Life Form	Traditional and Medical Use	Parts used
Euphorbiaceae			
Chrozophora tinctoria L.	herb	Emetic	Leaf
Fabaceae			
Acacia tortilis Forssk.	tree	Anthelmintic and antidiarrheal	Leaf
Alhagi graecorum Boiss.	shrub	Purgative	Whole plant
Astragalus spinosus Forssk.	shrub	Purgative	Leaf
Astragalus tribuloides Del.	shrub	To treat irritation of the stomach and colon.	Seed
Melilotus indica L.	herb	To treat bowel complaints and infantile diarrhea	Whole plant
Cassia holosericea Fres.	shrub	Purgative.	Leaf – fruit
Cassia italic (Mill) Lam	shrub	Purgative.	Leaf – fruit
Retama raetam Forssk.	shrub	To treat anthelmintic and purgative	Leaf (little amount)
Trigonella stellata Forssk.	herb	To treat abdominal pain, diarrhea and dysentery	Whole plant
Lamiaceae			
Lavandula pubescens Decne	herb	Carminative	New buds and stems
Mentha longifolia L	herb	Carminative	Seeds
Salvia lanigera Poir	herb	Carminative, and to treat indigestion.	Whole plant
Teucrium polium L.	shrub	Anthelmintic, to treat stomach and intestinal troubles.	Leaf
Thymus vulgaris L.	shrub	To treat abdominal pain, anthelmintic, and carminative	Whole plant

(Romans) and between Rhazes 865-1037 (Arab physicians) depended on plants for therapies (Al-Sodany et al., 2013, Akgül et al., 2016).

Saudia consists of almost 1200 flowering plants throughout the kingdom and 50% of plants are used for medical purpose. *J. procera*, Rumex nervosus and *Ziziphus spina*-christi are the commonly used medicinal plants for herbs in the Saudi Arabia. The list of available medicinal plants used in southwest region of Saudi Arabia has been documented in the prior studies. Numerous plants were used for preparing the herbal medicines in Saudia and majorly plant leaves were used for the preparation (Ali et al., 2017). It is well-known that leaves form a key part of the plant certification which can be visible effortlessly (Akerreta et al., 2007).

Saudi medicinal plants: The medicinal plants in Saudi Arabia is located in various regions in the kingdom. Most of the medicinal plants available in Jazan are shrubs, perennial herbs, annual herbs and trees (Umair et al., 2017). The information of Saudi medicinal plants was stored in the Saudi Herbal Plants information system (SHPIS) (Balaji et al., 2012) consists of the data for 120 unique variabilities of medicinal plants; which was distributed in 59 families of plants within the kingdom. SHIPS consist of local name, scientific name and family name of the specific plants within the database. SHIPS also provide information on precise plants used for medical or traditional use for treating human diseases as asthma, constipation, cold, diabetes, ear-eye problems, epilepsy, fever, rheumatism, stomach-ache, skin-allergy and toothaches (Syed et al., 2017).

Alharbi et al (2017) documented that the Tabuk city which is located at North-Western part of Arabian Peninsula, consists of 81 plant species which comes under 30 families; 9 species of Fabaceae, 8 species of each Asteraceae cum Plantigrade and 6 species of Zygophyllaceae are designated as high plant species which is used for traditional and medicinal purpose (Table 1). These plant species will be used in the medicinal purpose in human diseases for skin diseases, ulcers, diabetes and gynecology. Al-Said et al (al-Said, 1993) studies has confirms as traditional medicines visible in Saudia will be beneficial for potent and drug safety. Drugs will be incorporated in the clinical health sector once the clinical trial requires to complete successfully. Taif city has documented with 261 species of 178 genera which comes under 55 families; Asteraceae, Asclepiadaceae, Brassicaceae, Boraginaceae, Chenopodiaceae, Fabaceae, Poaceae and Zygophyllaceae (Al-Sodany et al., 2013).

The lowest medicinal plants with 28 families which involves 47 species have been recorded in Al-Rass domain at Qassim region (El-Ghazali et al., 2010). The detailed information about these Saudi plant species has been documented in the prior studies (Rahman et al., 2004, Saganuwan, 2010, Khatibi et al., 1989). Alharbi (Alharbi, 2017) studies confirm plant species from the Taif region can be used to treat the human diseases for the digestive tract, parasites, skin, fungal and antifungal activities. In South-western Saudi Arabia, Leguminosae, Labiatae, Compositae and Euphorbiaceae plants species were used to cure the large number of human diseases(Abulafatih, 1987), which have been listed by Syed et al (Syed et al., 2017). In this review, elaborated details were given towards Leguminosae, Compositae, Labiatae and Euphorbiaceae herbal plant species which are used for the human treatment.

Compositae: Mainly the family compositae comes under angiosperms group. Altogether, 104365 species were documented under compositae family. Numerous studies shows the role of compositae of medicinal herbs in both immune and non-immune diseases as reproductive system diseases, Alzheimer's, depression and anxiety disorders (Jafarinia and Jafarinia, 2019). *Achillea biebersteinii* (used for itching and revives from toothache), *Conyza incana* (relieves joint and muscle pains), *Euryops arabicus* (healing of the wound in the foot), *Psiadia arabica* (treated for rheumatoid), Senecio asirensis (for fever) and Targetes species (for cold and constipation) are involved in Compositae (Abulafatih, 1987).

Euphorbiaceae: The Euphorbiaceae are well-known for herbs, shrubs and trees. This family belongs almost 7300 species with 283 genera. It belongs to the similar family of Euphorbia bicolor. Genus Euphorbia is confirmed as world third largest flowering plants (Basu et al., 2019). This plant is visible globally and in Saudi Arabia is represented through Acalypha fruticosa, *Euphorbia schimperiana, Jatropha species and Ricinus communis* applied to treat skin disease, spine problems, laxative, vermifuge and also used to applied on the wounds. *Ricinus communis* will provide the castor oil (Abulafatih, 1987).

Labiatae: Labiate is also known as Lamiaceae known for mint which is firmly connected with Verbenaceae which has 236 genera between 6900-7200 plant species. Some of the largest genera are saliva, stachys, hyptis, vitex and nepeta. These plants are useful in preparing the drugs for Tulsi, peppermint, thyme, spearmint and coleus. The Lamiaceae family is known for its importance in the herbal medicines which involves large variety of plants in the medical purposes. Family members of these species are known to be aromatic which means mint, sage, basal, rosemary and lemon balm. It is also useful for the preparation of oils (Uritu et al., 2018, Raja, 2012).

Leguminosae: Leguminosae is also known as Fabaceae which incorporates the legumes as plants and fruits. Leguminosae is known for worlds third-largest group of plants have 730 genera in 19400 plant species. One of the major features of this family are the fruit of the plant and species ranges from dwarf herbs. Papilionodae, Caesalpinioideae and mimiosoideae are the sub-divisions of Leguminosae's family. Legumes are nominally used for fixation of nitrogen; as legumes can convert atmospheric nitrogen into beneficial nitrogen fixation (Ahmad et al., 2016). Acacia negrii is used for

treating retina issues, Cassia italica and Cassia Senna is for laxative, indigofera articulata is used for tooth problem and indigofera oblongifolia is used to remove dandruff(Abulafatih, 1987).

All the above plant species are available in the Saudi Arabia and majorly applied for the herbal medical purposes. Apart from these medicinal plants, there are many more are available and used for treating the human diseases. Till now in the Saudi Arabia, there is no update on complete valuable medicinal plants for positive factors. The present review can be helpful for the using the herbal medicinal plants for temporary use to stable the pain.

In conclusion, this review confirms the available of herbal medicines in Saudi Arabia and majorly, herbal medicines contain wide range of active pharmacological components which has the therapeutic effects. Lots of research shows that herbal medicines are used for the human treatment. Saudi Arabia has a great history in treating the diseases with herbal medicines. Future studies should be implemented in the remaining herbal plants which can be used for medical purpose.

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An Updated Literature Based Analysis on the Genetic Aspects of Coronavirus (COVID19) and its Connection with the Viral Genome

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ABSTRACT

World Health Organization has declared in 2020 "coronavirus 2019" (COVID-2019 or 2019-nCoV or SARS-CoV-2 or CoV) as a global endemic. A recent respiratory disease produced by a novel coronavirus was born in Wuhan city of China in late December 2019. CoV is connected with a large family of viruses commonly found in cat, camel, cattle and bats. Severe acute respiratory syndrome (SARS) virus is genetically interrelated with COVID 19 strains which infect bats. MERS-CoV, SARS-CoV and SARS-CoV-2 are affected by humans from the animals. The COVID 19 is known to be the human-to-human transmission. Presently, there is no medication for COVID19, and this disease has been inherited to another global country. Pharma companies will have a major role when genomic medicine goes at the mainstream, and presently, there is no medication for COVID 19. However, genome sequencing analysis can help to design medication (Rx) by pharmacists. The genome sequencing can be identified by the next-generation sequencing (NGS) in the whole viral genome. The advantage of NGS is to identify the disease-causing or novel variants in the human genome. Limited global studies have sequenced the human genome and confirm ~88% of the human genome is similar to the Bats sequence in an effected person of COVID 19. This review will be connected to the pharma companies in designing the Rx for COVID 19. Thus, NGS analysis can be helpful for the pharma companies for scheming medications.

INTRODUCTION

Human Coronaviruses are mostly 4 types (HCoV-OC43, 229E, NL63 and HKU1) as categorized with International committee for the Taxonomy of viruses (Lefkowitz et al. 2018; Zu et al. 2020). One of the types of pneumonia occured in 2019 novel coronavirus disease (COVID-19) is recognized to be highly infectious disease; declared by World Health Organization (WHO) as a global public health emergency (Chen et al. 2020). Several recent reviews since then have appeared in literature describing various aspects of the effects of the global pandemic such as biological, economical, social and psychological, (Gralinski and Menachery, (2020) and Miraj and Miraj 2020).

Coronaviruses (CoV) belongs to one of the large family group of viruses that cause starting from illness to common cold which is similarity for Middle-east respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS)-CoV, (Situational Report 2020). Coronavirus-2019 is known to be 2019 novel coronavirus outbreak, illness is termed as COVID-19, and virus is known to be SARS-CoV-2 or 2019-nCoV (Kim 2020). Right now, both WHO and centers for disease controls are monitoring the CoV disease (Lu et al. 2020). Presently, CoV disease is hugely expanding in China and its premises (Sun et al. 2020).

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CoV are documented as zoonotic; which indicates as the disease CoV has been transmitted from animals to people, (Gralinski and Menachery 2020).

CoV are termed as crown-like spikes on their surface; categorized as alpha, beta, gamma and delta sub-types. Between the 1960's, CoV was identified in humans and 229E (α-CoV), NL63 (β-CoV), OC43 (γ-CoV), (δ-CoV), MERS-CoV, SARS-CoV and SARS-CoV2 viruses can infect. Global people are currently affected with , β, γ and δ Coronaviruses (Xu et al. 2020). Appearance of common signs is cough, fever, shortness of breath, respiratory symptoms and breathing difficulties; amongst the rare cases, SARS, pneumonia, damage of kidney and at last death may also occur (Shanker et al. 2020).

Initially, CoV or COVID-19 is documented in Wuhan city at China in 2019 and is suspected as this disease may be allied with seafood market in Wuhan city. CoV possibly appeared initially from animal source but it seems now, it is dispersion from one affected person to another one (Albarello et al. 2020; Sohrabi et al. 2020a). COVID-19 is presently considered as more attention because it has been documented as novel virus; which was not documented earlier in the humans (Zhu et al. 2020). CoV is a respiratory viruses and others are influence; whereas, COVID-19 is not confirmed till now as droplet or air-borne transmission virus. Subsequently, diagnostic tests have been improved for CoV patients and applied these tests in the suspected cases in the worldwide, (WHO 2020).

Bats are whispered to be familial hosts of α and β CoV and for also SARS and MERS CoV(s) (Anthony et al. 2017). (Lam et al. 2020) studies report the confirmation of COVID 19 associated with CoV in Malayan Pangolins seized in anti-smuggling operations in south region of China. Metagenomic sequencing of pangolins confirms the two-sub lineage sequence in CoV associated with COVID19. Presently, COVID 19 is spreading globally 160 countries and showing no symptoms of slowing down. Worldwide, 2.5 million cases have been registered; USA has 0.8 million; the maximum registered cases in the globe, followed by Spain, Italy, France, Germany, UK, Turkey, Iran and 82,758 COVID cases has been confirmed and in China, till now >77,123 cases were recovered with COVID19 and >4600 deaths occurred whereas the rest of the world has registered 170044 deaths as of 21st April 2020, (WHO, 2020).

In South Korea 10,683 cases have been confirmed. Corona viruses are the largest RNA viruses that infect animals and humans in respiratory and enteric diseases. CoV causes almost one-third of common colds and the newly recognized SARS. Epidemiology of CoV in people harbors anti-corona virus antibodies; however, reinfection is documented as numerous circulating serotypes of viruses are present in the human population, (Sohrabi et al. 2020b). The pathogenesis of CoV is caused due to respiratory and enteric diseases in various animals. In humans, virus replication in the epithelial cells of the respiratory tract and one-third of colds are affected with CoV. The symptoms of CoV are sore throat, running nose, headache, cough, high grade fever, chills and the incubation period is for 72 hours. The spread of the viral is due to immune response with short life-span. Transmission is done through the transfer of nasal secretions in aerosols caused due to sneezes, (Cascella et al. 2020).

Viruses that infect epithelial cells of the enteric tract causes diarrhea. The infections for CoV are undiagnosed with self-limited diseases. The diagnosis can be confirmed through immuno-electron microscopy and serology. There are no routine anti-viral CoV infections. Severe acute respiratory syndrome (SARS) was documented in 2002 at Southern China, which was also reported in Africa, Asia, Europe and North America. The SARS CoV causes severe respiratory disease and an average incubation period is minimum of 14 days, (Lauer et al. 2020).

The common symptoms involve fever (>380C), malaise, chills, headache, dizziness, sore throat, cough and shortness of breath. The laboratory diagnosis for SARS are based on antigen as well as detection of nucleic acid. The antigen is perceived through Enzyme Linked Immuno Sorbent Assay (Elisa) tests with the high-quality of antiserum. Enteric CoV can be diagnosed using electron microscopy with the stool sample. Polymerase chain reaction (PCR) assays are useful to distinguish CoV nucleic acid in respiratory secretions and in stool samples. The RNA of SARS viruses was seen in plasma through PCR and it is difficult to observe human CoV with cell-culture technique. The SARS viruses are recovered from oropharyngeal specimens through kidney cells in Vero monkeys. Serology, Elisa and hemagglutination tests can also be used for the confirmation of SARS. Currently, there is no treatment for SARS; however, symptoms can be managed and drugs are under progress, (Ahmad et al. 2020; Al-Ahmadi et al. 2020; Albarello et al. 2020; Cleemput et al. 2020; Gallego et al. 2020; Huang et al. 2020; Sohrabi et al. 2020a; Yang et al. 2020). However, numerous vaccine trails have been implemented and none of the vaccines are available till now for COVID19. One of the complications with live virus vaccine is antigenic shift and unpredictable outcomes.

Sequencing analysis: CoV have RNA sequence with huge genomic structures through high error in repetition when equated with host genomics. Numerous coronaviruses can do effective recombination of their genomes after infecting host cells (Luo et al. 2018). Genome sequencing of pathogens is always critical for developing specific diagnostic tests and the identification of potential treatment options (Ma et al. 2019). Sequencing of viral genome, huge sample size with hundreds and thousands of genomes are required to analyze numerous phenomena in which genetics plays a role (Harris et al. 2008). High throughput or next-generation sequencing technique is known as powerful for identifying the disease causing or novel variants for human as well as viral genome sequences (Bogari 2016; Bogari et al. 2020).

Next-generation sequencing (NGS) has driven the research space and is insightful clinical applications with enormous impact. NGS technique can be used for DNA, RNA or methylation sequencing for large-scale genomic and transcriptomic sequencing because of the high-throughput production and outputs of sequencing data. NGS is lowered to cost compared with the traditional first-generation sequencing (FGS) or DNA-Sanger method. One of the differences between first and second-generation sequencing techniques are, FGS can screen the limited variants; whereas, second-generations sequencing (SGS) can be enormously high-throughput from several samples at lower cost (Kulski and Challenges 2016). Based on large sample size, it will be beneficial for detecting the large number of mutations through RNA sequence in the viral genome and will be useful in tracking the spread of COVID19. It is very important to screen towards phenotype for the spreading of viruses (Zhang and Holmes 2020).

The comprehensive diagnostic ability of NGS in documenting pathogens of unknown etiology during specific infections (Schilcher et al. 2019). Genome data is useful in understanding the transmission dynamics for expansion of rapid diagnosis (Cleemput et al. 2020). SGS data is very useful in diagnosis in confirming the rare or novel variants and which may be associate with the human disease. Currently, it is important to identify the basis of its replication, structure, and pathogenicity for discovering a way to the special treatment or the prevention (Mousavizadeh and Ghasemi 2020).

CoV and SGS studies: Lu et al (Lu et al. 2020) have documented 10 genome sequences of COVID19 NGS data which was attained from 9 patients. The COVID19 sequence was found to be similar with 88% in bat resulting from SARS like CoV (bats; SL-CoVZC45 and SL-CoVZXC21). Joyjinda et al (2019) initially studied the human coronavirus (HCoV) in 2004 from Hongkong population and its diagnosis was confirmed through PCR; targeting the CoV RNA dependent RNA polymerase gene. Tao and Tong (2019) have performed the complete genome sequencing SARS of Bats and recognized the CoV strain i.e., BtKY72 or Rhinolophus species in the Kenyan bats. This study also confirmed the sequence of BtKY72 is close to the BtCoV or BM48-31 in the European Bats. The SARS-CoV identified in Rhinolophus bats were from China, Europe and Kenya. Genetic sequence for BtKY72 will facilitate understanding the molecular evolutionary characteristics of bat SARS-related CoV (Tao and Tong 2019).

Recently, Wu et al (2020) have performed the Next-generation metagenomic RNA sequencing in 7 subjects allied with Sea food marker at Wuhan city from China and identified a novel RNA virus Coronaviridae family intended as WH-Human-1 coronavirus. This study also confirms as ~89% of nucleotide sequence was found to be common with Bats sequence in China for a group of SARS-CoV in the βCoV of Sarbecovirus.

Pharmacogenomics and SGS: SGS and RNA-S offers entire transcriptome and CHIP sequences for complete architecture of the genome. Discovering genetic or novel variants in the affected individuals may be predicted for the disease risk. NGS can also use for predicting the disease risk to predict the response to adverse effects of drugs. Presently, COVID19 is an essential disease has been grown up without any medication (Rx). There is no medication for COVID19 and there is a need for such a personalized medicine which needs to supply the possibility to treat COVID 19 transmitted disease. Pharmacogenomics will always play a major role in discovering the medication for novel diseases based on the SGS technologies and their precise applications in human diseases to foster human health care and personalized medicine (Rabbani et al. 2016).

CONCLUSION

From this current review, limited information is gathered for the pharmacists for developing the medicine using the available genome data from the humans affected with COVID19; as ongoing virus tumbles from animals to cause severe disease in humans. NGS analysis can be helpful for pharma companies for scheming medications. First of all, accurate diagnostic tests with PCR should be implemented to rule out the disease. Furthermore different researches should also be implemented on usage of medicinal plants or herbs for the future treatment perspectives of COVID 19 pandemic.

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Adhesive Bond Strength of Er Cr YSGG Laser Treated Deciduous Dentin to Resin Cements

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ABSTRACT

The present study was performed to assess the adhesive bond strength of Er Cr YSGG laser treated deciduous dentin to resin cements. Forty deciduous teeth were collected and stored in saline solution for 24 hrs. The prepared specimens were divided into two groups, group A (control, n=10) and laser treated groups (B, C, D) (n=30). The control group specimens were treated with 37% phosphoric acid (Rely-X-ARC) while specimens in remaining groups underwent Er Cr YSGG laser treatment. Furthermore, the three laser groups were divided based on the types of the cement build-ups employed; Rely X ARC, Rely X Unicem and GIC. Cement builds up were placed on each specimen using a putty mould followed by light cure in the resin based cements. After storage in distilled water (twenty-four hrs), the prepared specimens were tested for shear bond strength in a universal testing machine. Collected data was analysed using Analysis of variance (ANOVA) and Tukey multiple comparisons test. A significant difference in bond strength between the groups ($p< 0.05$) was observed; however, the control group presented with highest shear bond strength of 25.38 (3.81) MPa among all groups. Specimens treated with laser and bonded to Rely X ARC and Rely X Unicem showed comparable ($p>0.05$) bond strength outcomes. Lased specimens bonded to GIC exhibited significantly lower bond strength compared to other laser treated groups ($p<0.05$). Among all groups the most common type of observed failure was adhesive followed by the admixed and cohesive (GIC-D) respectively. Both surface treatment (laser) and cement type showed significant influence on their bond strength to deciduous dentin

KEY WORDS: ER CR YSGG LASER, DECIDUOUS TEETH, PRIMARY DENTIN, RESIN CEMENTS, BOND STRENGTH.

INTRODUCTION

Paediatric dentistry requires understanding and knowledge of restorative materials and techniques used in deciduous teeth (Koch et al., 2017). Conservative management is key in managing tooth destruction in children (Reston et al., 2011). Nevertheless, it is opted as the first step in the caries management continuum, which is based on the progressive philosophy to eliminate biofilm and demineralisation before tooth reconstruction (Hurlbutt and Young, 2014). Therefore, a proactive therapeutic approach is essential in managing early caries lesion in children (Hurlbutt and Young, 2014). The chronic lesions are followed up by minimally invasive restoration and conventional treatment. Composite resin and GIC are the most commonly used restorative materials in deciduous teeth (Frencken et al., 2012, Tellez et al., 2011). Minimally invasive treatment for management of carious deciduous teeth is the goal. Currently, lasers are gaining more

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attention in abrading the hard tissues of deciduous teeth to increase the retention of the restoration thereby reducing the need for local anaesthesia. Thus, the use of laser reduces time, improves efficiency and is comfortable for the patient (Tellez et al., 2011, Koch et al 2017).

Humans are diphyodonts who have sets of teeth that are unique in nature and characteristics (Neves et al., 2016). Retentive restoration in the primary teeth is essential as these teeth act as spacers and maintains masticatory function in children. Literature states that the primary teeth differ from permanent teeth in enamel and dentin quantity, pulpal anatomy, and tubular density (Lenzi et al., 2014). Studies have shown that the difference in physiological and morphological features influences the adhesive properties of the restorative materials. Presence of low inorganic content in deciduous teeth requires tooth conditioning for longer duration or strong surface acid etchant (de Siqueira Mellara et al., 2014, Sfondrini et al., 2011). However, the use of lasers recently as surface treatment has presented with improved bonding strength in deciduous teeth with resin cements (Neves et al., 2016, Kerkis and Caplan, 2012). Nevertheless, some studies have shown no change in the shear bond strength despite the differences with the permanent teeth (Lenzi et al., 2014, de Siqueira Mellara et al., 2014, Koch et al 2017).

Lasers are conceded as clinically practical and safe in children for preparation of deciduous teeth. Conventional lasers were limited due to their thermal effects on the large pulp chambers in the deciduous teeth; however low-level laser therapy with contemporary lasers has resolved this issue (Ansari et al., 2012). Studies have used lasers with variating wavelengths to accomplish optimum results with low adverse effects such as Er Cr YSGG. The erbium, chromium:yttrium-scandium-gallium-garnet(Er,Cr:YSGG) is a well-developed hydrophotonic pulsed laser system(Shafiei et al., 2013). The laser has the ability to abrade the surface efficiently without the deposition of the smear layer (Shafiei et al., 2013). In animal testing, Er Cr YSGG lasers have proved successful in producing low thermal effects and damage to the pulp, especially when used with water spray (Shahabi et al., 2013). It is hypothesised that the laser transfers the energy through the water droplets, which creates micro expansion to abrade the surface and removes debris (Giray et al., 2014).

Many studies have evaluated bond strength using lasers in comparing permanent and deciduous teeth (Bandéca et al., 2012, Ramos et al., 2014). It is suggested, that both deciduous and permanent dentition show similar shear bond strength outcomes in addition ot the topographical changes in both sets of teeth (Tseng et al., 2007, Giray et al., 2014). Literature suggests successful use of laser to improve bond integrity through an increase in surface resistance and reduced micro leakage (Shahabi et al., 2013).

However, limited data have been collected regarding the adhesive bond strength of resin cements to laser treated surface in deciduous teeth. Therefore, it is hypothesised that laser surface treatment will improve adhesive bonding strength of primary dentin with resin cements. The aim of the present study was to investigate the shear bond strength of Er,Cr:YSGG laser treated deciduous dentin when adhesively bonded to resin cements.

MATERIAL AND METHODS

After the approval from the institutional review board, the study was conducted to evaluate the adhesive bond strength of Er Cr YSGG laser treated deciduous dentin to resin cements. Forty deciduous premolars and molars were submerged in 0.9% saline solution immediately after the extraction and stored for 24 hrs at 25°C. The teeth were embedded into the acrylic teeth containing the polyvinyl chloride tubes with buccal surface exposed. The crown of each specimen was cut by the high-speed hand piece (Midwest Dental Products, Corp, IL) to a 3.5mm x 3.5 mm depth exposing a wide area of dentin. The drilled buccal surface was polished with the slow cutting polishing disc (3M™ ESPE™ Sof-Lex™ Diamond Polishing System, Canada) for 2 mins followed by a saline wash for 5 mins. The prepared specimens were divided into a total of four groups (n=10) with single bonded surface exposed to different surface treatment and cement applications.

Initially, the specimens were divided based on the surface treatment into two groups; control (n=10) and laser treated surface (n=30). The laser used for the surface treatment was Er,Cr:YSGG laser HKS (Waterlase system, BioLaseTechnology, Inc, San Clemente, CA.). The laser was operated at a wavelength of 2.78 mm with a pulse duration of 140µs and a frequency of 20 Hz. Subsequently, the laser treated group was further divided into three groups based on the type of cement build-up. Cement build-ups were performed using three different types of cement; self-etch cement (Rely-X Unicem), Resin cement (Rely-X ARC) and Glass ionomer cement (GIC). A polyvinyl siloxane putty mould (2mm x 3mm) was placed on the specimen to perform build-ups of cement.

The groups are as follows: Group A: 10 specimens in this group acts as control following the conventional surface treatment. All the teeth were etched with 37% phosphoric acid (Ivoclar Vivadent AG, FL-9494 Schaan/ Liechtenstein) for 60 seconds followed by rinse and air dried for one second. The micro brush was used to apply the bonding agent (Prime and bond NT, Dentsply Int. Inc USA) on the etched surface followed by a light cure for 10 seconds. A build-up of Rely-X ARC was performed and light cured for 40 secs from four sides (160 sec).

Group B: The surface of each specimen was laser treated. Rely X Unicem was auto mixed and applied on the bonding surface using a mould. Excess cement was removed and light cured for 40 secs from four sides (160 secs).

Group C: Following laser treatment, each specimen was coated with adhesive bond (Prime and bond NT Dentsply Int. Inc, USA.) light cured for 10 secs and build-up of

resin cement (Rely-X ARC) was performed as explained previously.

Group D: The laser treated surface was further treated with polyacrylic acid (Ketac molar, 3M, Germany) for 10 seconds. Glass ionomer cement (GIC) (Ketac Cem Aplicap, 3M, Germany) capsules were auto-mixed and applied to the deciduous teeth surface using a putty mould for build-up. After the setting of GIC, following an hour the mould was removed along with excess cement.

All the specimens were placed in distilled water following the build up for 24 hrs prior to shear bond testing. The shear bond strength was tested using an Instron testing machine (Instron Corp, Canton, MA.) the load applied was perpendicular to the composite build up tooth interface using a knife, at cross-head rate of 0.05 in/min. Interfacial bond failure assessment was performed after the fracturing of specimens using a light microscope. The failures were divided up into three categories adhesive, cohesive and admixed. Failure at the interface of build-up and tooth surface was termed as adhesive whereas fracture internally in the cement was termed as cohesive failure. Moreover, any remains of the cement on the fractured interface surface were defined as admixed failure.

Statistical software for social sciences (SPSS 20.0 version) was employed in the collected data analyses. The normality of data was assessed using Kolmogorov-Smirnov test. Mean and standard deviations (SD) of the observed data were assessed using descriptive statistics. Adhesive Bond strength was analysed using the ANOVA with $p < 0.05$ considered as a level of significance. A comparison of means and SD were performed with ANOVA and Multiple comparisons tests (Tukey-Kramer).

Table 1. Materials and product detail

Material	Product detail
Rely X ARC	3M™ Clicker™ Dispenser, USA.
Rely X unicem	Aplicap™ / Maxicap™ , 3M ESPE, USA.
GIC	(Ketac Cem Aplicap, 3M, Germany)
Poly acrylic acid	(Ketac molar, 3M, Germany)
Er,Cr:YSGG laser HKS	Waterlase system, BioLase Technology, Inc, San Clemente, CA.
37% phosphoric acid etch	Ivoclar Vivadent AG, FL-9494 Schaan/Liechtenstein
Primer-adhesive system	Prime and bond NT, Dentsply Int. Inc USA

RESULTS AND DISCUSSION

Kolmogorov-Smirnov test showed even distribution of the collected input portraying the normality of data. The mean comparison was made between the four groups. Group A acted as a control, while the laser treated groups were categorised based on the type of cement used B (Rely X Unicem), C (Rely X-ARC) and D (GIC). The computed result indicated that the control group displayed the highest mean shear value of 25.38 (±3.81) MPa, compared to the laser treated groups. Nevertheless, the comparison (ANOVA) presented a significant difference between the specimens among the study groups (A, B, C and D) ($p < 0.05$). There was no significant difference between the bond strength fo group B (Rely X Unicem) and C (Rely X ARC) ($p>0.05$). Moreover, the least mean shear bond strength was displayed in specimens of group D (GIC) (5.91±2.33 MPa). Table 1 shows the means and Standard deviations for shear bond strengths among study groups.

Table 2. Means and Standard deviations for shear bond strengths among study groups

Study Group	Mean	SD	p-value!
Group A (Control)	25.38[A]	3.81	
Group B (ECL-RelyX-Uni)	20.47[B]	3.11	< 0.05
Group C (ECL-RelyX-A)	21.65B	3.74	
Group D (ECL-GIC)	15.91[C]	2.33	

Different capital superscript alphabets denote statistical significant difference. (Multiple comparisons test)
! Analysis of variance (ANOVA) Test.

Two way ANOVA and Tukey HSD Post-hoc test revealed that the SBS strength was influenced more by the surface treatment than the type of cement (Table 2). This can be observed evidently while comparing group A (Etch-bond-Rely X- ARC) and Group C (Er Cr YSGG- Rely X-ARC), which uses similar cement for build up while the surface treatments were different. On the contrary, supporting this statement, there was no difference in groups B and C where the cements were different (Rely X Unicem and GIC), while the surface treatment remains the same. Nevertheless, group C showed a significant difference from other groups ($p<0.05$). Furthermore, observing the interfacial bond failure demonstrated that the majority of the bond failures were adhesive followed by admixed and cohesive respectively. Adhesive failure ranged from 80 – 70% in groups B, A and C whereas the admixed failure ranged from 30 – 20% in similar groups. However, group D presented 6 specimens with adhesive failure while 4 underwent cohesive failures. This indicates weak strength of the material. Table 2 presents with type of failures among the study groups.

The current study was based on the hypothesis that laser surface treatment will improve adhesive bond strength of primary dentin with resin cements. The study further compared the outcome of the laser treated deciduous

teeth to the conventionally etched control group. Thus, the comparison between the control (Etch and bond) and Er Cr YSGG laser treated primary dentin, presented better adhesive bond strength for control. The mean shear bond strength was highest in the control group compared to the laser treated groups. The type of the cement showed no significant difference as the bond strength between group A and C specimens were different, however there bond strength among group B (Rely X Unicem) and C (Rely X ARC) was comparable. All these outcomes suggest that the hypothesis was accepted. A multitude of explanations can be attributed for the outcomes observed.

Table 3. Type of failures among the study groups

Study Groups	Adhesive (%)	Cohesive (%)	Mixed (%)
Group A (Control)	70	0	30
Group B (ECL-RelyX-Uni)	80	0	20
Group C (ECL-RelyX-A)	70	0	30
Group D (ECL-GIC)	60	40	0

Researchers have identified that ER CR YSGG laser abrades the tooth surface and removes the intertubular dentin. This process creates surface irregularities and recrystallization with no smear layer, thus increases mechanical retention of restoration. Moreover, the literature has identified an evident difference between the chemical and morphological bonding of dentin in permanent and primary teeth (Shafiei et al., 2013, Scaminaci et al., 2013, Lenzi et al., 2012). The authors have stated that the number of dentinal tubules play an important role in the adhesive bonding system (Bandéca et al., 2012, Calvo et al., 2014). It has been observed that the primary dentin contains a high density of dentinal tubules per mm2, which leads to the presence of thick peritubular dentin. In addition, in primary teeth the content of the intertubular dentin is lesser compared to the permanent teeth, this indicates lower probability for a robust dentin bond due to the low amount of mineralised dentin in the primary dentin.

Multiple factors are also responsible for aiding the retention of restorative materials on tooth dentin, such as acid concentration, different types of laser (Er YAG, Er Cr YSGG) and etching time (Kensche et al., 2016). The primary dentin content mostly comprises of organic matrix thus longer etching time is necessary for deep penetration of the etchant and formation of resin tags; nevertheless, this may have a negative impact on the pulp. In addition, the ratio of dentinal tubules opening to intertubular dentin affects the bond strength due to the water content and collagen (Yildiz et al., 2013, Somani et al., 2016). Another barrier for reliable bonding as suggested by authors is the presence of smear layer while using the self-etching primer systems; however, the present study used total etch system that allows deeper penetration of etchant and resin tags compared to the lasers acting on the surface (Scaminaci et al., 2013, Mosharrafian and Sharifi, 2016). Therefore better bond strength was observed in the specimens treated with conventional (Etch-bond) dentin treatment.

The present study further compares three different types of cement (Rely X arc, Rely Unicem and GIC) based on three different bonding mechanisms, total etch, self-adhesive and chemical bonding, respectively. Rely X ARC presented with higher shear bond strength compared to other cement groups in the current study. Furthermore, comparing within the resin cement groups, Rely X ARC presented slightly higher bonding strength (21.65 ±3.74 MPa) than Rely Unicem (20.47 ±3.11 MPa); however, the multiple comparison test results were comparable. Rely X ARC is based on total etch adhesive system, which allows deeper penetration for resin tag formation in acid etched surface compared to laser abraded surface allowing limited bond formation. Authors have explained that self adhesive cement has a slight property of etching and does not require a prior bond application thus it modifies the smear layer before bonding and allows limited penetration of adhesive cement penetration (Kensche et al., 2016). Nevertheless, in the present study the difference in shear bond strength can be only appreciated under two different surface treatments where similar resin cements were employed.

The present study further displayed that GIC showed the lowest shear bond strength. Bonding for glass ionomer cements depends upon the ionic exchange at the interactive surface. The Polyalkenoate chains bind with the apatite crystals thereby displacing the phosphate ions (Calvo et al., 2014). Thus it builds an ion-enriched layer of cement attached to the treated surface. However, the poly-acid application on the tooth surface has a weak etching effect and low inorganic content, which creates a weak bond between the cement and tooth surface. Previous studies have evaluated the tensile bond strength of cements to deciduous tooth surface (Mazaheri et al., 2015, Calvo et al., 2014).

The present study evaluates the shear bond strength, which assesses the adhesive bond using a modified methodology. Rely X Unicem presented with highest adhesive bond failure indicating slightly less adhesive bond strength compared to Rely X ARC, whereas specimens in group A and C displayed comparable results, suggesting that surface treatment did not have a major impact on the failure mode of cement. Studies have displayed that bond strength for GIC is relatively low (3 to 7 MPa to the conditioned tooth surface (Mazaheri et al., 2015, Calvo et al., 2014). Thus, similar results were appreciated in the present study where GIC presented with the lowest adhesive bond failure.

It is pertinent to state that the study follows has certain limitations. Firstly, the findings can only be associated with the conditions and materials used in the present

study and should not be applied broadly. In addition, restorations intra-orally undergo exposure to a hostile environment with occlusal loads, acidic erosion and temperature changes. These conditions are absent in this in-vitro study. Ageing and occlusal loads have shown to reduce the durability of adhesive materials and their bonding (Ramos et al., 2014). Therefore, in-vitro studies assessing the influence of Er Cr YSGG laser dentin treatment for bond strength of adhesive cements with long-term ageing are recommended. Scanning electron microscopy for the assessment for surface topography of primary dentin with Er Cr YSGG laser treatment was not performed in the study; therefore, further studies should evaluate the fractured surface using SEM.

CONCLUSION

The study demonstrated that Er Cr YSGG laser treatment of primary dentin showed potential as a surface treatment regime for adhesive bonding to luting cements. Furthermore, comparable bond strength outcomes were observed for laser treated primary dentin when bonded to self-adhesive cement (RelyX Unicem) and resin cements (RelyX ARC). Primary dentin surface treatment displayed major impact on its adhesive bonding strength, in contrast to the type of cement.

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Anti-Inflammatory Potential of Marine Derived Compounds Xyloketal B and CEP 1347 for the Treatment of Ischemic Stroke

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ABSTRACT

A stroke happens when the blood supply to the brain regions is interrupted or diminished leading to loss of oxygen, supplements, thereby brain death. The limited treatment options have given a way for natural compounds. Xyloketal B and CEP 1347 are isolated from *Xylaria sp* and another marine bacteria *Nocardiopsis sp*, widely found in the South China Sea, having potent anti-inflammatory, anti-apoptotic and neuroprotective properties. In this study, the marine compounds Xyloketal B and CEP 1347 are screened for its pharmacokinetic profile of a drug molecule to define the pharmacodynamic mechanisms using ADME (absorption, distribution, metabolism, and excretion) properties. Whereas, the potential anti-inflammatory targets such as PGR, STAT3, VEGFA, MMP9, SRC, CDK2, ErbB4 were obtained from the previous study on the functional associations between the inflammatory responses and ischemia (which includes ischemic stroke, cardio embolic stroke and hemorrhagic stroke) via gene regulatory network and pathway analysis approaches. Then, the marine compounds (Xyloketal B and CEP- 1347) are docked with anti-inflammatory target such as PGR, STAT3, VEGF, MMP9, SRC, CDK2, and ErbB4. Based on the strong inter molecular interactions using docking approach (having Six or more hydrogen bonds) the highest dock score, we conclude that inhibiting the anti-inflammatory targets, its signals and corresponding pathways can improve the stroke recovery by reducing the loss of nerve function, apoptosis of neuronal cells to promote morphogenesis, to promote immuno modulation which provides a new target for the clinical treatment of ischemia conditions. Studies have shown that Xyloketal B and CEP-1347 are novel inhibitors of many vital pathways, regulates of apoptotic neuronal events, reduces cell and tissue damage, reduces the level of inflammatory cytokines during ischemia and can be represented as anti-stroke therapy for superior stroke retrieval in the future.

KEY WORDS: ISCHEMIA, ANTI-INFLAMMATORY TARGETS, XYLOKETAL B, CEP-1347, ADME DESCRIPTORS, RECEPTOR-LIGAND INTERACTIONS.

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INTRODUCTION

A stroke is a cerebrum assault, which happens when the blood stream to the regions of the brain is confined prompting limited oxygen supply that is required for cellular metabolism to keep the tissues alive. When the tissues become ischemic, except if the blood stream is quickly reestablished it leads to brain death. Ischemic stroke is brought about by the blockage of artery in the brain regions, representing around 87% of stroke cases, which adds to the major death cases and post-stroke inability in patients. Whereas, hemorrhagic strokes are caused when blood vessels blast inside (which is known as intracerebral drain) or on the surface of the brain (which is known as subarachnoid discharge). Hemorrhagic strokes are the most serious form and are related to higher danger within months. In cardioembolic stroke cases, the heart pumps unwanted materials into the brain circulating regions, bringing about the occlusion of the brain vessel and damage to the brain tissues (Arboix and Alió, 2010, Simats, et al., 2016; Chandra, et al.,2017, Agrawal et al.,2018).

According to the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC), 15 million people suffer stroke worldwide each year. In affected population, five million people die and a further five million have been permanently disabled. Stroke costs the United States an estimated $34 billion each year and £26 billion a year in the United Kingdom. By 2035, the prevalence of stroke will increase by more than 20% over 2016 and the direct medical costs are projected to reach $184.1 billion, which is a great challenge to human society and healthcare system. There are very limited number of FDA approved medications available for the treatment of stroke. A combination of medications is generally used to treat the condition and to prevent it happening again of such as alteplase (intravenous tissue plasminogen activator) that can dissolve the clots and restore the blood flow to brain is given via injection, antiplatelet, anticoagulants help to reduce the chances of another clot formation (such as aspirin, warfarin) is prescribed. Similarly, statins are prescribed to reduce the level of cholesterol in blood (French et al., 2016).

Figure1 ADMET result for Xyloketal B and CEP 1347 showing 95% and 99% confidence limit ellipse corresponding to Blood brain barrier and intestinal absorption models. Red and green color indicates absorption and blue and pink indicates BBB

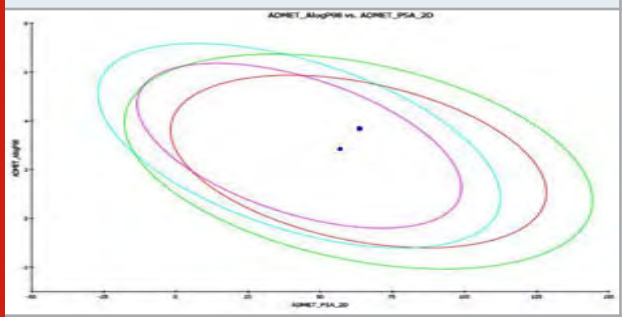
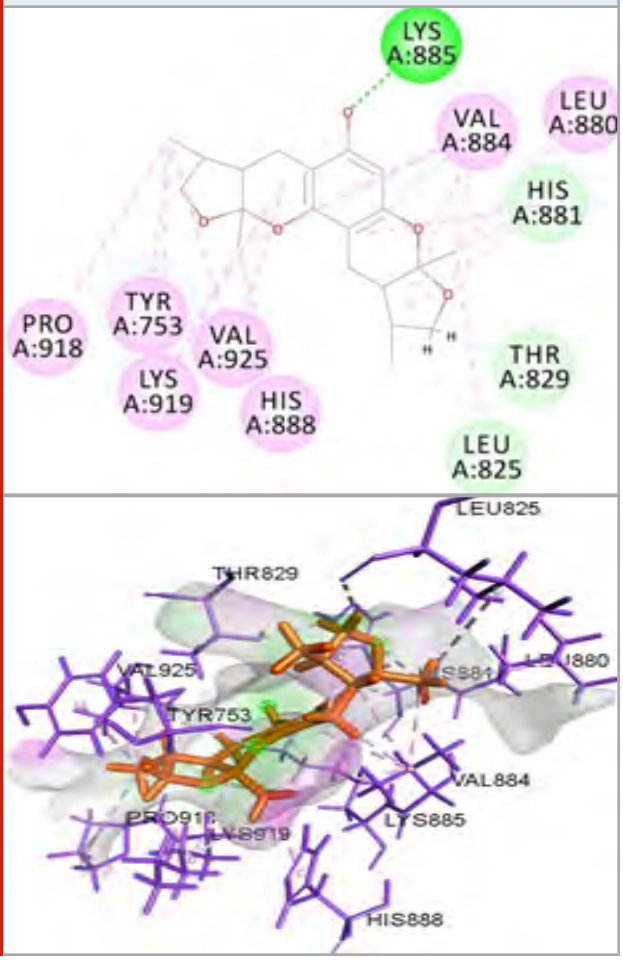


Figure 2: (Docked poses of PGR druggable cavity with xyloketal B. Interactions are shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Lys 885) mediates the hydrogen bond,then His 881,Thr 829,Leu 825 formed the carbon hydrogen bond and residues Leu 880, Val 884, Val 925, His 888, Lys 919, Tyr 753, Pro 918 involves in pi-Alkyl interaction with the receptor PGR)

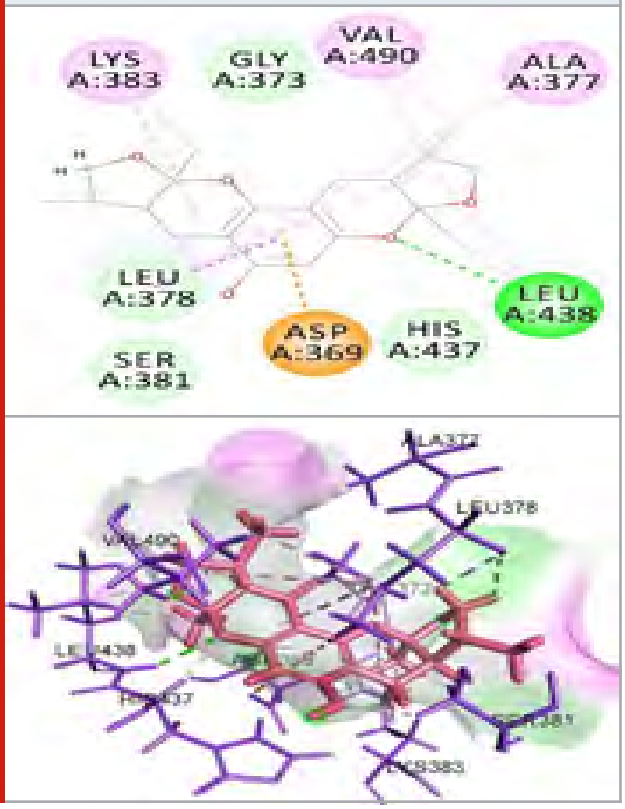


This gives wide opportunity for the natural compounds to act as protective agents, and which many experimental and clinical trials being performed. Marine compounds have diverse chemical structures, functions and therapeutic properties due to the physical and chemical marine environmental conditions (Agrawal et al.,2018) which have led to the successful clinical studies of Xyloketal B and CEP 1347 for the development of marine derived anti-inflammatory drugs targeting ischemia conditions. Xyloketal B is a natural compound isolated from the mangrove fungus, *Xylaria sp* and CEP- 1345, isolated from *Nocardiopsis sp* widely found in the South China Sea and the compounds Xyloketal B is been widely studied for free radical damage, atherosclerosis, and CEP- 1347 for Parkinson disease (Maroney et al., 2001 Gong et al., 2018 Huang et al., 2019).

MATERIAL AND METHODS

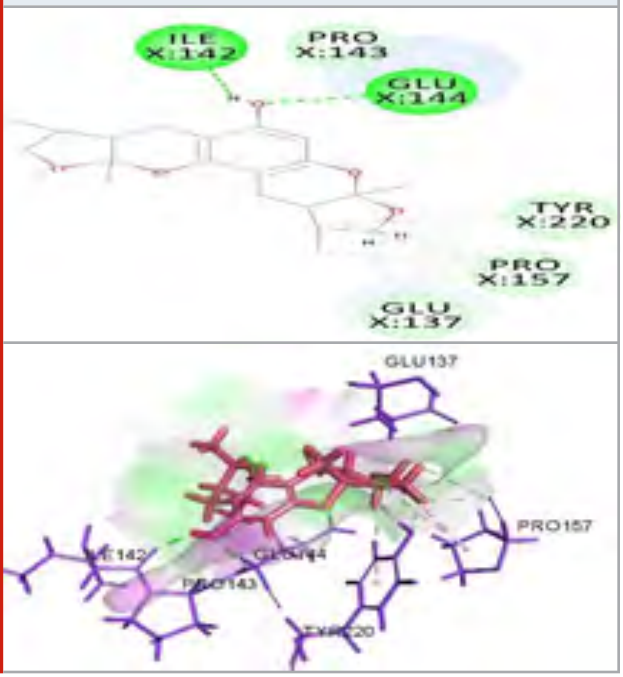
Identification of the potential anti-inflammatory targets: The differentially expressed genes were obtained for all the 3 ischemia datasets such as ischemic stroke (GSE37587), cardio embolic stroke (GSE58294) and hemorrhagic stroke (GSE13353) from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo)and analyzed using R programming, GEO2r, is a web tool based on t-test (ANOVA) or analysis of variance and is useful to compare two or more groups of samples in a GEO series, to identify differentially expressed genes. The DEG's are then screened out through the cut off criteria of adjusted Log2 (FC)| > 1 (Fan et al., 2017).

Figure 3: Docked poses of STAT3 druggable cavity with xyloketal B. Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Leu 438) mediates the hydrogen bond, then His 437, Ser 381, Leu 378 and Gly 373 formed the carbon hydrogen bond and residues Lys 383, Val 490, Ala 377 involved in pi-Alkyl interaction pink colored dashed lines,Asp 369 mediated pi cation interaction with the receptor STAT3.



Similarly, the inflammatory properties were also studied using inflammatory genes obtained from Gene cards and Harmonizome database. Then, the 2,422 overlapping genes were obtained using Venn diagram, for which protein- protein interaction reactome network has been constructed having 7,411 nodes and 61,158 edges. Subsequently, using graph theory approach 63 clusters were identified using MCODE analysis to detect the dense and closely related group of proteins that share similar functionality with Degree Cut-off as2, K-Core as 2(Christy and Priyadharshini,2018). Out of which, 10 clusters have been selected having nodes <= 10, which are classified as group of proteins that share a similar functionality (Myers et al.,2015). Now, pathway analysis and enrichment analysis are made to study the functional associations of the genes and the conditions. This has led to the identification of the potential anti-inflammatory targets based on further refinement and target proteins were listed in Table 1.

Figure 4: Docked poses of VEGFA druggable cavity with xyloketal B.Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Glu 144, Ile 142) mediated the hydrogen bond, then Pro 143,Tyr 220,Pro 157,Glu 137 formed the carbon hydrogen bond interaction with the



Target Protein retrieval and pre pre-processing: The structure of the anti-inflammatory targets such as PGR, STAT3, MMP9, VEGFA, CDK2, ErbB4, SRC were downloaded from PDB in PDB format. The hetero atoms and alternative conformers are then deleted to provide enrichment to the receptor- ligand interaction study. To make the protein stable, we have minimized the energy by applying force field CHARMm (Brooks et al.,2009) (Chemistry at HARvard Macromolecular Mechanics) and energy minimization algorithm conjugate gradient with the help of DS 2.1. The binding site of the proteins has also been cross validated with Cavity plus web tool, which provides potential binding sites on the surface of a given protein and rank them with ligandability and druggability scores based on structural geometry and CAVITY algorithm. The ligandability value represents the possibility of designing small ligands with high binding affinities and the druggability predicts the possibilities of the cavity being a good target.

Figure 5: Docked poses of SRC druggable cavity with xyloketal B. Interactions were shown as dashed lines between receptor residues and ligand atoms. Asp386 formed the carbon hydrogen bond interaction, Leu 407, Cys 277, Val 281, Phe 278 were the residues involved in pi-alkyl bonding pink colored dashed lines, then Lys 295 mediated pi cation interaction with the receptor SRC.

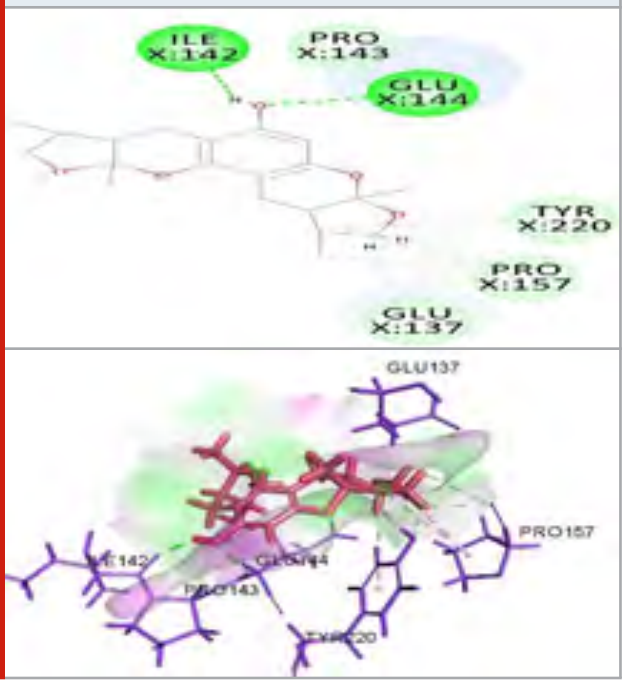


Figure 6: Docked poses of CDK2 druggable cavity with xyloketal B. Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Leu 54) mediated the hydrogen bond, then His 121 formed the carbon hydrogen bond and residues Leu 124, Val 123, Arg 150 involved in pi-Alkyl interaction with the receptor CDK2.

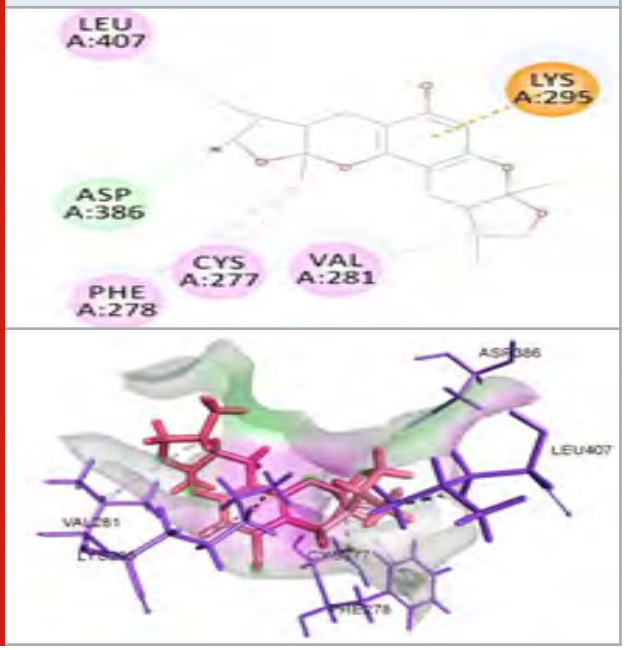


Figure 7: Docked poses of ErbB4 druggable cavity with xyloketal B. Interactions are shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Ala 749,Thr 860,Asp 861) mediated the hydrogen bond,Leu 794,Val 781,Leu 783,Val 732,Leu 850,Leu 864,Met 772 involved in pi-Alkyl interaction.Lys 751 mediates the pi-cation interaction with ErbB4.

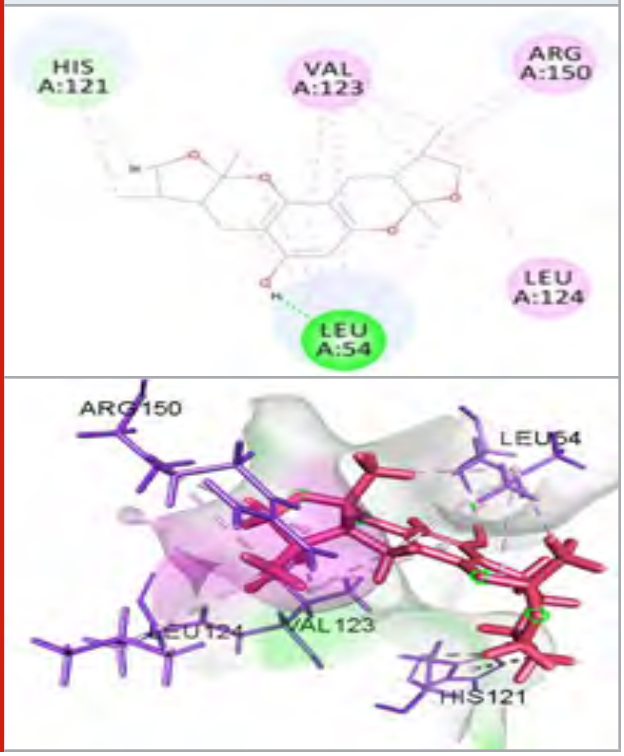
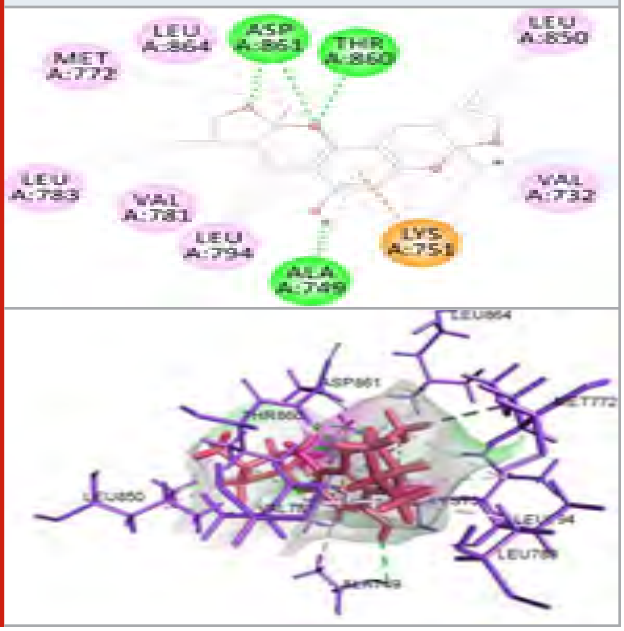


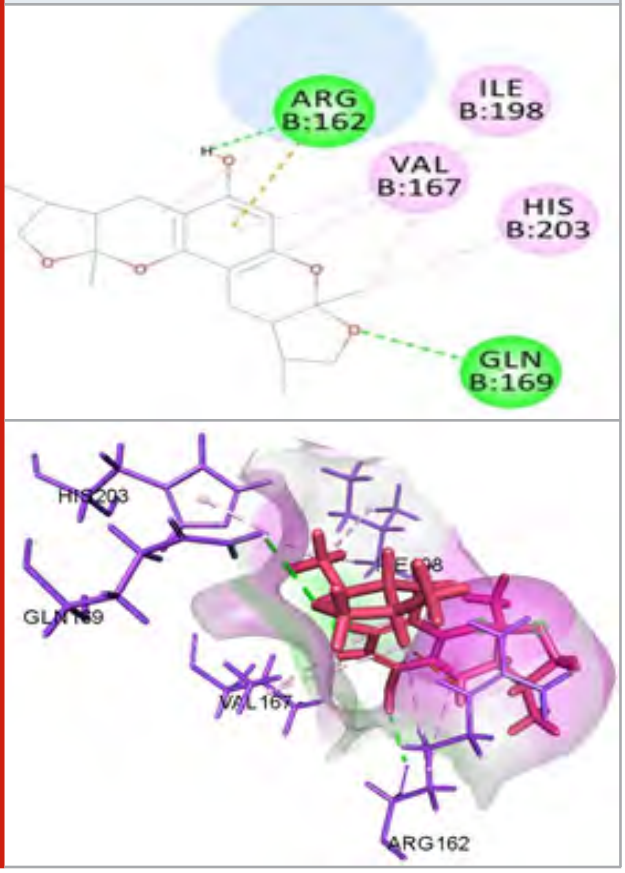
Figure 8. Docked poses of MMP9 druggable cavity with xyloketal B. Interactions are shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Arg 162,Gln 169) mediated the hydrogen bond, residues Val 167,His 203,Ile 198 involved in pi-Alkyl interaction with the receptor MMP 9.



It predicts maximum pKd, DrugScore and Druggability of all the detected cavities and this cavity score is influenced by cavity volume, pocket lip size, hydrophobic volume, cavity surface area, and hydrogen-bond-forming surface area (Xu et al.,2018)

Therapeutic ligand PK/PD property screening: The 3D structure of the Xyloketal B and CEP 1347 is obtained from PubChem in SDF format. The obtained structure is then screened for its drug likeliness property using Discovery studio 2.1. The properties such as absorption, distribution, metabolism, excretion and toxicity (ADMET), which are the important parameters to determine a compound for therapeutic use. These properties are co-related with the descriptors such as BBB, which determines the distribution of compounds in human body, low molecular weight for absorption. The output ellipses describe the regions where well absorbed compounds are expected to be found, and 95%, 99% confidence limit ellipse corresponding to BBB and intestinal absorption models are indicated (Ponnan et al.,2012).

Figure 9: Docked poses of PGR druggable cavity with CEP 1347.Interactions are shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Leu 921,Gly 923,Tyr 753,His881) mediated the hydrogen bond, then Thr 829,Ser 757 formed the carbon hydrogen bond and residues Ala922,Pro927,Val925,Pro918,Lys919,His888,Val884,Leu825, involved in pi-Alkyl interaction, Lys 885 mediated pi cation interaction with the receptor PGR.



Intermolecular Docking assessment: The targets are downloaded from Protein data bank in PDB format and the potential binding sites are identified using DS 2.1. A total of 7 proteins were docked with Xyloketal B and CEP-1347 using Ligand Fit protocol and CHARMm force field in DS which provides shape-based docking for accurately docking the ligand into the protein active sites. The active sites are predicted using grid search and ERASER algorithm. This method then combines monte carlo conformational search along with shape comparison filter for generating ligand poses consistent with active site shape. Then the docked poses are analyzed using potential of dock score and non-bonded interactions (Meng et al.,2011). The scoring functions are used to identify correct poses from incorrect poses, which is the sum of all non-bonded interactions such as hydrogen bond, hydrophobic effect, ionic interactions and binding entropy expressed in terms of like LigScore ,PLP, JAIN, PMF, and dock score (Ferreira et al.,2015).

Figure 10: Docked poses of STAT3 druggable cavity with CEP 1347.Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Thr 440,Lys 370,Asn 491,Lys 488) mediated the hydrogen bond, then Leu 438,Ser 372 formed the carbon hydrogen bond and residues Val 490,Leu 378,Ala 377 involved in pi-Alkyl interaction represented in pink colored dashed lines ,Asp 369 mediated pi cation interaction with the receptor STAT3.

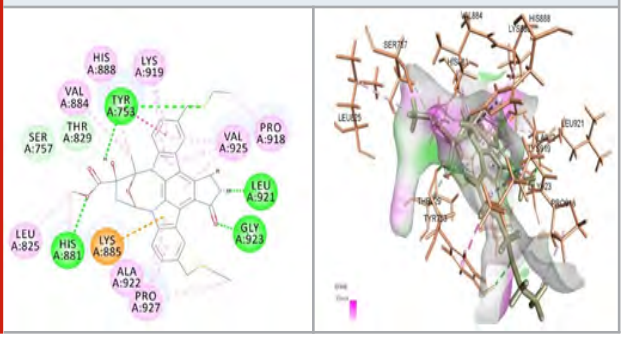


Figure11: Docked poses of VEGFA druggable cavity with CEP 1347.Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Gln 22) mediated the hydrogen bond, then residues Met 18,Tyr 139,Leu 204,Phe 172,Leu 174 involved in pi-Alkyl interaction,Lys 170,Glu 208,Lys 217 mediated pi cation interaction with the receptor VEGFA

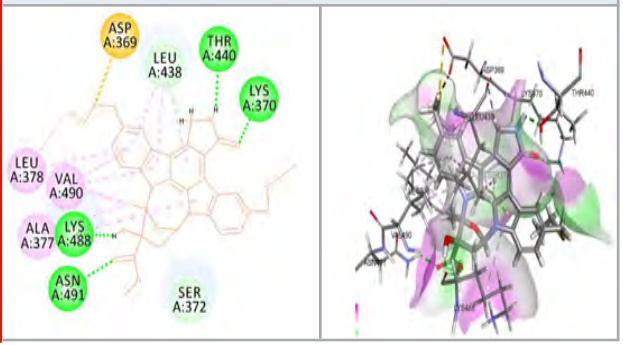


Figure 12: Docked poses of SRC druggable cavity with xyloketal B. Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Lys 295 ,Cys 277) mediated the hydrogen bond, then Arg 388,Asp 404 formed the carbon hydrogen bond and residues Trp 428,Pro 425,Phe 278,Ile 411,Val 281,Leu 393 involved in pi-Alkyl interaction with the receptor SRC.

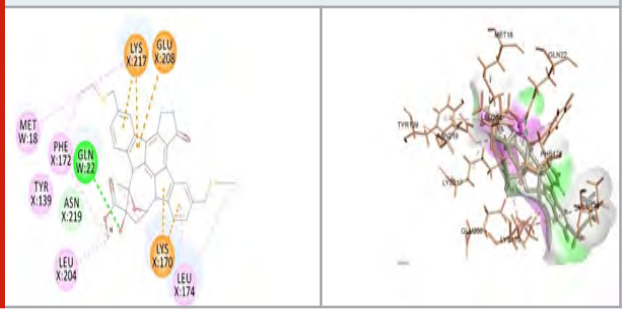


Figure 13: Docked poses of CDK2 druggable cavity with xyloketal B.Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Asp 145) mediated the hydrogen bond, then Ile 10 formed the carbon hydrogen bond and residues Ala 144,Phe 80,Val 64,Ala 31,leu 134,Phe 82 involved in pi-staking interaction with the receptor CDK2

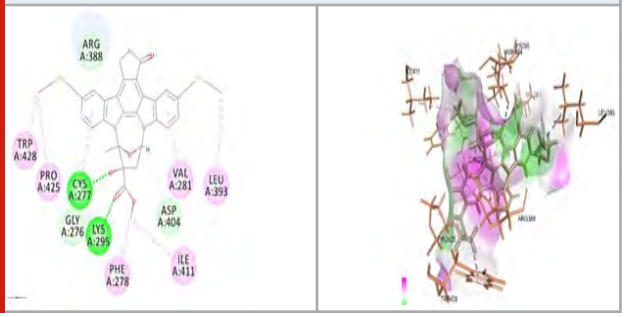


Figure14: Docked poses of ErbB4 druggable cavity with xyloketal B.Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Gln 797,Met 799) mediated the hydrogen bond, then Gly 725 formed the carbon hydrogen bond and residues Leu 798,Lys 734,Arg 847,Lys 751,Leu 794,Leu 850,Ala 749 involved in pi-Alkyl interaction,Cys 803 mediated pi cation interaction with the receptor ErbB4.

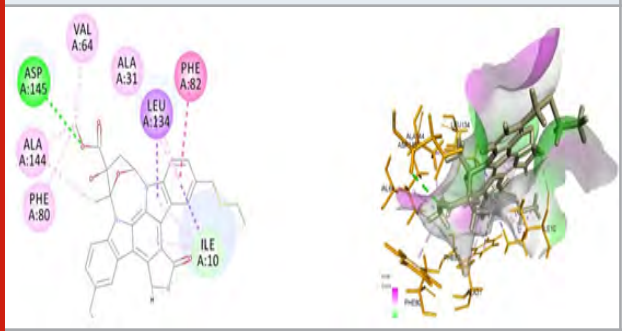
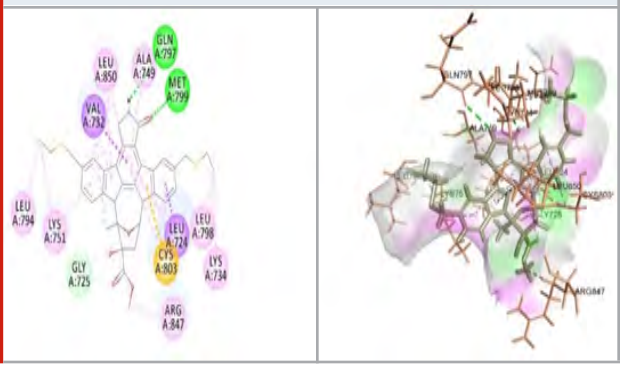


Figure 15: Docked poses of MMP9 druggable cavity with xyloketal B.Interactions were shown as dashed lines between receptor residues and ligand atoms. Residues highlighted in Green (Tyr 423) mediated the hydrogen bond, then His 411,Gly 186 formed the carbon hydrogen bond and residues Leu 188,His 401,Leu 187 involved in pi-Alkyl interaction with the receptor MMP9.



RESULTS AND DISCUSSION

ADMET Screening of ligands: In silico ADME analysis is performed using ADME Descriptors using Accelrys Discovery studio 2.1, in which various pharmacokinetics parameters such as Blood brain barrier (BBB), Hepatotoxicity, Plasma protein binding (PPB), cytochrome p450 inhibition were predicted for Cep-1347 and Xyloketal B. These levels influence the drug intensity and drug exposure to the tissues and thereby influencing the pharmacological activity of the compounds (Li ,2001). The results of the pharmacokinetic screening revealed that xyloketal B and CEP- 1347 followed Lipinki's rule of five for bioavailability. However compound CEP 1347 shows high lipophilic nature due to high log p value, whereas, xyloketal B has shown 99% and 95% confidence level for intestinal absorption and BBB and falls inside the ellipse model. Figure1 ADMET result for Xyloketal B showing 95% and 99% confidence limit ellipse corresponding to Blood brain barrier and intestinal absorption models. Red and green color indicates absorption and blue and pink indicates BBB. ADMET result for CEP 1347 showing 95% and 99% confidence limit ellipse corresponding to Blood brain barrier and intestinal absorption models. Red and green colour indicates absorption and blue and pink indicates BBB and the Figure 2 depicted the details. Table 2 ADMET descriptors for Xyloketal B and CEP 1347

Study of interactions between Xyloketal B, CEP 1347 and anti-inflammatory targets: Possible binding modes of Xyloketal B with the anti-inflammatory target receptors PGR, STAT3, VEGFA, SRC, CDK2, ErbB4, MMP9 were studied using Ligand Fit module in DS 2.1. The best docking pose and their dock score (Ligand/ Receptor interaction study + Ligand internal energy) as well as the non-bonded interactions (strong hydrogen bonds such as carbon and conventional hydrogen bonds) are noted to assess the affinity of the bonding. Higher the number of hydrogen bonds, more the solubility and more permeability for passive diffusion of the lead compounds. For cross validation of receptor ligand docking, cavity

plus results has been used to show the interactions more favourable for validation.

Table 1. Anti-inflammatory targets

Genes	Names	FDA Approval	PDB ID	Favourable Region
PGR	Progesterone receptor	Yes	2OVH	95.6%
STAT3	Signal transducer and activator of transcription 3	Yes	1BG1	80.6%
MMP9	Matrix metalloproteinase-9	Yes	IGKC	90.4%
VEGFA	Vascular endothelial growth factor A	Yes	1FLT	92.6%
ErbB4	Receptor tyrosine-protein kinase erbB-4	Yes	2R4B	81.9%
CDK2	Cyclin-dependent kinase 2	Yes	1URW	89.3%
SRC	Proto-oncogene tyrosine-protein kinase Src	Yes	2SRC	89.4%

Table 2. ADMET descriptors for Xyloketal B and CEP 1347

Parameters	Xyloketal B	CEP 1347
BBB	2	4
PPB	0	2
CYP450	0.30	0.33
Hepatotoxicity	0.6	0.6
Intestinal absorption	0	1
Aq. Solubility and drug likeliness	0	0

Glu 137 formed the carbon hydrogen bond interaction with the receptor VEGFA depicted in figure 5 and Table 6. Xyloketal B affinity to SRC analysed based on molecular docking and the interactions depicted in Figure 6 and residues involved in interactions are listed in Table 7.CDK2 intermolecular interactions with Xyloketal B mediated by hydrogen bonds and listed in Table 8, interacting residues and bonding distance were depicted figure 7.Similarly ErbB4 and MMP9 mediated interaction with Xyloketal B were depicted in Figure 8 and Table9 , Figure9 and Table 10 respectively.

Cavity module screening of anti-inflammatory targets revealed their draggability as well as ligandability nature. Xyloketal B intermolecular interactions with PGR listed in Table 4 and depicted in the Figure 3. Lys885, His881, Thr 829, Leu825 were the residues from druggable cavity mediated the hydrogen bond interaction within 2.5Ao distance. Within the same cavity pi-alkyl bonds interactions mediated pocket residues stabilize the intermolecular interactions. In general Pi-sigma interactions like Pi-alkyl and Pi-Sulphur which mainly largely comprise charge transfer aids in intercalating the drug within the druggable cavity of the receptor. Similarly, with STAT3 and other anti-inflammatory targets also mediated Hydrogen bond in addition to pi cation and pi alkyl bonds and interacting residues were listed in table 4 and depicted in figure 4. Residues Leu 438, His 437, Ser 381, Leu 378, Gly 373, Lys 383, Val 490, Ala377, Asp369 favoured the maximum number of interactions in the proposed target Table 5 and Figure 4. Glu 144, Ile 142 mediates the hydrogen bond, then Pro 143, Tyr 220, Pro 157,

Table 3. Output of cavity module for anti-inflammatory targets

Proteins	Druggability
PGR	Druggable
STAT3	Druggable
MMP9	Druggable
VEGFA	Druggable
ErbB4	Druggable
CDK2	Druggable
SRC	Druggable

Table 3a. Non-bonded interactions between Xyloketal B and PGR

Ligand	Dock score	Amino acid	Atom		Distance
		(PGR)	RECEPTOR	LIGAND	
Xyloketal B	66.019	LYS 885	HZ1	05	2.96989
		LYS885	HE2	05	2.28316
		HIS 881	HA	04	2.09333
		THR 829	OG1	H36	2.25979
		LEU 825	O	H37	2.76227

Table 4. Non- bonded interactions between Xyloketal B and STAT3

Ligand	Dock score	Amino acid	Atom		Distance
		(STAT3)	RECEPTOR	LIGAND	
Xyloketal B	52.471	LEU 438	HN	O4	2.5787
		LEU 378	O	H34	2.58422
		LEU 378	O	H35	2.67216
		GLY373	HA2	01	2.35742
		SER 381	HB2	05	2.23962
		HIS 437	HA	04	2.43909

Table 5. Non- bonded interactions between Xyloketal B and VEGFA

Ligand	Dock score	Amino acid	Atom		Distance
		(VEGFA)	RECEPTOR	LIGAND	
Xyloketal B	28.394	GLU 144	HN	O5	2.09583
		ILE 142	O	H51	1.68155
		PRO 143	HA	O5	2.36209
		PRO 157	O	H36	2.44219
		TYR 220	OH	H36	2.25501
		GLU 137	OE1	H37	2.59563
		PRO 157	O	H37	2.9771

Table 6. Non- bonded interactions between Xyloketal B and SRC

Ligand	Dock score	Amino acid	Atom		Distance
		(SRC)	Ligand	Receptor	
Xyloketal B	69. 839	ASP 386	H34	OD2	2.94002

Table 7. Non- bonded interactions between Xyloketal B and CDK2

Ligand	Dock score	Amino acid	Atom		Distance
		(CDK2)	Ligand	Receptor	
Xyloketal B	65.243	LEU 54	H51	O	1.86881
		HIS 121	H34	O	2.8994

Table 8. Non- bonded interactions between Xyloketal B and ErbB4

Ligand	Dock score	Amino acid	Atom		Distance
		(ErbB4)	Ligand	Receptor	
Xyloketal B	66. 695	THR 860	O1	HG1	2.08283
		ASP 800	O1	HN	2.52537
		ASP 861	02	HN	2.17772
		ALA 749	H51	O	2.57675

Table 9. Non- bonded interactions between Xyloketal B and MMP9

Ligand	Dock score	Amino acid (MMP9)	Atom		Distance
			Ligand	Receptor	
Xyloketal B	61.088	ARG 162	H51	O	2.36905
		GLN 169	O3	HE22	2.77257

Study of non- bonded interactions between CEP-1347 and anti-inflammatory targets

Table 10. Non- bonded interactions between CEP-1347 and PGR

Ligand	Dock score	Amino acid (PGR)	Atom		Distance
			Ligand	Receptor	
CEP-1347	70. 491	TYR 753	S1	HH	2.580589
		HIS 881	O5	HD1	2.79844
		GLY 923	O7	HN	1.93441
		TYR 753	H50	O	1.72248
		LEU 921	H57	O	2.14658
		SER 757	O6	HB2	2.51224
		THR 829	O4	HB	2.95172
		LEU 921	H52	O	2.06928

Table 11. Non- bonded interactions between CEP-1347 and STAT3

Ligand	Dock score	Amino acid (VEGFA)	Atom		Distance
			Ligand	Receptor	
CEP 1347	36.199	GLN 22	O4	HE21	2.65442
		ASN 219	H64	OD1	2.9773

Table 12. Non- bonded interactions between CEP-1347 and VEGFA

Ligand	Dock score	Amino acid (STAT3)	Atom		Distance
			Ligand	Receptor	
CEP-1347	57.725	LYS 370	O7	HZ2	1.65372
		ASN 491	O6	HD22	1.86214
		LYS 488	H50	O	1.76279
		THR440	H57	OG1	2.09157
		SER 372	O3	HB2	2.83771
		LEU 438	H51	O	2.37833
		LYS 370	H52	O	2.59893

Study on non- bonded interactions between CEP-1347 and anti- inflammatory targets: CEP-1347 is a indolocarbazole derivatives derived from Marine bacterial *sps Nocardiopsis*. It is considered as potent inhibitor of SAPK/JNK pathway which activated after neuronal toxic insults in neurons. In our studies CEP-1347 was assessed for their affinity with anti-inflammatory targets namely PGR, STAT3, MMP9, VEGFA, CDK2, ErbB4, SRC and MMP9.Pharmacophore features of this potent inhibitor showed better binding affinity and Figures 10,11,12,13,14,15,16 depicted the intermolecular interactions. Interaction mediated by hydrogen bonds, pi-cation, pi-alkyl and carbon hydrogen bonds. Interacting residues listed in Tables 11,12,13,14,15 and 16.

Table 13. Non- bonded interactions between CEP-1347 and SRC

Ligand	Dock score	Amino acid	Atom		Distance
		(SRC)	Ligand	Receptor	
CEP- 1347	99.144	CYS 277	O4	HN	1.81155
		LYS 295	O6	HZ2	1.62797
		GLY 276	O4	HA2	2.69631
		ASP 404	H44	OD2	2.85921
		ARG 388	-	HH12	2.85918
		ARG 388	-	HH22	2.69236

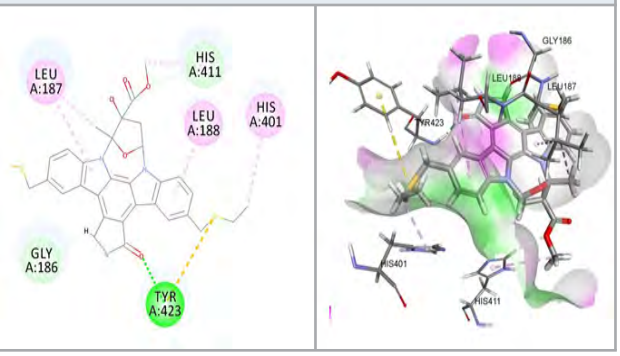
Table 14. Non- bonded interactions between CEP-1347 and CDK2

Ligand	Dock score	Amino acid	Atom		Distance
		(CDK2)	Ligand	Receptor	
CEP-1347	111. 05	ASP 145	O5	HN	2.87654
		ILE 10	H51	O	3.06647

Table 15. Non- bonded interactions between CEP- 1347 and ErbB4

Ligand	Dock score	Amino acid	Atom		Distance
		(ErbB4)	Ligand	Receptor	
CEP-1347	121.495	MET 799	O7	HN	1.91772
		GLN 797	H57	O	2.67839
		GLY 725	O3	HA2	2.92012
		GLY 725	O6	HA2	2.57601

Figure 16



Three different datasets on ischemia conditions such as ischemic stroke, cardio embolic stroke and haemorrhagic stroke were analysed. The control and diseased samples from each dataset are studied using techniques such as differential analysis of the genes, networking, pathway, functional analysis, hub genes validation, expression level studies using graph theory.

These analyses helped us to predict that, the potential anti-inflammatory targets, according to Human Protein Atlas, the proposed targets are FDA approved, such as PGR(Liu et al.,2012; Zhu et al.,2017; Gibson et al.,2009),STAT3(Wang et al., 2017;Liang et al., 2016), MMP9 (Chaturvedi et al.,2014; Christy,2013), VEGFA(Greenberg et al.,2013), CDK2 (Osuga et al.,2000), ErbB4 (Robert et al.,2013, Qian et al.,2018) and SRC plays vital role in the neuro immunology, neuronal development such as angiogenesis, apoptosis, motor performances, pathological events through activation of multiple signalling pathways, and by regulating many numbers of proteins that participate in the post-ischemic events to can attenuate the expressions(. The inter-molecular interaction study revealed that Xyloketal B has higher interactions with PGR, STAT3, VEGFA, and ErbB4 with dock score 66.019, 52.471, 28.394, 66.695 and CEP 1347 having higher interactions with PGR, STAT3, SRC, ErbB4, and MMP9 with dock score 70.491, 57.725,99.144, 121.495 and 62.72 respectively.

Through Cavity plus results, we ensured that the ligands are docked on the druggable cavities (having positive amino acids) of the receptor which has led to higher number of hydrogen bonds based on drug actions. This indicates that the intermolecular interactions are strong between the targets and the lead compounds, high solubility, high permeability across the membranes leading to good bioavailability of the compounds.

There are many experimental and clinical studies being performed on xyloketal B and CEP-1347 as an anti-oxidative, anti-apoptotic showing positive results, which gives us a possibility for bring better stroke recovery (Xiao et al.,2014).

Table 16. Non- bonded interactions between CEP- 1347 and MMP9

Ligand	Dock score	Amino acid	Atom		Distance
		(MMP9)	Ligand	Receptor	
CEP-1347	62.72	ASP 800	H61	OD2	2.5093
		ASP 800	H56	OD2	1.75975
		LEU 718	H57	O	2.90109
		LEU 788	H69	O	3.03434

CONCLUSION

Computational analysis of Xyloketal B and CEP 1347 and their interactions with anti-inflammatory targets such as PGR, STAT3, MMP9, VEGFA, CDK2, ErbB4, and SRC have shown higher binding affinity with strong favourable hydrogen bond interactions. Cerebral ischemia/reperfusion injury generates various cell apoptotic pathways, it's evident that ischemia-stimulated oxidative stress as well as inflammation are primarily responsible for the following cell death by necrotic or apoptotic processes. Reactive oxygen species (ROS) control cell survival/death by enabling several cells signaling process and related pathways, particularly JAK2/STAT3 stimulation makes a significant contribution to cell apoptosis subsequent ephemeral focal cerebral ischemia. In generic expression of VEGF-A in ischemic stroke group resulted in a decrease state as compared with the non-stroke group, thus VEGF signaling process correspond to vital targets for treatment of stroke.

CDK inhibitors blocks pRb phosphorylation and the increase in E2F1 levels and dramatically reduces neuronal death by 80%. These results suggest that CDKs are a promising therapeutic target for the treatment of ischemia. Here we have reported that the protective effects of Xyloketal B and CEP-1347 in brain injury and neuroinflammation by inhibiting STAT3 activation. PR is a key factor for endogenous neuroprotection; thus, they are deemed to be pharmacological target to treat stroke. The docking studies have shown that these compounds having anti-inflammatory, anti-apoptotic, anti-oxidative abilities can serve as treatment for ischemic conditions. Also, many experimental studies and clinical studies have been performed for reperfusion conditions, neurological disorders and extensive studies on stroke can be carried out for prevention and better recovery.

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Evaluation of Anti-HIV Activity of Selected Medicinal Plants: A Short Review

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ABSTRACT

Human immunodeficiency virus (HIV) causes the potentially life-threatening and chronic disease called acquired immune deficiency syndrome (AIDS). The main target of this viral disease is to suppress the immune system and make the body unresponsive to external stimuli. According to global health observatory data since epidemic, more than 78 million people were affected by HIV and 39 million people died globally. There were approximately 37.9 million people living with HIV at the end of 2018. Currently, antiretroviral therapy (ART) is available for the control of HIV but has serious associated side effects such as lipodystrophy. Because of the limitations, associated with ART, researchers throughout the world are trying to explore and develop more reliable and safe drugs from natural resources to manage HIV infection. A wide range of medicinal plants have been studied and have reported significant potential against HIV. Medicinal plants contain novel anti-HIV compounds. As it has been well reported that medicinal plants contain various types of phytochemical constituents including alkaloids, flavonoids, phenolic compounds, glycosides, tannins, and saponins, hence the medicinal plants could be potential sources of boosting immune responses, as well as halting the replication of HIV. A literature survey of medicinal plants from PubMed and plant literature database, was carried out to identify the plants with novel antiviral agents reported for the treatment of HIV/AIDS worldwide. Bioactive compounds from plants which play effective roles in the management of AIDS, which have been discussed in this review study. This could pave way for being taken up for active future in vitro and preclinical research studies to qualify as lead anti HIV molecules which is the need of the hour.

KEY WORDS: AIDS, ANTIRETROVIRAL THERAPY, PHYTOCONSTITUENTS, MEDICINAL PLANTS.

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INTRODUCTION

HIV continues to be a major global public health issue, having claimed more than 32 million lives so far. However, with increasing access to effective HIV prevention, diagnosis, treatment and care, including for opportunistic infections, HIV infection has become a manageable chronic health condition, enabling people living with HIV to lead long and healthy lives. There were approximately 37.9 million people living with HIV at the end of 2018.As a result of concerted international efforts to respond to HIV, coverage of services has been steadily increasing. In 2018, 62% of adults and 54% of children living with HIV in low- and middle-income countries were receiving lifelong antiretroviral therapy (ART).(WHO 2019). HIV is a retrovirus that can integrate its DNA into the host genome. The virus enters the host cell and affects the immune system mainly T lymphocytes, monocytes, macrophages and dendritic cells (Salehi et al,.2018).

Its genetic material RNA is made up of nine genes which contain all the instructions to make new viruses. Three of these genes – gag, pol and env – provide the instructions to make proteins that will form new virus particles. The other six genes rev, nef, vif, vpr and vpu, provide code to make proteins that control the ability of HIV to infect a cell, produce new copies of virus or release viruses from infected cells. The HIV-1 binds to the chemokine receptor 5 or the CXC chemokine receptor 4 by interacting with the envelope proteins to gain entry to the host cell (Salehi et al,.2018). Therapies are now available to inhibit various stages of viral infection such as entry inhibitors, reverse transcriptase inhibitors, integrase strand transfer inhibitors and protease inhibitors.Presence of antibody to HIV proteins is well accepted as indicative of HIV infection. Sometimes certain clinical conditions may also result in the presence of false-positive HIV antibody. Serologic tests for HIV includes ELISA, Western blot and HIV p24 antigen assay.

Types and Symptoms

1. Primary infection (Acute HIV): Some people infected by HIV develop a flu-like illness within two to four weeks after the virus enters the body. This illness, known as primary (acute) HIV infection, may last for a few weeks. Possible signs and symptoms include fever, headache, muscle aches and joint pain, rash, sore throat and painful mouth sores, swollen lymph glands, mainly on the neck, diarrhoea, weight loss, cough, night sweats. As the infection progressively weakens the immune system, they can develop other signs and symptoms, such as swollen lymph nodes, weight loss, fever, diarrhoea and cough. Without treatment, they could also develop severe illnesses such as tuberculosis (TB), cryptococcal meningitis, severe bacterial infections, and cancers such as lymphomas and Kaposi's sarcoma (WHO 2019).

2. Clinical latent infection (Chronic HIV): In this stage of infection, HIV is still present in the body and in white blood cells. However, many people may not have any symptoms or infections during this time.

Treatment: Despite challenges, new global efforts have meant that the number of people receiving HIV treatment has increased dramatically in recent years, particularly in resource-poor countries. In 2018, 62% of all people living with HIV were accessing treatment. Of those, 53% were virally suppressed. This equates to 23.3 million people living with HIV receiving antiretroviral treatment (ART) in 2018 – up from 7.7 million in 2010. However, this level of treatment scale up is still not enough for the world to meet its global target of 30 million people on treatment by 2020 (WHO 2019). Significant progress has been made in the prevention of mother-to-child transmission of HIV (PMTCT). In 2018, 82% of all pregnant women living with HIV had access to treatment to prevent HIV transmission to their babies – an increase of more than 90% from 2010.

Antiretroviral Therapy: The combination of drugs used to treat HIV is called antiretroviral therapy antiretroviral therapy (ART). ART is recommended for all people living with HIV, regardless of how long they've had the virus or how healthy they are. More than two dozen antiretroviral drugs has been approved by FDA to treat HIV infection. Different classes of antiretroviral drugs act at different stages of the HIV life cycle. Two nucleoside reverse transcriptase inhibitors (NRTIs; abacavir with lamivudine or tenofovir disoproxil fumarate with emtricitabine) and an integrase strand transfer inhibitor, such as dolutegravir, elvitegravir, or raltegravir; a nonnucleoside reverse transcriptase inhibitor (efavirenz or rilpivirine) or a boosted protease inhibitor (darunavir or atazanavir) are rcommended for initial regimens (Günthard et al., 2014).

Fostemsavir (entry inhibitor via gp120) and PRO140 (CCR5 monoclonal antibody) are the two additional viral entry inhibitors with novel mechanisms of action that are currently in phase 2 trials (Gravatt et al., 2017). A phase 3 study is currently ongoing (NCT02362503) to determine if fostemsavir is an effective treatment for patients with multidrug-resistant HIV. PRO140 (CytoDyn) is a humanized CCR5 monoclonal antibody with antiviral activity against CCR5-tropic HIV. Based on new evidence assessing benefits and risks, the WHO recommends the use of the HIV drug dolutegravir (DTG) as the preferred first-line and second-line treatment for all populations, including pregnant women and those of childbearing potential (WHO 2019).

Antiretroviral drugs for HIV infection has been classified into the following categories: Multi-class Combination Products, Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs),Protease Inhibitors (PIs), Fusion Inhibitors,Entry Inhibitors—CCR5 co-receptor antagonist and HIV integrase strand transfer inhibitors.

Herbal Medicine In The Treatment Of HIV/AIDS: The use of herbal medicine is increasingly becoming more popular in many countries (Sabde et al., 2011). This practice has continued to be a main source of health care in the rural communities especially in developing

countries, since modern medicine has not been able to reach the majority of the populace. In Africa, traditional herbal medicines are often used as primary treatment for HIV/ AIDS and for HIV-related problems including dermatological disorders, nausea, depression, insomnia and weakness. In North America, commonly used herbal dietary supplements have been found to impede on ARV drug effectiveness. Specifically, garlic supplements (Allium sativum) and St John's Wort (Hypericum perforatum) have been shown to have detrimental effects on the plasma concentrations of saquinavir and indinavir (Piscitelli et al., 2002).

Plants, produce numerous secondary metabolites as evolutionary responses to infections by fungi, nematodes, and other organisms, to avoid herbivory, and to complete for light and space, such as phenolics, glycosides, alkaloids, coumarins, terpenoids, essential oils and peptides. These metabolites have been identified with different biological activities. Some of them play an important role in immune system enhancement, exhibiting antiviral potential, including viral infections associated with Human Immunodeficiency Virus type 1 (HIV-1) and 2 (HIV-2) as genetic variabilities. An increasing number of patients with HIV infection cannot use the currently approved anti-HIV drugs including the reverse transcriptase and protease inhibitors, due to the adverse reactions, particularly liver diseases, that have been reported for antiretroviral drugs.

Some Chinese herbal preparation which consists of 14 plants (*Coptis chinensis, Jasminum officinale, Wolfiporia extensa, Sparganium stoloniferum, Polygonatum odoratum*, and *Scrophularia buergeriana* was investigated during 24 weeks and observed to have increased plasma CD4 count and also showed inhibition of HIV growth.

Table 1a. Plants with proven anti-HIV activity

S. no	Plant Name	Part of the Plant	Family	Assay	Reference
1	*Aegle marmelos*	Leaves Fruits	Rutaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
2	*Adhatoda vasica*	Leaves	Acanthaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
3	*Allium sativum*	Bulbs	Amaryllidaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
4	*Alstonia scholaris*	Stem bark Leaves	Apocynaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
5	*Argemone mexicana*	Leaves	Papaveraceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
6	*Asparagus racemosus*	Roots	Asparagaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
7	*Aconitum kusnezoffii*	Aerial	Ranunculaceae	MT-4 cell Assay	L M Bedoya, S Sanchez-Palomino, M J Abad et al., 2001
8	*Anemarrhena asphodeloides*	Rhizoma	Liliaceae	MT-4 cell Assay	Bahare Salehi, Nanjangud V. Anil Kumar, Bilge Sener et al., 2015
9	*Angelica sinensis*	Root	Umbelliferae	MT-4 cell Assay	Carolyn Williams-Orlando., 2017

Continue Table 1

10	*Artemisia caruifolia*	Whole plant	Asteraceae	MT-4 cell Assay	Chao-Mei MA, Norio Nakamura, Masao Hattori., 2001
11	*Andrographis Paniculata*	Leaves	Acanthaceae	MT-4 cell Assay	Mayur M Uttekar, Tiyasa Das, Rohan S Pawar et al., 2012
12	*Azadirachta indica*	Leaves	Meliaceae	Syncytium reduction assay, ELISA, Anti-HIV-1 RT inhibitory activity	David, Pedroza-Escobar Benjamín, Serrano-Gallardo Luis Delia et al., 2017
13	*Areca Catechu*	Seed	Piperaceae	-	Senthil Amudhan, V Hazeena Begum, K. B. Hebba, 2019
14	*Alchornea laxiflora*	Leaf, root, stem	Euphorbiaceae	HIV-1 Integrase inhibitory activity, Cytotoxicity activity	fD.Mnkandhla, M Issacs, F.M. Muganza et al., 2019
15	*Butea monosperma*	Roots Stem Bark	Leguminosae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
16	*Betula pubescens*	Bark	Betulaceae	anti-HIV-1 integrase assay	Prapaporn Chaniad , Teeratad Sudsai, Abdi Wira Septama et al., 2019
17	*Cassia occidentalis*	Leaves	Fabaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
18	*Catharanthus roseus*	Leaves	Apocynaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
19	*Cissampleos parriera*	Aerial part	Menispermaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
20	*Colchicum luteum*	Bulbs	Colchicaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
21	*Coleus forskohlii*	Aerial part	Lamiaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
22	*Cryptocarya chinensis*	Wood	Lauraceae	HIV growth inhibition assay	Tian-Shung Wu, Chung-Ren Su, Kuo-Hsiung Lee, 2012
23	*Coccinium fenestratum*	Stem bark	Menispermaceae	Integrase and Protease Inhibitor assay	J.J. Magadulai, H.O. Suleiman., 2010
24	*Calophyllum inophyllum*	Bark	Guttiferae	RT Inhibition assay	J.J. Magadulai, H.O. Suleiman., 2010
25	*Cinnamomun aromiticum*	Bark	Lauraceae	MT-4 cell Assay	Franklin Nyenty Tabe, Nicolas Yanou Njintang, Armel Hervé Nwabo Kamdje et al., 2015
26	*Cynanchum chinense*	Root	Asclepiadaceae	MT-4 cell Assay	Jian Tao, Jing Yang, Chaoyin Chen et al., 2011
27	*Cynomorium songaricum*	Stem	Cynomoriaceae	MT-4 cell Assay	Suvdmaa Tuvaanjav, Han Shuqin, Masashi Komata et al., 2016

Continue Table 1

28	*Dracocephalum rupestre*	Whole plant	Labiatae	MT-4 cell Assay	Qi Zeng, Hui-Zi Jin, Jiang-Jiang Qin et al., 2010
29	*Dryopteris crassirhizoma*	-	Aspidiaceae	MT-4 cell Assay	Ji Suk Lee, Hirotsugu Miyashiro, Norio Nakamura et al., 2008
30	*Embelica ribes*	Fruits	Primulaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
31	*Embellica officinalis*	Fruits	Phyllanthaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
32	*Erodium stephanianum*	Whole plant	Geraniaceae	MT-4 cell Assay	Chao-mei Ma, Norio Nakamura, Hirotsugu Miyashiro, 2002
33	*Eugenia jambolona*	Bark	Myrtaceae	-	Richa Sood, D Swarup, S Bhatia, D D Kulkarni et al., 2012
34	*Garcinia indica*	Leaves	Clusiaceae	MT-4 cell Assay	J.J. Magadulai, H.O. Suleiman., 2010
35	*Garcinia cambogia*	Leaves	Clusiaceae	Integrase and Protease Inhibitor assay	J.J. Magadulai, H.O. Suleiman., 2010

Table 1b. Plants with proven anti-HIV activity

S. no	Plant Name	Part of the Plant	Family	Assay	Reference
1	*Gentiana scabra*	Root	Centianaceae	MT-4 cell Assay	Bahare Salehi, Nv Anil, Bilge Sener et al., 2018
2	*Gossampinus malabarica*	Flower	Bombacaeae	MT-4 cell Assay	J A Wu, A S Attele, L Zhang et al., 2001
3	*Gymnadenia conopsea*	Root	Orchidaceae	MT-4 cell Assay	Xiaofei Shang, Xiao Guo,Yu Liu et al., 2017
4	*Glycyrrhiza glabra*	Glcyrrhyrine	Fabaceae	OKM-1, MT-4 cells	Cristina Fiore, Michael Eisenhut, Rea Krausse et al., 2008
5	*Glycyrrhiza glabra*	Roots	Fabaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
6	*Gentiana scabra*	Root	Centianaceae	MT-4 cell Assay	Bahare Salehi, Nv Anil, Bilge Sener et al., 2018
7	*Lygodium japonicum*	Spore	Schizaeaceae	MT-4 cell Assay	Xavier-ravi Baskaran, Antony-varuvel Geo Vigila, Shou-zhou Zhang et al., 2018
8	*Madhuca indica*	Bark	Sapotaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
9	*Morinda citrifolia*	Leaves	Rubiaceae	MT-4 cell Assay	P. Selvam, N. Murugesh, M. Witvrouw et al., 2009
10	*Moringa oleifera*	Leaves	Moringaceae	Vector based antiviral assay	Nworu CS, Ezeifeka GO, Ebele Okoye et al., 2013

Continue Table 1b

11	*Myrianthus holstii*	Root	Urticaceae	Synctia Formation assay	Michael J. Currens, Lewis K. Pannell, and Michael R. Boyd et al., 2000
12	*Ocimum sanctum*	Leaves	Lamiaceae	RT Inhibition assay, Gp120 Binding Inhibition assay	Kun Silprasit, Supaporn Seetaha, Parinya Pongsanarakul et al., 2011
13	*Oldenlandia diffusa*	Whole plant	Rubiaceae	MT-4 cell Assay	Bahare Salehi, Nanjangud V. Kumar, Anil Bilge Sener et al., 2018
14	*Polygonum divaricatum*	Whole plant	Polygonaceae	MT-4 cell Assay	Yu Zhong, Yoshiyuki Yoshinaka, Tadahiro Takeda et al., 2005
15	*Papaver somniferum*	Seeds	Papaveraceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
16	*Piper longum*	Fruit	Piperaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
17	*Phyllanthus amarus Schum*	Leaves	Phyllanthaceae	RT assay	F Notka, G R Meier, Ralf Wagner, 2003
18	*Phyllanthus emblica*	Fruit	Phyllanthaceae	p24 production assay	M Estari, L Venkanna, D Sripriya et al., 2012
19	*Pelargonium sidoides*	Root	Geraniaceae	HIV-1-cell attachment assays	Markus Helfer, Herwig Koppensteiner, Martha Schneider et al., 2014
20	*Rubia cordifolia*	Roots	Rubiaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
21	*Rhaponticum uniflorum*	Root	Compositae	MT-4 cell Assay	Hai-Li Liu,Yue -Wei Guo, 2008
22	*Rubia cordifolia* L	Root	Rubiaceae	MT-4 cell Assay	Yuanyuan Sun, Xuepeng Gong, Jia Y Tan et al., 2016
23	*Rauwolfia serpentina*	Roots	Apocynaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
24	*Papaver somniferum*	Seeds	Papaveraceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
25	*Piper longum*	Fruit	Piperaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
26	*Phyllanthus amarus Schum*	Leaves	Phyllanthaceae	RT assay	F Notka, G R Meier, Ralf Wagner, 2003
27	*Salacia oblonga*	Leaves	Celastraceae	Integrase and Protease Inhibitor assay	J.J. Magadulai, H.O. Suleiman., 2010
28	*Salvia miltiorrhiza*	Roots	Lamiaceae	MTT assay, Virus neutralization assay	Ibrahim S Abd-Elazem, Hong S Chen, Robert B Bates et al., 2002
29	*Silybum marianum*	-	Asteraceae	MT-4 cell Assay	Ching-Hsuan Liu, Alagie Jassey,
30	*Scorzonera glabra*	Root	Compositae	MT-4 cell Assay	Hsin-Ya Hsu et al., 2019 Chao-mei Ma, Norio Nakamura, Hirotsugu Miyashiro et al., 2002

Continue Table 1b

31	*Scutellaria barbata*	Whole plant	Portulacaceae	MT-4 cell Assay	Zi-Long Wang, Shuang Wang,
32	*Stellera chamaejasme*	Root	Thymelaeaceae	MT-4 cell Assay	Yi Kuang et al., 2018 Min Yan, Yan Lu, Chin-Ho Chen et al., 2015
33	*Stephania cepharantha*	Root, Tuber	Menispermaceae	MT-4 cell Assay	Chao-mei Ma 1, Norio Nakamura, Hirotsugu Miyashiro et al., 2002
34	*Sterculia scaphigera*	Seed	Sterculiaceae	MT-4 cell Assay	Moshera Mohamed El-Sherei, Alia Ragheb, Mona Kassem et al.,2016
35	*Tinospora cordifolia*	Stem bark	Menispermaceae	p24 antigen assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
36	*Terminalia sericea*	Leaves	Combretaceae	MTT assay	M A Chauke, L J Shai, M A Mogale et al., 2016
37	*Withania somnifera*	Roots	Solanaceae	p24 antigen assay, Gp120 Binding Inhibition assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011
38	*Withania somnifera*	Roots	Solanaceae	p24 antigen Gp120 assay, Binding Inhibition assay	Sudeep Sabde, Hardik S. Bodiwala, Aniket Karmase et al., 2011

Cytotoxicity of Anti-HIV Phytochemicals: Cytotoxic evaluation is very important and integral part of research involving discoveries of new and potent antiviral drugs. A novel formulation with potent antiviral activity have to be proven as not having any toxicity effects and cytotoxicity assays in a suitable cell culture system are only a part of primary step in this direction. For the purpose of testing, different plants active principals have to be extracted with suitable solvents. The list of commonly used solvents for extraction purpose is summarized in Table 2. Treating cells with these phytochemicals can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity.

Table 2. Solvents used for active components extraction

Water	Ethanol	Methanol	Chloroform	Di-chloro methanol	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Terpenoids	Alkaloids	Flavanols
Starches	Polyphenols	Terpenoids	Flavonoids		Terpenoids	
Tannins	Polyacetylenes	Saponins			Coumarins	
Saponins	Flavanol	Tannins			Fatty acids	
Terpenoids	Terpenoids	Xanthophyllines				
Polypeptides	Sterols	Totarol				
		Lactones				

Cytotoxicity can also be monitored using the MTT or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. Tetrazolium salts are reduced only by metabolically active cells. Thus, 3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide (MTT) can be reduced to a blue colored formazan32. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. In addition to using dyes to indicate the redox potential of cells in order to monitor their viability, researchers have developed assays that use ATP content as a marker of viability (Riss et al., 2004). Adenosine triphosphate (ATP) that is present in all metabolically active cells can be determined in a bioluminescent measurement. The bioluminescent method utilizes an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin. The emitted light intensity is linearly related to the ATP concentration (Weyermann et al.,2005). Neutral red (3- amino-m-dimethylamino-2-methylphenazine hydrochloride) has been used previously for the identification of vital cells in cultures. This assay quantifies the number of viable, uninjured cells after their exposure to toxicants; it is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red. Quantification of

the dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Weyermann et al., 2005).

Future Perspectives: In vitro studies of many plant phytoconstituents can be evaluated for various anti-viral activities including anti-HIV activity and COVID-19. Further studies can be carried out to know the mechanism of drug inhibition in virus. Synthetic drugs are proved to cause side effects. However, more exploratory research to prove the efficacy of medicinal plants including plant - drug interactions and their mechanism of action has to be explored so that plant compounds can be used to treat various viral infections including deadly COVID-19.

CONCLUSION

Many plant species have been investigated for anti-HIV potential and has shown promising activity. Azidothymidine, the first drug that was approved in the fight against AIDS in the 1980s, still a main component in the medication mix commonly prescribed to HIV patients today. But new research have found a plant-derived chemical compound that is much more effective than azidothymidine. The chemical compound is called "patentiflorin A" and is derived from a medicinal plant found in East Asia: Justicia gendarussa. Hence, plant based source drugs are non-toxic and work effectively unlike synthetic drugs. Many synthetic medicines are being used in the treatment of AIDS. Various medicinal plants or plant-derived natural products has offered alternatives to expensive medicines in future.

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Biological Production of Antimicrobial Peptides Against Plants as Well as Human Pathogens

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ABSTRACT

Antimicrobial peptides (AMPs) are one of the natural defense compounds grounded on drugs because of its vast potential as a novel biopharmaceutical product for both humans and plants. Antimicrobial Peptides (AMP) have gathered attention as a feasible clarification to this serious matter and are existence discovered for their prospective antimicrobial applications. These are small, cationic and characteristically have hydrophobic residues and play an important role in biological activities by creating interaction with the cell membranes. These compounds are extracted and purified by natural hosts like bacteria as well as fungi etc trough ribosomal or non-ribosomal synthesis .Plants and agricultural production are affected by various diseases that are caused by bacteria, fungi and viruses. Anti-microbial peptide are used as bio-pesticides which are concealed from many microorganisms alongside it about transgenic plants too rapid it to control the plant infections and plant pathogens but there are some social concerns and limitations because of less toxicity and less stability of these compounds that's why transgenic plants are not commercially marketed.Now a day, instead of using chemical peptides researchers are paying attention on numerous antimicrobial peptides which based on structural analog design and can overcome plant disease as commercial bio pesticides. This valuation will assess the work of bacterial cloning and its mechanism to supply the recombinant proteins in against of plant pathogens for instance bacteria, fungi, nematodes, and viruses that reason of plant disorder. It can help to exploit the recognition of peptide–antibiotic amalgamations that will meritoriously eradicate resistant bacteria.Various techniques such as gene editing, protein engineering or computational tools can provide help to face the industrial requirements which could stimulate development to squeeze proteins or peptides as well as can be used to screen latent peptide sequences with antimicrobial properties, and for computer-aided discovery of AMPs as well.

KEY WORDS: ANTIMICROBIAL PEPTIDES, BIOPHARMACEUTICAL, RIBOSOMAL SYNTHESIS, NON RIBOSOMAL SYNTHESIS.

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INTRODUCTION

Natural products are selected according to subordinate metabolites from an innumerous list of sources, just as creatures, oceanic living beings, organisms, earthly plants, and spineless creatures (Chin, et al., 2006). These synthetically and fundamentally various molecules act as an uncommon class of therapeutics to recuperate various maladies. The preliminary documentation of the use of normal items to recoup human constitution goes back to the ancient Mesopotamia's advanced restorative framework from 2900 - 2600 BCE, (Reisel et al., 2002, Rahman, et al., 2014). The natural product portion can't just zone that has encountered extensive development or utilizations useful items created in/from living creatures. Prokaryotic and eukaryotic bacterial cells, in blend with the advancement of recombinant DNA strategies, have been liable for a blast of biologics. Biologics are a lot of atoms whose active therapeutic materials are resulting from living beings, for example, creatures, microorganisms, plants, human blood items, and tissue transplants that are too multi-layered to even think about being delivered through organic synthesis (Revers, et al., 2010 Park et al., 2019).

Biologics can be separated into five superior classes: First one is monoclonal antibodies, as trastuzumab (HerceptinR) and rituximab (RituxanR); Second one is blood factor subsidiaries, similar to coagulation factor VIIa (NovoSeven RTR) and epoiten alfa (EpogenR); Third one is Immunization; Fourth one is Enzymes; and Fifth one is recombinant proteins, for instance immunomodulatory cytokines, and thrombolytic mediators (Lacana, et al., 2007).Bacteriocins are a protein delivered by microorganisms of one strain and dynamic against those of a firmly related these are specific peptides known as antimicrobial peptides which are formed by Gram-negative bacteria. These molecules have included generous concern; particularly those made by GRAS (Commonly Renowned as Nontoxic)) microbes, by way of normal nourishment preservers inside nutrient production fields. These peptides likewise indicate likely replacements to obsolete antibiotics which is utilized for cure of poisonousness within persons and food assembly creatures.(Cotter, et al., 2005; Desriac et al., 2010; Svetoch et al.,2010) besides, supplementary investigations, reveal probable such as medicines designed for tumor cure (Ravikant, et al., 2015).

The further most dynarnic requisite for this inspection is production of wide phases of energetic bacteriocin by using biological ways. Though specific peptides could be sterilized after their local making bacterial strains, these techniques required some additional time period, work besides bacteriocin productions are frequently decrease (Fernández, et al.,2003). Artificial manufacture is an important opportunity in few research works, nonetheless hassle of a number for the bacteriocins as well as cost for various manner restriction used for high amount production. (Dong, Chen et al. 2012, Park, Yoon et al. 2019,Dong et al., 2012; S. R. Park et al., 2019).

These straining, which might be food-grade creatures, provide an inoffensive preference for commercial foodstuffs, in addition to offer inherited as well as secretory apparatus for dynamic LAB bacteriocin assembly. Though, countenance bacterial strains accessibility are enormously certain and its effect in production are quiet unsatisfactory at manufacturing level, limiting variability and production of bacteriocin amount. Expected of these boundaries, *Escherichia coli*, has been the furthest generally utilized creature for heterologous protein manufacturing, it is a generous choice for representing the expression of heterologous bacteriocin owing to the situation, it shows quick development on low-price media, studying on its widespread inherited classification along with accessibility of multipurpose cloning techniques, structural expressions as well as various bacterial strains, (Fernández et al., 2003; Mergulhao, et al., 2004; Rosano et al., 2014 Li et al., 2016, Mesa-Pereira, et al.,2018).

This may cause an ease for efficient description besides establishing a beneficial formation method for production of different kinds of bacteriocins which are considered as tricky for their development, By adding toward the ones bacteriocins found out for elimination of its information through microbial genomes which arrange in particle order (Kuo & Huang, et al., 2013), Growing their capability for production and profit-oriented by using meals and therapeutic productions (Ongey & Neubauer, 2016). However, this technique isn't underprivileged of difficulties which may upward thrust throughout the expression, production or processing of these particle containing of two or further amino acids it's peptide in *E. coli* (J. Choi & Lee, et al., 2004).Advanced the powerful manufacturing of the recombinant human being insulin HumulinR , *E.coli* quickly developed the highest expression platform with in the 1980s when the bio-pharmaceutical area appeared and become monitored by means of yeast *Saccharomyces cerevisiae*, (Romero-Garcia, et al., 2016). Bacterial cells create the mainstream of hosts working in the production of currently allowable recombinant medicines for human being treatment, mostly due to their absence of uncommon post-translational modifications, terrible solubility and origination of cell stress responses, proteolytic ambiguity (Graumann et al., 2006; Mesa-Pereira et al., 2018).

This verifies that microbial hosts signify convenient and full of life systems for the well-prepared manufacturing of recombinant proteins despite a few blocks and complications. This assessment will evaluate the tasks of bacterial cloning and its mechanism to supply the recombinant proteins in contradiction of plant pathogens alike bacteria, fungi, nematodes, and viruses that reason of plant infections.

Mechanism designed for anti-microbial peptide protein production through microbial cloning: The host cell's exquisite qualities which prove as protein production device will yield treasured protein that will provoke define its entire procedure which designates understanding desirable for this approach; it's a

multiplicity of molecular apparatuses, methods, and substances. Amongst microbes, host schemes which are obtainable encompass microbes, algae, fungi, and yeast. All have strengths and weaknesses and their preference possibly concern to protein of interest (Adrio & Demain, 2010) Like, A prokaryotic expression scheme won't be suitable, uncertainty eukaryotic post-translational amendments are desirable. (Sahdev, et al., 2008). The benefits of use of the *Escherichia coli* for the reason that host entity are familiar. (i) lt contains unequaled reckless rate of growth.

In glucose and salts medium as well as assumed an optimum ecological circumstances, its repetition period is around 20min (Rosano, et al., 2014). So, its means that's culture injected through a 1/100 diluted of sterilized appetizer culture might also immobile stage inside rare times. Although, it need to remain as well-known which show expression of include are combinant protein might inform a metabolic load at mobe, producing a considerable reduction in gene proportion time (Pinfold, et al.,1996) (ii) Cell cultures with greater width are smoothly attained.Speculative thickness limit liquid culture of an *Escherichia coli* that is assessed around two hundered gram dry cellular weightiness /lorroughly1× 1013 possible microorganisms/ml.(Restaino et al., 2013). It is investigated that their physicochemical capabilities as well as, secondary structures, and appliances of action, and associated them with the peptides inside AMP database(Lei et al., 2019). Gram-positive bacteria that have habitat inside soil and 98% of identified AMPs likewise originated from natural bases such as skin excretions of frogs besides toxins from diverse species (Lei et al., 2019)

Existence a work horse creature, these plans ascended appreciations to the prosperity of understanding around its physiology like composition, functions and structures study. (iii) Opulent multifarious media may be crafted from keenly obtainable as well as reasonable mechanisms (Bakare, Fadaka, Klein, & Pretorius, 2020). (iv) Conversion through exogenic DNA remains reckless and calm as well. Plasmid conversion of Escherichia coli could implement tiny such as for five min, (Aranishi, Okimoto, & Izumi, 2005). Its structural physiognomies subsidize to their amphipathicity which permits for segregating obsessed by the membrane lipid bilayer (Bechinger & Gorr, 2017), in that way ornament their antimicrobial accomplishments, consequently impacting actual membrane absorbency on a assortment of cytoplasmic objectives, (Bakare et al., 2020).

Genetic factors that understanding repetition as free units, for instance plasmids, comprise are plicon. It consist of one supply of replication collectively with its associated cis-acting manipulate components A huge drawback is to having lack of awareness during selection of a suitable vector in duplicate amount. Duplication Controller amount be located in the replicon, (Turgeon, et al., 2008). Generally used vectors, like the pET collection, have the pMB1 foundation, 15 to 60 duplicates; (Bolivar et al., 1977) although a modified form of pMB1 starting place is existing within pUC sequences 500 to 700 duplicates for each cell; (Minton 1984). Alternative kind wild form ColE1 basis 15 to 20 duplicates to each cell; (Minton, et al., 1984). Alternative kind wild-kind ColE1 basis (15–20 duplicates (Lazzaro, et al., 2020) to each cell (Lin-Chao, et al., 1986; Lee et al., 2006). AMPs are ubiquitous among multicellular eukaryotes , with most plant and animal species expressing dozens of distinct AMP genes in epithelial tissues and in response to infection (Chen, et al., 2020; Waghu, et al., 2020). The diversity and potency of AMPs make them attractive candidates for translational application, and several are already in clinical trials (Lazzaro et al., 2020).

So it could inside the pQE vectors (Qiagen). These are totally suitable with identical mismatch institution which means that they can't be extent composed in the alike cell by way of their challenge with separately towards replication technology (Del Solar, et al., 1998; Joseph et al., 2010) . On behalf of twofold expression of specific recombinant proteins the usasge of double plasmids, schemes with pISA are reachable (pBAD and pACYC plasmids collection, ten to twelve duplicates per cell (Guzman, et al.,1995).

The foremost in research of prokaryotic promoter is irrefutably *luc promoter*, and rnost vital essential of loc operon (Müller, et al.,1996). Accrued material inside working of an organization is permissible because of its extensive usage for expression of vectors. For initiation of the system is Lactose sources and this sugar can be usage for production of protein. Though, overview remains challenging for existence of willingly resources of metabolized carbon for example glucose. If the both lactose or glucose are prevailing, lac promoter's expression is not entirely influenced till entirely the glucose has been used .At this estimation (small amount of glucose),cyclic adenosine monophosphate is designed (Bakare et al., 2020), that is vital intended for entire beginning of lac operon (Skrzypczak, et al., 1994; Wöhrl, et al., 1990).Many AMPs have the potential to fold into amphipathic a-helices with hydrophilic (Lei et al., 2019) and hydrophobic sides (top, left). This conformation is schematically represented as an amphiphilic cylinder (Lazzaro et al., 2020; Waghu et al., 2020), with hydrophobic (red) and hydrophilic (blue) halves. AMPs bind to the membrane surface with the hydrophobic side groups anchored in the hydrophobic lipidic core of the bilayer, leading to different outcomes (center) (Bakare et al., 2020).

This optimistic mechanism of expression is called catabolite repression .In agreement, cyclic adenosine monophosphate (cAMP) elevations are small in cells rising luc operon suppressing sucrose, as well as acquaintances by lesser charges of lac operon's expression. (Hsiao, et al.,1977). To sign the development of plasmid permitted cells, are confrontation indicator is added to support of plasmid. Inside system of *Escherichia coli*, resistance of antimicobial genome is naturally utilized for its resolution (Bhopale, et al., 2020). Ampicillin's confrontation is deliberated through bla gene which

causes enzyme (Nesa et al., 2020; Tincho, et al., 2020) Nevertheless, in place of b-lactamase is unremittingly covered, deprivation of antibiotic shadows as well a pair of times, ampicillin is closely fatigued (Rosano, et al., 2014). physicochemical properties and mechanism of action which governs their penetration into microbial cell (Boparai et al.,, 2020).

The budget of antibiotics and the distribution of antibiotic opposition are foremost uncertainties in plans deals with important values. Plentiful struggle has been pre in the progress about free of antibiotics plasmid schemes. These organizations are originated upon an idea of plasmid obsession, process which happens when plasmid cells are not accomplished for development or else alive (Peubez et al., 2010; Zielenkiewicz et al., 2001). In few cases, significant gene can detach from genome of microbe and positioned on a plasmid. Therefore, when division of cells is happened then, a microbe which is free from plasmid is died Various sub categories of plasmid-obsession schemes occur conferring to their purpose: (i) toxins or anti-toxin built schemes, (ii) metabolic rate- built systematic organization (iii) System of (ORT) operator repressor titration (Kroll et al., 2010).

However this favorable skill has been confirmed positive in extensive fermenters (Peubez et al., 2010; Pohlmann et al., 2006) Systems of expressions created on plasmid fascination are still not broadly disseminated. If physical, biological or chemical learning based on recombinant protein are essential, At the same time, synthesis companion necessity can eradicate from recombinant proteins. Identifiers of Peptide should must separate since it can impede structure as well as action of protein (Rosano, et al., 2014), then it can left inside residence level for learning of crystallography, (Falquet et al., 2002; Schmid et al., 2009). Identifiers might be abolished moreover enzymatic cleavage or chemical discontinuity occurs. A quick exploration in research paper or an appropriate *E. coli* straining for usage of it as host will produce for many potential contestants. Altogether it has many benefits as well as flaws. Aimed at an initially expression shade, solitary few of *E.coli* strains are vital: 21 (DE3) and approximately byproducts of K- 12 descent. At this detail, it would be richer in quantity of choices which scheming is knowingly up to an expression system.

Selecting the faultless mixture is not thinkable; consequently numerous circumstances must confirm the required protein achievement. lf there two protein are introduced to express duplication inside six unlike vectors of expressions, each partial in three unlike expressions sequences, then there would be 36 tribunals of expression. This quantity might be uniformly enhanced while additional variables are occupied inside version. This kinds of trials might be contains end errors as well as its time wasting more, experimental learning can be complete earlier. If micro expressional tests are attained at earlier level, then limited screens can accomplish within 96 fine plates and 2-m1 tubes (Li, et al., 2002). Extraordinary, through place procedures familiarizing involuntary fluid handling robots have been designated, creating it thinkable for a solitary individual to examine further than 1000 culture circumstances within a week.

Antimicrobial peptides produced by Bacteria against plant pathogens: Bacteriocins, a various collection of anti-bacterial ribosomaly formed peptides, contains probable such as biopreservers inside wide spreading diversity of diets besides forthcoming therapeutics for obstruction of antimicrobial confrontation microbes. However, numerous bacteriocins have been categorized, Many aspects border manufacture intimate enormous amounts, prerequisite to create their commercialized feasible for diet and uses of pharmacy as well. New bacteriocins are classified by databank removal has been bright; nonetheless their consideration remains stimulating toward assess inside non-appearance of appropriate manifestation organizations. *E. coli* has utilized as non-homologous toward construction of recombinant proteins for ages as well as devours a general set of manifestation vectors and bacterial strains reachable as well (Mesa-Pereira et al., 2018).

Peptaibols are linear peptides, collection of both C-terminal amino alcohol and an acyl N-terminus, that are preposterous in dialkylated amino acids for example a-diaminobutyric acid (Degenkolb, et al., 2003). The lipopeptaibols is the small peptide with anti-microbial action ensure an acylated N-terminus tranquil of a minor fatty acid chain (Toniolo, et al., 2001). Their production is attained by vast multi-purpose non-ribosomal peptide synthetase is an enzyme that has been duplicated (Jarvis, et al., 2002). Peptaibols is well-defined in numerous fungi. Their anti-microbial actions effects characteristically fungi as well as also plant infective agent Gram positive bacteria (pathogenic bacteria) through process of membrane interference. The explanations might either be the antibiotic resistance to the obtainable therapeutic particles or the sluggish rate of creating satisfactory therapeutic routines to challenge the fast development of novel infective syndromes, as well as the poisonousness of present behavior routines. (Lazzaro et al., 2020; Tincho et al., 2020).

Trichokonins be located dynamic in contradiction of plant infective bacterium is *Clavibacter michiganensis* (Bhopale, et al., 2020; Lazzaro et al., 2020) besides the fungi infective bacterium such as there are five kinds Bipolaris sorokiniana (Zhong et al., 2020), *Fusarium oxysporum, Rhizoctonia solani, Botrytis cinerea* in addition *Colletotrichum ssp*. (Zhuang, et al., 2006), antifungal association in contradiction of *Sclerotium cepivorum* by Trichorzins and harzianins (Rebuffat, et al., 1995). Recently, deeper evaluation of the molecular evolution and population genetics of AMP genes reveals more evidence for adaptive maintenance of polymorphism in AMP genes than has previously been appreciated, as well as adaptive loss of AMP activity (Lazzaro et al., 2020).

Table 1. Various antimicrobial peptides with specific characteristics obtained from different origins

Name	Specificity	Origin	References
Endolysins	Antimicrobial Compound	*Various bacteriophage*	(Challinor, et al.,2015; Mamo, et al.,2016)
Andropin	Antibiotic	*Drosophila melanogaster*	(Mookherjee, et al.,2020; Zhang et al., 2020)
Liamocin	-	*Aureobasidium pullulans*	(Price, et al., 2017)
Laparaxin	-	*Lactobacillus paracasei*	(S. Liu et al., 2012)
Harzianins	Compound	*Trichoderma harzianum*	(Phazang, et al.,2020)
Unknown	-	*Bacillus sp*	(Seal et al., 2018)
Erythromycin A	Antibiotics	*Saccharopolyspora erythraea*	(Alt, et al., 2013; McGuire et al., 1952)
Antifungal heliomicin	Compound	*Heliothis virescens*	(Lei et al., 2019)
NaD1	Antibiotics	*Pathogenic fungi*	(Breen, et al.,2015)
Tetracycline	-	*Streptomyces rimosus*	(Chopra, et al.,2001; Demain, et al., 2009)
Vancomycin	-	*Amycolatopsis orientalis*	(Marsboom et al., 2012; Schatz, et al., 1944)
Nisin	-	*Lactococcus lactis*	(Gyawali, et al., 2014; J. W.-H. Li et al., 2009)
Amphotericin B	Antifungal agents	*Streptomyces nodosu*	(Abu-Salah, et al., 1996; Tevyashova et al., 2013)
Lipopeptin A	Antibiotics	*Pyricularia oryzae*	(Tsuda et al., 1980; Zhang et al., 2020)
Coronamycins	Antibiotics	*Pythium ultimum*	(Zhang et al., 2020)
Ieodoglucomide C	-	*Bacillus licheniformi*	(Dahrouj, et al., 2015)
Inceptins	-	*Vigna Unguiculata*	(Wagh et al., 2020)
Bleomycin		*Anticancer, Antitumor Streptoalloteichus hindustanus, Streptomyces verticillus*	(Beck et al., 2002; Demain, et al.,2011)
Cahuitamycins(	Biofilm inhibitory agents	*Streptomyces gandocaensis*	(Park et al., 2016)
Plantaricin Pln1 (Class II)	Bacteriocin	*Lb. plantarum* 163	(Huang, et al.,2016)
Aurein	Antibiotic	*Litoria aurea*	(Bakare et al., 2020; Zhong et al., 2020)
Subtilosin A (Sactipeptide)	-	*B. subtilis* 168	(Himes, et al.,2016)
Plantaricin E (Class IIb, two-peptide bacteriocin)	-	*Lb. plantarum* 163	(M. Li et al.,2017)
Plantaricin EF (Class IIb, two-peptide bacteriocin)	-	*Lb. plantarum*	(Galluzzi et al., 2018)
Plantaricin K	-	*Soil metagenome*	(Arora, et al., 2014)
Abaecin	Antibiotic	*Apis mellifera L.*	(Bakare et al., 2020)

Almost reports have revealed that pathogen against tomato like *Clavibacter michiganensis* subsp. michiganensis conceals protein which is 14-kDa, *C. michiganensis* subsp. *michiganensis* AMP-I, which ends development of *Clavibacter michiganensis* sub *Sp sepedonicus*, significant intermediate of microbial sphere potato's deterioration. Through tryptic fragments achieved sequences, we have gene coding *CmmAMP*-I is acquainted then we necessity recombinant deliberate protein via an N-terminal intein (section of protein) tag. Sequence of gene is accessible to *CmmAMP*-I which includes distinctive N-terminal signal peptide on behalf of Sec-dependent excretion. The recombinant protein was enormously vigorous, per 50 percent progression reticence (IC50) around

10 pmol; on the other hand, it was not poisonous for potato shrubberies or else stems. CmmAMP-I does not appearance like slightly recognized protein and hence indicates a completely fresh thoughtful of bacteriocin. Owed to its rich in antimicrobial movement and its very tinny inhibitory spectrum, battling potato ring rot disease might be attention of CmmAMP-1 (Liu et al., 2013).

Considering to (Fouhy et al., 2012), laboratory bacteriocins are categorized in which they are changed post-transnationally (class I) and unaffected considerably changed peptides (class II). Class I could be distributed in many parts such as lantibiotics it's contain features of lethionine (with lanthionine channels), linaridins, proteusins, lined azoleor azoline-containing peptides (Az peptide) cyanobactins, thiopeptides its don't have action in contradiction of Gram negative bacteria, lasso peptides, sactibiotics (comprise sulfur a carbon associations), bottromycins, glycocins (anti-microbial peptide) , as well as more different microcins which won't fit more sub catogeries. Class II is additional detached inside class IIa is first sub class its pediocin alike the bacteriocins, Class IIb is second sub class in this dual peptide alike bacteriocins, ClassIIc is third sub-class it's like round bacteriocins, ClassIId is forth class unaffected, undeviating, non-pediocin alike, sole peptide bacteriocins which won't suitable to further sub category), and ClassIIe is the fourth sub class in this microcin E492- alike bacteriocins).

Herein taxonomy, enormous (more than 10 kDa), warmth labile anti-microbial peptides bacteria lysine and its class III bacteriocins remained disconnected through cluster of bacteriocin. Correspondingly, bacteriocins which is related to Gram-negative bacteria could alienate inside petty peptides, for instance microcins including [class I (alterations occurrence) and classII (unchanged)], besides huge peptides for instance colicins (Boparai, et al., 2011). In multipurpose (Chen et al., 2020), the manufacture of bacteriocins inside ordinary congregation wants many genes counting physical gene which codes pre-peptide (Waghu et al., 2020). Additional genes encrypt a protein which have resistance, specific excretion apparatus and in several investigations proteins expert implementation, alterations and monitoring systems (Nes et al.,1999; Tincho et al., 2020).Furthermore, the novel peptides also used high constancy to trypsin, serum, salts and diverse pH atmospheres. Furthermost particularly, the novel peptides presented a little propensity to grow bacterial resistance and they showed ideal antimicrobial activity in contradiction of the attained resistant strains (Zhong et al., 2020).

In furthest case, the appearance of physical gene and developed classification be located abundant to create vigorous bacteriocin. About instances comprise carnobacteriocin B2 (Jasniewski, Cailliez-Grimal, Gelhaye, & Revol-Junelles, 2008), divercin both AS7 as well as V41 (Bowman, et al.,2004; Drozdzynska et al., 2014; Ingham et al.,2005; Kendir, et al., 2007), epidermicin NI01also include (Sandiford & Upton, 2012), gassericin A isd another type (Choi et al., 2003), or sakacin P is the last one (Dong et al., 2012) Physical gene expressed together by genes intricate inside post-translational alterations on alike and also unlike plasmids exist obligatory for heterologous appearance of lantibiotics for instance lichenicidin (Al Toma et al., 2015; Lee et al., 2011), another example is nukacin ISK-1 (Hosomi et al., 2005), these are some more examples included like prochlorosin, haloduracin, nisin(Dabashi, et al., 2011), suicin(Jiang, et al., 2014).

Antimicrobial peptides produced by Fungi against plant pathogens: AMPs have been the entity of consideration in prior ages as candidates for defense products of plant. So they are minor peptides sequence, through regularly less than fifty amino acid remains designated in the living organizations, which are primaril defense line in animals or plant life. Valuations of Anti-microbial peptide have been approved out in the microbes (Jack et al., 2000; Montesinos, et al., 2007; Raaijmakers, et al., 2006; Thresh, et al., 2005), pests (Montesinos, 2007), aquatic invertebrates (Zhao et al., 2010), amphibians and mammals animals (Perron, Zasloff, & Bell, 2006; Toke, 2005), and plants. (Feng, et al., 2017; Lay, et al., 2005). Many filar fungi conceal AMPs of 51 to 58 amount of amino acid leftovers alike to protect from plants or animals, with a solid arrangement of anti-equivalent strands alleviated through disulphide networks. The peptides of AFP is belong to *Aspergillus giganteus* have anti-fungal activity (Montesinos, et al., 2007; Vila, Lacadena, et al., 2001),

PAF is belongs to *Penicillium chrysogenum* and another *Penicillium nalgiovense* both have anti-fungal activity (Carlsson et al., 2003; Lazzaro et al., 2020; Ramamourthy, Park, et al., 2020) and Anafp belongs to *Aspergillus niger* also have anti-fungal activity, (Lee et al., 1999; Waghu et al., 2020).AFP is energetic in illogicality of Botrytis cinerea, Pyricularia oryzae and *Fusarium spp.* (Mookherjee et al., 2020) and, but is sluggish in contradiction of microbes (Aniya et al., 2007; Meli, et al., 2001; Ramamourthy et al., 2020).. Antifungal peptides (AFPs) can be established as antibiotic to controller fungal toxicities in agriculture owing to their dissimilar antifungal appliances (Zhang et al., 2020).

There are various anti-microbial peptides which are secondary metabolites designated from fungi, bacteria as well as cyanobacteria which have remains of amino acids such as D or L-forms also allo- and di-amino byproducts. These are organized in the form of cyclical rings and frequently deprived of Disulphide association. Frequent plant-associated as well as soil-inhabiting bacteria which consists of antifunal, antibacterial, cytotoxic and surfactant characteristics plays an important role in formation of specific peptides known as Lipidic cyclopeptides (LCPs). Type of LCPs which are belonged with Pseudomonas ssp. which are generally of the depsipeptide sort as well as are confidential into seven foremost assemblages such as first amphisins, second is corpeptins novel bioactive lipodepsi peptides , third one is putisolvins biofilm development of dissimilar Pseudomonas, forth one is syringomycins, fifth one is

syringopeptins its new photo toxic lipodepsi peptides , six is tolaasins and last is viscosins), and they are beached on the structure of fatty acid and length of fatty acid, and the peptide ring also (Nesa et al., 2020; Raaijmakers et al., 2006).

Syringomicins and another one is syringopeptins both verify itself as virulence issues which are formed in the *Pseudomonas syringae*, nevertheless consistently press the type of bacteria its Gram positive bacteria (Grgurina et al., 2005) as well Botrytis cinerea (Lavermicocc, et al.,1997). The straining which create syringopeptin is the *Pseudomonas syringae* 508 was aggressive to Venturia insufficiencies, the important illustrative of apple coating (Burr, et al., 1996). Tolaasins origin inhibit of the Rhizoctonia solani also another bacteria like Gram-positive bacteria similar the other one is *Rhodococcus fascians*, on the other pointer not to Gram-negative bacteria equivalent to the Erwinia carotovora (Burr et al., 1996; Mookherjee et al., 2020). *Pseudophomins* are attained through the *Pseudomonas fluorescens* BRG100 it comprises anti-fungal ability in battle of *Leptosphaeria maculans* and another *Sclerotinia sclerotiorum* (Burr et al., 1996). Cormycin -A also corpeptins advanced active lipodepsipeptides from the cultures of *Pseudomonas corrugate*-syringomycins and another is syringopeptins which are robust poisonous compounds in illogicality of plants, microbes and animals (Scaloni et al., 2004).

Pseudo-peptides which is performed as plant disorder regulator and contains of intermittent bonds of peptide and multi-layered of amino acid alterations (like nucleosides) are molded by microbes (Mookherjee et al., 2020; Zhang et al., 2020). Pantocines are consequences from the alanine which frustrate transaminase its an enzyme which is catalyzed transamination reaction and its catalyzed amino acid bio development within the bacterium for example Erwinia amylovora, and the vital representative of potency syndrome of rosaceous plants, and they are created through strains of Pantoea agglomerans (Chahardoli, et al., 2018; Kwak et al., 2003; Montesinos, et al., 2007; Phazang et al., 2020). The nucleo-peptide revenues alike polyoxins its anti-fungal antibiotics formed through streptomycetes, nikkomycins it's also related to anti-fungal antibiotics formed through streptomycetes, blasticidin also mildiomycin are similarly pseudo-peptides through antifungal achievement (Copping, et al.,2000).

Polyoxins are oppressed for the mycological plant contamination regulator and are pyrimidine dipeptides that avoid chitin manufacture in fungi equivalent to *Alternaria spp*., another *Botrytis cinerea*, and last *Rhizoctonia solani*. The Nikkomycins are pyridine consequences like to the polyoxins. Blasticidin prevents protein bio-production in the prokaryotes and also signifies its ability in contradiction of *Pyricularia oryzae*. Mildiomycin is a serine derivative energetic in contradiction of powdery fungi like *Podosphera*, another *Sphaerotheca, Erysiphe* and last one Uncinula necator (Lazzaro et al., 2020; Mookherjee et al., 2020). The anti-fungal amide bond surrogates bacilysin,and an alanine-epoxycyclohexane reassured 2 peptides, also *rhizocticin*, a phosphonodipeptide, are formed after crabwise *Bacillus* subtilis straining (Stein, et al., 2005).

Antimicrobial peptides production through transgenic plants: Production of gene that contains of coding of sequence for Anti-microbial peptide which have been expressed on harvest plants provide numerous degrees of defense in inconsistency pathogens of plants and Antimicrobial medications resistant microorganisms have been detected wide-reaching and so alternative growth of antimicrobial peptides has increased attention in human and plants healthcare, (Bhopale, et al., 2020).

Numerous expressive genes of animals have been described as defensive genes exclusive plants. Cecropins A and Cecropins B expressed in the rice which measured resistance in contradiction of *Magneporthe grisea* (Campo et al., 2004) and *Xanthomonas oryzae* (Sharma, et al., 2000), magainin, it's a class of anti-microbial peptides uttered on tobacco deliberates conflict in contradiction of numerous fungi and also bacteria (Gray, et al., 2001), and tachyplesin peptide from crab quantified as protection in potato which was further functioning in contradiction of contagions initiated through *E. carotovora*, (Allefs et al.,1996; Lazzaro et al., 2020).

Plant defensing has been articulated in plants. The Rs-AFP2 radish protecting was conveyed in tobacco as well tomato and dissertations protection in contradiction of *Alternaria longipes* (Broekaert, et al., 1995), Alf-AFP alfalfa protecting uttered in potato defends in contradiction of V. dahlia (Zhou et al., 2000), SPI1 spruce articulates in the tobacco which is offer defense in contradiction of *Heterobasidium annosum* (Elfstrand, et al., 2001), DRR206 pea protecting articulated in canola and also tobacco protects in contradiction of *Leptosphaeria maculans* (Ma, Caldwell et al., 1999), Dm-AMP1 dahlia protecting articulated (Boparai et al., 2020; Tincho et al., 2020) in the eggplant which defend (Su, Wang, & Zhang, 2020) in contradiction of *Botrytis cinerea* and another *Verticillium alboatrum* (Ayi, Turrini, Piga, & Arese, 2004), and last one Mj-AMP1 jalapa protecting articulated in tomato acquires in contradiction of *Alternaria solani* (Piraino et al., 2005). The one another hevein Pn-AMP specified as protector in tobacco in contradiction of *Phytophthora parasitica* (Koo, Kim, & Jeon, 2002) and barley hordothionin expression confidential tobacco intricate it-self as the protector in contradiction of C. michiganensis and another *Pseudomonas syringae pv. Tabaci* (Carmona, et al., 1993). MSI-99 is uttered in the vine of grape protects in contradiction of *Agrobacterium tumefaciens* (Boyer et al., 2006; Chahardoli et al., 2018).

Simulated cecropin correspondents signify protection security to numerous pathogenic bacteria in plants (Zhong et al., 2020). SB-37 defends in difference to *E. carotovora* ssp. *Carotovora* on the potato (Arce, et al.,1999) also MB39 was functioning in contradiction of the plan pathogen bacterium *E. amylovora* on the Royal Gala apple (Liu, et al., 2001). Artificial *cecropin-melittin*

amalgams for instance MsrA2 have been expressed in the tobacco or potato and discuss resistance in contradiction of recurrent phyto-pathogens (Petrenko & Yevtushenko, 2005). The totally artificial peptide which is antimicrobial D4E1 has been articulated in tobacco or potato and poplar also, defensive in contradiction of numerous pathogens (Mentag, et al., 2003; Phazang et al., 2020).

The communicator of indolicidin Rev4 has been articulated in the tobacco and another *Arabidopsis* and was vigorous in contradiction of *Peronospora tabacina*, another one is *Pseudomonas syringae* pv. tabaci and last one is *E. carotovora* contagions (Xu et al., 2006). Artworks of antimicrobial peptides from bacterial basis uttered in the plants are irregular (Phazang et al., 2020; Su et al., 2020). The AFP fungiform protecting from the *Aspergillus* giganteus was uttered in the rice and intricate itself as the shell in contradiction of *M. grisea* (Coca, et al., 2006; Phazang et al., 2020).

It can help to exploit the recognition of peptide–antibiotic amalgamations that will meritoriously eradicate resistant bacteria and by understandings for its natural biology in respect to lesser possibility of security damage as well as circumvent the disaster of resistance. Various techniques such as gene editing, protein engineering or computational tools can provide help to face the industrial requirements which could stimulate development to squeeze proteins or peptides as well as can be used to screen latent peptide sequences with antimicrobial properties, and for computer-aided discovery of AMPs as well.

Future Perspectives: Antimicrobial peptides (AMPs) have a crucial role in natural defense compounds grounded on drugs because of its vast potential as a novel biopharmaceutical product for both humans and plants (Lehel, et al. 2020). There are many investigations which played an important role to provide fundamental resources for the expansion of newly discovered AMP-dependent therapeutics fewer prone to confrontation, a feature essential to circumvent any potential interference with our distinctive immune system (Spohn et al., 2019, Phazang et al., 2020).

Antibiotic-resistant bacteria recurrently demonstrate indemnity sensation to antimicrobial peptides, it will be further helped out to exploit the recognition of peptide–antibiotic amalgamations that will meritoriously eradicate resistant bacteria besides will slow down the novel evolution of resistance towards antibiotics (Lázár et al., 2018; Lei et al., 2019). Scientific based progress, predominantly in mass spectrometry (MS) as well as nuclear magnetic resonance (NMR), have been contributed inside recognizing and illuminating the assembly of innovative AMPs, particularly non ribosomal peptides that cannot be recognized by genomics tactics. Many non-plant AMPs are representing the prospective for plant disease immunity which are frequently verified by in vitro assays analysis. Categorization of AFPs with bio based activities has inordinate connotation for the improvement of novel antifungal therapeutic drugs in contradiction of antibiotic-resistant (Zhang et al., 2020).

Now, the utmost task is remained for the functional authentication of contender AMPs inside plants via transgenic experiments, mainly familiarizing non-ribosomal AMPs into crop yields (Breen et al., 2015; Zhang et al., 2020). Furthermore, these peptides highlighted the effective results towards its efficiency, wide specificity, low toxicity, fewer drug interfaces, biologically multiplicity as well as various characteristics for direct targeting. Pharmaceutical industries should demeanor specific clinical trials for better peptide drugs developments. There is a need to perform various de novo preclinical as well as clinical trials for effective peptides drug development so that modification of pre-treated peptides can be done at including its chemicals as well as physical characteristics (Boparai, et al. 2020).

It is essential to produce Imitation and enduring AMP analogs so that the drawbacks of their natural peptides should be overwhelmed besides the latent complications for the drug applicants can also be solved (Lei et al., 2019). The inclusive continuum of actions for these peptides will permit the chance to reconnoiter their paybacks as alimentary supplements as well as , (Bakare et al., 2020, Lazzaro et al., 2020). Peptides which have proved themselves as bacterial and fungal resistants by membranolytic as well as non-membranolytic mechanisms such as KW4 peptides, provide specific template progress of novel classes of antifungal or antibacterial drugs by showing themselves no cytotoxic activity to cultured humanoid keratinocyte cells. Nonlytic mechanism of action makes further open the doors toward the future anti-candidal agents, (Ramamourthy et al., 2020).

Natural AMPs of various bacteria are beneficial can demonstrate the strategy of AMP variants for undertaking the intensifying numeral of multi-drug-resistant contagions, as an appropriate supernumerary for conformist antibiotics (Nesa et al., 2020). It can also provide basic understandings for its natural biology in respect to lesser possibility of security damage as well as circumvent the disaster of resistance which is presently encrustation orthodox antibiotics. Various techniques such as gene editing, protein engineering or computational tools can provide help to face the industrial requirements which could stimulate development to squeeze proteins or peptides. These techniques and tools can e utilized screen latent peptide sequences with antimicrobial properties, as well as for computer-aided discovery of AMPs

CONCLUSION

Antimicrobial peptides can play their crucial role in field of agriculture to enhance the food production and crop development by overcoming the plant pathogens as well as controlling various diseases of plants. AMPs are environment friendly, less cost and can be utilized instead of chemical pesticides. AMPs are used as bio-pesticides

which are secreted from numerous microorganisms beside it some transgenic plants also express it to control the plant diseases and plant pathogens but there are some social concerns and limitations because of less toxicity and less stability of these compounds that's why transgenic plants are not commercially marketed. There is need to utilize various latent techniques of biotechnology so that toxicity of these compounds can be reduced as well as stability of it can be enhanced. Though, it can prove as a novel compound in the drug discovery.

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Helicobacter pylori leads to the Activation of Host Cell Intrinsic P53 Network Via DNA Damage: A Deterministic Model

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ABSTRACT

Helicobacter pylori (*H. pylori*) bacterium is widely studied risk factor of gastric cancer. The mechanism by which *H. pylori* induces gastric carcinogenesis, has been studied by various researcher. However, a molecular level mechanism by which *H. pylor*i enters into the cells causes p53 modulation leads to stress and gastric cancer developments, is still an open question. In the present studied we developed p53-ROS-*H. pylori* model integrated deterministic model to show the molecular level interaction between p53 proteins network and *H. pylori* bacterium via reactive oxygen species. The interactions among p53 network proteins, reactive oxygen species and *H. pylori* are described by system of a set of ordinary differential equations. The rate of the reaction has been set by using pervious experimental and theoretical works. These sets of differential equations further numerically solved by using standard Runge-Kutta fourth order method at high computational cost. Numerical simulation results showed that as the accumulation of *H. pylori* (kHP<0-0.5>) in the cells increases, the instability of p53 network systems are also increase. Various phases of the system have been observed viz normal phase, stable to damp phase, damped to stable phase and stationary phase. These phases correspond to various stages of cell cycle. The present model clarifies the molecular level interactions between *H. pylori* via reactive oxygen species. This model suggests that as the accumulation of *H. pylori* reaches at a particular threshold value, it leads to the cancerous progression. The suggested model will be very useful to understand the temporal dynamics of gastric cancer progression due to *H. pylori* bacterium. Further, study is needed in realistic environment.

KEY WORDS: HELICOBACTER PYLORY, P53, GASTRIC CANCER, DNA DAMAGE, TEMPORAL DYNAMICS.

ARTICLE INFORMATION

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INTRODUCTION

The expression of p53 tumor suppressor protein in the cell is reported as marker of the most of the cancer (Blagih et. al., 2020). It acts as a key regulator in the cellular network and response to a variety of cellular stress, including DNA damage, hypoxia, nucleotide depletion, nitric oxide and aberrant proliferative signals (such as oncogene activation) (Moll and Petrenko, 2003). But in most cancerous cells, p53 tumor suppressor signaling pathway usually found in inactivated condition (Blagih et. al., 2020). In normal cells, stress induced p53 leads to the participation in various key cellular processes, such as, cell-cycle arrest, senescence and most importantly tumor clearance to prevent cancer cell formation (Leeuwen, 2020, Zambetti, 2007). Moreover, it is reported that, activated p53 protein safeguards the organism against the propagation of cells that carry damaged DNA with potentially oncogenic mutations (Moll and Petrenko, 2003).

p53 act as transcription factor of MDM2 protein. Moreover, MDM2 act as negative feedback regulator of p53 protein which is also considered as an effective approach in cancer therapy (Leeuwen, 2020, Michael and Oren, 2003). MDM2 physically blocking its ability binding itself to p53 leads to the transactivation of p53 gene expression, and stimulating its degradation (Fang et. al., 2000). Further, the interaction of N-terminal domain of MDM2 with transactivation domain of p53 (p53TAD) performs a significant role in the regulation of the G1 checkpoint of the cell cycle and cell function (Boyd et. al., 2000). ROS (Reactive oxygen species) are chemically reactive molecules containing oxygen ions and peroxides (Amendola et. al., 2013). They are synthesized from normal metabolism of oxygen as a natural byproduct and play important roles in cell signaling and homeostasis (Devasagayam et. al., 2004; Rowe et. al., 2008 and Poetsch, 2020).

However, ROS level inside cell can be elevated by UV irradiation or heat exposure which can drive the cell at different stress states (Devasagayam et. al., 2004) High level of ROS can promote DNA damage, and may probably lead the cell to mutagenesis, carcinogenesis and aging (Poetsh, 2020, Amendola et. al., 2013; Rowe et. al., 2008; Proctor and Gray, 2010). *H. pylori* is a structurally spiral shaped bacterium. It generally present in digestive tract of the human. It is reported that in developing country children are commonly infected by *H. pylori* (Horvat et.al., 2018; Kakelar et.al., 2019, Choi, et. al, 2020).

Studies suggests that control of *H. pylori* leads to the control of gastric cancer (Ford et. al., 2020, Hu et. al., 2020; Sablet et.al., 2011). The inclusion of *H. pylori* in the host cells increases the production of ROS, which further leads to the suppression of the endogenous level of p53 protein via DNA damage and represses to apoptosis in human gastric cells (Handa et. al. 2011; Polk and Peek, 2010). Various studies have been performed to understand the mechanism by which *H. pylori* induces gastric carcinogenesis (Wang et. al., 2020, Handa et. al., 2011, Achanta et. al., 2004). However, how the dynamical behaviour of p53 protein changes at molecular level due to *H. pylori* via reactive oxygen species, is still an open question. In the present study, we try to answer these fundamental questions based on basic deterministic model.

MATERIAL AND METHODS

p53-MDM2-*H. pylori* model.- The model we consider (Fig. 1) is integration of p53-Mdm2 regulatory network (Proctor and Gray, 2008) with stress inducers ROS via DNA damage (Rowe et. al., 2008) and *H. pylori* (Handa et. al., 2011). In this model we assume that *H. pylori* are supposed to be constantly entered in the nucleus after infection. ROS synthesis due to *H. pylori* is assumed to occur with a rate of k12. This ROS synthesis triggers DNA damage with a rate of k14 (Rowe et. al., 2008). Then this DNA damage leads to the activation of ARF with a rate k16 (Lee et. al., 2005) followed by the degradation of ARF with a rate of k17. Further, the activated ARF protein binds to MDM2 with a rate of k18 to control ubiquitination of p53 (Zhang and Xiong, 2001) The ARF and MDM2 interaction results into to the formation of ARF_MDM2 complex (Khan et. al., 2004).

The formation of ARF_MDM2 complex reduces the concentration level of MDM2 in the system which in turn alters the behaviour of p53 (Khan et. al., 2004). On the other hand, dissociation of ARF_MDM2 complex with a rate k19 helps the degradation of MDM2 population and recruit activated ARF. The synthesis of p53 takes place through transcription of p53_mRNA with a rate k2. Further, this p53 synthesis depends on the available p53_mRNA concentration level. At normal condition p53 is generally bound to MDM2 with a rate k8 recruiting a complex p53_MDM2 and after which the dissociation of the complex ubiquitinates p53 with a rate k9 and MDM2 with a rate k12 (Zatorsky et. al., 2006) exhibiting oscillatory behavior of p53 in the model. Further, rate of MDM2 transcription by p53 via production of MDM2_ mRNA, at k5 (Proctor and Gray, 2008).

Hence, the MDM2_mRNA provides intermediary link between p53 and MDM2. The self ubiquitination of MDM2_mRNA is assumed to be with a rate k7. MDM2_ mRNA synthesize MDM2 protein with a rate k6. The self ubiquitination of MDM2 is assumed to be with a rate k11. The molecular species involved in this model are listed in Table 1, and the biochemical reaction channels involved in the model network with their descriptions, kinetic laws and values of the rate constants used in our simulations are given in Table 2.The model biochemical network (Fig. 1) described by the twenty two reaction channels (Table 2) can be described by the following coupled ordinary differential equations (ODE) using Mass action law of chemical kinetics,

$$\frac{dx_1}{dt} = k_2 * x_4 - k_8 * x_1 * x_2 + k_{10} * x_5$$

$$\frac{dx_2}{dt} = k_6 * x_3 - k_8 * x_1 * x_2 + k_9 * x_5 + k_{10} * x_5 - k_{11} * x_2 - k_{18} * x_2 * x_8$$

$$\frac{dx_3}{dt} = k_5 * x_1 - k_7 * x_3$$

$$\frac{dx_4}{dt} = -k_3 - k_4 * x_4$$

$$\frac{dx_5}{dt} = k_8 * x_2 * x_1 - k_9 * x_5 - k_{10} * x_5$$

$$\frac{dx_6}{dt} = k_{12} * x_{10} - k_{13} * x_6 - k_{14} * x_6$$

$$\frac{dx_7}{dt} = k_{14} * x_6 - k_{15} * x_7$$

$$\frac{dx_8}{dt} = k_{16} * x_7 - k_{17} * x_8 - k_{18} * x_8 * x_2 + k_{19} * x_9$$

$$\frac{dx_9}{dt} = k_{18} * x_8 * x_2 - k_{19} * x_9 \qquad \frac{dx_{10}}{dt} = k_1 - k_{20} * x_{10} - k_{12} * x_{10}$$

The set of ODEs can be written in compact form as in the following,

$\frac{dx(t)}{dt} = F(x_1, x_2, \ldots, x_N)$ Where $F = [F_1, F_2, \ldots, F_N]^T$ is the functional vector.

The time evolution of the state vector $\vec{x}(t)$ can be obtained by numerically solving the non-linear coupled differential equations (1)-(11) using standard 4th order Runge-Kutta algorithm for numerical integration (Press, 1992).Consider the state of the system be described by a state vector given by $x(t) = [x_1(t), x_2(t), \ldots, x_N(t)]^T$, where, {x} is the set of concentrations of the respective molecular species, N=10 and T is the transpose of the vector. The model biochemical network (Fig. 1) described by the twenty reaction channels (Table 2) can be described by the following coupled ordinary differential equations (ODE) using Mass action law of chemical kinetics,

$$\frac{dx_i(t)}{dt} = F_i[x_1(t), x_2(t), \ldots, x_N(t)]$$

where, i=1,2,...,N and Fi is the ith function whose form is given in table. The non-linear coupled N ODEs of p53-MDM2-*H. pylori* model are solved using 4th order Runge-Kutta method which is the standard algorithm for numerical integration (Press, 1992) to find the dynamics of the system variables. The simulation is done for 10 days using the parameter values given in Table 2 and starting from an initial condition.

RESULTS AND DISCUSSION

Role of *H. pylori* on p53 dynamics: The impact of *H. pylori* on p53 was studied by keeping fixed k6=0.0001 and allowing to change the values of k10 (Fig. 2). We got three different states namely stable, damped with sustain oscillation and again stable state of p53 driven by *H. pylori* (Fig. 2). The small values of k10 (k_{10} <0>) could not able to provide significant stress to p53 dynamics, and maintains at stabilized state (Fig. 2 E). The further increase in k10 values (<0.005>k_{10} <0.1) Fig. 2 A). This suggests that the increase in concentration of *H. pylori* (k10 coresponds to kHP) in the system drives the system at various stress states, lowering p53 concentration level (Wei et. al., 2010; Polk and Peek, 2010; Handa et. al., 2011). Moreover suggests that extreme values of k_ROS may cause very high DNA damage, such that the damage could not able be repaired back, which could be the condition of apoptotic phase. The excess kHP values induce lowering of p53 concentration level even below normal stabilized p53 state indicating the possibility of switching stress state to cancerous state (Handa et. al., 2011).

Table 1. List of molecular species

S.No.	Species Name	Description	Notation
1.	p53	Unbounded p53 protein	x_1
2.	MDM2	Unbounded Mdm2 protein	x_2
3.	MDM2_ mRNA	Mdm2 messenger mRNA	x_3
4.	p53_ mRNA	p53 messenger mRNA	x_4
5.	p53_ MDM2	Mdm2 with p53 complex	x_5
6.	ROS	Reactive Oxygen Species	x_6
7.	Dam_DNA	Damage DNA	x_7
8.	ARF	Alternative Reading Frame protein	x_8
9.	ARF_ MDM2	ARF and Mdm2 complex	x_9
10.	HP	*Helicobacter pylory* factor	x_10

Modulation of p53 dynamics by *H. pylori*: The impact of *H. pylori* on the amplitude and time period of p53 dynamics is studied (Fig. 3) in order to understand modulation of p53 dynamics driven by stress inducer. The impact of k_HP on amplitude and time period of p53 dynamics is shown (Fig. 3). The lower values in k_HP could not able to make significant change in amplitude of p53 dynamics, and maintains at stable state with high value of constant p53 amplitude (Fig. 3). Further increase in k_HP drives the p53 amplitude to decrease monotonically, and then reach a stable state with minimum amplitude. The behaviour of the time period T_p53 as a function of k_HP shows three regimes, stable, monotonically decreased with increase in k_HP and stable state again. However the different behaviour i.e. the decrease in A_p53 as increase in k_HP may trigger cancer state from stress condition.

Co-existence of states: The phase transition like behaviour of the system dynamics induced by *H. pylori* concentrations available in the system can be well characterized by analyzing the nature of transition time of the p53 dynamics. We define T_1s to be the transition time below (<T_1s>) which the dynamics shows stable state (does not show any oscillation) and above which the dynamics shows oscillatory behaviour. We further define second transition time, T_ds which separates increasing damped and sustain oscillations (Fig. 2). Similarly, T_sd and T_2s are taken as transition times separating sustain and damped oscillation, and damped oscillation and stabilized state. We then calculated T_1s, T_ds, T_sd and T_2s as a function of kHP (Fig. 4 upper panel) where the regimes for T< T_{1s} > and T < T_{2s} > corresponds to stabilized states, regimes between T_{ds} < T < T_{1s} and T_{2s} < T < T_{sd} corresponds to damped states and T_{sd}< T < T_{ds} indicates the sustain oscillation state regime.

Table 2. List of chemical reaction, Kinetic Laws and their rate constant

S. No.	Name of the process	Kinetic Law	Rate Constant	References
1.	Inclusion of *Helicobacter pylor*	k_1	$1*10^{-4}sec^{-1}$	(Handa et. al., 2011)
2.	p53 mRNA translati	k_2x_4	$8*10^{-2}sec^{-1}$	(Moll and Petrenko, 2003; Proctor and Gray, 2008)
3.	p53 mRNA synthesis	k_3	$1*10^{-3}sec^{-1}$	(Moll and Petrenko, 2003; Proctor and Gray, 2008)
4.	p53 mRNA degradation	k_2x_4	$1*10^{-4}sec^{-1}$	(Moll and Petrenko, 2003; Proctor and Gray, 2008)
5.	Mdm2 mRNA synthesis	k_5x_1	$1*10^{-4}sec^{-1}$	(Moll and Petrenko, 2003l; Proctor and Gray, 2008)
6.	Mdm2 synthesis	k_6x_3	$495*10^{-5}sec^{-1}$	Moll and Petrenko, 2003; Proctor and Gray, 2008)
7.	Mdm2 mRNA degradation	k_7x_3	$1*10^{-4}sec^{-1}$	Moll and Petrenko, 2003; Proctor and Gray, 2008)
8.	p53 Mdm2 complex forma-tion	$k_8x_1x_2$	$1155*10^{-5}sec^{-1}$	Moll and Petrenko, 2003; Proctor and Gray, 2008)
9.	Mdm2 creation	k_9x_5	$825*10^{-5}sec^{-1}$	Moll and Petrenko, 2003; Proctor and Gray, 2008)
10.	Dissociation of p53 Mdm2 complex	$k_{10}x_5$	$1155*10^{-5}sec^{-1}$	Moll and Petrenko, 2003; Proctor and Gray, 2008)
11.	Mdm2 degradation	$k_{11}x_2$	$433*10^{-5}sec^{-1}$	Moll and Petrenko, 2003; Proctor and Gray, 2008)
12.	ROS formation	$k_{22}x_{10}$	$1*10^{-4}sec^{-1}$	(Proctor and Gray, 2010; Proctor and Gray, 2008)
13.	Degradation of ROS	$k_{18}x_6$	$2*10^{-2}sec^{-1}$	(Proctor and Gray, 2010; Proctor and Gray, 2008)
14.	Initiation of DNA damage	$k_{14}x_6$	$2*10^{-2}sec^{-1}$	(Proctor and Gray, 2010; Proctor and Gray, 2008)
15.	DNA repair	$k_{15}x_7$	$2*10^{-5}sec^{-1}$	(Proctor and Gray, 2010; Proctor and Gray, 2008)
16.	Activation of ARF	$k_{16}x_7$	$33*10^{-5}sec^{-1}$	Zhang and Xiong, 2001; Proctor and Gray, 2008; Khan et. al., 2004)
17.	Degradation of ARF	$k_{17}x_8$	$1*10^{-4}sec^{-1}$	(Zhang and Xiong, 2001; Proctor and Gray, 2008; Khan et. al., 2004)
18.	ARF Mdm2 complex for-mation	$k_{18}x_8x_2$	$1*10^{-2}sec^{-1}$	(Zhang and Xiong, 2001; Proctor and Gray, 2008; Khan et. al., 2004)
19.	Dissociation of ARF Mdm2 complex	$k_{19}x_9$	$1*10^{-3}sec^{-1}$	(Zhang and Xiong, 2001; Proctor and Gray, 2008; Khan et. al., 2004)
20.	Degradation of Micro RNA	$k_{20}x_{10}$	$5*10^{-2}sec^{-1}$	(Handa et. al., 2011)

Figure 1: A schematic diagram of p53-Mdm2-*H. pylori* network model.

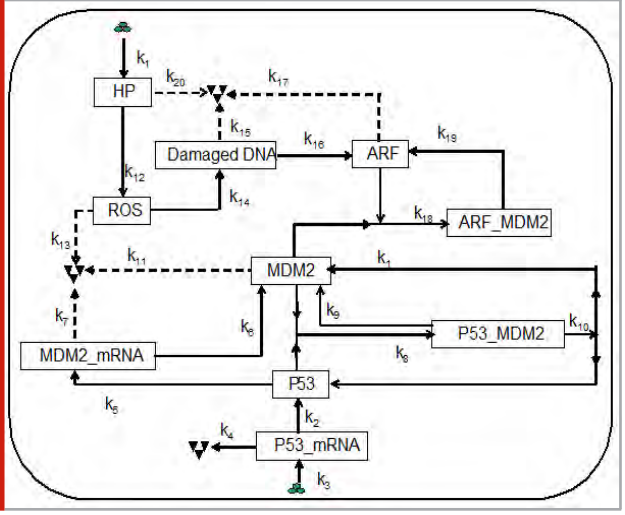


Figure 2: The p53 temporal behaviour when *H. pylori* act as a stress inducer

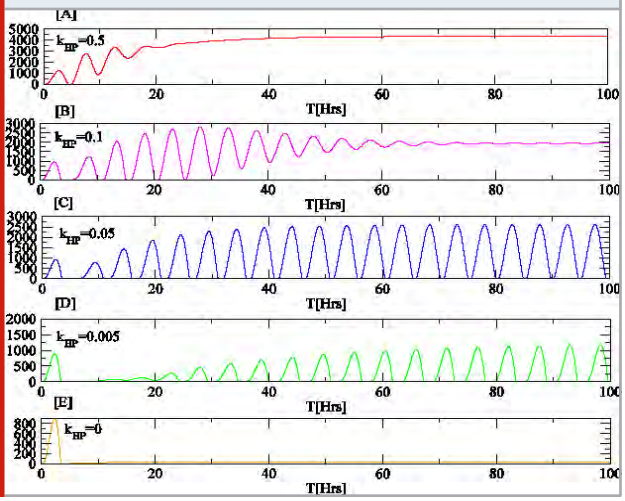


Figure 3: A comparative plot for the amplitude verses k_HP and the time period variation verses k_HP.

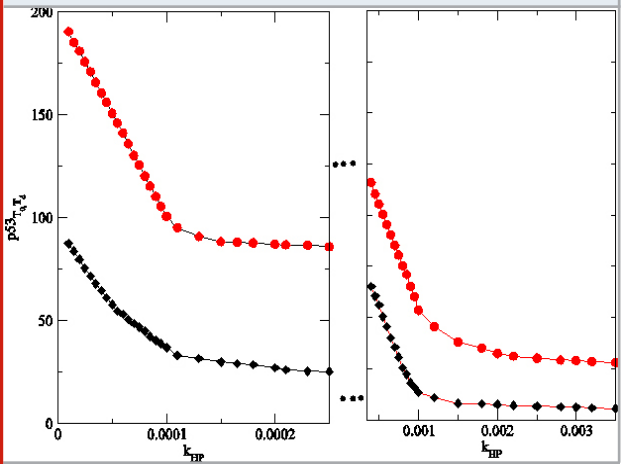
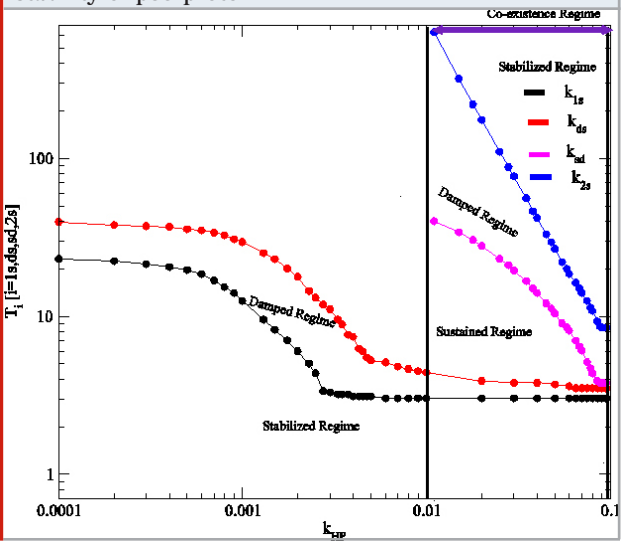


Figure 4: A phase diagram showing impact of k_HP on stability of p53 protein



The co-existence of the four states can be obtained in the case of *H. pylori* induced p53 dynamics. Within this co-existence regime, the regions of damped, sustain and stabilized states are observed (Fig. 4). The results indicate that there is a certain range of kHP (region bounded by two lines) where one can find the four states together including two stable states for any value of kHP (Fig. 4). This means that for any concentration of ROS which is due to *H. pylori* in the system corresponding to any values within this range, the p53 dynamics will stay stable for some interval of time, then it will start activated to reach maximum activation within certain interval of time and after sometime it will stay stable again. In the other regimes, at most we can find three states. This co-existence of the states indicate that exposure of the system to constant *H. pylori* concentration can drive the system from normal to stress and then to apoptosis.

CONCLUSION

The variation in concentration of reactive oxygen species in cellular system triggers to the changes in the p53 dynamics (various stress states). Further, the introduction *H. pylori* to the system shows inhibitory effect on p53 production and switching of stress states by varying *H. pylori* concentration. Present model suggests that *H. pylori* accumulation in host gastric cells, triggers cancerous progression. The obtained results are quite interesting and provide hidden molecular level information regarding the activity of *H. pylori* that it can probably switch the system to cancerous state. Moreover, the impact of the *H. pylori* on p53 regulatory pathway should be further studied in realistic system in order to capture the state switching mechanism quantitatively and to understand the role of noise in the cellular process.

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Insilico Docking Studies of Phytomolecules as Anti-Breast Cancer Agents

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ABSTRACT

Breast cancer is the most frequent cancer among women. The present study focuses on the comparative in-silico based investigation of the plant natural compounds namely Cubenin, Curcumin, Delta Cadiene, Eugenol, Terpene and Quercetin binding efficacy towards HER2 and their intermolecular interactions were compared with the commercial drugs:Cyclophosphamide, Doxorubin, Letrozole, Methotrexate and Tamoxifen.The comparative molecular docking was performed with the natural compounds and the synthetic drugs used in breast cancer treatment against the target HER2. The molecular docking analysis was done using Discovery Studio. The ADME properties were also studied. The observation of the common binding site for all the ligands confirms the binding pocket; where the isolated compound agrees well with the binding residues and thus can be optimized further to arrive at a molecule that has a high binding affinity and low binding constant. The results of the docking studies carried out on HER2 provide an insight for the natural compounds having druggable properties. These results are supportive to confirm the natural compounds from plants as a better lead for cancer therapeutics. Thus, the results of the docking studies carried out on HER2 corroborate to the findings that the most suitable drug like properties are possessed by the compound. In comparison with other compounds natural compounds are better and it is druggable. This provides evidence of how a natural compounds from plants can be a source of potential anti- cancer agent. The preclinical studies will pave way for a potential anti-cancer compound.

KEY WORDS: DOCKING; ADME; HER2; DISCOVERY STUDIO.

ARTICLE INFORMATION

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INTRODUCTION

Cancer is the leading causes of death globally where 1 in 6 die. Low economic prosperity and lack of awareness of prevention strategies are noted to be vital factors contributing to the burden and incidence for cancer ERBB is abbreviated from erythroblastic oncogene B, a gene isolated from avian genome. Heterodimerization of this receptor with other members of the EGFR family, typically owing to HER2 overexpression, results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the heterodimer and initiates a variety of signalling pathways leading to cellular proliferation and tumorigenesis (Yarden, et al.,2001)).Amplification or over-expression of this oncogene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer. According to TCGA (The cancer Genome Atlas) data portal HER2 aberration studied in various solid tumours associated with cervical, bladder and were have limited therapeutic options (Cancer Genome Atlas,2013). Approximately, 70% of death due to cancer occurs in low- and middle-income countries. Receptor tyrosine-protein kinase erbB-2, otherwise known as HER2, a significant member in the EGFR family of receptor tyrosine kinases family, (Pegram et al.,2020 Siegel et al.,2020).

So, outspreading the HER2-based therapeutic option beyond breast cancer to other solid tumours with HER2 overexpression will be beneficial to the affected individuals. In recent years, the protein has become an important biomarker and target of therapy for approximately 30% of breast cancer patients. In this study, the natural compounds from plant source is considered based on their pharmacological properties. The compounds have been characterized in detail for breast cancer. The natural source would overcome the existing synthetic drugs in mode of action and also reduce the side effects caused by the commercial compounds (Christy and Swetha 2019). The present study focuses on the comparative in-silico based investigation of the plant natural compounds namely Cubenin, Curcumin, Delta Cadiene, Eugenol, Terpene and Quercetin binding efficacy towards HER2 and their intermolecular interactions were compared with the commercial drugs:Cyclophosphamide, Doxorubin, Letrozole, Methotrexate and Tamoxifen. The in-silico approach enables one to screen for ADMET properties of vast number of molecules within short span thus reducing the time and is a non- expensive and non-tedious process with great accuracy, which is not possible in standard experimental methods.

MATERIAL AND METHODS

HER2/ERBB2 expression datamining: The cancer genome data repository was used to assess the HER2 positive cancer types and the Figure 1 revealed that HER2 expression was seen in other solid tumors associated with bladder, uterine, cervical regions also (TCGA GDC portal).Human pathology atlas module of human protein atlas also revealed the HER2 varied expression with breast, colorectal, cervical, renal and urothelial cancers and dataset related information's listed in Figure 2. (Uhlen et al.,2017)

Figure 1: HER2 aberrations in various cancer types

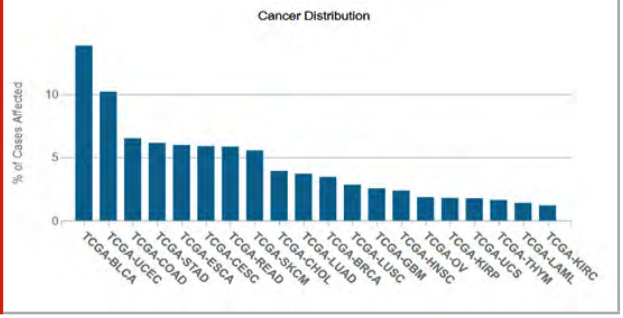
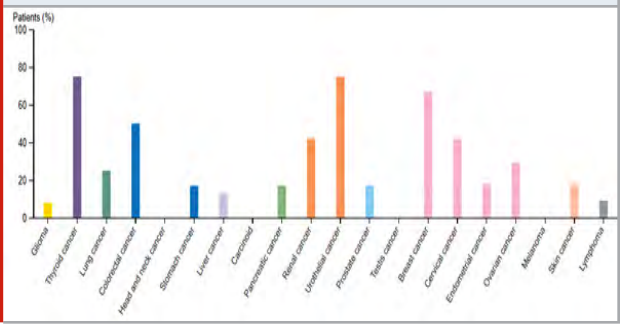


Figure 2: HER2 profile based on human pathology atlas



HER2 protein structure quality assessment: The target is retrieved from the Protein Data Bank, RCSB-PDB (H.M Berman et al.,2000). Upon searching the protein structure for the HER2 protein, the structure 3RCD has been chosen the fitting protein model (Ishikawa et al.,2011). Stereochemical properties based assessment revealed that nearly 90% of residues are residing in the allowed region( ).Before proceeding with the docking the protein structure has been energy minimized to find an arrangement in space of a collection of atoms where, the net inter-atomic force on each atom is acceptably close to zero and the position on the potential energy surface (PES) is a stationary point . the energy value of the protein molecule after Energy minimization is-44093.590. The energy minimisation was done in Swiss PDB viewer, it is a downloadable software (Swiss PDB viewer). Then after this the protein structure was uploaded in the Discovery Studio, where the force field CHARMM was applied and then receptor binding pockets where determined so as the ligand fits in.

Preparation of Ligand: The commercial compounds Cyclophosphamide, Doxorubin, Letrozole, Methotrexate and Tamoxifen and the natural compounds Cubenin, Curcumin, Delta Cadiene, Eugenol, Terpene and Quercetin three dimensional structures were retrieved from Pubchem database (Kim et al.,2016). Then the ligands were analysed if they are druggable or not using the online tool, which is based on the Lipinski's rule of 5 (Jayaraman et al.,2012).

ADMET Profiling: The ADME profiling was done using the Accelrys Discovery Studio 2.5 software. The lead compounds from natural resources fail to enter the market due to the poor pharmacokinetic properties. So, designing ligands satisfying the Adsorption, Distribution, Metabolism Elimination and Toxicity (ADMET) properties will go through the market as a good drug. The drugs should be orally absorbed and distributed to the site of action and eliminated from the body without leaving any traces, which produces adverse effects. Hence, the tools and computer-aided methods, nowadays, have become popular in identifying good drug candidate molecule.

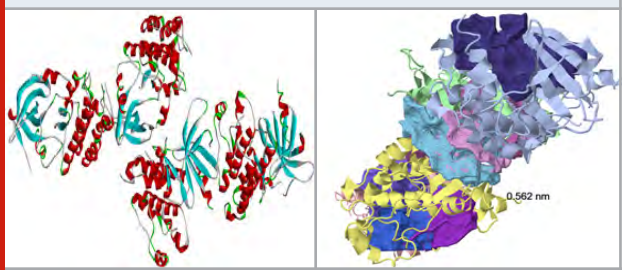
Molecular Docking: Molecular docking is a key tool in structural molecular biology and computer-assisted drug design (Venkatachalam et al.,2003). The goal of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. The receptor-ligand interaction uses Ligand Fit protocol, which docks the ligands into the binding site of receptors using shape-based searching and Monte Carlo sampling of ligands (Gangopadhyay et al,2017).

The parameters used are PLP1 algorithm for energy grid and conjugate gradient for energy minimization. The scores for docked poses are obtained by LigScore1, LigScore which predicts the binding affinities, -PLP1, -PLP2 known as Piecewise Linear Potential scoring function calculates both the shape and hydrogen bond complementarity of poses to the active site and Jain scoring function which scores the non-covalent protein-ligand interactions(Krammer et al.,2005;Jain 1996).

RESULTS AND DISCUSSION

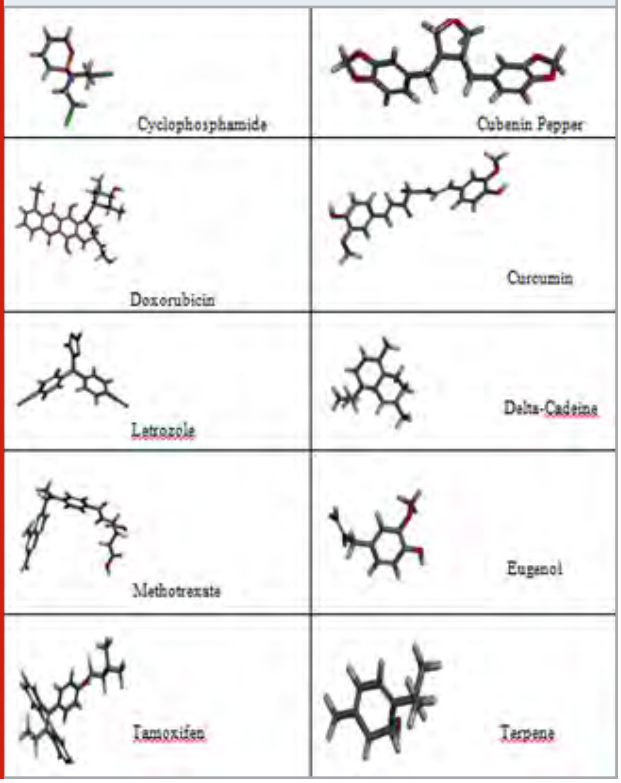
The protein structure retrieved from RCSB-PDB: There were eight druggable cavities populated in the chains of HER2 three-dimensional structure and were ranked based on the cavity score and drug score. In general The ligandability score used to assess the possible small ligands affinity to the specified cavity, whereas druggability scores reveal and rank the good target for HER2 binding .Specified caities of HER2 were predisposed based on cavity volume, pocket lip size, hydrophobic volume, cavity surface area, and hydrogen-bond-forming surface area of the chosen HER2 cavity.

Figure 1a: Three-dimensional structure of HER2 and druggable cavities



The Ligand Structures retrieved from Pubchem: PubChem an open source data repository for chemical substances and their related biological activities. This structure based search module compiled with variety of chemical structure format like SMILES, SMARTS, InChI, CID, molecular formula and SDF format. Since our ligandfit module accepts the SDF format we have downloaded all the Plant based natural compounds and synthetic compounds in SDF format. HER2 specific synthetic drugs as well natural compounds were listed in the Figure 2.

Figure 2: Natural compounds and synthetic compounds three dimensional structures



ADME Studies: ADME profiling of the natural compounds aids in proving the compounds are less toxic and it passes through various barriers.

Lipinski Rule: Lipinski rule of 5 helps in distinguishing between drug like and nondrug like molecules. It predicts high probability of success or failure due to drug likeness for molecules complying with 2 or more of the following rules:

Molecular docking studies using ligandfit module: The docking studies where done to find the Receptor-Ligand Interactions LigandFit assess the small molecules affiinity into the HER2 protein active site by applyinng its shape complementary screening. In general docking process list the top conformations of ligand after the energy minimization method based on steepest descent method and conjugate gradient method. Ligandfit uses the consensus scoring to minimize the false positive. Proposed consensus scoring method was depends on LigScore1, LigScore2, piecewise linear potential 1 (PLP1),

piecewise linear potential 2, potential mean force (PMF), Jain score.

The above are the docking 2D & 3D images of the receptor with the ligand, with their docked positions and the respective amino acids. The dock scores are evaluated based on the parameters of lig score, PLP1, PLP2,JAIN score and PMF score.

Table 1. ADME Profiling of Natural Compounds

Compound	ADMET-BBB	Solubility	Hepatotoxicity Probability	CYP2D6 Probability	AlogP98	ADMET_PSA_2D
Cubenin	-0.203	-4.448	0.708	0.227	3.194	65.466
Curcumin	-0.544	-3.537	0.887	0.376	3.554	94.092
Delta Cadien	1.373	-5.713	0.258	0.178	4.939	0
Eugenol	0.172	-2.416	0.35	0.029	2.579	29.745
Terpene	0.242	-2.285	0.37	0.029	2.346	20.815

Table 2. ADME Profiling of Synthetic Compounds

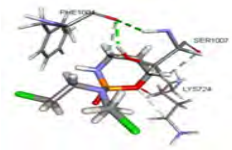
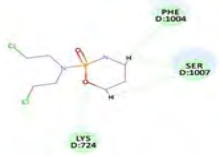
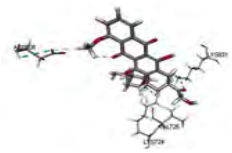
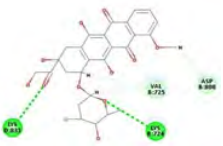
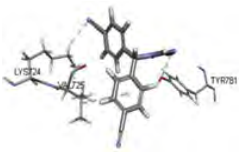
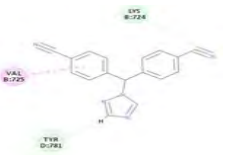
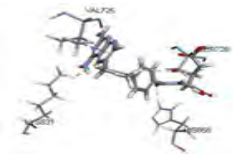
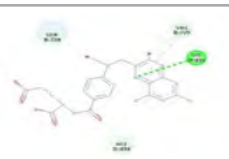
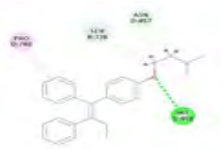
Compound	ADMET-BBB	Solubility	Hepatotoxicity Probability	CYP2D6 Probability	AlogP98	ADMET_PSA_2D
Cyclophosphamide	-0.723	-1.296	0.589	0.059	0.33	42.393
Doxorubicin		-4.798	0.9	0.455	-0.044	209.31
Letrozole	-0.471	-3.828	0.841	0.663	2.749	73.74
Methotrexate		-4.018	0.741	0.178	0.376	207.82
Tamoxifen	1.605	-6.711	0.894	0.702	6.319	12.282

Table 3. Lipinski Rule of Druggability for Synthetic Compounds

	MASS	HBD	HD A	Log P	MR
Cyclophosphamide	356	1	6	2.5102	91.47678
Doxorubicin	368	2	6	3.369898	102.0166
Letrozole	204	0	0	4.725199	66.74298
Methotrexate	164	1	2	2.1293	48.55979
Tamoxifen	152	1	1	2.2797	47.30179

Table 4. Lipinski Rule of Druggability for Natural Compounds

	MASS	HBD	HD A	Log P	MR
Cubenin	356	1	6	2.5102	91.47678
Curcumin	368	2	6	3.369898	102.0166
Delta Cadeine	204	0	0	4.725199	66.74298
Eugenol	164	1	2	2.1293	48.55979
Terpene	152	1	1	2.2797	47.30179

Table		
Synthetic compounds	Intermolecular interaction of HER 2 and synthetic drug compounds	
Cyclophosamide Doxorubicin		
Letrozole		
Methotrexate		
Tamoxifen		
		

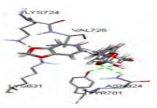
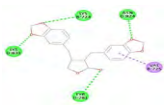
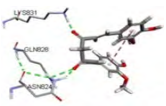
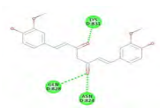
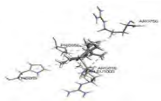
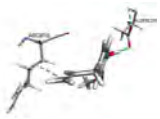
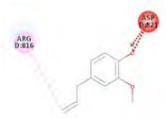
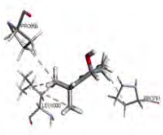
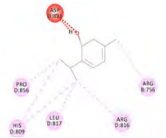
Natural compounds	Intermolecular interaction of HER2 with plant-based compounds	
Cubenin		
Curcumin		
Delta cadeine		
Eugenol		
Terpene		

Table 5. Dock scores of Synthetic compounds

Compounds	Lig score 1	Lig score 2	PLP1	PLP 2	JAIN	PMF
Cyclophosphamide	-0.168	1.652	30.288	32.14	1.035	13.01
Doxorubin	2.961	4.52	45.541	41.176	1.873	41.452
Letrozole	1.375	3.749	50.645	46.388	-0.093	19.141
Methotrexate	2.883	4.105	38.079	30.442	-2.452	34.579
Tamoxifen	1.878	3.238	34.473	32.632	-1.709	41.811

Table 6. Dock scores of natural compounds

Compounds	Lig score 1	Lig score 2	PLP1	PLP 2	JAIN	PMF
Cubenin	3.563	4.065	47.011	48.088	0.271	37.173
Curcumin	3.235	4.174	45.657	44.984	-1.815	50.533
Delta Cadiene	6.009	2.93	42.196	41.498	1.496	39.294
Eugenol	1.321	2.519	19.224	20.169	1.367	30.905
Terpene	1.368	2.549	26.964	29.576	-0.044	33.962

CONCLUSION

Thus, the results of the docking studies carried out on HER2 corroborate to the findings that the most suitable drug like properties are possessed by the compound. In comparison with other compounds natural compounds are better and it is druggable. This provides evidence of how a natural compounds from plants can be a source of potential anti- cancer agent. The preclinical studies will pave way for a potential anti-cancer compound. The study aimed in finding the compatible natural lead molecules from plants and it shows that the compounds are druggable based on the Lipinski's rule and the Insilico toxicity study studies are also positive, adding advantage for the compounds to be druggable. With the docking studies , the compounds that dock with the target has been found. The dock scores are also supporting for the further study. The work will be continued on breast cancer cell line study invitro and then in vivo studies.

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Adhesive Bond Strength of Resin Cements to CAD-CAM Hybrid Ceramic Materials

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ABSTRACT

The present study was conducted to assess the adhesive bond strength of the resin cements to hybrid:computer-aided design (CAD) / computer-aided manufacturing (CAM) ceramics under the standard surface treatment (hydrofluoric etching). Two types of hybrid ceramic: Vita Enamic (VE) and Lava Ultimate (LU) were used to prepare a total of 30 specimen blocks for each ceramic. Each material was divided into three different subgroups depending on the type of cement used [Rely X Unicem (Gp A), Rely X ARC (Gp B) and GIC (Gp C)] (n= 10). Each specimen surface was polished and treated with hydrofluoric acid (10%). Subsequently, silane coupling agent was applied to the specimen in group A and B. Using a putty mould index, cement was build up on each specimen block. After high intensity light cure of group A and B specimen and settling down of GIC in group C, each block was tested for shear bond strength under a load in universal testing machine. Ten samples from each group were assessed for modes of failure. Data was assessed using analysis of variance and Tukey multiple comparisons test. Comparison between the cements in each hybrid ceramic revealed that the highest mean value was for groups B-VE (18.22 ± 1.25) and B-LU (16.56 ± 1.31) and least values were for group C-VE (11.44 ±1.84) and C-LU (10.68±2.17). Further comparison between the two types of hybrid ceramics presented VE to have significantly higher ($p<0.05$) bond strength. The mode failure mostly observed was adhesive followed by the cohesive and admixed. The study displayed a significant influence of different types of cements on SBS of CAD/CAM hybrid ceramics ($p<0.05$). Therefore, to achieve maximum adhesive bonding strength among hybrid ceramic materials, a compatible luting cement and surface treatment is critical.

KEY WORDS: VITA ENAMIC, LAVA ULTIMATE, HYBRID CERAMIC, CAD/CAM CERAMICS AND HYDROFLUORIC ACID.

INTRODUCTION

Modern era of restorative dentistry uses the computer-aided design (CAD) / computer-aided manufacturing (CAM) technology for deliverance of swift production and services (Kassem et al., 2012 Li et al 2014). This chair side service allows faster, improved and efficient quality of products. The system has scanners that capture the structures and displays virtual image of the tooth anatomy for the computer system to design exact similar 3 D restoration to be replaced in the mouth (Elsaka, 2016, Awada and Nathanson, 2015). The computer imaging captures the precise marginal outline and presents with an optimum internal fit for the fixed prosthodontics (Awada and Nathanson, 2015).

Currently, a broad range of materials are available for digital manufacturing process. The decision to use the appropriate CAD/CAM material for the restorative

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material is challenging at times. The materials are divided into two categories, ceramics and composites, including aluminium-oxide, yttrium tetragonal zirconia polycrystals, feldspathic glass ceramics, leucite-reinforced glass ceramics, lithium disilicate glass ceramics, and composite blocks (Elsaka, 2015 Barutcigil et al., 2019).

Composite materials are usually softer, easy to mould, finish and adjust and are less abrasive to opposing teeth; however, show increased wear and tear (Kassem et al., 2012). Nevertheless, ceramic has higher esthetics property, more biocompatibility, resistant to discoloration and wear resistance (Awada and Nathanson, 2015). However, these materials are more susceptible to fractures. Therefore, authors have suggested hybrids for ceramics to stabilise the material.Hybrid ceramics are formulated by mixing two types of materials to enhance the properties of the material and longevity of the restoration. The hybrid ceramic consists of a principal ceramic complex (86 weight %) supported by an acrylic polymer meshwork (14 weight %)(Vita Enamic; VITA Zahnfabrik, Bad Sackingen Germany; Lava Ultimate LAV; 3M ESPE, St. Paul, MN, USA), comprising of a highly cured resin matrix, which is heavily filled with nanoceramic particles (up to 80% by weight) and ceramic nanoclusters (Mörmann et al., 2013, Duarte et al 2016).

These are also known as Polymer-infiltrated ceramic network (PICN), a dual phase ceramic and composite combination (Elsaka, 2016). These materials offer improved biocompatibility and translucency with reduced brittleness and increased flexibility (Kassem et al., 2012, Güngör et al., 2016). Adopting the CAD/CAM material accounts for accuracy in restoration and improves the fracture toughness compared to the ceramics. Ceramic prosthesis have conventionally been cemented with water based cements including, glass-ionomer cement or resin-modified glass-ionomers, particularly in case of zirconia ceramic (Egbert et al., 2015). However due to the failure of water based cements, the use of resin-based cements is recommended for long-term retention and durability (Duarte et al., 2016, Selz et al., 2016, Acar et al., 2016).

Resin cements are common adhesives used to cement indirect all-ceramic restorations. A critical factor in the longevity of indirect restoration is the prevention of micro leakage and positive marginal adaptation, which depends on the adhesive bond strength (Cekic-Nagas et al., 2016). Clinical trials conducted to assess bond strength, has revealed that majority of failures were outcomes of fragmenting, fracture, secondary caries, and debonding of cements (Cekic-Nagas et al., 2016, Flury et al., 2016). Multiple techniques and surface treatments to increase the surface energy creating durable bonds have shown to improve bonding outcomes (Elsaka, 2015, Güngör et al., 2016). Hydrofluoric (HF) acid is considered an effective method for chemical and micromechanical retention of indirect ceramic restorations to cements (Barutcigil et al., 2019).

HF acid dissolves the glassy phase ceramic to create minute interlocking retentive areas (Peumans et al., 2016). Moreover, silane coupling agents are applied to increase the surface wettability through the formation of silane covalent bonds between silica particle in ceramic and methacrylate groups of resin cements (Peumans et al., 2016). At present in current literature, limited data is available in relation to the bond strength of resin cement to hybrid CAD/CAM ceramics. It is hypothesized that no significant difference between the shear bond strengths of the different cements bonded to the hybrid CAD/CAM ceramics will be observed. Therefore, the aim of the present study was to evaluate the adhesive bond strength of the resin cements to the hybrid CAD/CAM ceramics under standard surface treatment.

MATERIAL AND METHODS

The present study was conducted after the approval from the institutional board to demonstrate the adhesive bond strength of resin cements to hybrid CAD-CAM ceramic material. The study uses two types of hybrid CAD/CAM ceramics namely, Lava Ultimate (LU) and Vita Enamic (VE).

Specimen preparation: 30 specimens of each material; group A (VE) and group B (LU) with dimensions 10x10x2mm were prefabricated into clear cut rectangular blocks using a diamond saw (Isomet 1000; Buehler Ltd., Lake Bluff, IL) under a water coolant. Each block was embedded into the acrylic resin dough (Panacryl, Arma Dental, Istanbul, Turkey) to create a flat base for the specimen block followed by carbide polishing. One surface of each block was prepared for a wet ground surface suitable for attachment using a 600 grit silicon carbide (SiC) paper. Subsequently, the specimens were stored in distilled water for 24hrs. Each prepared block surface was etched with 10% of HF acid (Angelus Dental, Londrina, Brazil) for 2 minutes, rinsed in cold distilled water and air dried for 1 minute. Thirty specimens for each hybrid ceramic material (VE and LU) were divided into a total of three groups based upon the three types of cements [Rely X Unicem (Gp A), Rely X ARC (Gp B) and GIC (Gp C)] used (n=10). The materials used in this study are presented in Table 1.

Each group was divided into three Subgroups:Group A–VE: Specimens were coated with a silane coupling agent (SingleBond Universal; 3M ESPE, St.Paul, MN), applied with a microbrush for 60 seconds over the etched surface followed by an air dry for 10 seconds. After the surface preparation, the Rely X unicem cement is build-up using a teflon mold (2 mm diameter, 4 mm thickness). The cement was polymerised with a high-intensity light cure (3M ESPE, St. Paul, USA) for 20 s on each side (total 40 secs) for the RelyX Unicem groups, unit calibrated at 1,900 mW/cm^2.

Group B–VE: Similar procedure was followed as group A-VE; however, the light cure polymerization was for 40 secs (160 secs total) for each side for the Rely X ARC. Group C–VE): Glass-ionomer cement (Vivaglass Cem, Ivoclar Vivadent AG) was homogenously mixed and smeared onto the treated surface of each specimen

Table 1. Materials, composition and manufacturer details.

Material	Composition	Product description
Vita Enamic	Vita Enamic (75 wt% Hybrid feldspar ceramic (resin infiltrated ceramic network) : silicon dioxide 58–63%, aluminum oxide 20–23%, sodium oxide 9–11%, potassium oxide 4–6%, boron trioxide 0.5–2%, zirconia and calcium oxide. Polymer part (25%): UDMA and TEGDMA	Vita Enamic; Vita Zahnfabrik, Bad Sackingen, Germany
Lava Ultimate	Lava Ultimate nanoceramic particles (80%) comprising of silica nanomers (20 nm), zirconia nanomers (4–11 nm), nanocluster particles , progressively cured resin matrix (20%) BisGMA, Bis-EMA, UDMA and TEGDMA and silane coupling agent.	(3M-ESPE, Seefeld, Germany)
RelyX ARC	PASTE A: Silane-treated ceramic, TEGDMA, BisGMA, silane-treated silica, functionalized dimethacrylate polymer, triphenylantimony PASTE B: Silane-treated ceramic, TEGDMA, BisGMA, silane-treated silica, functionalized dimethacrylate polymer, 2-benzotriazolyl-4-methylphenol, benzoyl peroxide	3M™ Clicker™ Dispenser, USA.
RelyX Unicem	Base paste: silane-treated glass powder, 2-propenoic acid, 2-methyl-, reaction products with 2-hydroxy-1,3-propanediyl dimethacrylate and phosphorus oxide, TEGDMA, silane-treated silica, sodium persulfate, glass powder, tertbutyl peroxy-3,5,5- trimethylhexanoate, cooper acetate monohydrate 588286 Catalyst paste: Silane-treated glass powder, substituted dimethacrylate, 1-benzyl-5-phenyl-barbic-acid, calcium salt, silane-- treated silica, sodium	Aplicap™ / Maxicap™, 3M ESPE, USA.
GIC	Liquid: Polialquenoic acid, tartaric and water. Powder: fluorosilicate glass, Al-Ca-La polymer (5% acrylic acid and malic acid).	3M/ESPE , St. paul, USA.
Universal bonding agent (Silane coupling agent)	10 Methacryloyloxydecyl dihydrogen phosphate, HEMA, silane, dimethacrylate resins, Vitrebond copolymer, filler, ethanol, water, initiators	SingleBond Universal; 3M ESPE, St.Paul, MN

and allowed to air dried before the application of the load.Group A- LU: A similar procedure to group A-VE was performed for build-up using RelyX Unicem on LU samples.Group B-LU): A similar procedure to group B-VE was performed for build-up using Rely X ARC on LU samples.Group C-LU: A similar procedure to group B-VE was performed for build-up using GIC on LU samples.

After the completion of polymerisation process, the cement was allowed to set before the removal of CAD/CAM resin-ceramic hybrid-composite resin from the mould. Subsequently, the bonded specimen blocks were placed in a water bath for thermocycling. The blocks were thermocycled for 3000 cycles at 5°C and 55°C for a 20 seconds dwell time in a thermocycler (MTE 101; MOD Dental, Esetron Smart Robotechnologies, Ankara, Turkey). Each block was secured with the help of a jig for the shear bond strength testing using a universal testing machine (Lloyd LF Plus; Ametek Inc., Lloyd Instruments, Leicester, England). Each specimen was subjected to a standard force at 0.5 mm/min crosshead speed untill failure. The recorded failure at a maximum load was measured in Newton (N) which was divided by bonding surface area to calculate the shear bond strength in megapascals (MPa). The debonded surface was visualised through a stereomicroscope (DV4; Stemi, G¨ottingen, Germany) to identify the fracture pattern.

Failure mode classification includes three distinctive types namely: type I, adhesive failure, debonding at the interface; type II, mixed failure partially hybrid ceramic, partially resin cement, consisting of both parts and type III, cohesive failure, fracture occurring in the cement. Data were statistically analysed through the statistical program for social science (SPSS). Normality was assessed using Kolmogorov–Smirnov test. Shear bond strength was analysed and tabulated using one-way ANOVA and Tukey multiple comparisons test (α= 0.05).

RESULTS AND DISCUSSION

The assessed data was normally distributed. The maximum shear bond strength was observed in group B –VE [18.22 (1.25)], whereas the minimum shear value strength was exhibited in specimens of group C–LU [10.68 (2.17)]. A significant difference was observed among the outcomes of cements (Gp A, Gp B and Gp C) within the hybrid ceramic groups (VE and LU) ($p<0.05$) (Table 2). An overall significant difference was observed among the shear bond strengths between hybrid ceramic materials (VE and LU) ($p<0.05$) (Table 2).

Table 2. Means and SD for shear bond strength among the study groups.

Study groups	VE	LU	ANOVA
Rely U-Gp A	16.28 (1.68)$^{A\,a}$	14.37 (1.47)$^{B\,a}$	
Rely ARC-Gp B	18.22 (1.25)$^{A\,b}$	16.56 (1.31)$^{B\,b}$	<0.01
GIC- Gp C	11.41 (1.84)$^{A\,c}$	10.68 (2.17)$^{A\,c}$	

*Dissimilar superscript capital letter in same row show significant difference
*Dissimilar superscript small letter in same column show significant difference
* Tukey multiple comparisons test

Comparing the two types of resin cements Rely X ARC and Unicem; the shear bond strength value measured was higher in the Rely X ARC in both hybrid ceramic groups. However, GIC showed very low shear bond strength in comparison to resin based cements. Nevertheless, there was a prominent difference between all three types of cement (Rely-X Unicem, Rely-X ARC and GIC) in each CAD/CAM ceramic; VE (16.28 vs 18.22 vs 11.41) and LU (14.37 vs 16.56 vs 10.68) respectively. Moreover, assessment between the CAD/CAM groups showed there was a significant difference between means of SBS for VE and LU for resin cements [Rely-X ARC (VE 18.22 vs LU 16.56) and Rely-X Unicem (VE 16.28 vs LU 14.37)] respectively. However, SBS for VE and LU when bonded to GIC (VE11.41 vs LU .68) was comparable ($p>0.05$).

Table 3. Distribution of failure modes in the tested groups.

Study Groups	Adhesive (%)	Cohesive (%)	Mixed (%)
Gp A-VE	70	0	30
Gp B- VE	50	10	40
Gp C-VE	80	0	20
Gp A-LU	60	10	30
Gp B-LU	20	50	30
Gp C-LU	30	50	20

The modes of failure as observed among the study groups are presented in Table 3. Adhesive failure was most commonly observed among study groups [Gp C–VE (80%), Gp A–VE (70%), and Gp A-LU (60%). However, specimens in Gp C-VE and Gp B- LU both demonstrated 50% of the cohesive failures in cement. Admixed failure mostly ranged from 20–30% except for Gp B-VE, which showed 40% admixed failures. The lowest adhesive failure was noted in Gp B- LU (20%). The present study assessed adhesive bond strength of resin cements to the CAD/CAM hybrid ceramics using standardized technique. Shear bond strength outcomes for different cements on hybrid ceramics were significantly different. Therefore, the hypothesis that different cements will show comparable bond strength outcomes with hybrid ceramic materials was rejected. The study outcomes suggested that the resin cements exhibited comparatively better shear bond strength than the GIC cements. Furthermore, a comparison between two types of hybrid ceramics displayed better adhesive bond strength outcomes for VE than LU. Multiple factors including material properties, adhesive potential, material composition and surface topography are implicated for the observed outcomes.

For the validation of the present study outcomes, thermocycling was performed to age the specimen and mimic oral conditions that are responsible for compromising the adhesive bond strength among tested materials (Campos et al, 2016). The comparison between polymer-infiltrated ceramic materials, Vita Enamic and Lava Ultimate, exhibited a stronger adhesive bond for the former material, particularly with Rely-X ARC (18.22 MPa) cement. This difference in adhesive bonding is explained by the difference in the modulus of elasticity among the restorative materials. Previous studies have specified that the modulus of elasticity (MOE) of LU (12.8 GPa) and VE (30.1 GPa) are closer to the MOE of dentine and resin cement (16 - 20.3 GPa) (Lawson et al., 2016, Belli et al., 2017). The similarity in the MOE allows the homogenous distribution of stress under loads; hence allows long-term retention of indirect restoration withstanding continuous load over a longer. In addition, the resilient polymer matrix base of both hybrid ceramics, exhibits the phenomenal capacity to bond with the resin cement (Belli et al., 2017).

Previous studies have recommended separate surface treatment for each type of hybrid ceramic i.e. HF acid etching for VE and sandblasting for LU (Güngör et al., 2016, Barutcigil et al., 2019). VE contains 86 weight % of feldspar in ceramic filler, which preferably dissolves on application of HF acid compared to LU (80 weight % nanoceramic (Andrade et al., 2018, Sabri et al , 2016). The microstructure gets altered due to the partial dissolution of the polymer and feldspar ceramic glass phase by the acid thus forming micro porosities (El-Damanhoury and Gaintantzopoulou, 2018). By contrast, LU has a high content of 80% silica and zirconia nanoparticles with hard and rough texture which is resistant to surface treatment. This is a possible explanation for the lower bond strength of LU samples in the present study (Andrade et al., 2018).

Earlier studies have assessed the adhesive bond strength by comparing the different surface treatment in the hybrid ceramic materials (Barutcigil et al., 2019, Güngör et al., 2016). Micromechanical retention was suggested as a priority for improvement in the retention of the restorations. Application of hydrofluoric acid as a standard surface treatment for all specimens created a baseline to evaluate the impact of individual cement. The results revealed higher bond strength in the resin cement groups because the acid etching dissolves the glassy particles to create interlocking areas over the surface like a honeycomb (El-Damanhoury and Gaintantzopoulou, 2018).

In addition, the application of the silane coupling agent increases the surface energy for bonding. The outcomes of the present study exhibited better adhesive bond strength for Rely-X ARC than other cements (Rely-X Unicem and GIC). It is known that the silane containing a universal adhesive system is responsible for greater bond strength. Cement selection is the most critical factor in adhesion between the indirect restoration and the tooth structure. Resin cements have demonstrated high bond strength with micromechanical retention grooves aiding in adhesive bond strength (Secilmis et al., 2016). Studies have pointed out that conventional cements such as the zinc phosphate and GIC have a limited capacity for adhesion; however, certain authors recommended zinc phosphate because authors observed that zirconia based cements form a bond with only MDP containing resin cement (Peumans et al., 2016, Duarte et al., 2016).

Thus it limits the maximum bond strength formation. The present study compares resin-based cements (Rely X Unicem and ARC) to conventional cement (GIC). Rely X Unicem is known for its ease in application and low technique sensitivity. The base paste of the Rely X Unicem contains methacrylate monomers comprising of acid phosphoric groups that exhibit self-etching property (Weyhrauch et al., 2016). The property is evident on complete ionisation in a water medium that is present within the paste. The alkaline environment of the catalyst paste allows for neutralisation reaction leading to low pH and low surface interaction leading to the formation of the hybrid layer (Weyhrauch et al., 2016). This hybrid layer allows a desirable bond strength at the interface; nevertheless, the diffusion is limited, which created a lower adhesive bond strength compared to total-etch Rely X ARC with low solubility and high mechanical properties.

Shear bond strength test is the most commonly used bond assessment method in the literature (Flury et al., 2016). Shear bond strength testing in the present study displayed a uniform and homogenous distribution of stress under the load. However, specimen preparation is considered a challenge, as construction of blocks can result in irregularities in small-unbounded area. This could be observed in the form of cohesive failure. The majority of the cohesive failures were observed in Gp C-LU and Gp B–LU, which signifies the material strength. This could also be because of the inaccuracies during the procedure. Despite the fact that aging of the material compromises the adhesive bond strength; Gp B–VE group samples revealed lesser adhesive bond failures, which indicates that the adhesive bond strength was higher than the others. In conventional cementation, GIC does not require pre surface treatment before the application; hence, no interlocking mechanism and limited silane bond formation lowers their adhesive bond strength in comparison to resin cements. (Jevnikar et al, 2012; Sayam et al, 2017)

The present study does posses few limitations that are required to be addressed in future studies. Firstly, there was no variation in the pre surface treatment to distinguish the performance of the cements under various surface roughness with different cement choice. Secondly, the trial performed was relevant to only the two types of CAD/CAM hybrid ceramic. Further trials are necessary to assess the capacity for adhesive bond strength among other contemporary materials. Lastly, there were few of the specimens that displayed cohesive failures, which adds discrepancy; therefore, the adhesive bond may need further evaluation. Therefore,

it is recommended to conduct further studies with considerations given to the polymerisation shrinkage and evaluation of surface topography of hybrid ceramics after surface treatments.

CONCLUSION

Within the limitation of the study, it is concluded that the type of CAD-CAM hybrid ceramic material has a significant influence on the quality of adhesive interface it produces with resin, resin modified glass ionomers and glass ionomer cements. Therefore, to achieve maximum adhesive bonding strength among hybrid ceramic materials, a compatible luting cement and surface treatment is critical.

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The Variation of Sensorial, Physicochemical and Microbiological Quality Index in Indian Mackerel (*Rastrelliger kanagurta*) in Ice Storage Procedure.

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ABSTRACT

The study aimed to evaluate the variation of sensory, physicochemical, and microbiological quality indices with storage time of Indian mackerel preserved on ice. The research was done on Indian mackerel caught in one offshore fishing trip, stored on ice traditionally within 25 days. The quality indices were assessed and recorded on the 0, 3rd, 5th, 10th, 15th, 20th, and 25th day of the storage period. Quality Index (QI), pH, PVB-N, NH_3, TMA, NPN, PV, TBA, and TPC were experimented and recorded. Data were analyzed using descriptive statistic method. QI varied from 0-31 points. Visually significant quality changes appeared between 10-15 days of storage. The pH range was from 6.07 ± 0.02 to 7.16 ± 0.04 ($P<0.05$). The NH3 content was from 16.25 ± 0.04 to 119.03 ± 0.05 percent; the TVB-N value was from 119.03 ± 0.05 to 152.57 ± 2.89 mg per 100g; the TMA value ranged from 6.07 to 20.39 mg per 100g; the NPN content was from 0.24 to 0.77 percent. The PV range was 0.91 ± 0.06 to 5.14 ± 0.09 meq per kg, while TBA was from 63.01 ± 0.51 to 571.87 ± 3.50 µmol per kg. The TPC ranged from 12.4 to 4.1×10^5 CFU per g. All indices showed a linear increment relationship with time of storage. QI scheme suggested for Indian mackerels was 0-15 QI points, and the estimated shelf-life was ten days for whole fish storage on ice. The pH and some nitrogen related quality indices (NH_3, TVB-N, and NPN) had a slight relationship to the acceptable sensory quality limit while the lipid-related index did not. They could be indicators or estimators to spoilage of fish under the studied conditions. The TPC showed similar to prior studies. Indian Mackerels were found to have 10-15 days shelf-life under the studied storage conditions, which was not enough for offshore fishing time, but it could be acceptable in VietNam fishing industry condition. The sensory, physicochemical, and microbiological indices were extensively assessed and formed a foundation to further research on the preservation and quality evaluation of the Indian mackerels on the Vietnamese market.

KEY WORDS: SENSORY; PHYSICOCHEMITRY; MICROBIOLOGY; QUALITY INDEX; INDIAN MACKEREL; RASTRELLIGER KANAGURTA; ICE STORAGE.

ARTICLE INFORMATION

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INTRODUCTION

Fresh fish is an essential seafood product, accounting for more than one-third of total fish products on the world trade market (FAO 2012). Fish and seafood are considered rich and economical animal protein sources and play the primary role in nutritional prevention, especially in coastal underdeveloped and developing countries (Alasalvar et al., 2011; Humaid & Jamal 2014). Indian mackerel (*Rastrelliger kanagurta*) is a species of Scombrid family (Scombridae). In Vietnam, the total yield of Indian mackerels in the fishing industry is 4842 ton/year in the Northern Sea, 4050 ton/year in Central, and the highest in East Southern 6560 ton/year (Nguyen, 2020).

Indian mackerel is one of the top yield food fish species among small pelagic fish and is the most consumed fish in the Vietnamese domestic market. Freshness is an essential parameter in the quality assessment of human consuming foodfish in the market that not only closely related to food safety but also the economic benefits of fishers. Without proper storage and preservation practices, fish start their spoiling process very shortly after being cached. The spoilage has two leading causes: the biochemical cause from the digestive enzymes in the fish and the microbiological reason from the microorganism from the fish intestine and surface, as well as from the storage environment. Both causes are highly dependent on temperature, in which the closer to ice melting temperature (0 ^{o}C), the slower the spoiling process. For this reason, ice in various forms is used all around the world for fresh fish storage and preservation and could be applied in Viet Nam as recommended by TCVN 3696:1981. Various prior studies proved the relationship among temperature and time to the changes in Indian mackerel quality in different storage and preservation conditions, (Gopalakrishnan et al.,, 2016; Humaid & Jamal, 2014 Chudasama et al., 2018).

Studies mainly assessed biological characteristics (Chu et al., 1998), resources, and reserves (Nguyen, 2020). Still, there was not yet any research on the changes of quality in Indian mackerel during processing and preservation in Viet Nam. The biochemical transformation in fish species is highly differentiated, depending on the stage of mature, method, season and time of fishing, and conditions of storage and preservation. In the fishing ships, trading markets or processing facilities, long term storage, and exposure to underqualified hygienic procedures might lower the quality of fresh fish. Considering the importance of keeping the highest fresh fish quality possible, this study aimed at evaluating the changes in the sensory, physicochemical, and microbiological quality index in Indian Mackerel (*Rastrelliger kanagurta*) in ice storage procedure. The quality indices included sensory grading, pH, total volatile basic nitrogen (TVB-N) values, trimethylamine (TMA), ammonia (NH_3), non-protein nitrogen (NPN) content, peroxide value (PV), Thiobarbituric Acid (TBA) value, and total plate count (TPC) of microorganism.

MATERIAL AND METHODS

The study was conducted on Indian mackerel fish at size 5-7 individuals per kg, collected from offshores fishing boat in April 2019. The samples were taken from the first over 100kg Indian mackerel batch cached offshores. Sample fish at the size of 5-7 individuals per kg were collected randomly from the batch. Fish was stored in-tray of 5kg with 5 kg ice in alternated layering, one layer of fish one layer of ice. Experimental lots were marked 0; 3; 5; 10; 15; 20 and 25 respective to the number of days, each lot had three repetitions. At each testing period respective to the lot number, the fish was checked for sensory grading; after that, the processed and gutted based on the method described in TCVN 5106-1990 (according to Codex CAC/RCP 9-1976); and deep-frozen for offshore testing.

The samples were then transferred to the Lab of Marine Sciences – Research Institutes for Marine Fisheries for physicochemical indices after the fishing trip had finished (within 25-30 days). All analytical grade chemicals using for evaluating physical-chemical and microbiological parameters were supplied by Merck chemical company, Germany, through a dealer in Viet Nam (THANG LONG Science Technique., JSC).

Sample preparation:Samples for sensory grading were the whole fish randomly selected from respective lots. Samples for physicochemical evaluation were the whole clean fish without internal organs, minced into a fine powder, and tested within 4 hours after processing (according to TCVN 5276-90). The sensory assessment was done using the Quality Index Method (QIM) (Bernardi et al., 2013). The sensory attributes to be evaluated were skin, eyes, gills, and texture. Each quality was scored from 0 to 3, the lower, the better. All merit scores were then summed to form the demerit score or the QIM points. The pH value was obtained using pH meter (Mi 150 – Martini – Rumania) according to the method described in TCVN 4835:2002 - ISO 2917:1999.

TVB-N was measured according to TCVN 9215:2012 (Commission Regulation (EC) No 2074/2005 of 5 December 2005, Annex II, Section II, Chapter III Determination of the concentration of TVB-N in fish and fishery products). Trimethylamine (TMA) value, NPN content and Thiobarbituric acid (TBA) value were determined according to Woyewodal (1986). NH3 value was measured according to TCVN 3706 – 90. Peroxide value (PV) was determined using iodometric (visual) endpoint determination (TCVN 6121:2018 -ISO 3960:2017). Total bacteria were identified using the total plate count method, according to TCVN 4884-1:2015-ISO 4833-1:2013. Data collected were analyzed using descriptive statistic method (averaging, standard deviation, p-value) using Anova and Statgraphic XV statistical software. Each experiment was done three times.

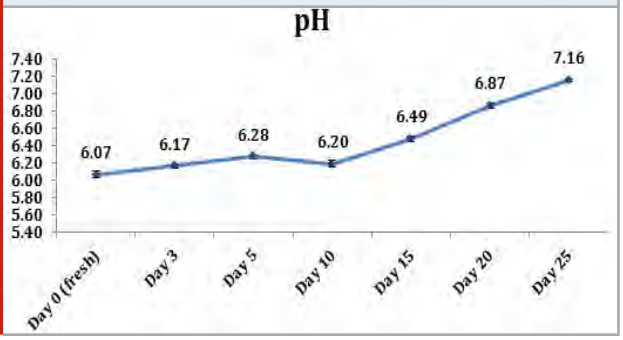
RESULTS AND DISCUSSION

The Quality Index points of Indian mackerels stored in ice for 0, 3, 5, 10, 15, 20, and 25 days were presented in Chart 1. The fish got the highest quality (0 points) on day 0 and the QI increase linearly with the storage time till reaching its highest points of 31 on day 20 till 25. The fish remained good quality within the initial five days with slow QI increment and reached its transformation point on day 10 with a sharper increase in its QI points.

Figure 1: The Quality Index points of Indian Mackarel stored in ice

Fresh fish had pH value of 6.07 ± 0.02 ($P < 0.05$) on day 0. The pH noticeably increased to 6.27± 0.02 ($P < 0.05$) after 5 days, and slightly decreased to 6.20± 0.03 ($P < 0.05$) on day 10. After day 10, the pH value sharply increased to 7.16± 0.04 at the end of the storage period on day 25, see Chart 2.

Figure 2: pH value by storage time



NH_3, TVB-N, TMA, and NPN variation were presented in Chart 3, 4, 5 and 6, respectively. NH_3 content in the fish's flesh advanced from 16.25 ± 0,04 percent on day 0 to 119.03± 0,05 percent after 25 days preserved on ice ($P<0.05$). TVB-N value was from 13.17 ± 0.53 mg per 100g on day 0 to 152.57 ± 2.89 mg per 100g on day 25. The acceptable quality value of 34-35 mg per 100g fell in between day five and day 10 of the storage period. TMA value presented a flat curve between 6.07 on day 0 and 20.39 mg per 100g on day 25 with a slight increase by time. The significant increase was noted on day five and day 25 of the storage period. NPN content increased linearly with storage time, from the initial value of 0.24± 0.005 % to 0.77 ± 0.003 % at the end of the storage period on day 25.

Figure 3: NH_3 content by storage time

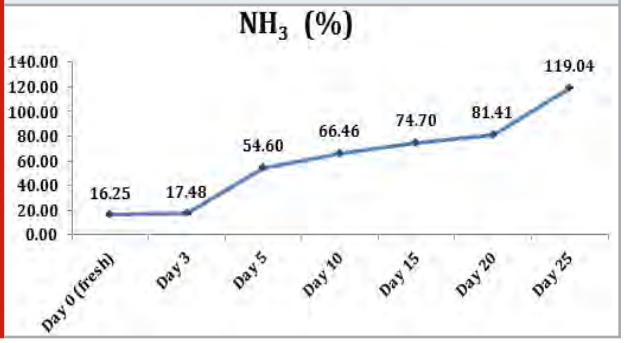


Figure 4: TVB-N value by storage time

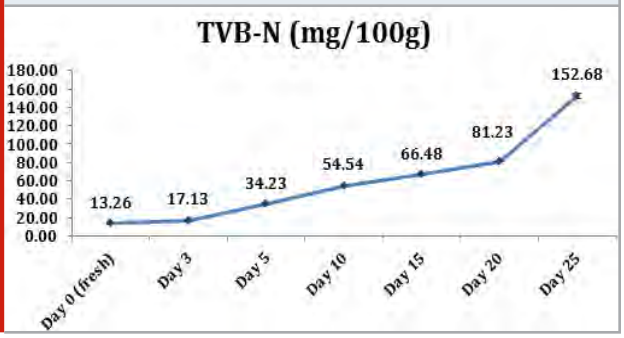


Figure 5: TMA value by storage time

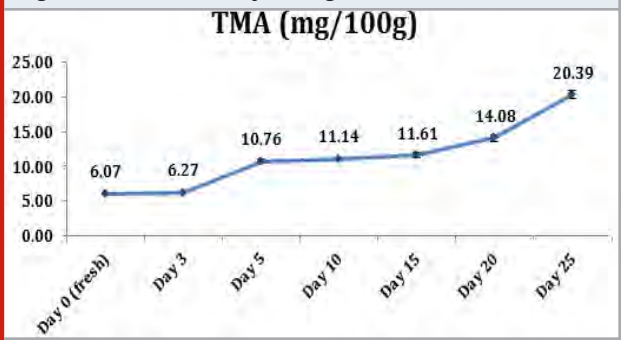
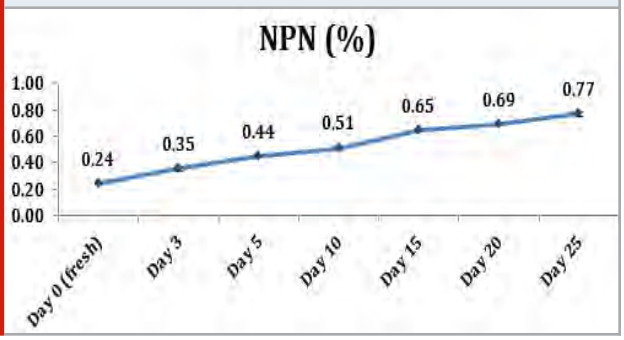


Figure 6: NPN content by storage time



PV and TBA value results were shown in Chart 7 and Chart 8, respectively. PV increased linearly with storage time. On day 0, PV was 0.91 ± 0.06 meq per kg lipid and slowly rose to 1.70±0.03 on day 15. PV showed a sudden turn after day 15 and quickly increased to 5.14±0.09

on date 25 of the storage period. TBA value showed a significant difference (P<0.05) during the storage period. Initial TBA value was 63.01 ±0.51 µmol per kg, and then it increased linearly by time to 571.87 ± 3.50 µmol per kg on day 25.

Figure 7: PV value by storage time

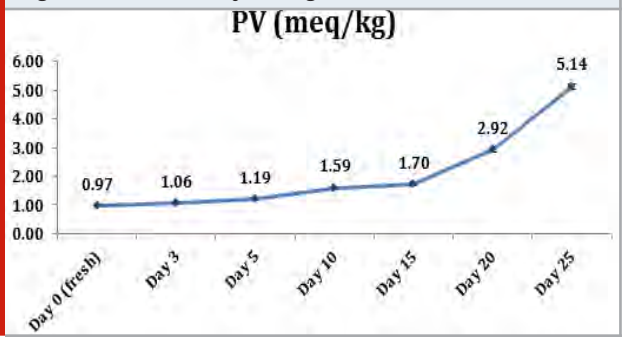
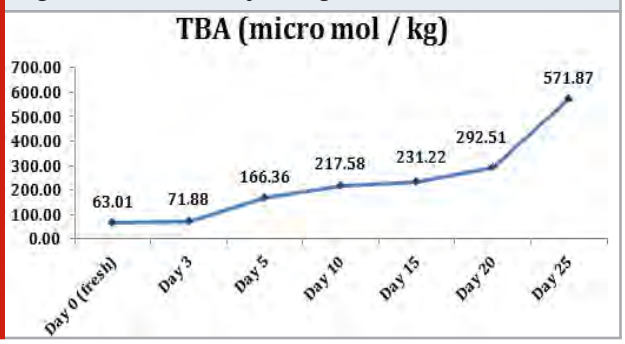
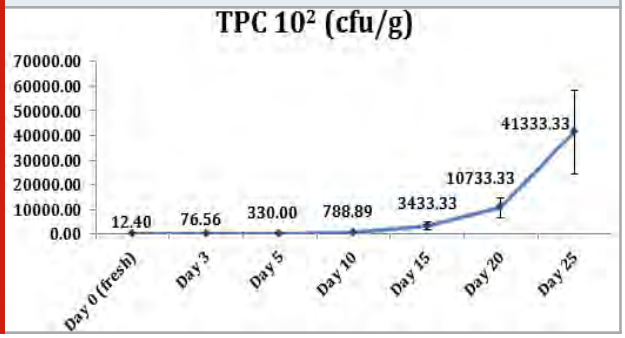


Figure 8: TBA value by storage time



There was a clear increment in TPC among the time marks. When freshly caught, the fish have shown TPC of only 12.4 CFU per g. TPC escalated rapidly to $4.1x10^5$ at the end of the storage period. The total plate count (TPC) result was shown in Chart 9.

Figure 9: TPC by storage time



As the aim of the QIM system, a linear relationship between QI and storage time on ice was established and observed for whole Indian mackerels in this study. QI point variation was between 0-31 with a significant increment between days 5 and 10. The entire Indian mackerel fish have normally shown noticeable spoilage in skin and belly within 10-15 days. The result was comparable to the previous report of Indian mackerel recommended good quality after 5-6 days of storage at 4°C (Humaid & Jamal, 2014) and estimated seven days shelf life for gutted Horse mackerel chilled on ice (Bernardi et al., 2013). In our study, we suggested the QI scheme of 0-15 QI and an estimated ten days shelf –life for Indian mackerel.

The pH value in raw fish had small variations, but significant implications in terms of technology. However, pH could not be used as the only indicator of the freshness of fish. The pH results in our study for Indian mackerels were between 6.08 – 7.17, similar to the range of 6.07-7.27 reported by Chudasama et al. (2018) for Indian mackerels stored chilled in ice. In another report, pH value of caught fish, depended in season, species and other factors, varying from 6.0-7.1, of which freshly caught fish value was 6.0-6.5, acceptable quality fish was 6.8 and spoiled fish was over 7.0 (except some fish with basal flesh such as shark or ray)(Simeonidou et al., 1998). In our results, the pH value had high variation between day 10 and 15, similar to the period with high differentiation of sensory quality, as pH is the most critical factor that affects the meat structure and the meat stiffness, an activity of the connective tissues (Huss, 1988).

Ammoniac (NH_3) forms in cold storage fish from two pathways and is not considered an essential indicator for freshness. The NH_3 increases during the storage and preservation period of seafood, forming due to the deposition of protein, amino acid and other nitrogen compounds under the bacterial activities (Lougovois and Kyrana, 2014) or the self-decomposition of adenosine monophosphate (AMP) in chilled seafood (Huss, 1995). Although no study in Indian mackerel was reported, the variation of NH_3 in our study represents the linear increment with storage time, similar to studies in most of the other fish species (Huss, 1995). According to Etienne et al. (2005), NH_3 is an indicator of the lack of freshness but not the spoilage in fish (Etienne & Ifremer, 2005)

The TVB-N is a standard method to determine the usable limit of fish in wet preservation and a spoilage estimation indicator (Boee et al., 1982; Fatih & Yesim, 2000). TVB-N included the measurement of trimethylamine, dimethylamine, ammoniac, and other volatile essential compounds formed during seafood spoilage. TVB-N values increase linearly with time and storage temperature. Seafood is in the best quality when TVB-N value is 25 mg per 100g, the limit of good quality is 30 mg per 100g, the acceptable limit is 35 mg per 100g and over that is considered spoilage according to Amending regulation (EC) No 2074/2005 (2008). In our study, TVB-N value increased from the best quality level of 13.17 ± 0.53 mg/100g on day 0 to acceptable limit of 34-35 mg/100g on day 5-7, comparable to Chudasama et al. (2018). It showed a close estimation of the time of the significant sensory change at day 10. The observation was also similar to reports from other authors that TVB-N value had a close correlation to the sensory points and number of microorganisms (Amegovu et al., 2012) and was related to the later phase of spoilage (Rehbein & Oehlenschlager, 1982).

TMA is an odorous reduction product of trimethylamine oxide (TMAO) in most marine species due to bacteria activities. TMA is often used as an indicator of bacterial spoilage in fish but due to the different initial amount of TMAO in each fish species as well as other seasonal effects, the correlation between TMA and sensory quality of seafood is not significant (Woyewodal, 1986). In our study, the range of TMA was from 6.07 to 20.39 mg per 100g, with a slightly steeper increase on day five and day 25 of the storage period.

The nature and composition of non-protein nitrogen (NPN) in fish affect the taste, quality and preservative potential, which plays a vital role in deciding the eatability of the fish. NPN accounts for 9-18% of total nitrogen content in fish. In living fish, the main composition of NPN is creatine and a large number of free amino acids (Vivekanandan & Jayasankar, 2008). In preserved fish, the NPN content included compounds formed during preservation or products of the decomposition process, such as ammonia, trimethylamine, derivatives of guanidine and imidazole, urea, amino acids, purine and pyrimidine. The changes in concentration of these compositions reflect changes in the fish muscles before spoilage appears in sensory measures. Therefore, NPN can be used as an estimator for fish quality. The distributions of different nitrogen composition are distinct among fish species and depended to the environment, reproductive cycle, season, size and age of the fish (Petricorena, 2015; Velankar NK and Govindan TK, 1958). In this study, the NPN value showed a slight decrease in day five and continuously increased from day 10. The variation theme might present the change in the composition of NPN in the relationship with the decomposition process and line with sensory observation.

Peroxide value in fish is considered an index to evaluate the spoilage that very sensitive to the environment and time and mostly applied to fatty fish (Hras et al., 2000). In our study, the PV gradually increased with storage time and significantly higher at the end of the storage period, comparable to a prior report of PV value in Indian Mackerels (Binsi et al., 2016)

Similarly, the TBA is an indicator of the decrease of lipid in fish muscle. The TBA value in Indian mackerels is higher than other fish species thanks to the concentration of unsaturated fatty acid and antioxidants in the meat. In this study, the TBA value increases linearly with storage time, comparable to prior research on Indian mackerel (Sathishkumar et al., 2017). The two lipid-related indices, PV and PVA, had no swift changes at day 10-15 that marked the significant transforming in the sensory quality of the fish. Therefore, we assumed that these two indices have a weak correlation with the sensory index.

Total plate count results showed rapid growth in the number of bacteria after 25 days of storage, similar to other studies on Indian mackerels (Humaid & Jamal, 2014; Sathishkumar et al., 2017). The storage of the whole fish is one of the reasons to increase the risk of bacterial spoilage as bacteria in the gut and internal organs will quickly develop and speed up the spoiling process (Lougovois & Kyrana, 2014). The TPC is affected by the hygienic conditions of the storage cellar, the quality of ice or chilled water used to preserve and other environmental factors.

CONCLUSION

In this study, we assessed the indices to evaluate the quality of Indian mackerels in traditional ice storage conditions within 25 days, equivalent to a regular offshore fishing trip. The sensory variety of the fish showed significant step down after 10-15 days of storage. These results suggested that the current storage method could not cope with the traditional offshore fishing period and prosed high risk of quality and economy lost. Still, it could be acceptable in Viet Nam industry fishing condition. Some physicochemical indices such as pH and nitrogen related indices (TVB-N, PNP) showed an anticipated relationship to the signification sensory quality changes time while the lipid-related indices and TPC did not. Our study as extensively assessed the sensory, physicochemical and microbiological indices on Indian mackerels. It formed a foundation to further research on the preservation and quality evaluation of the specie on the Vietnamese market.

ACKNOWLEDGMENTS

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Endothelial Functions in People with High Normal Blood Pressure Experiencing Regular Exercise

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ABSTRACT

In order to determine the effect of athletics on endothelial function, 75 middle-aged patients with this disorder were examined for 2 years at high normal blood pressure. The first observation group consisted of 37 patients who had normalized diet and daily routine. The second group consisted of 38 patients who additionally experienced regular physical activity in the form of regular runs. The observation period is 90 days. Everyone underwent dopplerography of the brachial artery in a sample with reactive hyperemia on the Sonos-1000 apparatus and biochemically examined the level of nitric oxide metabolite -NO^{2-} in the blood. In patients, a decrease in plasma nitric oxide concentration and accumulation of its metabolite in erythrocytes, a decrease in endothelium-dependent vasodilation, a tendency of vessels to spasm, a vasoconstrictor reaction in a sample with reactive hyperemia were found. Normalization of the diet and day regimen led to an unbalanced activation of the NO system, endothelial hyperstimulation while maintaining its dysfunction. The use of regular jogging in combination with basic recovery provided the normalization of the NO-system and the restoration of the vasoregulatory function of the vascular endothelium, which increased the effectiveness of the treatment.

KEY WORDS: ENDOTHELIAL DYSFUNCTION, HIGH NORMAL BLOOD PRESSURE, DAILY ROUTINE, DIET, EXERCISE.

INTRODUCTION

High normal blood pressure is increasingly found in industrialized countries and is one of the pressing medical and social problems (Medvedev, Gamolina, 2008; Medvedev, Kumova, 2007a). According to modern concepts, it is considered one of the leading pathogenetic factors of chronic heart failure, hypertension, and coronary heart disease, due to impaired endothelial function (Medvedev, Kumova, 2007b; Makhov, Medvedev, 2018a). Endothelium becomes a new target for therapeutic effects in individuals with developing cardiovascular pathology (Zavalishina, 2018a; Mal, Vorobyeva et al., 2018). The influence of the basic treatment of high normal blood pressure on the functional state of the endothelium is being actively studied (Vorobyeva, Medvedev, 2018; Zavalishina, 2018b). The optimization of energy metabolism in the myocardium, using feasible physical activity, the purpose of which is very appropriate for such people, is considered a promising approach to eliminating high normal blood pressure (Mal, Kharitonov,

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2018; Makhov, Medvedev, 2018b). The mechanism of action of muscle loads provides for their positive effect on the state of vascular endothelium through activation of nitric oxide synthesis (Zavalishina, 2018c), which is still poorly understood. The objective of the present study was to determine the effect of physical exertion on vascular endothelial function during treatment of high normal blood pressure.

MATERIAL AND METHODS

75 patients with high normal blood pressure, without concomitant pathology, were examined. The average age of the patients was 34.2±1.2 years. There were 30 men among the examined, 45 women. Depending on the health-improving effect, the patients were divided into two comparable groups: the first was 37 patients whose normalized diet and work and rest; the second - 38 patients, who additionally ran an additional 2 km at a free pace daily. The observation period was 90 days. The control group consisted of 22 healthy individuals, similar in gender and age. All patients underwent echodoplercardiography and dopplerography of the brachial artery in a sample with reactive hyperemia to assess the state of cardiohemodynamics and vasoregulatory function of vascular endothelium using the Sonos-1000 ultrasound complex (Correti, 2002). The level of NO was estimated by the concentration of its stable metabolite, NO^2- in plasma and in red blood cells using a biochemical method (Green, 1982). The results were processed by the method of variation statistics with one-way analysis of variance. The significance of differences was assessed by Student's t-test and Fisher's F-test using Microsoft Excel.

RESULTS AND DISCUSSION

In the examined patients, the systolic function of the left ventricle was preserved: the ejection fraction was 6.14±1.56%. All patients showed signs of vascular endothelial dysfunction: a decrease in the content of nitric oxide in blood plasma by 17.7% and an increase in the accumulation of NO metabolite in red blood cells by 7.8% compared with those in the control group. The examined patients also recorded a decrease in blood flow velocity, shear stress on the endothelium, a positive increase in blood flow velocity with reactive hyperemia, which was negative in healthy patients (Table 1).

The effectiveness of including physical exertion in the patient recovery program in relation to the functional state of the endothelium was determined by analysis of variance. A significant effect of the applied recovery on the shear stress on the endothelium was revealed (Fisher's F-test was 6.57, exceeding the critical level F for this sample (4.67) ($p<0.05$)).

In the first group of patients, against the background of basic improvement, the shear stress on the endothelium increased - by 72.0% in the initial state and by 50% with reactive hyperemia, in the second group, when athletics run included in the rehabilitation scheme, it decreased by 1% in In the initial state and by 6% with reactive hyperemia (Table 2). It is known that the shear stress on the endothelium regulates the transcription of the NO synthetase gene (Glagoleva, Medvedev, 2018; Oshurkova, Medvedev, 2018a).

Table 1. Indicators of the functional state of vascular endothelium in the examined

Index	Sick, n=75	Almost healthy, n=22
Vo, m/s	0.66±0.09*	0.77±0.05
D_o,x10^{-2}, m	0.43±0.06*	0.37±0.04
τ_o, x10^{-6} dean x m^2	530.4±5.65	548.4±3.84
V_1, M/C	0.66±0.07	0.75±0.10
D_1 , x 10^{-2}, m	0.44±0.04*	0.42±0.05
τ_1, 10-6 dean x m^2	573.6±6.9	596.2±7.23
NO2- in plasma, nmol/ml	104.5±3.18*	123.0±4.17
NO2- in red blood cells, nmol/ml	80.1±1.20	74.3±0.99

Note: V– is the blood flow velocity in the brachial artery, D – is the diameter of the brachial artery, τ – is the shear stress on the endothelium, index 0 – is at rest, index 1 – is for reactive hyperemia; the significance of differences is *p<0.05, n is the number of subjects. In the following table, the notation is similar.

The concentration of NO^2- in the blood plasma of patients of the first group during therapy was increased to a greater extent (by 8.8%) than in patients of the second group (by 4.2%). The concentration of NO^2- in erythrocytes against the background of basic recovery increased in the first group (by 8.9%) and decreased in the second with additional prescribing runs (by 14.0%), which indicated the activation of NO metabolism. The dynamics of NO^2-concentrations in plasma and erythrocytes against the background of basic recovery can be regarded as an unbalanced activation of the NO-system while maintaining its dysfunction (Zavalishina, 2018d; Makhov, Medvedev, 2018c), and when jogging is included in the recovery scheme, as a balanced normalization of the NO-system.

By the end of the observation period, the vascular tendency to spasm remained in the first group of patients, manifested by an increase in blood flow velocity against the background of an increase in the diameter of the brachial artery by 19.6% at rest and by 26.4% with reactive hyperemia. This reflects the presence of hyperstimulation of the endothelium while maintaining its dysfunction 90 days after the start of recovery with basic drugs. In the second group of patients, the dynamics of observation recorded a decrease in blood flow velocity against the background of an increase in the diameter of the brachial artery by 1.6% at rest and by 4.7% with reactive hyperemia, which indicated an improvement

in vascular endothelial function during regular runs (Zavalishina, 2018e; Vatnikov, 2019).

Patients experiencing regular muscle loadings noted a decrease in the number of cases of destabilization of blood pressure, headaches, increased physical performance in a shorter time compared with patients who received only basic recovery. The inclusion of regular physical activity in the rehabilitation scheme, according to the results of analysis of variance, increased the effectiveness of treatment (Fisher's F-test was 12.86, exceeding the critical level F for this sample (3.98) ($p < 0.001$)).

The positive effect of physical activity on the vascular endothelium is due to their weakening of the activity of systolic influences and the activation of acetylcholine receptors (Makhov, Medvedev, 2018d; Zavalishina, 2018f), which causes an increase in the synthesis of NO (Oshurkova, Medvedev, 2018b). An important feature of physical activity is their ability to stimulate the production of the physiologically necessary amount of NO, which exerts a normalizing effect on vascular tone (Zavalishina, 2018g; Zavalishina, 2018h).

The synthesis of NO with the participation of NO-synthase mainly comes from the amino acid L-arginine, and as suggested from L-citrulline, since it can increase the level of L-arginine. L-arginine is a part of proteins and is present in ordinary protein foods. A lot of this amino acid in seafood, nuts, seeds, seaweed, rice and soy. In addition, L-arginine can be synthesized in the body from citrulline, for example, in the kidneys. It is also synthesized in the liver, although it is completely reutilized in the urea cycle. The content of L-arginine in plasma depends on age and metabolism, and mainly depends on catabolism-the intake of this amino acid in the body. Extracellular L-arginine is rapidly absorbed by vascular endothelial cells and, in the presence of molecular oxygen and NADP, is rapidly oxidized to NO.

Table 2. Endothelial activity on the background of health effects

Index	First group of patients, n=37		Second group of patients, n=38	
	Before treatment	after treatment	Before treatment	after treatment
V_o, m/s	0.63±0.07	0.70±0.06**	0.68±0.08	0.74±0.06
D_o, x 10^{-2}, m	0.45±0.08	0.44±0.05	0.42±0.09	0.40±0.04
τ_o, x10^{-6} dean x m^2	527.0±4.86	534.7±6.12**	539.6±3.46	541.5±4.27*
V_1, M/C	0.63±0.05	0.69±0.04	0.69±0.03	0.73±0.08*
D_1, x 10^{-2}, m	0.42±0.04	0.40±0.02*	0.46±0.06	0.41±0.05
$\tau 1$, 10^{-6} dean x m^2	570.5±5.60	577.6±4.72**	578.9±6.03	589.7±3.20*
NO^{2-} in plasma, nmol/ml	102.4±4.10	114.5±2.23	106.3±2.75	120.3±1.83
NO^{2-} in red blood cells, nmol/ml	83.2±1.34	79.6±0.94	77.6±0.86	75.6±0.63*

This is a whole chain of events that is catalyzed by the enzyme NO-synthase, which has an arginine binding site. There are several forms of NO-synthase - neuronal, inducible and endothelial. Endothelial and inducible NO-synthases are constitutional and are controlled by intracellular calcium and calmodulin. Neuronal NO-synthase requires gene transcription, is calcium independent and is expressed during muscle activity during development, as well as by macrophages and other tissues in response to inflammatory mediators. In addition, L-arginine is involved in other metabolic processes unrelated to NO-synthase, for example, in the urea cycle, and it also has a strong Sokogonny effect.

Infusion of arginine at rest increases the level of insulin, glucagon, growth factor, prolactin and catecholamines. Such hormonal changes affect metabolism, in particular glucose and fat levels. It is assumed that the growth factor influences exercise performance, increasing fat oxidation and saving glycogen stores, as well as stimulating the release of an insulin-like growth factor, contributes to the absorption of amino acids and protein synthesis. It also improves exercise through increased muscle mass and strength. Arginine affects some indicators of physical performance in untrained and low-trained people. The decrease in metabolic products (ammonium, potassium) observed in some articles is associated with an increase in dilution of these products due to an increase in blood flow due to the synthesis of NO. However, there is no evidence of the effect of high doses of arginine consumed with food on blood flow in healthy people.

Studies on well-trained athletes have shown that a combination of arginine with aspartate can cause biochemical changes in the blood, which is not always confirmed by other researchers, but nevertheless a general conclusion is made - these additives do not significantly affect exercise. The lack of a pronounced effect in trained athletes is associated with physiological and metabolic adaptations due to regular physical activity. The fact of the effect of exercises on endothelial function is well established - regular exercises during the week increase the activity of endothelial nitric oxide. It is likely that activation of endothelial function is a systemic rather

than a local reaction, with significant muscle load. And probably the activation of the pulmonary, cardiovascular and nervous systems due to regular training significantly exceeds the possibility of activation caused by taking arginine with food. However, there are other factors that can reduce the effectiveness of arginine - this is the ratio of arginine/lysine. Lysine competes with arginine for entry into the cell (intracellular active transport) and inhibits arginase; under normal conditions, the food ratio of arginine / lysine should not exceed 2.5.

CONCLUSION

Persons with high normal blood pressure are characterized by signs of vascular endothelial dysfunction: a decrease in the concentration of nitric oxide in blood plasma and the accumulation of its metabolite in red blood cells, a decrease in endothelium-dependent vasodilation and a tendency of vessels to spasm. Improvement of these individuals through normalization of the diet and the regime of work and rest leads to an unbalanced activation of the NO system, hyper-stimulation of the endothelium while maintaining its dysfunction. An additional purpose of physical activity leads to the normalization of the NO-system and the restoration of the vaso regulatory function of vascular endothelium, increasing the effectiveness of the treatment of high normal blood pressure.

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Biosynthesis of Alpha-Amylase and Other Crude Enzymes in *Bacillus subtilis* Culture Rich with Banana Organ Specific Cultures

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ABSTRACT

The α-*amylase*s (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) are very important commercial industrial enzyme produced by various microorganisms. Aim of present study is to analyze the production of crude *amylase* including other hydrolytic enzymes (*xylanases* and *pectinases*) in *Bacillus subtilis* LC4 nutrient culture supplemented with banana agriculture waste as a fermentation substrate. The *B. subtilis* culture was grown on TY [trypton (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1})] medium as used as a control medium (LB_0). Two levels of extracts (15% and 30%) of different banana parts (leaf, leaf sheath, floral leaflets, banana peel and sucker) were maintained in LB_1 (1/8LB_0) medium. Maximum cell growth was observed in $LB_{a,z}$ (2.167±0.120) supplemented with leaf blade as a substrate and LB_a (1.570±0.069) control TY-medium. The cultures were harvested after 12-hrs of incubation. Production of enzymes and complex banana substrate saccharification into simple soluble sugars was observed. Maximum soluble sugars (10.06±0.188 mg ml^{-1}) in $LB_{s,a}$ and reducing sugars (6.217±0.097 mg ml^{-1}) in $LB_{s,a}$ noted than other cultures ($p \leq 0.05$), while total proteins in $LB_{t,a}$ and prolines in $LB_{3,a}$ which remined lower than LB_a (TY-medium). The hydrolytic enzymes like as α-*amylase* showed highest activity in LB_a (leaf blade) and LB_a (fruit peel), *xylanase* in $LB_{z,a}$ (leaf blade) and $LB_{t,a}$ (sucker), while pectinase in $LB_{s,a}$ (fruit peel) and in $LB_{t,a}$. The above outcomes have shown that cheapest agro-banana industry wastes are promising fermentation substrate to produce low cost industrial enzymes as well as secondary metabolites.

KEY WORDS: BACILLUS SUBTILIS LC4, SACCHARIFICATION, AGRO-BANANA WASTES, AMYLASES, SUB-MERGED FERMENTATION.

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INTRODUCTION

Banana (*Musa spp*.) is grown extensively since earliest human agriculture (Frison and Sharrock, 1998). It produces agriculture wastes in the form of fruit peel, pseudostem, leaves and sucker, which are posing serious environmental problems due to its improper management (Essien et al 2005). Meanwhile, its wastes are rich with fermentable proteins and starch (Mohiuddin et al., 2014) could be useful in production of biogas (Tewari et al., 1986), ethanol (Itelima et al., 1992), methane (Li et al., 2016) and lactic acid (López-Bac and Gómez, 1992). Banana waste may also be useful for production of low-cost industrial enzymes (Barredo, 2005; Patel et al., 2012 Ahmad, and Danish, 2018).

The *amylases* are the most important textile industries enzymes it is also useful in other industries including food, paper, pharmacy and detergent (Walker and Dundee, 2009; Demirkan, 2011). Its in-vitro production with synthetic cell nutrient medium is appeared costly that's why short-cut in cost of amylase production is required. Agriculture wastes are the major low-cost available plant polysaccharide such as starch, lignocelluloses and chitins on earth. Being hydrolyzed with extra-cellular α-amylase or glycogenase into dixtrins than smaller oligomers and finally glucose molecules (Demirkan et al., 2005). These extra-cellular enzymes including amylases are produces by microorganisms for their nutrition. Indeed, the secretion of amylases has been reported as in archae (Kwak et al., 1998), actinomycetes (Kar et al., 2008), bacteria (Agüloglu et al., 2014: Slavic et al., 2016) and fungi cells (Sahnoun et al., 2012) under aseptic conditions.

The α-*amylase* could be obtained from higher organisms such as plants and animals. Like as, it has been isolated from barley and rice (Oboh, 2005) but commercially not available because of complex downstream purification from the cultures of higher organisms. The α-amylases isolated from various microbial genotypes have shown diverse performances under normal to harsh physical condition (Vengadaramana, 2013). However, microorganisms are playing very important role in efficient production of α-amylases due to their rapid growth rates and being genetically manipulate-able (Dumorné et al., 2017).Microbial enzymes are useful under industrial conditions because of their low cost availability, large productivity and stability under harsh chemical conditions with variable plasticity in their activity (Rana et al., 2013). The *B. subtilis* is well known soil growing, non-pathogen (Baysal and Yıldız, 2017) and is being horse for numerous extra-cellular heterologous proteins secreting micro-organism (Huang et al., 2018).

A large variety of industrial amylases have been derived from very wide and diversified *Bacillus genus* (Barros et al., 2013). A number of studies are performed with better α-amylase production either in wild or recombinant form in different *B. subtilis* strains (Yang et al., 2011). As it has been widely used for α-*amylases* production with different nutrition rich in-vitro cultures. The agriculture wastes are the available low cost microbial fuel, which could be utilized for the growth of microbial cultures as well as to gain their by-products including extra-cellular enzymes (Haq et al., 2018).

The fate of agriculture wastes may be on choice either allowed to decompose itself with the passage of time or fired-up. Meanwhile, there is not proper management or utilization of banana agro-wastes. Even its all parts especially fruit peel is rich with minerals, carbohydrates and proteins (Subedi and Walsh, 2011; Fahrasmane et al., 2014). It could be utilized as a raw material for biosynthesis of various bio based products. The *B. subtilis* is able to utilize banana wastes either in fresh or dry mass and could produce extra-cellular hydrolytic enzymes while may be helpful to reach the industrial need (Silpa et al., 2018). There has not been much work done for the selection of a specific part of banana agro-wastes except fruit peel is useful for the production of α-amylase or other enzymes with *B. subtilis*. The aim of the present study is to search out a proper organ of banana plant which plays a significant role in the production of enzymes specially α-amylases. The *B. subtilis* as being non-pathogenic industrial micro-organism could make it possible to produce low cost α-amylases, which is a prime need of the time to boom the economy of a country in food to garment industries.

MATERIAL AND METHODS

Preparation of fermentation substrate: The banana agriculture wastes were collected from open banana fields. Farmers excises the plants banana fields at the time of plant harvest as well as diseased plants. Different parts of banana plant were targeted such as leaf blade, leaf midrib, pseudostem, floral leaflets, fruit peel and sucker. The collected material was washed with tap-H2O to remove dirt, dried with filter-paper and crushed into fine pieces (looks like powder) with Molineux blender. Its exact 50 g was mixed with 50 ml dH2O and again crushed with blender for 5 min. The crushed mixture was squeezed first with muslin cloth and then filtered with filter-paper. The extracted fluid was stored at 4˚C for next use (Gomes et al., 2008).

Preparation of agriculture nutrient based fermentation medium: The tryptone yeast extract-based TY or Schaeffer [tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), NaCl (5 g L^{-1})] medium was used for batch mode fermentation (Sambrook et al., 1989). It was used as a control culture denoted as LB. Two levels of concentration (15% and 30%) of each extract of banana parts were maintained in LB (1/8LB_0) medium (Table 1). Total volume of each fermentation culture maintained 25 ml in 200 mL Erlenmeyer flasks. The cultures were fermented in an orbital shaker (250 rpm) at 37°C after its sterilization through autoclave at 121˚C for 15 min.

Pure Culture of B. subtilis and Preparation of Inoculum:For *B. subtilis* LC4 cell activation, almost 20 µl of glycerol

stock was used to inoculate 5 ml TY-medium. The culture was incubated with 250 rpm shaking at 37°C for overnight in dark. Its 0.5 ml was sub-cultured in 50 ml LB medium and incubated at same conditions for 1 hour. The O.D of culture was checked it's around OD600 0.5). From this master culture, other cultures were inoculated to raise an initial OD600 equivalent to 0.05. All these cultures were incubated at 37°C with constant 250 rpm shaking for 12 hours *Bacillus* fermentation period. After O.N., cultures were harvested after taking culture's OD.

Table 1: Composition of *Bacillus Subtilis* Lc4 Growth Cultures Supplemented With Agro Banana-Wastes As Fermentation Substrate

S. #s	Medium	Composition of medium
1	LB_0	Tryptone 10 g L^{-1}, NaCl 5 g L^{-1} and yeast extract 5 g L^{-1}
2	LB_2	$1/8LB_0 + dH_2O$
3	$LB_{2.0}$	LB_1+ 15 % leaf blade extract + dH_2O
4	LB_3	LB_1+ 30 % leaf blade extract + dH_2O
5	LB_3	LB_1 + 15 % leaf midrib + dH_2O
6	LB_4	LB_1 + 30 % leaf midrib + dH_2O
7	LB_6	LB_1 + 15 % pseudostem + dH_2O
8	LB_0	LB_1 + 30 % pseudostem + dH_2O
9	$LB_{0.2}$	LB_1 + 15 % floral leaflets + dH_2O
10	LB_a	LB_1 + 30 % floral leaflets + dH_2O
11	LB_a	LB_1 + 15 % fruit peels + dH_2O
12	LB_τ	LB_1 + 30 % fruit peels + dH_2O
13	$LB_{\tau\varepsilon}$	LB_1 + 15 % sucker + dH_2O
14	LB_τ	LB_1 + 30 % sucker + dH_2O

Note: LB: TY-medium; Volume of each culture was maintained 25 ml in 200 ml conical volumetric flask

Extraction of Crude Enzyme: For crude enzyme extraction, over-night (O. N.) fermented medium was diluted with 50 ml dH_2O. The mixture was agitated for 1 hr flask with 160 rpm shaking. The mixture was filtered with cheesecloth for removal of cells. It was also centrifuged at 5000 rpm for 10 minutes at 4°C. Supernatant was transferred in new tube and used as crude enzyme (Singh et al., 2009). Determination of Organic Contents in B. subtilis Culture: Total proteins measured by mixing sample (0.5 ml) with 2.5 ml alkaline copper reagent at room temperature than 0.25 ml Follin-Ciocalteau reagent was added after 10 min and absorbance read at 750 nm (Lowry et al., 1951). Reducing sugars were also analyzed in same sample by mixing 1 ml supernatant and 1 ml DNS (3,5-dinitrosalicyclic acid). After heating the mixture in boiling H_2O for 5 min, absorbance was read at 540 nm (Miller, 1959), while total sugars were determined according to (Hansen and Moller, 1975). For free proline determination, 0.5 ml sample was mixed with equal volume of glacial acetic acid in a test tube. The 0.5 ml acidic ninhydrin and 1 ml toluene were also added and mixture was incubated for 1 hour in a boiling water-bath. The reaction was cooled down at room temperature and absorbance was read against blank at 540 nm on spectrophotometer (Marin et al., 2010).

Performance of Hydrolytic Enzyme Assays: The α-amylase activity of B. subtilis enzyme crude was measured with DNS assay (Worthington, 1993). In brief, reaction mixture was prepared by mixing 2 ml 20 mM phosphate buffer (pH 7), 0.2 ml crude enzyme, 0.2 ml freshly prepared substrate (5 % starch). The mixture was incubated at 37°C for 15 minutes. The 2 ml of DNS was added to develop a complex of α-amylase product as well as to stop the enzyme reaction. The liberated amount of reducing sugars was estimated by reading OD at 540 nm with UV-VIS spectrophotometer. For *xylanase* activity, 1 ml of crude enzyme sample was mixed with 1 ml substrate [1 g xylose dissolved in 100 ml citrate buffer (pH 5.3)] in the test tubes. The mixture was incubated at 60°C for 15 minutes. Exactly, the equal volume of DNS was added to stop the reaction. Absorbance was noted at 540 nm against blank (Hansen and Moller, 1975).

For *pectinase* assay, 167 µl crude enzyme was mixed with 1 ml buffer [0.1 M citric acid or phosphate Buffer (pH 5.0)] than add 167µl substrate (0.5% D-galacturonic acid). The mixture was incubated at 37°C in water bath for 60 min. Exact 0.334µl of reagent A (40 g anhydrous Na_2CO_3 dissolved in 600 ml ddH_2O than stirred after adding 16 g glycine until dissolved. The 0.450 gm $CuSO_4$ $5H_2O$ was also added during stirring and volume raised to 1 liter) and B (1.2 g neocuprine-HCl dissolved 1-liter ddH_2O and stored in brown bottle at 4°C) were added separately. Mixture was heated in boiling water bath again for 13 min. tubes were cool down at room temperature then 2ml H2O added and mixed by inversion. Absorbance was read at A450 against water blank and activity was determined from D-galacturonic acid standard curve (Holtzhauer, 2006).

Statistical Analysis: Cultures of this experiment were maintained with three replicates. Collected data of different parameters of experiment were subjected for its significance analysis and Duncan's Multiple Range test at 5 %. It was computed with COSTAT (CoHort software, Berkeley, USA) software, a statistical package.

RESULTS AND DISCUSSION

The increasing human population acquires variant qualities in food to textile products. Both depend on annual production of agriculture crops to their processing ways either chemical and or enzymatic based. Specifically, enzymatic processing is considered as safe, while is being costly when enzymes are produced with the utilization of synthetic nutrient medium. Micro-organisms are efficient for the production of extracellular enzymes, while costs remain high if secreted in commercial synthetic medium. It is quite possible to reduce the production costs of extracellular hydrolytic enzymes with the utilization of cheap agro-wastes as raw materials for the cell growth of microorganisms (Yang et al., 2011). The agriculture wastes like as peels of potato, banana, orange, molasses and cane bagasse including their plants or aqueous

extracts are useful carbon sources either used in minimal or synthetic growth medium. Such medium could be more workable, if they are used for *Bacillus subtilis* for the production of industrial important hydrolytic enzymes (Haq et al., 2018).

The *B. subtilis* is the subject for production of α-amylases including other hydrolytic enzymes and its efficiency of saccharification for banana agro-industrial wastes used as fermentation carbon source. For this experiment, a 12-hrs *B. subtilis* culture on seven organs of banana agro-waste was maintained in LB_1 (1/8LB) medium including LB_0 (control) TY-medium. After 12 hours of culture incubation, maximum cell growth (OD) was observed in $LB_{z,a}$ (2.167±0.120) supplemented with leaf blade as a substrate and LB_0(1.570±0.069) with standard TY-medium. The distinct *B. subtilis* growth phases observed among the organ specific cultures, which depends on the type or part of banana agro-waste materials supplemented as nutrient source (Sharma et al., 2017).

Table 2. Adjustment of Various Biochemical In *Bacillus Subtilis* LC4 Growth Cultures Supplemented With Agro Banana-Wastes As Fermentation Substrate

#s	Nutrient medium	Culture growth OD	Soluble sugars (mg m^{-1})	Reducing sugars (mg ml^{-1})	Total proteins (mg ml^{-1})	Proline contents (mg ml^{-1})
1.	LB_1	1.570±0.069^{bc}	12.66±0.276^{a}	6.821±0.236^{b}	15.63±0.045^{a}	2.177±0.045^{a}
2.	LB_2	0.653±0.017^{h}	4.360±0.552^{b}	2.644±0.195^{f}	12.06±0.023^{j}	1.540±0.062^{bc}
3.	LB_{2a}	1.753±0.068^{b}	7.328±0.138^{f}	3.924±0.171^{de}	11.20±0.158^{e}	1.608±0.080^{abc}
4.	LB_3	2.167±0.120^{a}	9.114±0.227^{cd}	6.189±0.569^{b}	13.53±0.102^{bc}	0.843±0.057^{de}
5.	LB_{3a}	1.155±0.009^{efg}	8.200±0.187^{g}	5.387±0.284^{ef}	11.16±0.023^{g}	1.441±0.119^{bcd}
6.	LB_a	1.112±0.039^{fg}	8.045±0.081^{ef}	3.249±0.089^{c}	13.63±0.208^{d}	1.549±0.057^{bc}
7.	$LB_{'a}$	1.339±0.062^{cde}	7.215±0.284^{def}	6.217±0.162^{de}	13.06±0.057^{h}	1.471±0.034^{bcd}
8.	LB_σ	1.412±0.093^{cd}	9.508±0.162^{bc}	3.924±0.154^{b}	13.29±0.260^{C}	0.980±0.023^{cde}
9.	LB_a	1.400±0.049^{cd}	6.793±0.154^{g}	5.331±0.073^{de}	12.77±0.045^{f}	1.549±0.091^{bc}
10.	$LB_{\sigma,\alpha}$	1.506±0.117^{c}	10.08±0.138^{b}	3.924±0.365^{c}	13.96±0.442^{b}	0.618±0.040^{e}
11.	LB_{0a}	1.233±0.035^{def}	8.200±0.171^{ef}	4.402±0.008^{d}	12.77±0.113^{d}	1.461±0.063^{bcd}
12.	LB_τ	1.530±0.015^{c}	10.06±0.188^{b}	6.217±0.097^{a}	12.55±0.260^{bc}	0.892±0.096^{de}
13.	$LB_{\tau\alpha}$	0.983±0.070^{g}	5.513±0.179^{g}	2.921±0.219^{d}	12.45±0.226	0.667±0.125^{e}
14.	LB_τ	1.482±0.110^{c}	6.751±0.114^{cde}	3.260±0.049^{b}	13.75±0.340^{bc}	1.382±0.006^{ab}
p -significance		24.844***	40.132***	43.513***	225.87***	5.962***

Lowest growth was noted in $LB_{3,a}$ (1.112±0.039) and LB_a (1.412±0.093) cultures, when cells were growing on medium supplemented with leaf midrib and pseudostem respectively (Table 1 and 2). Variable amounts of total soluble sugars and reducing sugars observed among the cultures. This fluctuation among these parameters might be due to the structural variation in substrate type of the nutrient medium (Table 1). In this study, maximum total soluble sugars were noted as 10.06±0.188 mg ml^{-1} and reducing sugars 6.217±0.097 mg ml^{-1} in LB_{0a} and LB_α respectively (Table 2). The total proteins observed higher in $LB_{\tau,a}$ and prolines in $LB_{3,a}$ ($p \leq 0.05$), while remained lower than LB (TY-medium) *B. subtilis* cultures.

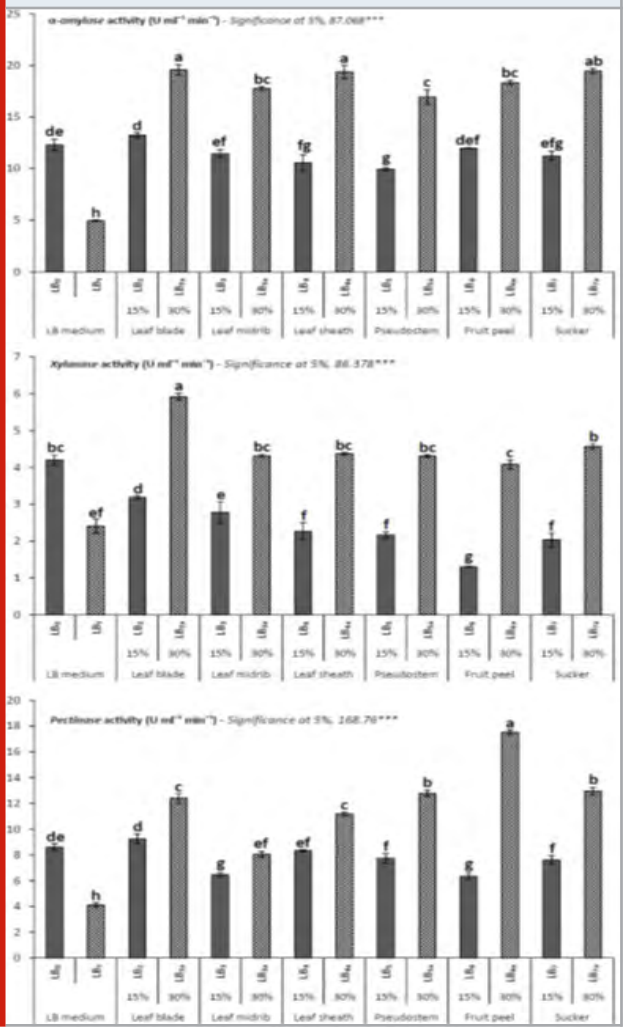
The production of lower to higher concentrations of the soluble sugars or reducing sugars basically depends on the added value of nutrient substrates. Type of a substrate controls the growth rate of an organism with production of desired secondary products. It could be led to stimulate the production of supplemented substrate relevant enzymes by *B. subtilis* LC4, which results into good conversion of complex to small molecules simply like as reducing sugars (Bell et al., 2005; Shahrim et al., 2008). Meanwhile, prolonged fermentation time may not be a key for significant increase in reducing sugar concentration depends on the secretion of its associated enzymes. The *B. subtilis* growth is coupled with the production level of amylases in the culture medium. This suggests that growth of Bacillus is crucial for the secretion of extra-cellular proteins (Table 2).

The concentrations and type of plant organ based raw materials are taken as major fermentation substrate for estimation of hydrolytic enzyme productions. The α-*amylase* yield was maximum at 30 % concentration of leaf blade extract ($LB_{2,a}$) and sucker ($LB_{\tau,a}$) with 12.91 U ml^{-1} min^{-1} and 12.91 U ml^{-1} min^{-1} activities (Fig 1). The α-*amylase* production was variable among the cultures which depend on type of substrate. In LBand LB_a cultures, yield was lower as due to targeted floral leaflets used as substrate. Similar trend in the production of *xylanase* was observed among the cultures as shown by α-amylase (Fig 2). However, production of *pectinase* has shown variation in yields among the maintained cultures from both α-*amylase* and *xylanase* (Fig 3). It means that the required energy source of microbes

for high extracellular protein secretion is retained in the type of substrate source and its available concentrations. Since it could be belonging to category of agro-lignocellulosic raw materials with capability of providing many essential nutrients for growth of microbes for useful productions.

In general, banana waste is comprised on 50-60% cellulose, 25-30% hemicelluloses, 12-18% lignin, 3-5% pectin, 3-5% fat and wax, 2-3% water soluble sugars and proteins (Mukhopadhyay et al., 2008). The rate of saccharification and enzymes production depends on the available type of substrate in the culture medium. Apparently, enzyme production could be repressed due to surplus saccharification and availability of catabolite. One enzyme could be reached to maximum production limit while others entered to decline phase. It results into lowering the levels of total soluble sugars and reducing sugars. In this regard, catabolite base repression of amylases (Vinuselvi et al., 2012), xylanases (Coleri et al., 2009; Shulami et al., 2014) and pectinase (Kumar et al., 2009; Sharma et al., 2012) have been reported in various fermentation organisms including bacteria and fungi.

Figure 1: Production of Different Hydrolytic Industrial Enzymes In *Bacillus subtilis* Lc4 Growth Cultures Supplemented With Agro Banana-Wastes As Fermentation Substrate



The rate of enzyme production is limited with growth phases like as activities of xylanase and pectinase slightly decline after log phase, while amylases increases. It might be possible that these enzymes are degraded by proteases, which are released from autolyzing cells during or after stationary growth phase (Berg and Pettersson, 1977). At early fermentation culture hours (log phase), increasing phase of reducing sugars is observed, which is due to the hydrolysis of xylan and pectin by xylanases and pectinases (Gaewchingduang and Pengthemkeerati, 2010). At the stationary phase, *B. subtilis* secreted numerous heterologous proteins in the culture medium, while dying cells and deficiency of nutrition in medium triggers the production of proteases in the medium. Due to this reason, fluctuations in the amount of total proteins in cell cultures are observable.

CONCLUSION

Production costs of hydrolytic enzymes could be reduced with the specific selection of free energy rich organs from agriculture waste materials. Banana plant is also one with 100% wasted vegetative materials including fruit peels. Its leaf blade, pseudostem and fruit peels are energy rich source and could be useful to establish an optimum growth of the fermentation microorganism. Like as *B. subtilis* is able to grow with saccharification of complex to simple soluble sugars and reducing sugars as well as produces various enzymes including *amylase, xylanase* and *pectinase*. Optimization of cell culture growth depends on the mode of substrate saccharification, which in final results into efficient enzyme production.The banana can provide enough nutrients for microorganism growth without additional supplement of carbon or nitrogen sources. The above system could be useful in future to reduce the overall production costs of industrial enzymes including other secondary metabolites.

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Exploiting the Genetic Diversity of *Rhizobia* to Produce Universal Inoculants with High Quality

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ABSTRACT

This study was done to study the possibility of producing high quality, universal or regional commercial inoculants. Sequences of 16SrRNA, glnII and recA genes of rhizobia isolated from different sites in Gadarif State, Sudan were aligned using A plasmid Editor (APE) software to choose the most genetically related strains. The result showed that *Rhizobium sp*. Haw1 and *Rhizobium sp*. G6-11 are identical according to the 16SrRNA and glnII genes sequences. They are also almost identical in their recA sequences because they were found differ in one position only. At the same time, these two strains were found differ at 9 positions when compared with *Rhizobium sp*. UoG27 also isolated from groundnut grown in another location. On the other hand, the sequences alignment of *Rhizobium sp*. Sab13 (isolated from nodules of Bambara groundnut) and *Rhizobium sp*. Umk34 (isolated from nodules of cowpea) also isolated from different locations showed that there are one and five positions of differences in the sequences of 16SrRNA and glnII, respectively. The study concluded that the relationship between rhizobia strains isolated from different sites and different related legumes can be exploited to produce universal or regional high quality inoculants

KEY WORDS: LEGUMES, STRAINS, ISOLATES, GROUNDNUT, BAMBARA GROUNDNUT, BACTERIOPHAGE.

INTRODUCTION

Variations in bacterial genes occur by deletion or addition of element like plasmids and through bacterial viruses which called bacterio-phages. This variation leads to genetic diversity due to differences in gene content and nucleotide variation in or between structural genes (Ochman et al. 2000). Variation among allels arises from nucleotide mutation, horizontal gene transfer, and intragenic recombination events (Reid et al., 2001).There are two types of substitutions occur in protein encoding genes; synonymous and non-synonymous single nucleotide polymorphisms (SNPs). Non synonymous SNPs (nsSNPs) result in amino acid replacements and hence provide substrate for evolutionary selection. While synonymous SNPs (sSNPs) do not alter the structure of proteins and are evolutionarily neutral (Kimura 1983; Schork et al. 2000; Gut 2001).

This type provides useful targets for large-scale molecular population genetic studies examining evolutionary relationships among bacterial strains. It is also permits

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all closely related strains to be assigned to lineages that are identical or related by descent and removes a critical barrier to population-based studies of the relationships between strain genotypes (Gutacker et al., 2002) and the host.

Rhizobial inoculants have been used successfully in world agriculture for about 100 years. The area inoculated is about 20 million ha in the world each year. This area can increase if inoculants with high-quality were available to all farmers. Therefore the future of the inoculant industry, and its potential benefits for world agriculture, depends on improving the quality inoculants (Herridge et al., 2002) and producing inoculants valid to be used for the related legumes and in a wide range in the world or at least suitable to group of countries in one geographical region or environmental zone. However, after over a century of rhizobial inoculation, most of the inoculants produced in the world are still of relatively poor quality (Lupwayi et al., 2000; Stephens and Rask, 2000, Santos et al., 2019).

In many countries in Africa biological nitrogen fixation is critical to the agricultural sustainability, but is often constrained by the absence of efficient and competitive rhizobia in the soil which need to improve its quality for every cropped legume (Hungria et al., 2005). So the poor inoculant quality, in those areas of the world where inoculation is ineffective or little used needs to be addressed (Hall and Clark, 1995; Marufu et al., 1995 Santos et al., 2019).

Agricultural Microbial Genetic Resources (AMiGR) usage in developing countries is often limited by lack of manufacturing capacity and quality control which also needs to be addressed (Howieson and Committee, 2007). It was stated that "the whole question of inoculants and their use starts with quality. If the quality is poor, then everything else is irrelevant" (Herridge et al., 2002). More than that, there is need to develop rapid methods to identify genetic relationships among all strains. However, it depends on the availability of partial and whole genomes sequencing of the different strains of rhizobia. Therefore this study was conducted to study the possibility to produce high quality, universal or regional commercial inoculants by exploiting the genetic diversity of different strains.

MATERIAL AND METHODS

Bacterial strains: The isolation of the strains used in this study and the amplification of the genes were described before elsewhere (Idris et al., 2012).

Nucleotide accession numbers: The accession numbers of the different strains genes used in this study were as follow: MN211542, MN211544, MN211545, MN211546 and MN211548 for 16SrRNA. The accession numbers for glnII were: MN218340, MN218342, MN218343, MN218344 and MN218346 and for recA the accession numbers were: MN218348, MN218350, MN218351, MN218352 and MN218354 for the different genes isolated from *Rhizobium sp*. Haw1, *Rhizobium sp*. G6-11, *Rhizobium sp*. UoG27, *Rhizobium sp*. Sab13 and *Rhizobium sp* Umk34, respectively.

Data analysis:Each two sequences of 16SrRNA, glnII and recA genes of *Rhizobium sp*. Haw1, *Rhizobium sp*. G6-11, *Rhizobium sp*. UoG27, *Rhizobium sp*. Sab13 and *Rhizobium sp* Umk34 were aligned using APE (A plasmid Editor) version 4.0.49.0 soft ware to detect the nucleotide variations.

RESULTS AND DISCUSSION

In this study sequences of the most related rhizobia isolates were chose and aligned to detect the polymorphism. The alignment of the 16SrRNA gene sequences of *Rhizobium sp*. Haw1 and *Rhizobium sp*. UoG27 (both isolated from groundnut) revealed that they are differ at 9 positions. The same result was obtained when *Rhizobium sp*. UoG27and *Rhizobium sp*. G6-11 sequences were aligned. However, *Rhizobium sp*. Haw1 and *Rhizobium sp*. G6-11 alignment showed that they are identical although they were isolated from groundnut grown in different sites (western and eastern parts of Gadarif state, Sudan). While the alignment of the same gene of *Rhizobium sp*. Sab13 isolated from Bambara groundnut and *Rhizobium sp*. Umk34 isolated from cowpea resulted in difference in one position only although they were isolated from different hosts and different locations (Tables 1 and 2).

Table 1. 16srRNA gene single nucleotides polymorphisms between *Rhizobium sp*. Haw1 and *Rhizobium sp*. UoG27

Positions	Isolates/Nucleotides	
	Rhizobium sp Haw1	*Rhizobium sp* UoG27
64	C	T
68	A	G
111	C	T
116	T	C
120	G	A
136	A	C
881	C	T
914	T	G
936	C	G

Table 2. 16srRNA gene single nucleotides polymorphisms between *Rhizobium sp* Sab13 and *Rhizobium sp* Umkh34

Positions	Isolates/Nucleotides	
	Rhizobium sp. Sab13	*Rhizobium sp*. Umk34
1028	G	A

The sequences alignment of glnII of *Rhizobium sp*. Sab13 and *Rhizobium sp*. Umk34 showed that there are five positions of differences, although 16SrRNA sequences

alignment showed that there is difference in one position only. This difference in one position clustered these two isolates in one group in the phylogeny tree (data not shown). The clustering in one group in the phylogeny tree is an indication that these isolates are at least of the same ancestor (Table 3). This difference in the five positions resulted in changes in five positions in the amino acids composition of glutamine encoded and synthesized by glnII gene (Figures 1 and 2). Despite this, we detected that *Rhizobium sp*. Haw1 and Rhizobium sp. G6-11 are identical in their glnII sequences. Accordingly, the glutamine encoded and synthesized was identical.

Table 3. GlnII gene single nucleotides polymorphism between *Rhizobium sp*. Sab13 and *Rhizobium sp*. Umk34

Positions	Isolates/Nucleotides	
	Rhizobium sp. Sab13	*Rhizobium sp.* Umk34
224	C	T
254	C	T
365	C	A
398	G	A
422	C	A

Although *Rhizobium sp*. Haw1 and *Rhizobium sp*. G6-11 were typically the same in their 16SrRNA and glnII genes, they were found differ in one position in recA sequences alignment (Table 4). This difference in one position in recA resulted in addition of one amino acid at the end of the recombinaze synthesized by *Rhizobium sp*. G6-11. This addition modified the synthesized protein structure and may modify its function. So the recombinaze synthesized by *Rhizobium sp*. G6-11 differed from that synthesized by Rhizobium sp. Haw1 (Figures 3 and 4).

In this study we aligned sequences of different genes isolated from different legumes grown in different locations to detect the genetic variation which may be useful in inoculants production. DNA sequencing is a viable method for assigning alleles of polymorphisms (Gut, 2001). The sequences alignment in this study showed that the most related isolates were found identical in some genes sequences and differ in others as in the case of Rhizobium sp. Haw1 and *Rhizobium sp*. G6-11 in which single nucleotide polymorphism was detected in recA sequences in spite of the identity of 16SrRNA and glnII sequences. This indicates that recA is characterized by "good capability to identify and classify strains" (Martens et al., 2008).

Figure 1: Glutamine II synthesized by *Rhizobium sp*. Sab13

Arg-Asn-Arg-Ala-Thr-Arg-Leu-Arg-Arg-Val-Leu-Thr-Thr-Arg-Ala-Ser-Ala-Ser-Arg-Thr-Ser-Val-Arg-Leu-Pro-Ala-Lys-Ser-Leu-Lys-Ser-Thr-Leu-Ile-Ser-Ala-Ser-Lys-Pro-Ala-Ser-Thr-Thr-Lys-Ala-Ser-Thr-Pro-Lys-Trp-Pro-Arg-Ala-Ser-Gly-Asn-Ser-Arg-Phe-Ser-Ala-Arg-Ala-Pro-Ser-Ala-Pro-Pro-Thr-Arg-Ser-Gly-Ser-Leu-Ala75-Thr-Cys-Cys-Cys-Val-Phe-Ala-Asn-Ser-Thr85-Ala-Ser-Thr-Ser-Asn-Ser-Ile-Ala-Ser-Arg-Ser-Ala-Thr-Pro-Thr-Gly-Thr-Val-Arg-Ala-Cys-Thr-Ala-Thr-Ser-Pro-Pro-Ser-Thr-Cys-Val-Lys-Leu-Ala-Ala-Arg-Thr122-Ile-Ser-Lys-Pro-Ser-Trp-Pro-Leu-Leu-Pro-Arg133-Thr-Gly-Lys-Ser-Thr-Ser-Thr-Phe141-Thr-Val-Arg-Thr-Thr-Thr-Phe-Ala-End

Figure 2: Glutamine II synthesized by *Rhizobium sp*. Umk34

Arg-Asn-Arg-Ala-Thr-Arg-Leu-Arg-Arg-Val-Leu-Thr-Thr-Arg-Ala-Ser-Ala-Ser-Arg-Thr-Ser-Val-Arg-Leu-Pro-Ala-Lys-Ser-Leu-Lys-Ser-Thr-Leu-Ile-Ser-Ala-Ser-Lys-Pro-Ala-Ser-Thr-Thr-Lys-Ala-Ser-Thr-Pro-Lys-Trp-Pro-Arg-Ala-Ser-Gly-Asn-Ser-Arg-Phe-Ser-Ala-Arg-Ala-Pro-Ser-Ala-Pro-Pro-Thr-Arg-Ser-Gly-Ser-Leu-Val-Thr-Cys-Cys-Cys-Val-Phe-Ala-Asn-Ser-Met-Ala-Ser-Thr-Ser-Asn-Ser-Ile-Ala-Ser-Arg-Ser-Ala-Thr-Pro-Thr-Gly-Thr-Val-Arg-Ala-Cys-Thr-Ala-Thr-Ser-Pro-Pro-Ser-Thr-Cys-Val-Lys-Leu-Ala-Ala-Arg-Asn-Ile-Ser-Lys-Pro-Ser-Trp-Pro-Leu-Leu-Pro-Lys-Thr-Gly-Lys-Ser-Thr-Ser-Thr-Tyr-Thr-Val-Arg-Thr-Thr-Thr-Phe-Ala-End

In addition, this verifies that identification of SNPs as a molecular marker is extremely valuable in phylogenetic studies (Flores et al., 2005). Also the wide range of inconsistency of the different genes may act as an indication of genetic instability which is considered as important characteristic in strain selection for inoculants production (FAO, 1991).

Table 4. recA gene single nucleotides polymorphism between *Rhizobium sp.* Haw1 and Rhizobium sp. G6-11

Positions	Isolates/Nucleotides	
	Rhizobium sp. Haw1	*Rhizobium sp.* G6-11
438	G	C

Figure 3: Recombinaze synthesized by *Rhizobium sp.* Haw1

Pro-Thr-Thr-Ser-Ser-Arg-Ser-Leu-Thr-Glu-Pro-Met-Arg-Arg-Met-Ser-Arg-Arg-Thr-Glu-Ala-End-Asn-Phe-Ser-Ala-Leu-Pro-Pro-Val-Val-Val-Ser-Gly-Glu-Pro-Asn-Met-Thr-Pro-Ile-Phe-Met-Arg-Ile-Trp-Leu-Met-Asn-Ile-Thr-Met-Gln-Phe-Asp-Leu-Glu-Ile-Glu-Ala-Val-Ser-Leu-Arg-Ser-Ala-Trp-Leu-Ile-Ser-Arg-Ala-Cys-Met-Pro-Gly-Arg-Leu-Ser-Pro-Ile-Ser-Pro-Ser-Ile-Ser-Ala-Arg-Gly-Val-Ser-Ala-Ala-Thr-Glu-Ser-Thr-Thr-Arg-Thr-Ser-Thr-Ala-Pro-Glu-Arg-Thr-Ser-Val-Ser-Val-Ile-Ser-Ser-Ala-Cys-Ser-Pro-Val-Ser-Gly-Cys-Glu-Ile-Arg-Arg-Phe-Cys-Arg-Ser-Thr-Pro-Ser-Leu-Arg-Ala-End-Thr-Gly-Ser-Ser-Ala-Cys-Ser-Ala-Ser-Thr-Lys-Ala-Gln-Met-Pro-Pro-Phe-Phe-Cys-Asp-Ser-Ala-Met-Val-Cys-Ser-Ala-Ser-Val-Val-Leu-Pro-Glu-Leu-Ser-Gly-Pro-End-Ile-Ser-Met-Ile-Arg-Pro-Phe-Gly-Arg-Pro-Pro-Met-Pro-Ser-Ala-Ile-Ser-Arg-Pro-Ser-Glu-Pro-Val-Glu-Thr-Val-Ser-Ile-Ser-Thr-Thr-Phe-Ser-Leu-Glu-Pro-Ser-Phe-Met-Ile-Glu-Pro-Leu-Pro-Asn-Asp-Arg-Ser-Ile-Cys-Glu-Ser-Ala-Ala-Ser

Figure 4: Recombinaze synthesized by *Rhizobium sp.* G6-11 (150)

Pro-Thr-Thr-Ser-Ser-Arg-Ser-Leu-Thr-Glu-Pro-Met-Arg-Arg-Met-Ser-Arg-Arg-Thr-Glu-Ala-End-Asn-Phe-Ser-Ala-Leu-Pro-Pro-Val-Val-Val-Ser-Gly-Glu-Pro-Asn-Met-Thr-Pro-Ile-Phe-Met-Arg-Ile-Trp-Leu-Met-Asn-Ile-Thr-Met-Gln-Phe-Asp-Leu-Glu-Ile-Glu-Ala-Val-Ser-Leu-Arg-Ser-Ala-Trp-Leu-Ile-Ser-Arg-Ala-Cys-Met-Pro-Gly-Arg-Leu-Ser-Pro-Ile-Ser-Pro-Ser-Ile-Ser-Ala-Arg-Gly-Val-Ser-Ala-Ala-Thr-Glu-Ser-Thr-Thr-Arg-Thr-Ser-Thr-Ala-Pro-Glu-Arg-Thr-Ser-Val-Ser-Val-Ile-Ser-Ser-Ala-

Other important of using single nucleotide polymorphism in inoculants production is that it is delineates relationships among closely related strains and allows construction of genetic frameworks for examining the distribution of host range and provides new insight into genetic relationships (Gutacker et al., 2002). More than that single nucleotide polymorphism of single-strain inoculants will act as facility in quality control (Thompson, 1980), it also enhance "the tendency to use single-strain inoculants in countries with strong inoculant quality-control programs as well as in those with a tendency to recommend specific strains for each ecosystem" (Date, 2001). In addition, now the issue of inoculants quality has centered on some problems one of them associated with genetic instability of rhizobial strains (Herridge et al., 2002). Single nucleotide polymorphism can plays crucial role to detect this genetic instability.

Some previous studies addressed inculant quality control as final step after "choosing and processing the carrier, culture maintenance and growth at increasing scales of production, in addition to aseptic injection of broth culture into the peat, proper maturation, and adequate packing" (Hungria et al., 2005). This quality control regulated in most countries in which mainly concentrates in some criteria one of them is the quality of the strain (Herridge et al., 2002). Isolating of different strains from different sources and comparing the different genes of the inoculants to determine the degree of the differences at single nucleotide polymorphism level will contributes in the quality of the inoculants strains by selecting strains related genetically, effective, capable to adapt to different environments, with wide host range, and therefore it will be suitable to be used as inoculants in different regions over the world. This will help to overcome the problem of searching for effective rhizobial strains for each legume which was described as "a labor- and time-consuming process" (Binde et al., 2009).

The importance of this study is that it can be applied to produce a universal inoculants or at least effective inoculants to be used in different regions, for example inoculants suitable for the tropical regions, others for the temperate or cold regions and so on. This can be done by surveying in the gene bank data base and search for the most related isolates from different sources over the world and select the most related strains to be used as inoculants. Before inoculants production, different genes especially symbiotic genes of the selected strains should be aligned to detect the degree of the relationship; in addition they should be tested in different sites in the world to authenticate their tendency to adapt to the different and adverse environmental conditions.

The model of *Rhizobium sp*. Haw1 and Rhizobium sp. G6-11 in this study supports the idea of the possibility of producing universal or regional inoculants because as mentioned that the isolates were obtained from groundnut grown in different locations far away from each other, despite this they were found identical in their nucleotide sequence of some genes. Like that, the model of Rhizobium sp. Sab13 and Rhizobium sp. Umk34 alignment is in this study also supports the above mentioned idea and indicates to the possibility to produce inoculants from the most related rhizobia strains which can establish symbiotic relationship among the most related hosts.

These latter strains were isolated from legumes belong to the same host (Vigna) but different species. So for example in the first model mentioned above, one of the strains can be used to manufacture inoculant suitable to be used in the two sites from which the isolates were obtained instate of manufacturing two inoculants, the same is true for the second model in which inoculant produced of one isolate will be more general because it can be used in different sites and different legume

species. However, the non synonymous (SNP) found in the glnII and recA sequences which resulted in change in amino acid composition of the glutamine and recominase mentioned in the result section leads to change in the synthesized protein structure and function which may reduce the chance of these isolates to replace each other as inoculants. The assumption of manufacturing inoculants suitable to different places and hosts depends on the degree of the relationship in the symbiotic genes and the ability of the strains to tolerate the different environmental conditions. This assumption may be logic because it was stated before that the genomic diversification is a result of recombination, transposition and horizontal transfer of genes between the most related strains (Flores et al., 2005).

The process of producing universal or regional inoculants with high quality requires isolation of rhizobia from different countries over the world, amplify different genes with concentration in symbiotic genes, aligning different genes to select the most related strains and test their ability to tolerate different and adverse environmental conditions. To do this a network and collaboration between researchers over the world should be conducted. Finally, improving inoculants quality will guarantee brilliant future for inoculant industry and its potential benefits for world agriculture as it was stated by Herridge et al. (2002).

CONCLUSION

There is strong genetic relationship between rhizobia strains isolated from different sites and different related legumes. This relationship makes producing universal or regional high quality inoculants possible if the different strains have the susceptibility to achieve effectively in the different environmental conditions. However, more studies required to collect different genes sequences of different effective rhizobia strains over the world and study their relationship with concentration in the symbiotic genes.

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Some Variables Influencing The Jackfruit, *Artocarpus heterophyllus* Beverage Production

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ABSTRACT

Ripe jackfruit pulp containeds high amount of sugar with specific aroma and taste. There is a growing market for minimally processed jackfruit. In order to increase the added value of jackfruit, we decided to turn jackfruit into one instant beverage. We performed the extraction (ratio of water: jackfruit pulp, temperature and duration of pasteurization); juice formulation; storage. Our results revealed that the jackfruit drink having the highest overall acceptance when extracting with water: jackfruit pulp ratio (50:50) at 90oC in 3 minutes. Jackfruit juice was usually cloudy and colloidal suspensions that have an unstable cloud or the turbidity. The mixture was then cooled to 37°C before the supplementation of pectinase enzyme at 0.06%. The mixture was then incubated in a water bath at 37°C in 60 minutes. After the incubation, the mixture was heated at 100°C for 3 minutes to inactivate all existing enzymes. The hydrolyzed jackfruit juice will be formulated with sugar 3.5%, citric acid 0.06%, carrageenan 1.0%. Jackfruit beverage would be extended its shelf-life for 6 months. The use of pectinase in jackfruit juice processing is essential to get better juice yield, improve filtration rate and produce clear juice.

KEY WORDS: *JACKFRUIT, BEVERAGE*, CARRAGEENAN, PECTINASE, STABILITY.

INTRODUCTION

Jackfruit, Artocarpus heterophyllus is a tropical climacteric fruit, belonging to Moraceae family. Jackfruit tree has a relatively high productivity (Loizzo et al., 2010). Jackfruit has a green to yellow brown exterior rind (Prakash et al., 2009). Jackfruit colour alters from yellowish green to yellow owing to the conversion of chlorophylls, anthocyanins, and carotenoids during ripening (Tiwari and Vidyarthi, 2015). Jackfruit pulp is rich in nutrients including carbohydrates, proteins, vitamins, minerals, and phytochemicals such as carotenoids, favonoids, volatile acids sterols, tannins Arung et al., 2007; Chandrika et al., 2009; Lin and Lu, 1993; Ong et al., 2006; Venkataraman, 2001; and Wong et al., 1992). These functional components have capability to control high blood pressure, heart diseases, strokes, bone loss, anti-infammatory, anticancer, antiulcer, antiaging etc (Swami et al., 2012; S.-C. Fang et al., 2008; Wei et al., 2005). Owing to numerous health benefits, the utilization of jackfruit pulp has increased in recent years (Ruiz-Montanez et al., 2014 Ranasinghe et al., 2019).

The shelf life of the ripe jackfruit pulp is quite short at ambient condition (Andri et al., 2012). It is highly perishable and ofen undergoes favour loss, tissue sofening, and cut surface browning.The fruit softening makes it more susceptible to bruising and mechanical injury. Huge amount of ripe jackfruits undergo quick decomposition owing to lack of proper knowledge on postharvest practices resulting in poor handling and

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inadequacy of sanitary practices and storage facilities (Mondal et al., 2013, Jagtap et al., 2011). Application of high yielding jackfruit varieties, together with proper harvesting and postharvest manipulations such as appropriate handling, transportation and preservation, innovation of novel processing technologies, and finding for new implementations to limit postharvest and production losses as well as conversion of jackfruit waste into value-added products would be better options for popularizing the jackfruit cultivation and consumption along with waste management of jackfruit processing industries. Its pulp has been converted into various forms such as jam, jellies, marmalades, and ice creams to improve its economic value (Shamsudin et al., 2009). Fresh-cut jackfruit bulbs may remove the difficulty in separating the bulbs from the rind and conserve time (Saxena et al., 2008). The edible coating prolongs the stability of precut jackfruit (Vargas-Torres et al., 2017).

The efficacies of calcium soaking, osmo-blanching and drying methods on physico-chemical and organoleptic properties of jackfruit slices were studied (Saxena et al., 2015). Salty snacks or chips from jackfruit flesh may attract the consumers. Various products such as jam, jelly, pickle, and squash have tried to developed (Mondal et al., 2013; Shwetha and Ranganna et al., 2018). Jackfruit wine is demonstrated to have good antioxidant properties and protective effects (Jagtap et al., 2011). In order to turn jackfruit pulp into instant healthy beverage, we tried to verify the enzymatic extraction (ratio of water: jackfruit, temperature and duration of pasteurization); formulation; and preservation.

MATERIAL AND METHODS

Material: We collected jackfruit fruit in Hau Giang province, Vietnam. They must be cultivated following VietGAP to ensure food safety. Only technical ripen jackfruit without any defect was selected. After collecting, they must be conveyed to laboratory as soon as possible for experiments. The jackfruit pulp were dehusked, separated from seeds and collected by hand. Apart from jackfruit we also used other ingredients such as sugar, citric acid, carrageenan, petrifilm. Lab utensils and equipments included thermometer, waterbath, viscometer, weight balance, refractometer, colony counter.

Effect of primary juice extraction: Jackfruit pulp was separated from seed by hand. Water was added by various ratio of 30:70, 40:60: 50:50, 60:40, 70:30 to primarily extract juice from jackfruit pulp. The mixture was filtered using a cotton cloth to collect juice. Total soluble solid (oBrix) was indicator to determine the optimal dilution ratio.

Effect of temperature and time in pasteurization: Jackfruit pulp was separated from seed by hand. Water was added by the ratio of 50:50 to primarily extract juice from jackfruit pulp. The mixture was filtered using a cotton cloth to collect juice. This juice will then be pasteurized at different temperature and time (80oC in 5 minutes, 85oC in 4 minutes, 90oC in 3 minutes, 95oC in 2 minutes). Beta-caroten (mg/100g) was indicator to determine the optimal temperature and time in pasteurization

Effect of enzymatic hydrolysis on jackfruit juice: After choosing the suitable dilution ratio (50:50), pasteurization (90oC in 3 minutes) in the primary juice extraction; the sample was then cooled to 37°C before supplementation of pectinase enzyme at different level 0%, 0.03%, 0.05%, 0.07% and 0.09%. The mixture was then incubated in a water bath at 37°C in 60 minutes. After the incubation, the juice was heated in a water bath at 100°C for 3 minutes to inactivate all existing enzymes. Juices were taken to verify sensory, yield, total soluble solids, and viscosity.

Effect of jackfruit juice formulation: The hydrolyzed jackfruit juice will be formulated with sugar (3.5%) different contents of citric acid (0.02%, 0.04%, 0.06%, 0.08%), carrageenan (0.5%, 0.75%, 1.0%, 1.25%). In each sample, we conducted the sensory evaluation to define the optimal contents of citric acid and carrageenan

Preservation: In order to verify the stability of jackfruit beverage during storage, we sampled the microbial (TPC, Coliform, *E. Coli*) at different intervals (2 month, 4 months, 6 months, 8 months). We also carried out the overall acceptance evaluation for jackfruit drink.

Physico-chemical and biological analysis: Jackfruit juice was filtered on a cotton cloth on the volumetric flask. Total soluble solids (TSS) were measured by refractometer. Beta-caroten (mg/100g) was measured by on-line near-infrared spectroscopy (Tamburini et al., 2017). The viscosity (cP) measurement was made by using a viscometer. Overall acceptance was evaluated by a group of panelists using the 9-point Hedonic scale. 3M-Petrilm was utilize to measure TPC, Coliform, *E. coli*.

Statistical analysis: The experiments were run in triplicate with three different lots of samples. Statistical analysis was performed by the Startgraphics Centurion XVI.

RESULTS AND DISCUSSION

Nutritional composition in jackfruit juice: We performed the primary analysis in ripen jackfruit. Our results showed in table 1. From table 1, we could see that durain had a good source of protein (1.51%), low fat (0.23%), high fibre (1.62%) and hihg carbohydrates (23.75%). Our results were similar to data from Ranasinghe et al., (2019), the moisture (72-94%), protein (1.2-1.9%), fat (0.1-0.4%), carbohydrate (16.0-25.4%), fiber (1.0-1.5%), vitamin A (175-540 IU). Rahman et al., (1995) have reported the presence of a high percentage of starch in jackfruit perianth. According to Goswami et al. (2011), the protein content of the flesh of different varieties of ripen jackfruit has ranged from 0.57 to 0.97%. The fiber

content of jackfruit was 0.33-0.40% with no significant changes in different portions of the fruit at different ripening stages (B. T. Ong et al., 2006).

Table 1. Nutritional composition in ripe jackfruit pulp

Composition	Value
Moisture (%)	72.89±0.03
Protein (%)	1.51±0.00
Fat (%)	0.23±0.02
Fibre (%)	1.62±0.01
Carbohydrate (%)	23.75±0.03
Beta-caroten (mg/100g)	17.36±0.02

Note: the values were expressed as the mean of three repetitions;

Effect of primary juice extraction Effect of dilution ratio: According to Jadhav et al. (2018), the jackfruit juice contained total soluble solid, protein as 25oBrix, 1.5% respectively. In our research, jackfruit pulp was separated from seed by hand. Water was added by various ratio of 30:70, 40:60: 50:50, 60:40, 70:30 to primarily extract juice from jackfruit pulp. The mixture was filtered using a cotton cloth to collect juice. Total soluble solid was indicator to determine the optimal dilution ratio. Our results were elaborated in table 2. From table 2, we noticed the dilution (water: jackfruit pulp) at ratio of 50:50 appropriated for juice extraction. Comparing to other research, jackfruit must was prepared by diluting the juice as per the treatments (1:1, 1:2. 1:3 and 1:4), dilution level (1:1) recorded highest (11.7 mg/100ml) ascorbic acid content (Jadhav et al., 2018).

Table 2. Effect of dilution ratio in jackfruit juice extraction

Dilution ratio (water:jackfruit pulp)	Total soluble solids of jackfruit juice (oBrix)
30:70	9.85±0.03^{a}
40:60	9.24±0.00^{ab}
50:50	9.02±0.01^{b}
60:40	8.31±0.00^{c}
70:30	7.43±0.02^{d}

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

Effect of temperature and time in pasteurization: Beta-carotene in jackfruit pulp increased gradually with the progress of ripening. The antioxidant activities of jackfruit fresh extracts were correlated with the total phenolic and flavonoids content (Jagtap et al., 2010). Jackfruit pulp was separated from seed by hand. Water was added by the ratio of 50:50 to primarily extract juice from jackfruit pulp. The mixture was filtered using a cotton cloth to collect juice. This juice will then be pasteurized at different temperature and time (80oC in 5 minutes, 85oC in 4 minutes, 90oC in 3 minutes, 95oC in 2 minutes). Beta-carotene (mg/100g) was indicator to determine the optimal temperature and time in pasteurization. Our results were elaborated in table 3. From table 3, we presumed that optimal temperature in jackfruit juice pasteurization should be kept at 90 oC in 3 minutes. Norjana, and Aziah (2011) proved that after the incubation process, the puree was again heated in a water bath at 90°C for 10 min to inactivate the enzyme present.

Table 3. Effect of temperature and time of Pasteurization on beta-carotene (mg/100g) in jackfruit juice

Temperature (°C) and time (minutes) in Pasteurization	Beta-carotene (mg/100g)
80°C, 5 minutes	9.07±0.00^{c}
85°C, 4 minutes	13.75±0.03^{ab}
90°C, 3 minutes	14.59±0.00^{a}
95°C, 2 minutes	12.04±0.0^{2b}

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

After choosing the suitable dilution ratio (50:50), pasteurization (90oC in 3 minutes) in the primary juice extraction; the sample was then cooled to 37°C before supplementation of pectinase enzyme at different level 0%, 0.03%, 0.05%, 0.07% and 0.09%. The mixture was then incubated in a water bath at 37°C in 60 minutes. After the incubation, the juice was heated in a water bath at 100°C for 3 minutes to inactivate all existing enzymes. Juices were taken to verify sensory, yield, total soluble solids, and viscosity. From table 4, we notice that jackfruit juice should be incubated with 0.07% pectinase at 37 °C and in 60 minutes of incubation. Norjana, and Aziah (2011) proved that the juice treated with 0.05% pectinase concentration and 3 hour incubation time was the most preferred. In another report, juice and water ratio for preparation of jackfruit must should be 1:1 and for obtaining the better recovery of wine pectinase enzyme quantity should be 0.10% (Jadhav et al., 2018).

Effect of jackfruit juice formulation: There are different types of stabilizers or thickener suitable for fruit beverage production such as alginate, pectin, carrageenan, gellan, gelatin, agar, modified starch, methyl cellulose and hydroxypropyl methylcellulose (Banerjee and Bhattacharya, 2012). In our research, the hydrolyzed jackfruit juice will be formulated with sugar (3.5%) different contents of citric acid (0.02%, 0.04%, 0.06%, 0.08%), carrageenan (0.5%, 0.75%, 1.0%, 1.25%). In each sample, we conducted the sensory evaluation to define the optimal contents of citric acid and carrageen. The present results are depicted in table 5 where it can be noted that 0.06% citric acid with 1.0% carrageenan would give the best jackfruit beverage.

Preservation: In order to verify the stability of jackfruit beverage during storage, we sampled the microbial (TPC, Coliform, *E. coli)* at different intervals (2 month, 4 months, 6 months, 8 months). We also carried out the overall acceptance evaluation for jackfruit drink. We noted that jackfruit beverage could be stable for 6 months (see table 6).

Table 4. Effect of enzymatic hydrolysis on jackfruit juice

Pectinase (%)	Sensory (score)	Yield (ml)	Total sluble solids (oBrix)	Viscosity (cps)
0	4.18±0.00^{c}	325±0.01^{c}	9.02±0.01^{c}	3.86±0.02^{a}
0.03	7.01±0.01^{b}	474±0.03^{b}	10.65±0.01^{b}	2.85±0.03^{b}
0.05	7.23±0.03^{ab}	491±0.00^{ab}	11.17±0.02^{ab}	2.56±0.00^{bc}
0.07	7.64±0.02^{a}	514±0.02^{a}	11.86±0.00^{a}	2.21±0.01^{c}
0.09	7.67±0.00^{a}	520±0.01^{a}	11.92±0.03^{a}	2.19±0.02^{c}

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

Table 5. Effect of jackfruit juice formulation on sensory evaluation

Citric acid (%)	Carrageenan (%)				Average
	0.5	0.75	1.0	1.25	
0.02	7.49±0.03	7.84±0.02	8.16±0.00	8.19±0.03	7.92±0.02^{b}
0.04	7.73±0.00	7.97±0.00	8.31±0.02	8.35±0.00	8.09±0.01^{ab}
0.06	7.98±0.02	8.21±0.03	8.49±0.01	8.52±0.02	8.30±0.02^{a}
0.08	8.00±0.01	8.25±0.01	8.63±0.03	8.63±0.01	8.37±0.02^{a}
Average	7.80±0.02^{c}	8.07±0.02^{b}	8.40±0.02a	8.42±0.02^{d}	

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

Table 6. Stability of jackfruit beverage during storage

Storage (months)	TPC (cfu/ml)	Coliform (cfu/ml)	E. coli (cfu/ml)	Sensory score
2	1.18x102±0.03^{c}	0	0	8.21±0.01^{a}
4	2.35x102±0.00^{bc}	0	0	8.02±0.03^{ab}
6	3.58x102±0.01^{b}	0	0	7.94±0.02^{b}
8	8.63x102±0.02^{a}	0	0	7.15±0.01^{c}

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

CONCLUSION

At peak harvesting season, the overproduction of jackfruit (*Artocarpus heterophyllus*) and its perishability have caused significant damage for farmers. High sugar content of the jackfruit pulp makes its possibility for beverage production. We have successfully optimized some technical parameters in jackfruit drink production. Generally jackfruit drink products show more viscosity, low separation and stable shelf-life. Advanced processing technologies and sustainable waste management strategies should be considered when processing jackfruit in commercial scale.

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Homelessness and Neglect of Children in Modern Russia: Literature Based Analysis

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ABSTRACT

Adversity in Russian society and the Russian family has led to increased child abuse, psychological stress, illness, suicide, and prostitution. It should be borne in mind that many of the rules of law governing family and state relations, came into conflict with modern life realities, and ceased to adequately meet the needs of the family and society. An important factor in child neglect in Russia, in addition to a dysfunctional family producer, is the stage of outgoing violation of the distinctive rights of children's elements; delivery in the field of education, rehabilitation, the system for obtaining a profession and housing, the degree and measures of the growth of the unemployment system, an activity that is developing more and more worsening the financial situation Russian families. According to the legislation of Russia, children and adolescents who have lost family and family ties, abandoned by their parents, or who voluntarily leave families that have not provided the child with the minimum necessary conditions for life and full development, are neglected. The hallmarks of homelessness include the complete loss of any ties with family, parents, relatives, living in places not intended for human habitation, obtaining funds to provide for life in ways that are not recognized by society as socially positive, submission to caste criminal laws prescribed by street children as adult criminals.

KEY WORDS: HOMELESSNESS, NEGLECT, CHILDREN, RUSSIA, THE LAW.

INTRODUCTION

Modern studies in the social sphere indicate crisis situations in many areas of the life of Russian people, which affect their consciousness and behavior. A serious social danger is that the negative consequences of such changes affect children as the most vulnerable category of the population. This leads to a violation of their physical and mental health, gives an impetus to the development of so-called social diseases, including neglect and homelessness (Kholostova, 2011). Significant reasons for the increase in neglect in modern conditions are the ongoing deterioration in the standard of living of Russian families, the decline in its moral principles, the unwillingness of many parents to raise their children, and the increase in the number of divorces and single-parent families (Vorobyeva et al., 2018).

Social problems in society and in the family was the cause of frequent ill-treatment of children, psychological stress, diseases, suicides, prostitution. Because note that many of the rules of law regulating the relations of the family and the state, came into conflict with modern life's realities,

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have ceased to adequately meet the needs of the family and society (Lublinsky, 2012). Additional factors of child neglect, in addition to the disadvantaged producer families, step is leaving the distinctive violation of the rights of children elements of delivery in education, health, the system of obtaining the establishment of a profession and the same housing, degree and measures the growth of unemployment, more activities, more developing worsening financial situation of families.

Determining how many street children are in Russia is quite difficult. According to estimates by the Ministry of the Interior in Russia, there are from five hundred thousand to two and a half million street children today. Their number constantly fluctuates and develops on the basis of data received from internal affairs bodies. The figures given by official bodies differ from those used by non-governmental organizations, sometimes twice. So, according to the Russian Children's Fund, the country has three million homeless children, according to the Russian movement "In Defense of Childhood"- four million. Conducted sociological studies give other figures. The media also operate on various indicators. The difference in the estimates is explained by the fact that the authorities only consider children who have become the object of police attention, as they have committed various offenses and were put on the wanted list. At the same time, according to the Ministry of Internal Affairs, a negative tendency remains in Russia to affect the growth process of the number of domestic street children represented, (Merdeshev, 2019).

There is also a need for a radical increase in the associated effectiveness of the work representing all the progressive structures of a developing state, establishing responsible for the retail solution to the division of the problems of street children. Necessary being a search include non-standard evolving ways of reducing elements providing the acuteness of the trade and the negative consequences of the deviating commercial behavior of children and adolescents. The purpose of the establishment of the work features: to consider the problems of street and street children in Russia.

MATERIAL AND METHOD

The research material was 31 literature sources listed in the list of references. As research methods, analysis and synthesis methods, induction and deduction methods, as well as a generalization method were used. With their help, the available information presented in the results of the study was generalized and critically processed.

RESULTS AND ITS DISCUSSION

Homelessness and neglect as a social phenomenon is the object of study in many areas and fields of science. This circumstance is due to the complexity of the phenomenon itself, which requires an intersubjective approach to it, a combination of scientific, axiological, regulatory, ethological and other levels of its analysis (Bolshakova and Khamadeeva, 2012).In the Russian legislation on the family, in the legislation on the protection of the rights and legitimate interests of children and in the scientific literature, the term "children deprived of parental care" has become widespread. In this regard, the concepts of "neglect" and "homelessness" often began to be used as equivalent. For quite a long time, researchers considered the phenomenon of neglect as one of the significant reasons for adolescents committing unlawful actions (Nevarov, 2014).

The Russian Federal Law of June 24, 1999 "On the Basics of the System for the Prevention of Homelessness and Juvenile Delinquency" prevented the definition of "homelessness" and "homelessness" as various phenomena for the first time, but hereinafter in the text of this law there are no real differences between these concepts not traceable. Because of this, difficulties ensued in the selection and determination of ways to prevent homelessness, which, in our opinion, shows the highest degree of social danger for minors and for the whole society. This law provides the following definitions of these concepts: "neglected - a minor whose control over the conduct is absent due to non-fulfillment of silt and improper fulfillment of obligations to educate him, about teaching and (or whether) with the help of parents or equestrian representatives, or officials "Homeless - homeless, with no place of residence and place of stay" (Federal Law of the Russian Federation 120 of 24.06.1999).

A street child, unlike a street child, usually lives under the same roof with his parents, maintains ties with his family, he still has emotional affection for one of the family members, but these bonds are fragile and are at risk of atrophy and destruction. Left to their own devices, children abandon their studies, give free time to the street, aimless pastime. Child neglect is often the first step towards homelessness, social maladaptation, and the destruction of the normal process of child socialization.

Neglected include children and adolescents: those who have lost family and family ties; abandoned by parents or voluntarily abandoned from families that did not provide the child with the minimum necessary conditions for life and full development, who tolerated ill-treatment, escaped from boarding schools, graduates of boarding schools who found themselves without work and livelihood, engaged in vagrancy, begging, committing petty theft; consuming alcoholic beverages, toxic and narcotic substances; who have received a delay in serving their sentences, are victims of sexual crimes, are involved in illegal activities, and are sentenced to probation.

The hallmarks of homelessness include: the complete loss of any ties with the family, parents, relatives, living in places not intended for human habitation, obtaining funds to ensure life in ways that are not socially recognized as socially positive; submission to caste criminal laws prescribed by homeless "authorities". Lack of own housing usually turns street children

into vagabonds, wandering from place to place. The combination of all the above signs distinguishes a street child from other children (Kholostova, 2017).

Homelessness is often associated with unlawful behavior. Street children do not have parental or state care, a permanent place of residence corresponding to the age of socially approved occupations, necessary care, proper upbringing and systematic education. Among wandering, homeless adolescents, depending on the duration of their stay in the street environment, three levels are distinguished. The first level includes adolescents who have been outdoors for less than a month. He had not yet managed to adapt in this world, did not lose hope of returning to his family (especially younger adolescents).

The second level includes adolescents who have been outdoors for more than a month (sometimes up to a year). As a rule, he was exposed to the additional risk of ill-treatment and violence after escaping from home or a child care facility. Such adolescents have already gained experience in drinking alcohol, toxic agents, and often sexual relations. The spectrum of their antisocial behavior is significantly expanded in comparison with children of the first level. Begging, and he prefer theft.

The third level includes teenagers who left home more than a year ago. Such adolescents have already gained criminal experience; they are often involved in various activities by criminals. The transition of a teenager to this group increases the risk of his socio-psychological deformation and reduces his chances of getting into a social rehabilitation institution. In today's conditions, this group has a tendency to increase.

According to the Russian Federal law "the homeless" – it's the same street but are in the most difficult life situation due to their lack of residency. Speaking about the mismatch of the terms "neglect" and "neglect" should be aware that neglect is mainly determined by the pedagogy. Its distinctive and essential features, in particular, are the subject of pedagogical science, calling attention to the supervision of minors, which is not limited to the control over its behavior, pastime, as is the maintenance and preservation of inner spiritual connection with a child, a teenager, a relationship that allows you to save even at a distance contact the parents and persons substituting them with his pupil. The lack of such oversight is fraught with trouble, when problems arise of a legal nature, including those associated with homelessness (Bogdanov,2016).

Between neglect and homelessness, of course, there is a strong link. Neglect is a fertile ground for homelessness. Homelessness consider how long the disease goes through several stages, or phases, of development. The initial phase of this social "disease" is precisely the neglect and final, already very advanced, on the verge of irreversibility becomes homelessness as such, which determines the position of the child, in the peculiar socio-psychological status, which he acquires at his own request or due to a confluence of any circumstances. Among them was dominated by neglect, that is, the lack of supervision of parents or persons substituting them (Feonychev, 2015).

To understand the causes and factors influencing the occurrence of child neglect in Russia, it is necessary to consider socio-psychological portrait of the Russian street children.Modern neglected child resembles a children's home: it is distinguished by the underdevelopment of the emotional sphere, the mental retardation and short attention span. Living conditions on the street make the child to survive and realize itself, forming mental flexibility, reaction time, rigidity of the decisions made, acuity at the household level (Vorobyeva et al., 2018). Such children are creative and resourceful, able to unite to face the danger. They are good psychologists and know in adults, have high communication skills. The presence of these qualities allow the homeless to earn money to life in ways not recognized in society (begging, stealing) (Bolshakova and Khamadeeva, 2012).

Russian street child is characterized by a steady violations of psycho-emotional sphere, abnormalities in social behavior and a gross distortion of group norms of interaction. Prolonged lack of attention and supervision by parents, schools, families and other social institutions of society, these violations lead to the inevitable changes in the child's personality and affect his transition from the category of street children in the street. Significant difference in the socio-psychological characteristics of the above two categories is the presence of street children psychosomatic diseases and mental disorders caused by prolonged use of psychoactive substances and the specific conditions of existence. Among street children and adolescents, there are only preconditions for such violations due to inattention on the part of adults (Pishchulin, 2007).

From the point of view of criminologists children continue to lead a so called normal way of life differ from other also by the fact that life goals have shifted in the direction of psychological comfort, social - group, short-term pleasures of consumerism and greed. They have fixed the weakening of the sense of shame, indifferent to the feelings of others, aggressiveness, rudeness, deceit, nezametnoe. Added to that, clicking on the position of the outcast, homeless minors losing the initiative in the assimilation of more complex forms of social behavior. From children in normal families homeless in Russia is characterized by a stronger instinct and habit to the artificial agents (drugs, alcohol, and toxic substances). Some of them prematurely begin a sex life. Constant, at times severe physical stress, making such children are physically fit, providing them with an ideal body weight. The possibility of development of this effect was confirmed in experiment indicating the possibility of preservation of good health on the background of regular physical activity, despite the negative impact from the external environment, (Vatnikov et al., 2018).

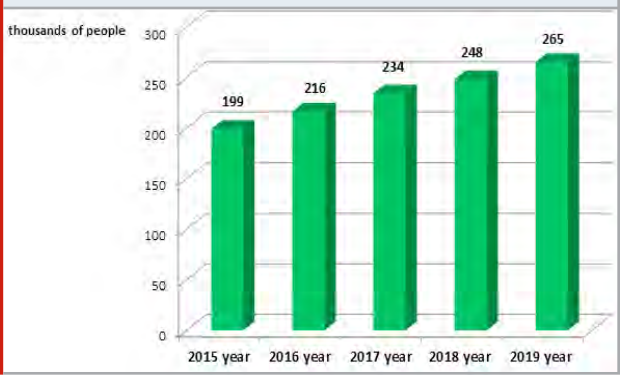
Most often, this category of children can be found in the busiest places where you can get food or earn money bazaars, markets, squares, supermarkets, expensive night clubs. Their day usually begins closer to lunch, and ends well after midnight. Livelihood is earned by washing cars, begging, removal of debris from the stalls, theft, prostitution. In many cases, their work is heavy, dangerous and long-lasting (Demidova, 2013).As a result of rising male mortality at young age, divorce, and extramarital birth rate, the number of single-parent families with less opportunities for maintaining and raising children is increasing in Russia. Every seventh Russian child is brought up in an incomplete family.

In conditions of disorganization of family life, the prevailing moral and ethical standards and traditions of the family structure are being destroyed. The disorganization of the life of families is expressed in strengthening the conflict of relations between spouses, parents, children, their depressed state as a result of legal, economic, moral insecurity. Dysfunction, poverty of families, unfavorable psychological atmosphere adversely affect the upbringing of children, their moral and physical development. For most of them, the most necessary consumer goods, leisure services, sports, and culture services were not available,(Abdacim, 2017). In children who have not been given due attention from the moment of birth, there is an insufficient formation of observation, obedience and diligence, moral ideas and a sense of respect for others. The absence of the necessary moral ideas and skills prevents them from orientating themselves correctly in communication with peers, leading to conflicts and alienation. The child becomes "difficult" for peers and for educators, pedagogical neglect arises (Erman, 2007, Bespalov et al., 2018).

There's also the reverse situation – the child, are not educationally neglected, may cause persistent resistance to pedagogical influences in the power of the inept approach of the teacher, the impact of the unfavorable current situation, the personality of the teenager. It can be assumed that the requirements of the teacher, is unfair, are a manifestation of its arbitrariness and Caprice. Internal dislike of a student to the teacher can be easily transferred to all outgoing from him ideas and can make the student strong enough internal resistance that the traditional pedagogical means cease to operate, and sometimes give results opposite to the expected. In such conditions, relationship conflicts there is often a conscious rejection of the child norms and values that come from the caregiver, and consequently the student becomes pedagogically neglected (Komarov, 2014).

For quite a large period of time in Russia there is a growth of the total crime in General, and that combined with the quantitative increase in juvenile crime. Street children, being so-called risk group, not having a livelihood, they seek affordable way. Not always by legal means. And as a consequence permanently join the ranks of juvenile delinquents. The number of teenagers taken to the police for various offenses in 2019 exceeded 1 million 140 thousand Ten years ago - there were exactly two times less. Among delivered to 310 thousand – Teens as young as 13 years old, and 295 thousand, neither working nor students. While homeless and neglected of them were 265 thousand, and 45 thousand of them the numbers were generally not literate (figure 1) (Mikirtichan et al., 2010).

Figure 1: Five-year dynamics of the number of street and street juvenile offenders in Russia



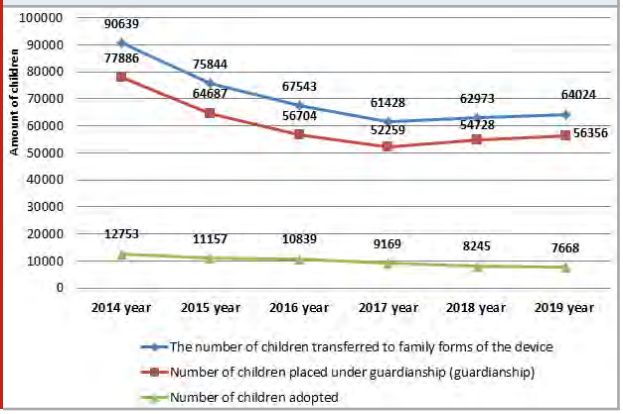
Recently in Russia more than in 2 times increased the number of minors who committed murder and attempted murder, 1.5 times committed a minor robbery, 2.4 times minors who were detained for illegal purchase and manufacture of drugs. The number of street children with syphilis, other sexually transmitted diseases, and acquired immune deficiency syndrome has increased tenfold. Over the past three years, the number of children registered in drug addiction clinics has increased almost three times, for substance abuse - 3.5 times, and the number of adolescents annually recognized as drug addicts has increased 13 times over ten years. A specific problem of minors is substance abuse and addiction. Adolescents abuse drugs 7.5 times, and intoxicants 12 times more often than adults (Ivanova et al., 2017).

Statistics show that Russian juvenile delinquency has a steady tendency to "rejuvenate". Therefore, the most acute problem is the crime of minors who have not reached the age of 14, as well as adolescents who are not subject to criminal liability. There is an active process of involving adolescents in the criminal business, in extortion, in prostitution. In Russia, child crime is now highly organized. Group nature is today one of the specific features of juvenile delinquency. In recent years, the proportion of minors who have committed crimes in groups has consistently exceeded 70% (Samoylova and Sidukova, 2014).

The development of work on the social adaptation of street and street minors includes the identification of such children and adolescents and their placement in specialized institutions for minors, the organization of activities aimed at adapting each time phase of a child's life to reality. Minors in difficult situations experience a lack of internal resources to change both themselves and the situation as a whole. That is why, for their adaptation and integration, they need a socially organized influence aimed at active inclusion in society, corresponding to the

needs of the child. Such socially-organized influence aimed at the successful process of social rehabilitation, adaptation, integration and socialization of street and homeless minors is carried out, in accordance with Federal Law *No*120 "On the Basics of the System for the Prevention of Street Childhood and Juvenile Delinquency", in specialized institutions for minors in need of social rehabilitation (Abramov, 2006).

Figure 2: Shows the features of the device of the identified street and street children



To solve this problem, state programs are being developed in the country to transfer children from shelters to guardianship or to raise a family. The modern system of state institutions for such children is ineffective from the point of view of adaptation to independent life in the new socio-economic conditions and the conscious choice of their adult social role. For this reason, their number tends to decrease (table).

Table 1. Dynamics of the number of social institutions for children in Russia

Institutions for orphans	2016 year	2017 year	2018 year	2019 year
Baby houses, units	243	227	218	207
They have children, thousands of people	17.8	17.5	16.3	16.0
Children's homes, units	1095	1048	1012	976
They have children, thousands of people	52.3	50.0	48.3	45.9
Boarding schools for orphans and children without parental care, units	106	90	83	72
They have children, thousands of people	12.1	9.8	9.0	7.8

To improve the current situation, to increase the efficiency of the process of social adaptation of street and street minors in Russia, the development of programs and methods aimed at the professional adaptation of adolescents in specialized institutions is being implemented; closer interagency interaction, coordination and cooperation of social institutions, organizations and services in working with the category of street and street minors is ensured (Forms and Fokin, 2008).The above measures are implemented in Russia at the municipal level of government, directly at the institution level. However, for a more successful process of social adaptation of street and homeless minors in specialized institutions, improvements are needed, including the legal and regulatory framework, at all levels of government. The activities of specialized institutions are referred to the competence of social protection authorities. This provision was enshrined in the Federal Law "On the Basics of Social Services for the Population", an order of the Government of the Russian Federation and a decree of the Government of Russia "On the Approval of Model Provisions on Specialized Institutions for Minors in Need of Rehabilitation". In accordance with the adopted standards, social rehabilitation centers for minors, social shelters for children and adolescents, and territorial centers for social assistance to families and children were created (Burgoeva, 2014).

The fundamental document for solving the problems of social deviations in children's environment in Russia is the Law "On the Basics of the System for the Prevention of Neglect and Juvenile Delinquency." Developed and approved a program of measures to overcome social orphan hood and improve the situation of children left without parental care. The program provides for improving the regulatory framework, supporting families in the upbringing and education of children, social protection of orphans and children left without parental care. Depending on what categories of children and adolescents, and socially vulnerable families have to work the specialists, their areas of activity are built in a particular system, in which the measures of primary, secondary and tertiary prevention (Oliferenko and Shulga, 2002).

Primary prevention and early correction of the child's behavior and the parent-child relationship are educational institutions, leisure socio-pedagogical centers, counseling centers and educational rehabilitation. The primary objects of the work are children and adolescents, which was not seen in the manifestation of antisocial acts, but are a long time in a socially dangerous situation and have problems in school learning, intellectual development, communication, have fragile social ties with family, relatives, which can further lead to the escape of the child from the family or educational institution (Malerova and Nesmeyanov, 2004).

The subjects of rehabilitation space, operating on the first level aim at the humanization of society of the child, harmonious development, prevention of school and social maladjustment (Namereny, 2003).Profound socio-pedagogical correction of behavior of the child and his/her family members, as well as early rehabilitation of children who have a tendency to deviation, is carried out by: the Commission on Affairs of minors and protection of their rights formed by local government, designed to reveal violations of the rights of minors to education,

work, recreation, and other rights. The guardianship and guardianship that identify minors left without parental care or in the environment that pose a threat to their health and development; health Authorities responsible for examination, monitoring and treatment of neglected and homeless children who use alcoholic drinks, narcotic drugs, intoxicants or psychotropic substances (Machulskaya, 2007).

The object of secondary prevention are street children and teenagers of various ages, from pre-school to youth. They're not involved in criminal activity, the suppression of which should be engaged in law enforcement, but, nevertheless, their social development is adversely and is characterized by various behavioral problems of anti-social character: an introduction to alcohol, drugs, aggression, self-serving misconduct, the shirking study, work, prone to vagrancy. Work in relation to such children in Russia aimed at the development of forms of social and public impact of behavioral change and social adaptation of children in the area of social risk (Krivonosov, 2007).

Work with homelessness on the third level is the correctional institutions, as well as the specialized social rehabilitation institutions, which play a crucial role in crime prevention. These institutions accepted emergency measures aimed at providing assistance, rehabilitation, correction, protection of rights and legal interests of juveniles who are in a critical situation. It can be street children, a long time outside of adult supervision absconded from families or educational institutions, have undergone various forms of violence and cruelty, commit illegal acts, prosecutable (Komarov, 2014).The actors in Russia solution to the problems of homelessness at the third level, realize their activity in the system of stationary and semi-stationary institutions (Abramov, 2006).

Work on solving the problems of child neglect and homelessness is impossible without the suppression of the initial origins of vagrancy most children in dysfunctional families. The main objective of activities aimed at optimizing the social situation of homeless children is the improvement and optimization of social relations in the immediate environment of the child, family, school, peer group. In this regard, on the territory of the Russian Federation, there are local centers of assistance to family and children. Data centers carry out measures of control and social support in the rehabilitation of a teenager with the experience of vagrancy, criminal activity, substance use. The activities of the specialists of the center are aimed at maintaining the educational functions of the family in the juvenile and at preventing the negative consequences of child neblagopoluchnomu (Harichkin, 2001).

In Russia often use social patronage - form is the most close interaction with the family. This method of operation has the goal of creating a family of optimal conditions for the full development of the child's personality, and also implies the establishment of contacts with maladjusted children and motivate them to socially acceptable activities. The center also runs a day (evening) of the children, which operates year-round. In the process of rehabilitation activities the group is the identification of sources and causes of social maladjustment of minors, promote the development of creative abilities of children (Kuzmin, 2004).

On the basis of what categories of children have to work to specialists of the social rehabilitation institutions there are the following main objectives: prevention of neglect and homelessness among minors; providing free social services to juveniles in a socially dangerous situation; identify sources and causes of social maladjustment of minors; the development and provision of individual programmes for social rehabilitation of children and adolescents; provision of temporary accommodation of street children in a normal domestic environment; the provision of psychological, rehabilitative and other assistance; participation jointly with the relevant departments to decide the destiny of minors in their device (Ershov, 2003).

CONCLUSION

According to the legislation of Russia to the street include children and adolescents, by losing family and kinship, abandoned or absconded from families that do not provide the child the minimum necessary conditions for the life and full development. The distinctive signs of neglect include complete loss of all ties with family, parents, relatives, living in places not intended for human habitation, obtaining funds for maintenance of life in ways not recognized by society socially positive, submission of caste criminal laws specific to homeless adult criminals.Homelessness and neglect cannot be called a social phenomenon in the strict sense of the word, since they have a non-rooted, socially determined, and themselves homeless and neglected are certain groups of teenagers, with their usual values, norms, rules, relationships and psychological characteristics.

In the Russian Federation currently has developed a system of correctional-rehabilitation work with juveniles in difficult life situations. This system includes various state and public organizations, which are within its competence, implement measures for the elimination of deviant behavior among juveniles. Support and assistance to children of "risk group" is in the Russian Federation through social services, shelters, social rehabilitation centers, territorial centres for social assistance to families and children. They allow to solve problems of correction and rehabilitation work, focusing it on the child caught in difficult life situations, and the environment in which it is located, including the family home.

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Studies on Various Technical Parameters Affecting Production of Wine from Lucuma, *Pouteria lucuma*

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ABSTRACT

Lucuma (*Pouteria lucuma*) is an important fruit in Vietnam. The fruit pulp exhibits an intense yellow colour and characteristic aroma. It is rich in carotenoids; minerals, vitamins, dietary fibres, triterpenes, phenolic compounds with significant pharmaceutical values. Possible spoilage occurred in the ripen lucuma fruit would be due to inappropriate storage condition. Wine is an alcoholic beverage made by fermenting fruit. In order to diversify this valuable fruit into value-added product like wine, purpose of the current research penetrated on the investigation of lucuma pulp fermentation under several technical variables such as sugar addition (4%, 6%, 8%, 10%, 12%), yeast inoculation (0.3%, 0.4%, 0.5%, 0.6%, 0.7%), fermentation temperature (30°C, 33°C, 35°C, 37°C, 40°C) in the primary fermentation; numerous clarifying agents (gelatin, chitosan, casein, wheat gluten) in the secondary fermentation or aging. Results revealed that sugar added at 8%, yeast inoculated at 0.6%, fermentation temperature conducted at 35oC in the initial must were significantly affected to the ethanol, residual soluble solid and organoleptic property of wine. Chitosan showed the best clarifying effectiveness in fining step to control haze turbidity in lucuma wine. From this research, consumers had more chance to inherit a functional food drink come from natural source.

KEY WORDS: *LUCUMA* WINE, YEAST INOCULATION, SUGAR ADDITION, FERMENTATION TEMPERATURE, CHITOSAN, ETHANOL, RESIDUAL SOLUBLE SOLID, ORGANOLEPTIC PROPERTY

INTRODUCTION

Lucuma (*Pouteria lucuma*) is a tropical fruit belonging to Sapotaceae family (Marianela et al., 2019). Its pulp has an intense yellow colour, sweet pleasant taste and characteristic aroma. Its sweet taste is utilized as a natural food sweetener (Banasiak, 2003). This fruit has a low moisture content compared to other fruits, however protein and reducing sugar contents are high (Erazo et al., 1999; Brizzolari et al., 2019). It's a rich source of carotenoids; minerals, vitamins, dietary fibres, triterpenes, phenolic compounds with variety of functional benefits (Rojo et al., 2010; Fuentealba et al., 2016; Albena et al., 2019).

Lucuma has been successfully processed in the production of ice cream, juices, cakes, biscuits, yogurt, chocolate, baby food and pies (Dini, 2011). It has been reported to treat antihyperglycemia and antihypertension (Marcia et al., 2009). Extracted lucuma nut oil can accelerate wound healing properties (Leonel et al., 2010). Lucuma fruit has been classified as one of super fruits (Mukta et al., 2017).

Fermentation is a relatively efficient, low-energy preservation process. Fruit wines are undistilled alcoholic

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beverages undergoing a period of fermentation and aging. During fermentation, yeast interacts with sugars in the juice to create ethanol, commonly known as ethyl alcohol and carbon dioxide as a by-product. They contain 8–11% alcohol and 2–3% sugar. The nutritive value of wine is increased due to the release of amino acids and other nutrients from yeast during fermentation (Pazhani et al., 2017).

After fermentation was completed, the wine is separated from the sediment by racking or using fining agents. At least 6 months should be allowed for aging. This aging enhanced the taste, aroma, and preservative properties of the wine (Van and Tromp, 1982). There are not many researches mentioned to processing of *Pouteria lucuma* fruit into wine. Hence, the purpose of this research focused on the identification of main technical variables such as sugar addition, yeast inoculation, fermentation temperature in the primary fermentation; numerous clarifying agents such as gelatin, chitosan, casein, wheat gluten in the secondary fermentation or aging influencing to the wine quality making from Pouteria lucuma pulp.

MATERIAL AND METHODS

Lucuma fruits were naturally collected from Soc Trang province, Vietnam. After collecting, they must be moved to laboratory quickly for experiments. They were washed under tap water to remove foreign matters. They were set on stainless tray to drip the remaining water. After that, they were opened by sharp knife to collect their pulp. Sugar and Saccharomyces cerevisiae were added into lucuma pulp in different ratio for the primary fermentation at different temperature in 4 weeks. The aging would be continued in fining by treating with different clarifying agents for 6 weeks at 9.5°C.

Effect of sugar addition in the main fermentation: *Pouteria lucuma* pulp was mixed with sugar addition at various ratio: 4%, 6%, 8%, 10%, 12% with Saccharomyces cerevisiae 0.3%. After 4 weeks of fermentation at 30°C, we examined the residual soluble dry matter (oBrix), ethanol (%v/v), and organoleptic attribute (sensory score) in wine.

Effect of yeast inculate in the main fermentation: *Pouteria lucuma* pulp supplemented 8% sugar combined with Saccharomyces cerevisiae was inoculated at various ratio (0.3%, 0.4%, 0.5%, 0.6%, 0.7%). After 4 weeks of fermentation at 30°C, we examined the residual soluble dry matter (oBrix), ethanol (%v/v), and organoleptic attribute (sensory score) in wine.

Effect of temperature in the main fermentation: *Pouteria lucuma* pulp supplemented 8% sugar combined with Saccharomyces cerevisiae was inoculated at 0.6% in various temperature (30°C, 33°C, 35°C, 37°C, 40°C). After 4 weeks of fermentation, we examined the residual soluble dry matter (oBrix), ethanol (%v/v), and organoleptic attribute (sensory score) in wine.

Effect of clarifying agent in the aging to wine quality: *Pouteria lucuma* wine was stored at 9.5oC for 6 weeks as aging with the supporting of numerous fining agents such as gelatin, chitosan, casein, wheat gluten at 0.2%. We monitored organoleptic attribute (sensory score) in wine.

Chemical and sensory analysis: Soluble solid (oBrix) was examined by hand-held refractometer. Ethanol (%v/v) in wine was measured by spectrophotometer. Sensory score was evaluated by a group of 13 panelists using 9 point-Hedonic scale.

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

RESULTS AND DISCUSSION

Effect of sugar addition in the main fermentation: Sugar is one of the most common substrate of fermentation to produce ethanol, lactic acid, and carbon dioxide (Giri et al., 2013). Sugar is an essential precursor nutrient affecting to the wine flavor, aroma taste and quality of fruit wine. It shows a correlation to the degree of fermentation (Xiao et al., 2017). Higher sugar concentration inhibits the growth of microorganisms (Pino et al., 2015). Sugar may need to be added to spur the fermentation process in the event that the fruit does not contain enough natural sugar to ferment on its own in the presence of yeast (Pazhani et al., 2017).

Table 1. Effect of sugar addition on the main fermentation

Parameter	Sugar addition (%)				
	4	6	8	10	12
Residual soluble solid (oBrix)	2.79±0.02^{d}	3.39±0.03^{cd}	4.04±0.00^{c}	6.97±0.02b	8.43±0.01^{a}
Ethanol (%v/v)	3.11±0.00^{d}	4.65±0.01^{c}	7.09±0.00^{a}	5.72±0.03^{b}	4.03±0.00^{cd}
Sensory score	4.09±0.03^{c}	5.25±0.02^{b}	6.14±0.01^{a}	5.68±0.00^{ab}	4.83±0.03^{bc}

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

In the present research, *Pouteria lucuma* pulp was mixed with sugar at various ratios: 4%, 6%, 8%, 10%, 12% with Saccharomyces cerevisiae 0.3% at 30°C. The results are presented in table 1. We could clearly see that 10% of sugar addition was appropriate for wine making to obtain the best wine quality.

Effect of yeast inoculate on the main fermentation: Saccharomyces sp. is important in food fermentation as its ability to reproduce much faster (Pazhani et al., 2017). Yeast primarily requires sugars, water, and warmth to stay alive. Yeast flourishes in habitat where sugar exists. The alcoholic fermentation of fruit must is initiated by a complex yeast community comprising a high proportion of oxidative and weakly fermentative yeasts (Bahareh et al., 2017). In pure fermentation, the ability of inoculated Saccharomyces cerevisiae to suppress the wild microflora is one of the most important feature determining the starter ability to dominate the process (Maurizio et al., 2016).

The inoculation of musts using selected Saccharomyces strains does not ensure their dominance at the end of fermentation (Capece et al., 2010). In our current study, *Pouteria lucuma* pulp was inoculated with Saccharomyces cerevisiae at different ratios (0.3%, 0.4%, 0.5%, 0.6%, 0.7%). Our results were presented in table 2. We could clearly see that 0.6% of yeast was suitable for wine making to obtain the best wine quality.

Table 2. Effect of yeast inoculate on primary fermentation

Parameter	Yeast ratio (%)				
	0.3	0.4	0.5	0.6	0.7
Soluble dry matter (oBrix)	4.04±0.00^{a}	3.71±0.02^{ab}	3.01±0.01^{b}	2.62±0.03^{bc}	2.31±0.00^{c}
Ethanol (%v/v)	7.09±0.00^{b}	7.42±0.01^{ab}	7.68±0.02^{ab}	7.81±0.00^{a}	7.90±0.03^{a}
Sensory score	6.14±0.01^{b}	6.39±0.03^{ab}	6.67±0.00^{ab}	6.79±0.01^{a}	6.82±0.00^{a}

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

Effect of temperature in the main fermentation: A lower temperature is preferred as it increases the formation of esters, other aromatic compounds, and alcohol itself. This causes the wine easier to clear and less susceptible to bacterial infection (Akubor et al., 2013). Temperature control during alcoholic fermentation is essential to promote yeast growth, extract flavors and colors from the skins, allow accumulation of desirable by-products, and limit undue rise in temperature that might kill the yeast cells. The low temperature and slow fermentation favor the retention of volatile compounds. High temperature is necessary to extract the pigment from the fruit skins (Fleet, 2013). In our research, *Pouteria lucuma* pulp supplemented 8% sugar combined with Saccharomyces cerevisiae inoculated at 0.6% in various temperature (30°C, 33°C, 35°C, 37°C, 40°C). Our results were presented in table 3. We could clearly see that fermentation should be conducted at 35°C to obtain the best wine quality.

Table 3. Effect of temperature on the main fermentation

Parameter	Temperature (oC)				
	30	33	35	37	40
Soluble dry matter (oBrix)	2.62±0.03^{ab}	2.11±0.01^{b}	1.63±0.00^{c}	1.98±0.03^{bc}	2.97±0.01^{a}
Ethanol (%v/v)	7.81±0.00^{bc}	8.03±0.02^{b}	8.37±0.01^{a}	8.23±0.00^{ab}	7.54±0.02^{c}
Sensory score	6.79±0.01^{c}	7.42±0.03^{b}	7.81±0.02^{a}	7.60±0.01^{ab}	.01±0.00b^{c}

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

Effect of clarifying agent in the aging to wine quality: The clarifying agents bound the target components to form insoluble aggregates that were subsequently removed from the wine (Matteo et al., 2019). Gelatin preferentially removed high molecular weight tannins (Sarni et al., 1999; Maury et al., 2001; Smith et al., 2015). The gluten preparations were reported to remove highly galloylated tannins in similar quantities as gelatin, while gelatin was more effective in removing total tannins (Maury et al., 2003). Gluten used as fining agent had less impact on the color and anthocyanin content of red wines (González et al., 2014). Fining the red wines

with gluten had a small impact on proanthocyanidins (Granato et al., 2014).

Casein was much more effective at low addition rates (Marchal et al., 2002). Being nontoxic and biodegaradable, chitosan may be used as an alternative agent for clarification. Chitosan has been reported to prevent protein haze by the partial precipitation of excess proteinaceous matter (Ricardo et al., 2012). Chitosan as a clarifying agent complexes with protein, polyphenols, and others insoluble solids inducing flocculation and sedimentation thus resulting in removal of these potential haze precursors (Soto et al., 1989). In our research, *Pouteria lucuma* wine was preserved at 9.5oC for 6 weeks as the aging with the supporting of different fining agent such as gelatin, chitosan, casein, wheat gluten at 0.2% (see table 4). Our results revealed that chitosan was superior to other fining agents. One study compared the effectiveness of gelatin and kaolin in clarifying wine variously produced from locally available fruits (pawpaw, pineapple, cashew and banana). Gelatin was a better clarifier than kaolin (Awe, 2018).

Wheat gluten was used as clarifying agent of musts and white wines (Richard et al., 2002). One study compared gluten to gelatin for clarification of young red wines (Iturmendi et al., 2010). Gluten and gelatin were similar in reducing turbidity, but gluten had the advantage of producing less lees and reduced the content of polyphenolic material less than gelatin did. According to Noriega et al. (2010) wine treated with gluten had residual wine post-filtration turbidity that was lower than that achieved using gelatin. Gluten was very fast in lowering wine turbidity upon application (Granato et al., 2018). Gluten showed a similar clarification ability to that of casein while it produced a lower amount of lees (Fernandes et al., 2015). Some residues can be present in gluten fined wines (Simonato et al., 2011; Cattaneo et al., 2003).

Table 4. Effect of clarifying agents on the secondary fermentation to wine quality

Parameter		Fining agent 0.2%		
	Gelatin	Chitosan	Casein	Wheat gluten
Sensory score	8.24±0.02^{ab}	8.87±0.04^{a}	7.95±0.03^{b}	8.41±0.030^{ab}

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

CONCLUSION

Fermentation diversified new products of fruits with modified physicochemical and sensory qualities, especially flavor and nutritional constituents. Lucuma is most common in the form of flour. It's a good source of biologically active substances especially beta-carotene, having an excellent antioxidant activity with antihyperglycaemic characteristics. Lucuma could be considered as a good substitute for rational nutrition because its powder is a good source of beneficial nutrients with various therapeutic advantages. Our research demonstrated that ripen lucuma fruit could be exploited for wine fermentation. From this approach, the commercial value of this lucuma fruit would be improved respectively.

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Impact of Factor V Leiden (G1691A) Variant in Saudi Women with Gynecological Disorders

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ABSTRACT

Gynecological disorders are defined as a condition which disturbs the female reproductive organs. Recurrent Pregnancy Loss (RPL) is one of gynecological disorder which is ostensibly defined as more than couple of consecutive miscarriages before 20 weeks of gestation. After the abnormalities found in chromosomes, thrombophilia is known to be the one of the major genetic factors that may prone the RPL disease. Factor V Leiden (FVL) mutations are well-known for one of the thrombophilia and eminent for essential clotting factor in coagulation cascade. The aim of this study was to investigate the genetic association of G1691A mutation in FVL gene at RPL in the Saudi women. In this study, 113 women were involved with RPL. Collected blood was stored in an EDTA tube to extract the genomic DNA using kit-based method. Real-Time Polymerase chain reaction was carried out using the probes. The mean age of the involved 113 women were 34.4±5.79 years and Body Mass Index were established as 30.06±7.05. In this study, 99.1% of genotypes has confirmed as GG genotypes, heterozygous (GA) was documented in single RPL women (0.09%) and AA was not documented in any women. In conclusion, this study showed only single heterozygous in G1691A mutation in FVL gene. This could be due to the small sample size.

KEY WORDS: RECURRENT PREGNANCY LOSS; FVL, G1691A, THROMBOPHILIA.

INTRODUCTION

Gynecological disorders are defined as a condition which disturbs the female reproductive organs. Endometriosis, polycystic ovarian syndrome, Fibroids, female infertility (FI), ovarian cysts, premenopausal syndromes are some of gynecological disorders and recurrent pregnancy loss (RPL) is categorized under FI. Nearly 1:20% of reproductive women experiences couple of consecutive miscarriages and <1% experience triple or more miscarriage completely is known to be RPL (Park et al. 2020; Rohilla 2020; van Dijk et al. 2020). RPL can be not essentially for spontaneous demises of pregnancy; although 15% of first trimester pregnancies ends in miscarriages (Leduc-Robert et al. 2019). It has been assessed that 01-02% of second trimester pregnancies involves miscarriages before 24 weeks of gestation (Maddirevula et al. 2020).

RPL aetiology involves numerous factors like chromosome abnormalities in the couples, infections, uterine alterations, endocrinological disorders, autoimmune diseases and randomly, 50% of RPL cases remains to be idiopathic(Arias-Sosa et al. 2018). The major reason for developing RPL is due to the genetic and non-genetic factors (Dean et al. 2019). However, maternal-paternal ages, endocrine, uterine anatomic abnormalities, sperm-quality, infections, metabolic/hormonal disorders, environmental and immunological factors have been associated with RPL(Bhatt et al. 2020).

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Maternal age and previous histories of miscarriages at outset are confirmed to be couple of major and important risk-factors for consequent miscarriage. Maternal age is also connected with the risk of miscarriage when the women turns to be 35 Years (11% risk in 20-24 years and 51% risk in 40-44 years) (Bhatt et al. 2020). The prevalence of RPL is in between 1-5% and documented in reproductive medicine and modern diagnostics. Both maternal and paternal inheritances are connected with in development of RPL in the initial stages of the pregnancies(Trifonova et al. 2019). RPL are categorized as primary RPL is defined as women without any effective pregnancies and secondary RPL is denotes as women with fruitful pregnancy interm of live new-infant(s) with the histories of miscarriages(Michita et al. 2019). After the abnormalities found in chromosomes, thrombophilia is known to be the one of the major genetic factors that may prone the RPL disease (Fesahat et al. 2020).

Thrombophilia is a generic term defines an increased propensity towards thrombosis and its associated morbidities (Favaloro 2019a). Factor V Leiden (FVL) mutations are well-known for one of the thrombophilia and eminent for essential clotting factor in coagulation cascade. FVL acts as co-factor for permitting factor X to rouse, conversion of prothrombin to thrombin (Ajmeri et al. 2020). Activated protein C is known as active anticoagulant which extends the clotting through terminating factor V and lowers the thrombin formation (Heeb et al. 2009). Factor V Leiden (FVL) epitomizes single nucleotide polymorphism (SNP) in the F5 gene causes missense mutation; substitutes from Guanine-Adenine at 1691 position and amino-acid modifies from arginine-glutamine (Kamineni 2015). Heterozygous mutation in G1691A is popularly known to increase relative risk of thrombosis between 1.8-2.6 folds of increase in common population and significance of FVL mutation with RPL disease is controversial. Global studies showed significant and non-significant associations between RPL and FVL mutation (Reddy et al. 2019). There are no studies which have been carried out in the capital city of Saudi population and current study aims to investigate the general association between G1691A mutation in FVL gene and RPL in the Saudi women.

MATERIAL AND METHODS

In this study, 113 cases of RPL cases have been recruited from Gynecology Dept in King Saud University (KSU) Hospital. In this study, only RPL womens were recruited and the selection of RPL is defined as per the prior study carried out in the Saudi population(Turki et al. 2016). Ethical approval was obtained from IRB at KSU (E-19-4445) and inform consent was signed by the women who has participated in this study. Clinical details were obtained from the involved women and 4 mL of the EDTA blood was collected and used for molecular analysis. Genomic DNA was extracted using DNA isolation kit as per the company's instruction. DNA was quantified with NanoDrop, a spectrophotometer and 20ng/ul of each genomic DNA was used to perform the real-time polymerase chain reaction (RT-PCR) using Roche (LightCycler, version 2.0) instrument was used with VIC and FAM probes was used to analyse the alleles for G1691A mutation in FVL gene. The protocol of RT-PCR was performed as per the company protocol. Statistical analysis was applied using Openepi software (Khan et al. 2019). Variable data was expressed in mean±standard deviation. Allele and genotype frequencies were distributed in the form of percentages.

RESULTS AND DISCUSSION

The current study involves 113 women confirmed with RPL from Dept. of obstetrics and Gynecology from KSU clinic. The baseline characteristic details were involved in Table 1. The mean age of the involved 113 women were 34.4±5.79 years. All the involved subjects were women with 85.8% were documented as Saudi nationalities. BMI was confirmed as 30.06±7.05. The overall prevalence of chronic disease was found to be 44.2% and 5.3% was documented as the prevalence of diabetes. The prevalences of Hypertension, hypothyroidism, asthma, anemia, PCOS and stroke were found in the RPL women was 1.7%, 15.1%, 4.4%, 0.9%, 7.1% and 0.9% respectively. None of the women were found to be the prevalence for dyslipidemia (0%) and 92.9% of involved women were had the miscarriages. 7.9% of RPL women had family history. Genotype and allele frequencies of G1691A mutation of FVL gene in RPL women has been documented in Table2. GG genotype was confirmed as homozygous; GA as heterozygous and AA as homozygous variants. In this study, 99.1% of genotypes has confirmed as GG genotypes; GA (heterozygous) was documented in single RPL women (0.09%) and AA was not documented in any women and the prevalence was found to be 0%. The prevalence of G allele was 0.96 and 0.04% was found to be A allele.

Thrombophilia is documented as one of the common causes for RPL which can be observe in 40-50%. Thrombophilia in mother could ripen the hypercoagulable state of pregnancy; which generates prethrombotic vasculopathy at the placental level. This hypercoagulable stage becomes more worst and impair blood flow by the maternal veins, further which leads to deep vein thrombosis. This will clot in the vessels of placenta which leads to fetal growth restriction or demise (Favaloro 2019b; Garrido-Barbero et al. 2019). Thrombophilic gene polymorphism is known to be a risk factor in RPL women and FVL gene is one of thrombophilic gene (Farahmand et al. 2016). In this study, G1691A mutation from FVL gene has been performed in RPL women and 99.1% of GG and 0.09% of GA genotypes has been documented in 113 RPL women. AA genotype was not documented in this study. However, Turky et al (Turki et al. 2016) studies showed prevalence of heterozygous variant as 14.9% and homozygous as 0.5% in Saudi population with RPL couples. The present study was carried out in RPL women in 85.8% of Saudi women.

RPL is a real disenchantment for couple who failed to conceive the child. Most of the studies have confirmed

RPL as cytogenetic and molecular abnormalities which leads to further recurrent miscarriages and pregnancy demises (Jain and Malik 2014). RPL in general was named habitual abortion; defined as minimum of 3sequential miscarriages before 20th week of gestation. This definition was confirmed by both Royal college of obstetrics and gynecologists and European society of human reproduction and embryology. Spontaneous miscarriages occur randomly in 15% of clinically confirmed pregnancies in <35 years of age. The prognosis in RPL couples is not confirmed through single parameter but established with risk factors along with the precise characteristics. Maternal age is connected with RPL through the cellular mechanisms governs meiotic spindle formation and function have the huge rate of error. It was assumed that 30% of embryos are aneuploid in the women whose age is 40 (Koifman et al. 2016). RPL is known to be multifactorial in nature and numerous risk-factors have been linked up with its pathogenesis (Bahia et al. 2020).

Numerous global studies have been linked up with the G1691A mutation and RPL globally. This case-control studies have been carried out in various ethnic populations in the global world. Maximum studies have been performed with PCR-restriction fragment length polymorphism method. However, present study was performed with RT-PCR which is known to be one of the strengths of this study. The difference between thermal PCR and RT-PCR are; thermal PCR require the validation to cross-check the study results, whereas, RT-PCR studies doesn't require any validation. The results were found to be accurate by involving couple of probes. These probes are known to be labelled fluorescently in DNA oligonucleotides and bind to the sense and antisense primers. Global studies have showed both the positive and negative associations (Balajewicz-Nowak et al. 2015; Farahmand et al. 2016; Jusic et al. 2018; Karadag et al. 2019; Kardi et al. 2018; Kashif et al. 2015; Reddy et al. 2019; Sharma et al. 2015; Turki et al. 2016).

The global results may vary depends on the ethnicity of specific countries. Meta-analysis studies should be implemented for the accurate results. Meta-analysis of case-control data can be expanding the association of confirmed analysis from the prior studies (Khan et al. 2016). A couple of meta-analysis studies have been performed in G1691A mutation in FVL gene with RPL and both the studies shows the significant association (Kovalevsky et al. 2004; Sergi et al. 2015). So, G1691A mutation has a prominent role in RPL globally. The strength of the present study was implemented in the RPL women and genotyping was performed with RT-PCR analysis. The limitation of this study was skipped the control subjects, performed only single SNP and lower subjects were involved. In conclusion, this study showed only single heterozygous in G1691A mutation in FVL gene. This could be due to the small sample size. Future studies should be performed with large sample size in various ethnic populations.

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Etiology and Prevalence of Permanent Tooth Extraction Among Group of Yemeni Population

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ABSTRACT

The objective of the present study was to investigate the reasons of the permanent tooth extraction and its relationship with age and gender, for which 662 participants, divided into five age groups 14–23, 24–33, 34–43, 44–53, and ≤54-years-old were studied. Oral and radiographic examinations were done for each participant. Causes of tooth loss, age group, gender, Khat chewing, Shammah use, smoking, teeth brushing and Miswak using were recorded. The data were statically analyzed with SPSS program using Chi-square tests. The p value ≤ 0.050 were considered statistically significant. From the total number of the participants 335(50.6%) were males. The highest age group was in the 14–23 age-group (43.1%). Dental caries was represented by (49.53), while the periodontal disease was (23.3%). The failure of root canal treatment, orthodontic and other causes were (3.6%, 11.3%, 11.3%), respectively. There were significant differences between genders and different age groups in relation to causes of tooth extraction.

KEY WORDS: EXTRACTIONS, PERMANENT TEETH, DENTAL CARIES, PERIODONTAL DISEASE, TOOTH LOSS.

INTRODUCTION

Many surveys on the causes of tooth loss in different countries have been conducted and have concluded several controversies regarding whether periodontal and /or dental caries diseases are the main reasons for tooth loss. In-addition, failure of previous endodontic treatments, orthodontic causes and other reasons such as trauma, iatrogenic or preapical pathosis and combinations of these, have been cited as some of the common reasons for extraction of teeth in the available literatures (Richards et al. 2005, Reich and Hiller 1993).

Khat-chewing habit in Taiz, Republic of Yemen is widely spread and practiced by most of the population (Al-Sharabi 2011). Khat is fresh leaves of the shrub Catha edulis, which are chewed like tobacco in the lower

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buccal pouch unilaterally (left or right) in a bolus form for several hours per day (Hattab and Al-Abdulla 2011, Imran and Murad 2009). Due to continuous khat contact with mucosa and gingiva by daily khat chewing, there is an increase in periodontal problems with membrane attachment loss, being the the most common causes of khat chewing, (Hassan etal. 2007, Al Moaleem 2017, Ali et al., 2018, Noman et al. 2019 Salman et al., 2019).

Table 1. Summary of some studies in different countries regarding the causes of tooth extractions

Researcher Names/ Year of the Study	Country	Sample Size	Causes and Percentages	Gender / Significant	Highest % in age group / Significant
CURRENT STUDY	Taiz, Republic of Yemen	662	Caries (49.53), P A (23.3%), Orthodontic (11.3%),	Failure RCT (3.6%), Others (11.3%) Male (49.4%)/ Female (50.6%) Significant	14-23 Dental caries was the most common cause in (14-23 and 24-33). PD↑ gradually from (34-43) to ≥ 54-year-olds.
Salman et al.2019	Pakistan	520	Caries (50.53), P A	Female (53%)	45-54
			(38.4%), Orthodontic (0.0%), Failure RCT (0.3%), Others (10.8%)	Male (47%)/ Significant	Most lost their teeth due to caries, followed PD
Shah et al. 2019	Gujarat,	869	Caries (77.3), P A	Female	44-45 & 55-64
	India		(16.6%), Orthodontic (0.5%), Failure RCT (5.0%), Others (0.6%)	(50.4%) Male (49.6%)/ Non-significant	Caries (53.7%), in patients (15-44 years group). P D 81.4% of (45-84 years group). Young patient
Ali et al.2018	Aden,	450	P D (51.1%),	Male (72.2%)	
	Yemen		Caries (33.1%), Other & Orthodontic (15.8%)	Female (27.8%)	caries P D ↑with age
Al Ameer &	Al- Madinah,	1589	Caries (63.4%),	Females	Over 40 (40.8%)
Awad. 2017	KSA		P D (14.6%) Failed root canal (2.7%), Orthodontic (1.3%), Trauma (0.2%)	(57.6%), Male (42.3%) /Significant	Female all age groups
Al Moaleem et al.	Jazan,	579	P D (37.1%),		Over 40 (50-59)
2016	KSA		Caries (30.1%) Trauma (12.1%)		caries P D ↑50 (70%)
Kaira et al.	India	1506	Caries (43.95%),	Females (55.9%),	(51-70), then
2016			P D (31.34%), Orthodontic (10.4%), Failure RCT (0.45%), Trauma (0.29%)	Male (44%)	(11-20)
Sahibzada et al.	Turkey	8355	Caries (85.3%),	Females (59.1%)	Over 50 years
2016			P D (7.6%) Orthodontic (2%), Failure RCT (1%), Trauma (1%)	Male (40.9%)/ Significant	
Al-Shammari et	Kuwait	2783	Caries (43.7%)	Female (50.2%)	31 - 40 (29.9%)
al. 2015			P D (37.4%) Orthodontics (4.3%).	Male (49.8%)/ Significant	Caries ↓in young patients (60.7%; 20-29)

Continue Table 1

			Failed RCT (2.7%)		P D (63%; 30-50)
Chrysanthakopulos 2015	Greek	1231	P D (34.4%) Caries (32.2%) Others (33.4%)		P D ↑with age
Gossadi et al. 2015	Jazan, KSA	691	Caries (33.3%), P D (31%) Orthodontic (17.1%)	Female (28.5%) Male (25.9%) / Non-significant	Young (20-29) caries P D ↑ 40 years
Lee et al 2015	Taiwan	4811	Caries (55.3%); all age group P D ↑ 35 years	Female	Over 60 years
Kashif et al. 2014	Pakistan	6251	Caries (51.8%), P D (19.2%), Orthodontics (2.9%), Failed RCT (2.8%)	Female (56.7%), Male (43.3)	Age 50 years
Alesia & Khalil 2013	Riyadh, KSA	1554	Caries (50.2%) Orthodontics (18.2%) P D (8.2%)	Female (55.5%) Male (44.5%)	10-30
Jafarian & Etebarian. 2013	Iran	1,382	Caries (51%), P D (14.4%), Orthodontic (7.2%)	Female (51.3%) Male (47.8%) / Significant	(41-60) Caries ↑20, P D ↑40 years. Male (55.3%), Female (43.9%)/ Significant
Anyanechi & Chukwuneke 2012	Eastern Nigeria	3998	Caries (55.2%)	Females (62.3%) Males (37.7%)	(11- 30) Both males & females
Haseeb et al. 2012	Pakistan	1178	Caries (63.1%), P D (26.2%), Failure RCT (4.6%), Trauma (3.2%)	Male (59.6%), Females (40.4%)	Over 51-80 years
Montandon et al. 2012	Brazil	437	Caries 45 years P D ↑ with 45-82		35-65 years
Yousaf et al. 2012	Pakistan	1500	Caries (41.2%), P D (34.8%), Orthodontic (4.3%), Others (3.2%)	Male (70%) Female (30%)	Over 40 years
Nasreen & Haq 2011	Bangladesh	110	Caries (68.2%), P D (12.7%), Orthodontic (4.5%)	Female (53.6%) Male (46.4%)	(20-39)
Preethanath, 2010	Al Baha, KSA	820	Caries P D	Female (19.71%) Male (12.57%)/ Non-significant	Young (20-29) caries P D ↑ with age
Anand & Kuriakose 2009	India	1791	Caries (39.5%), P D (28.4%), Orthodontic (19.4%), Others (2.5%)	Males (53%) Females (47%)/ Significant	(55-64), (15-24)
Baqain et al. 2007	Jordan	2435	Caries (63.8%), P D (22.9%), Orthodontics (2.4%), Trauma (2.4%)	Male/ Significant	21 - 30 years/ Significant P D ↑40 Orthodontic/ Significant
Sayegh et al. 2004	Jordan	2200	Caries (46.9%), P D (18%) Orthodontics (4%), Trauma (0.7%)		≤ 40 years of age (Caries), ↑40-year-old group (P D)
Aderinokun & Dousmu 1997	Nigeria	1301	P D (61.9%) Caries (34.1%) Trauma (4.0%)	Females (51.5%) Male (49.5)/ Non-Significant	(21-31) caries P D ↑45 years

Continue Table 1

Murray et al. 1996	Canada	6143	Caries Orthodontic in chilled hood	All age P D ↑40 years

PD; periodontal diseases RCT; root canal treatments Increase; ↑ Decrease; ↓
Significant; * Non-Significant #

It is clear from a number of earlier studies that dental caries is a main cause of tooth loss among young age and in both gender as well as elder groups) Kashif et al.2014, Montandon et al.2012 (Anyanechi and Chukwuneke 2012 Noman et al. 2019, but other cross-sectional studies have concluded that both periodontal diseases and caries are the main reasons of permeant teeth extractions in males and females (Gossadi et al. 2015, Murray et al.1996, Aderinokun and Dosumu 1997), even though a quite number of studies mentioned that teeth loss were totally related to periodontitis (Ali et al. 2018, Murray et al.1996). Studies pointed to some reasons such as orthodontic causes (Noman et al. 2019, Nasreen and Haq 2011, Chukwuneke 2012, Yousaf et al. 2012, Al-Shammari et al. 2006, Baqain et al., 2007), while others said it is related to root canal failures (Al Ameer and Awad 2017, Chukwuneke 2012, Kashif et al.2014 , Al-Shammari et al. 2006), or other causes were observed to be the causes of tooth loss (Al Ameer and Awad 2017, Nasreen and Haq 2011, Chukwuneke 2012, Kashif et al.2014 , Al-Shammari et al. 2006, Baqain et al., 2007, Aderinokun and Dosumu 1997). Table 1; shows summary of some studies in different countries regarding the causes of tooth loss.

Tooth loss may affect communication, as well as produces some masticatory difficulties and could end in poor facial aesthetic outcome. In-addition, it is an indicator of the overall general oral health of any population (Brodeur et al.1996, Stratton and Wiebelt 1988). Thus the aim of this study was to investigate the causes of tooth loss and the effect of several social habits that causes tooth loss its relationship with age, and gender.

MATERIAL AND METHODS

Study design: This cross-sectional study was conducted among subjects seeking dental extractions and oral treatments at different clinics in Taiz city, Republic of Yemen. The current study was conducted in full accordance with the World Medical Association Declaration of Helsinki, and after a signed of the ethical approval of the study.

Study participants, data collections and questioner:The data collections were carried out during the period from February 2018 to March 2019 for participants who were requiring teeth extraction. A total of 662 participants (335; males and 327; females) were involved in the present study. The participants were selected through non-probability convenience sampling. The data were collected by general practitioners dentists after a short period of training using a pre-designed questionnaire. After a written consent had been signed by each participant, the clinical and radiographic examinations of dental arches were performed on a dental chair using the regular examination kit. The questioner form was simple and consisted of a single page. The chartings were done to record the causes of tooth loss in relation to participant's gender and age.

Table 2. Descriptive of participants in relation to gender and social habits

Parameter	Gender		Khat Chewers		Frequency of Khat Chewing			Khat Chewing Side		Teeth Brushing		Miswake Using		Shammah Users		Shammah Using Side		Smoking	
	Male	Female	Yes	No	Daily	Weekly	Monthly	Left	Right	Yes	No	Yes	No	Yes	No	Right	Left	Yes	No
Number	335	327	377	285	287	42	48	260	117	308	354	142	520	71	591	31	40	421	241
Percentage	50.6	49.4	56.9	43.1	43.4	6.3	7.3	39.3	17.7	46.5	53.5	21.5	78.5	10.7	89.3	4.7	6.0	63.6	36.4

Participants grouping and social habits: Khat chewing, Shammah using, Miswake, toothbrushes, and smoking were recorded. The questions of the sides of khat chewing and Shammah use were recorded, also the chewing durations were registered as daily/week/month. Patients of both genders, above the age of 14 years and without any systemic diseases, were involved in the current study. All data related to the causes of teeth loss were recorded and collected in a self-designed preform. According to the age, the selected subjects were divided into five groups, 14–23, 24-33, 34-43, 44-53, and ≥54-years-old, respectively.

Classification of causes and criteria recording: With some modification all the data classifying the causes of missing teeth were recorded using the criteria

mentioned by Mc Caul LK et al. 2001 and Cahen PM et al.1985 . The criteria were: Dental Caries (A tooth was concerned as requiring extraction due to dental caries when caries had destroyed the crown so that it cannot be restored, if there were carious exposure of the pulp or a septic roots. Periodontal Disease (extraction due to periodontal disease if it tended to satisfy the score criteria of Russell's PI index (Russell, 1956), namely the presence of considerable mobility according to the Miller Mobility Index Miller, 1956). Orthodontic Treatment Causes (whenever a tooth is removed under the request from the orthodontist); Other Causes which included trauma (when a non-carious associated trauma to the tooth is the reason for its extraction); or iatrogenic (due to incorrect treatments done in dental clinics).

Statistical analysis: All the data were recorded then summarized as frequencies and percentages, after that analyzed descriptively using Statistical Package for Social Sciences (SPSS) software (version 20.1 SPSS, Chicago, Illinois, USA). An association and comparison with different variables were performed using the Chi-square test. The p-values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

From table 2; a 662 participant were included in this study, (335; 50.6% males and 327; 49.4% females). The highest age group was in the 14–23 age group (285; 43.1%), followed by 198 (29.9%) among the 24–33 age group, while the lowest participants were in the age group with ≥ 54 and represented (16, 2.4%). The number and percentage of khat chewer participants were 377 (56.9%), with 287 (43.4%) were daily chewed khat. The participant's number with Shammah user were 71; 10.7% only. But, the highest number and percentage regarding the sides for khat chewing and Shammah using were the left side in both parameters (260 [39.3%] and 31 [4.7%], respectively). Finally the number and percentages of the participants using toothbrush, Miswak and smoking were (308; 46.5%, 142; 21.5%, 421; 63.6%), respectively.

Table 4. Frequency and percentages of the study variables in relation to gender

Gender	Male N %	Female N %	Total N %	P value
Khat Chewers				0.000*
Yes N (%)	266 (74.4)	111 (39.9)	377 (56.9)	
No N (%)	69 (20.6)	216 (66.1)	285 (43.1)	
	Frequency of Khat Chewing			0.000*
Daily	199 (59.4)	88 (26.9)	287 (43.3)	
weekly	32 (9.6)	10 (3.1)	42 (6.3)	
Monthly	29 (8.7)	19 (5.8)	48 (7.3)	
No	75 (22.4)	210 (64.2)	285 (43.1)	
		khat Chewing Side		0.000*
Left	206 (61.5)	204 (62.3)	410 (61.9)	
Right	73 (21.8)	44 (13.5)	117 (17.7)	
No	56 (16.7)	79 (24.2)	135 (20.4)	
		Teeth Brushing		0.102
Yes	145 (43.3)	163 (49.8)	308 (46.5)	
No	190 (56.7)	164 (50.2)	354 (53.5)	
		Miswake Using		0.257
Yes	78 (23.3)	64 (19.6)	142 (21.5)	
No	257 (76.7)	263 (80.4)	520 (78.5)	
		Shammah Users		0.000*
Yes	60 (17.9)	11 (3.4)	71 (10.7)	
No	275 (82.1)	316 (96.6)	591 (89.3)	
		Shammah Using Side		0.000*
Left	33 (9.9)	7 (2.1)	40 (6.0)	
Right	27 (8.1)	4 (1.2)	31 (4.7)	
No	275 (82.0)	316 (96.6)	591 (89.3)	
		Smoking		0.053
Yes	201 (60.0)	220 (67.3)	421 (63.6)	
No	134 (40.0)	107 (32.7)	241 (36.4)	

*Statistically significant if p ≤ 0.05 from Chi-Square tests

Table 4. Association between different age groups and cause of tooth loss

Cause/ Age Group	14-23 N %	24-33 N %	34-43 N %	44-53 N %	≥ 54 N %	Total N %	P value
			Dental Caries				0.000*
Yes	150 (52.6)	126 (63.6)	30 (28.3)	16 (28.1)	6 (42.9)	328 (49.5)	
No	135 (47.4)	72 (36.4)	76 (71.7)	41 (71.9)	10 (57.1)	334 (50.5)	
			Periodontal Disease				0.000*
Yes	17 (6.0)	50 (25.3)	48 (45.3)	29 (50.9)	10 (71.4)	154 (23.3)	
No	268 (94.0)	148 (74.7)	58 (54.7)	28 (49.1)	6 (28.6)	508 (76.7)	
			Failure of Root Canal Treatment				0.060
Yes	9 (3.2)	13 (6.6)	0 (0.0)	2 (3.5)	0 (0.0)	24 (3.6)	
No	276 (96.8)	185 (93.4)	106 (100)	55 (96.5)	16 (100)	638 (96.4)	
			Orthodontic Cause				0.000*
Yes	65 (22.8)	9 (4.5)	0 (0.0)	1 (1.8)	0 (0.0)	75 (11.3)	
No	220 (77.2)	189 (95.5)	106 (100)	56 (98.2)	16 (100)	587 (88.7)	
			Other Causes				0.000*
Yes	21 (7.4)	19 (9.6)	19 (17.9)	11 (19.3)	5 (21.4)	75 (11.3)	
No	264 (92.6)	179 (90.4)	87 (82.1)	46 (80.7)	11 (78.6)	587 (88.7)	

*Statistically significant if p ≤ 0.05 from Chi-Square tests

Table 4. Association between different age groups and cause of tooth loss

Gender	Male N %	Female N %	Total N %	P value
		Dental Caries		0.000*
Yes	97 (29.0)	231 (70.6)	328 (49.5)	
No	238 (71.0)	96 (29.4)	334 (50.5)	
		Periodontal Disease		0.001*
Yes	96 (28.7)	58 (17.7)	154 (23.3)	
No	239 (71.3)	269 (82.3)	508 (76.7)	
		Failure Root Canal Treatment		0.681
Yes	11 (3.3)	13 (4.0)	24 (3.6)	
No	324 (96.7)	314 (96.0)	638 (96.4)	
		Orthodontic Cause		0.000*
Yes	19 (5.7)	56 (17.1)	75 (11.3)	
No	316 (94.3)	271 (82.9)	587 (88.7)	
		Other Causes		0.000*
Yes	54 (16.1)	21 (6.4)	75 (11.3)	
No	281 (83.9)	306 (93.6)	587 (587 (88.7)	

*Statistically significant if p ≤ 0.05 from Chi-Square tests

The relation and association between the frequency and percentages among gender in the khat chewing and Shammah using (side or frequency) parameters were significant with p value 0.000. However, we did not detect an association between participants from both gender and teeth brushing, Miswak using and smoking and the results of these parameters were not significant with p values 0.102, 0.257, and 0.053, respectively (Table 3).

Table 4 shows the relation between the different age groups and the resons of tooth loss. Dental caries was the most common cause of teeth loss in the young age groups (14-23 and 24-33 years; 150 [52.6%] and 126 [63.6%] respectively). The rate of periodontal disease increased gradually from the middle age group 34-43 (45.3%), and reached 71% among ≥ 54-year-olds. Among the 14–23-year-olds, all extractions of permanent teeth were for orthodontic causes. The failure of RCT was recorded in the middle and elder age groups. All the previous results

were significant differences with p values < 0.000 except in the cause of failures in root canal treatment which was not significantly difference.

Comparing the causes of tooth loss among gender, among the females participants the number and percentages of tooth loss were more due to dental caries and orthodontic causes (231; 70.6% and 56; 17.1%), while in males it was higher among the periodontal diseases participants (96; 28.7%), and all the parameters were significant differences P < 0.001. The other causes of tooth loss were more among males and recorded 54 (16.1%). All the variables were significantly differences among gender except in the failure of root canal treatment cause (Table 5).

The participants recruited in the current study were carried out at different private clinics in Taiz city. The objectives of this study were to investigate the reasons of the permanent tooth extraction and its relationship with age and gender. World Health Organization (WHO) in its report pointed a good oral health as an indicator of overall good health and recommended many steps in order to improve oral health globally (The World health Report 2002-2003).

It is important to include a good number from both genders in a prevalence study. In the current study the participant's males to females percentages (table 1) were near to each other 50.6% -49.4%, this percentages were close to numbers mentioned by other studies conducted in Yemen (Taiz), India, Iran and Nigeria(Noman et al. 2019, Shah et al.2019, Jafarian and Etebarian 2013, Aderinokun and Dosumu 1997). In other hand this percentage were less than that obtained in other worldwide studies as in Pakistan (Salman et al. 2019, Kashif et al.2014, Yousaf et al. 2012), in Yemen (Aden), in Saudi Arabia cities (Riyadh, Al-Madinah, Jazan), in Bangladesh, in Nigeria, in Turkey, in India (Ali et al. 2018,Al Ameer and Awad 2017, Nasreen and Haq 2011, Chukwuneke 2012, Gossadi et al.2015, Sahibzada et al. 2016, Kaira et al. 2016). These differences may relate to the selected place from where the samples were collected.

From the demographic data of this study, the highest participant numbers were among the 14-23 years-age-group (43.1%), followed by the 24-33 years-age-group (29.9), those age-groups were closed to the same age-groups registered by studies in Asia (Noman et al. 2019, Anyanechi and Chukwuneke 2012, Montandon et al.2012, Gossadi et al.2015, Baqain et al.2007 Aderinokun and Dosumu 1997), but this was in contrast with the results of other international studies (Salman et al. 2019, Shah et al.2019, Nasreen and Haq 2011, Jafarian , Etebarian 2013, Kashif et al.2014, Montandon et al.2012, Kaira et al. 2016 (Salman et al., 2019).

The major cause of tooth extraction among participants from Taiz city, Republic of Yemen was dental caries in the younger age group 14–23 and 24–33 and it is significantly differences. In-addition the periodontal disease was gradually increased from the middle to elder age groups 34 – over 54. These results coincided with results found in Yemen (Noman et al. 2019, Ali et al. 2018), in Saudi Arabia (Alesia and Khalil 2013, Gossadi et al.2015, Preethanath2010), in Iran (Jafarian and Etebarian 2013), in Jordon (Baqain et al.2007). Other results concluded that periodontal diseases are the common cause of tooth loss as obtained by (Ali et al. 2018) in India, (Al Moaleem et al. 2016)in Saudi Arabia, (Chrysanthakopoulos 2011) in Greek and in Nigeria (Aderinokun and Dosumu 1997).

Dental caries is the most oral diseases leads to extraction of the permanent teeth. From this prospective study, we found that nearly half of the teeth in the all age-groups (49.5%) were extracted due to dental caries and its sequelae (table-4). This is in parallel with the finding of other research in different countries (Noman et al. 2019, Salman et al. 2019, Alesia and Khalil 2013, Jafarian and Etebarian 2013, Kashif et al.2014, Sayegh et al. 2004). In-addition extraction of teeth due to dental caries diseases were more than 50% in the researchers conducted in other countries(Shah et al.2019, Al Ameer and Awad 2017, Nasreen and Haq 2011, Chukwuneke 2012, Haseeb et al. 2012, Lee et al.2015, Baqain et al.2007, Sahibzada et al. 2016), but it does not reach 40% in a other group of studies (Ali et al. 2018, Anand and Kuriakose 2009, Yousaf et al. 2012, Gossadi et al.2015, Al Moaleem et al. 2016, Al-Shammari et al.2006, Chrysanthakopoulos 2011, Kaira et al.2016, Aderinokun and Dosumu 1997) as showed in table 1. This can be explained by the type of social habits regarding type of foods.

From table 4 and 5 in the present study, the results the cause of tooth loss ''failures of root canal treatments'' were not significantly differences among the different age groups or gender. This is in association with previous results mentioned by(Noman et al. 2019, Salman et al. 2019, Al Ameer and Awad 2017, Kashif et al.2014, Al-Shammari et al. 2006, Sahibzada et al. 2016, Kaira et al.2016). The frequency of the same factor registered near to or more than 5% in other research(Ali et al. 2018, Haseeb et al.2012, Yousaf et al. 2012). Among the orthodontic cause of tooth extraction our results were agreed with that mentioned those types of extraction were totally related to the younger age groups (Noman et al. 2019, Salman et al. 2019, Nasreen and Haq 2011, Jafarian and Etebarian 2013, Kashif et al.2014, Yousaf et al.2012, Al-Shammari et al.2006, Baqain et al.2007, Sahibzada et al.2016, Murray et al.1996), but it was less 2% in (Salman et al. 2019, Al Ameer and Awad 2017) , and reach near to 20% in a studies (Salman et al. 2019, Ali et al. 2018, Alesia and Khalil 2013, Anand and Kuriakose 2009, Haseeb et al. 2012). This wide range of differences can be related to many factors such as the socioeconomic status of the patient, governmental services of such type of treatments and the education level of their parents as well as educational level.

One of the limitation of this study is its designed by researchers but, the data were collected by many general dental practitioners after a demonstration for

participant examination and recording the clinical and radiographically findings on the examination sheet. On the other hand the strength of this study is its participants selections were collected from different areas of Taiz city, Republic of Yemen.

CONCLUSION

Within the limitation of this cross-sectional study the following conclusions can be drawn: The major reason of tooth loss among participants from Taiz city, Republic of Yemen was dental caries and in the younger age group 14–23 and 24–33. Periodontal disease was gradually increased from the middle to elder age groups 34 – over 54. There were significant differences between genders and different age groups in relation to causes of tooth extractions.

Conflict of interest: None

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Annonaceous Acetogenin Annonin – I Inhibits HCT116 Colorectal Cancer Cells Proliferation by Acting on Mitochondrial NADH Dehydrogenase-1

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ABSTRACT

Plant secondary metabolites are being extensively screened for various therapeutic purposes with the help of virtual screening. Annonaceous acetogenins are a large class of secondary metabolites known to possess various therapeutic activities. These have proven to efficiently regulate cancer in brine shrimp. Molecular modeling plays a pivotal role in computer aided drug design. In this study, based on literature survey, three major acetogenins were tested for their binding affinity to the target *Homo sapiens* mitochondrial NADH Dehydrogenase subunit-I. The i-Tasser 3D model was energy minimized. Acetogenins Annonin-I, Squamocin-C & Squamocin-D were docked with target using Schrodinger's Glide and the analysis was done based on docking score and hydrogen bonding between the molecules. The acetogenin annonin-I had the best binding affinity and a molecular dynamics simulation using Desmond package was done for 30 ns (NPT ensemble). Trajectories were plotted against time to assess potential energy, RMSD and RMSF between apo and NADH1-annonin-I complex. Hydrogen bonding involving residues Thr108 and Ser159 facilitating the binding of NADH1 and annonin-I were maintained throughout the simulation and the system was nicely equilibrated. The ADME properties were also predicted. Annonin-I was tested against HCT-116 cancer cells for its efficacy with the help of MTT assay and the in-vitro results showed anticancer activity in favour of annonin-I. At the 100 µM concentration of the annonin-I, only 46% growth of the HCT-116 cells was observed.

KEY WORDS: MTT ASSAY, ACETOGENINS, ANNONIN-I (SQUAMOCIN A), MOLECULAR MODELING & DOCKING, NADH DEHYDROGENASE SUBUNIT-I (ND1), HCT-116.

ARTICLE INFORMATION

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INTRODUCTION

Computer aided drug design (CADD) is one of the tools which can be used to increase the efficiency of the drug discovery process (Ooms, 2012). Molecular modeling describes the generation, manipulation or representation of three-dimensional structures of molecules and associated physico-chemical properties (Nadendla, 2004; Wade and Salo-Ahen, 2019). The orientation of a ligand and its affinity to the receptor helps to predict the different binding modes of a ligand – receptor complex; this complex could further be assessed for its behavioral changes by molecular dynamics simulation (Andrew, 2001). Application of CADD has already resulted in discovery of novel therapeutic compounds in the treatment of variety of diseases (Baig et al., 2017; Yang et al., 2018; Dar et al., 2019).

Cancer is a fatal disease attributed to life style and environmental factors which are responsible for the mutation in vital genes related to cell regulatory proteins. This leads to abnormal cell growth surrounding the normal tissues and destroys vital organs(Ruddon W, 2007; Hassanpour and Dehghani, 2017). With an estimation of over 1 million new diagnosed cases; colorectal cancer is the second most familiar malignancies in the United States and clocks approximately 608700 deaths worldwide every year (Jemal et al., 2011) and it is forecast approximately 147,000 may die in 2020 (Siegel et al., 2020). Diagnosis of cancer has proven to be a daunting task for the scientific community over the years. Chemotherapy and anti-hormone therapies have shown good results in treating cancer (Huang et al., 2017; Stevanato Filho et al., 2017; Falzone, Salomone and Libra, 2018; Ji, Sundquist and Sundquist, 2018).

Present studies focus on gene manipulating and molecular genetic analysis. Nowadays, a group of biologically active plant secondary metabolites termed as phytochemicals are known to reduce the carcinogenicity (Wang et al., 2012; Seca and Pinto, 2018; Shin et al., 2018). Phytochemical substances such as Resveratrol (Feng et al., 2016) and Bufalin have been proven to be inducing apoptosis in human colorectal cancer (Wang et al., 2015).

Similarly, the custard apple family Annonaceae, consists of various biologically active acetogenins. Structurally, they belong to a series of C-35/C-37 natural products, which possess a terminal g-lactone ring and a terminal aliphatic side chain connected with some oxygen-bearing moieties and several hydroxyl groups (Alali, Liu and McLaughlin, 2010; Liaw et al., 2010, 2016). They are known to inhibit mitochondrial NADH Dehydrogenase family of enzymes leading to ATP synthesis (Pomper et al., 2009; Alali, Liu and McLaughlin, 2010). Structure activity relationship (SAR) studies for acetogenin compounds have been found out on cancer cell cultures, brine shrimp, mosquito larvae, isolated mitochondria and mitochondrial fragments, MDR human mammary MCF7/Adr cells (Oberlies, Chang and McLaughlin, 1997) and their positioning in the cell membranes are predicted by nuclear magnetic spectroscopy (Shimada et al., 1998). Specifically, squamocin-C, D and annonin-I are proven to possess significant cytotoxic activity when tested upon brine shrimp and five in-vitro cell lines (Abdel-lateff et al., 2009; Md Roduan et al., 2019).

Studies have also reported the selective action of these acetogenins on the target NADH Dehydrogenase subunit I (ND1) between the normal and tumor cells (James Morré et al., 1994; González-Coloma et al., 2002; McLaughlin, Benson and Forsythe, 2010). As the mitochondria of tumor cells are not involved in the cellular ATP production; tumor cells which rely hugely on glycolysis for energy production and consume approximately 19 times more glucose than the normal cell, therefore, normal cells may not get affected due to acetogenins (González-Coloma et al., 2002).

In this study, the efficacy of squamocin-C, squamocin-D and annonin-I are tested against the target Homo sapiens NADH Dehydrogenase subunit-I (ND1) for anti-cancerous activity by molecular docking studies. The ADMET properties of the selected compounds was predicted and compared with that of the available drugs in the market. The best performing docked complex was subjected to molecular dynamics simulation to further understand the behavioral changes in the docked complex. This compound was further tested for its efficacy by performing MTT cell line assay against HCT-116 cell lines in-vitro.

MATERIAL AND METHODS

Sequence and structure retrieval: The sequence data for *Homo sapiens* mitochondrial NADH Dehydrogenase subunit1 (ND1) was obtained from the NCBI database (Accession #: ABH03920.1) consisting of 318 amino acids. Acetogenins Annonin-I (CID 441612), Squamocin C (CID 44307147) and Squamocin D (CID 164503) (Fig 1a) were downloaded from the pubchem database (Kim et al., 2019) and were converted to the required format by using Ligprep module of Schrodinger.

Molecular Modeling, Docking & Dynamics: The 3D structure of the target sequence was modeled by threading approach using a web server iTasser (Yang et al., 2014). This structure was used as a target for our study and the binding site of the target was predicted with the help of the Sitemap. Molecular docking of the target structure and the three acetogenins selected for the study was performed using Glide. ADME properties were predicted using the Qikprop module of Schrodinger.

Molecular dynamics simulations for the human ND1-Annonin-I docked complex was performed using Desmond (version 3.2, D. E. Shaw Research, NY, 2011), implemented in Schrodinger package with 30ns simulation time. OPLS2005 force field was used to perform initial steps of the simulation. Water molecules were added to ND1- Annonin-I complex using the SPC and system was neutralized with ions and periodic boundary conditions were used.

The full system of 43089 atoms was simulated through the multistep MD protocols of Maestro. The relaxed system was simulated for a simulation time of 30000ps, NPT ensemble using a Berendsen thermostat at 310K and velocity resampling for every 1ps. Trajectories after every 0.300ps were recorded. Energy fluctuation and RMSD of the complex in each trajectory were analyzed with respect to simulation time. The intermolecular interactions of ND1-Annonin-I complex were assessed for stability of the docked complex.

MTT assay: The growth inhibition property of Annonin-I was assessed against the HCT-116 (Human Colon Adenocarcinoma) cell lines by the MTT assay (Carmichael et al., 1987). The HCT-116 cell lines purchased from NCCS, Pune, India; were cultured in 25 t-flasks in DMEM medium containing, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO2, 95% air and complete humidity. When the cell density in a culture flask reached 70-80% confluence, they were trypsinized and seeded into 96-well plates at a density of 2500 cells per well in 100µl and incubated for 24 hours in CO2 incubator.

The cultivated cells were treated with Annonin-I, the test compound, which was dissolved directly in to the RPMI media at 100, 10, 1.0 and 0.01 µM/ml concentrations. Untreated wells were kept as controls. The plates were further incubated for 48 hours in the CO2 incubator. MTT solution was added to each well and the plates were further incubated for 2.5 hours. The medium was carefully decanted and the formazan crystals were air dried in dark place and dissolved in 100µL DMSO; the plates were mildly shaken at room temperature and the OD was measured using Synergy H4 microplate reader at 570nm.

From the optical densities the percentage growths were calculated using the following formula:
Percentage growth= $100\times [(T-T_0)/(C-T_0)]$
if T is greater than or equal to T_0, and if T is less than T_0,
Percentage growth = $100\times [(T-T_0)/T_0)]$,
Where T is optical density of test; C is the optical density of control; T_0 is the optical density at time zero.

From the percentage growth a dose response curve was generated and GI50 values were interpolated from the growth curves. After 48 hours of incubation the images were captured prior to the adding of MTT. Cells treated at different concentrations were observed under the microscope and images of each concentration were captured and recorded.

RESULTS AND DISCUSSION

Amongst five models predicted by the iTasser server; one with the best C-score (Confidence Score) of -3.92 was chosen. More negative the Z-score for a particular model better will be its overall quality. The modeled structure mainly consists of alpha helices and an odd beta strand connected by intermittent loops (fig 1b). The stereochemistry of this model was verified by submitting to Procheck validation server and Ramachandran plot showed 96% of the total residues to be aggregating in the most favorable and allowed regions. Minor corrections which were reported by the procheck server were corrected by energy minimization technique before the model could be considered for further study.

Sitemap helped us to find the binding cavity in the ND1 and therefore docking of Annonin1 was performed with Glide module and the best docked pose with respect to glide score of ND1 with Annonin-I is shown in fig 1c. The mode of interaction of Annonin-I with ND1 was due to H-bond formation with Thr65 through a water molecule intermediate, and directly by Thr108 & Ser159 of the ND1. Table 1 shows the energy contribution of various parameters for the formation of receptor - ligand complex. It can be seen from the table that Annonin-I to possess the lowest glide score -10.108kJ/mol in comparison to the other two compounds, marginally higher than squamocin C. The glide score involving Squamocin C was -10.103kJ/mol, whereas -10.108 in the case of Annonin-I.

Table 1. Glide docking results of Squamocin A, C & D with ND1

Compound	Gscore[a]	Gevdw[b]	Gecoul[c]	Genergy[d]	Geinternal[e]	Gemodel[f]
Squamocin A	-10.108	-54.653	-9.656	-64.309	10.490	-87.835
Squamocin C	-10.103	-50.513	-9.282	-59.795	5.1160	-74.590
Squamocin D	-6.4154	-54.878	-5.194	-60.073	8.0975	-78.714

All terms are expressed in Kcal/mol. a - Glide Score; b - Van der Waals energy; c - Coulomb energy; d - Modified Coulombic - Van der Waals interaction energy; e - Internal torsional energy; f - Model energy (Emodel combines GlideScore, the non-bonded interaction energy, and, for flexible docking, the excess internal energy of the generated ligand conformation).

Annonin-I - ND1 complex was predicted to have overall non bonded glide energy of -64.309 kJ/mol. This was relatively better than the other two acetogenins by approximately -4.00KJ/mol (squamocin C) and -5.00 kJ/mol (squamocin D). Model energy which combines glide score, the non-bonded interaction energy and the excess internal energy was better in case of Annonin-I at -87.835 kJ/mol much higher than squamocin C (-74.590 kJ/mol) and squamocin D (-78.714 kJ/mol). These results were an indication for Annonin-I possessing the best binding affinity among the three acetogenins.

Molecular Dynamics Simulation of ND1- Annonin-I complex: Though molecular docking resulted in Annonin-I having best affinity to ND1, it was necessary to understand major conformational changes, if any, between the unbound state and bound state of Annonin-I with ND1. Molecular dynamics simulation was performed for 30 ns for the apo ND1 and ND1-Annonin-I complex to study the thermodynamics fluctuations between the two states. The stability of the complex was assessed and the RMSD values for the protein Cα atoms calculated by aligning the MD production phase trajectories to their initial structures. Examination of the data presented in the plots (fig 2) revealed the exact binding interaction of the docking complex with system embedded with water molecules, temperature and pressure. Interactions responsible for holding the ligand and the receptor complex together were found to be constantly present over the period of dynamics study as approved by the molecular docking results. No major change in the interactions before and after dynamics study of the complex was witnessed (fig 2a & b).

The ND1 – Annonin-I docking interactions were reproduced during the entire simulation period. The two hydrogen bond interactions involving the residues Thr108 and Ser159 responsible for the complex formation were examined during the entire simulation and were observed to be intact. Interestingly, at few steps these interactions were found to be competing with the water molecule for the hydrogen bond formation. And it was during these instances, that the RMSD of the Ser159 for hydrogen bond was higher, otherwise a very stable interaction. In case of Thr108, similar conclusion could not be reached, because of the increasing hydrogen bond RMSD with the increasing timestep (fig 3a).

RMSD which is measured relative to the reference structure for the backbone atoms was found to be stable from 18000 ps attaining equilibrium for both the protein and the docked complex. Initial randomness in the system during the simulations generally results in higher fluctuations however; they tend to get equilibrated over the simulation period. In our study both the trajectories reported equilibration from the 18000 ps and the behavior was maintained until the end of the simulation up to 30000 ps. Final RMSD found to be aggregating at 0.5 to 0.6 nm and 0.4 to 0.5 nm for the protein and ligand complex respectively. Similar pattern was observed in the RMSD analysis involving C alpha atoms as well (fig 3b).

Potential energy of the system as a function of time was plotted to study the behavior of the complex and the apo (ND1 only) protein, indicated equilibration over the entire simulation. Potential energy of the docked complex was calculated to be -533954 kJ/mol (fig 3c) and the total energy at -434583 kJ/mol maintained at a temperature of 300 (K) and pressure at -0.7689 bar. This when compared with the apo protein was -537455 kJ/mol, -437518 kJ/mol, 299.999 (K) and -0.6038 bar of potential energy, total energy, temperature and pressure respectively.

ADME Properties: ADME - toxicity and lipinski assessment for Annonin-I, squamocin C & D and that of the most commonly prescribed drugs in the treatment of cancer such as Temsirolimus, Vinorelbine, Cabazitaxel and Goserelin was predicted. This was done to find the relative efficacy of acetogenins with that of the existing market drugs. Table 2 lists the results of this exercise and

Table 2. Comparative Qikprop properties of acetogenins with the market cancer drugs.

Molecule	MW[a]	dHB[b]	aHB[c]	QPlogPo/w[d]	PHOA[e]	QPlogHERG[f]
Temsirolimus	1030.3	0	20.7	7.286	58.368	-6.74
Vinorelbine	778.944	1	11.5	6.184	50.754	-8.076
Cabazitaxel	835.944	2	17.35	5.312	66.323	-6.704
Goserelin	1269.43	11.5	25.7	-2.267	0	5.003
Squamocin C	622.924	3	11.5	7.386	89.543	-7.056
Squamocin A	622.924	3	11.5	7.386	89.543	-7.056
Squamocin D	622.924	3	11.5	7.386	89.543	-7.056

a - Molecular Weight (g/mol); b – Hydrogen Bond Donors; c – Hydrogen Bond Acceptors; d - Predicted octanol/water partition coefficient; e - Predicted human oral absorption on 0 to 100% scale (acceptable range: <25% is poor, >80% is high); f - Predicted IC50 value for blockage of HERG K+ channels (concern below –7).

it could be seen none of the 7 compounds including the market drugs seem to obey the lipinski's criteria. When analyzed at the level of molecular weight acetogenins (622.924 g/mol) were found to have lesser molecular weight than others in the range of 778 to 1269 g/mol.

Similar conclusion could be made with respect to other parameters such as partition coefficient and acceptor hydrogen bonds. With an exception to Goserelin all the compounds had the donor hydrogen bonds to be well in the acceptable limits as was enunciated by lipinski's criteria. Poor human oral absorption was recorded for Goserelin and higher for acetogenins which was in the range of 89%. In general, the pharmacokinetics of the drugs must be taken into consideration, as it determines the concentration of drug reaching the heart and therefore the hERG channel, which implicates cardiac functioning. These acetogenins were calculated to have QPlogHERG as -7.056, negligibly higher by 0.056 units. All these results of Qikprop study therefore, point to the fact that these acetogenins are better in comparison to other commonly used drugs.

Goserelin which is classified as a leutinizing hormone releasing hormone (LHRH) agonist, used to cure prostate and breast cancer is known to result in testesterone imbalance coupled with 5% chances of heart failure (Goserelin - Drug Information - Chemocare, 2019). Cabazitaxel used in the treatment of prostate cancer has side effects such as fatigue, respiratory issues, low blood count, and diarrhea in more than 30% of population (Cabazitaxel - Drug Information - Chemocare, 2019). Temsirolimus (Temsirolimus - Chemotherapy Drugs - Chemocare, 2019) and Vinorelbine (Vinorelbine - Chemotherapy Drugs - Chemocare, 2019) are other common drugs used to treat renal cell cancer and other types of cancer also reported to have some side effects. Annonin-I is found to have better and satisfactory predictions relative to the drugs earlier. Annonin-I is already reported to be one of the potent compounds that has the pesticidal properties by targeting the ND1 (Habeeb and Sanjayan, 2015).

The drugs we took here for reference and comparison are to support our findings that annonin-I is almost having properties similar to few of the known and existing drugs though they might have their own side effects or the existence of better ones than these. Based on the observations made from the results obtained, we proceeded further for assessing the growth inhibition property by MTT studies.

MTT Assay: Table 3 shows the MTT assay growth inhibition results. A 46% growth inhibition was witnessed when the concentration of the Annonin-I was 100 µM. The next concentration chosen for the study was 10 µM, 1.0µM, 0.1µM and 0.01µM and the corresponding growth inhibition was 78%, 89%, 85% and 90% respectively (Fig 4). From figure 4 differences in the cellular growth could be clearly seen as the control was highly packed with a greater number of cells than the 100 µM treated. These results therefore indicate that there was a good growth inhibition property of the compound Annonin-I in HCT-116 cell line.

Table 3. MTT assay growth inhibition results

Compound	Percentage Growth					GrowthInhibiton µM		
	100 µM	10 µM	1.0 µM	0.1 µM	0.01 µM	GI50	TGI	LC50
Annonin-1	46	78	89	85	90	76.13	100.0	100.0

Figure 1: (a) Structure of the three acetogenins Squamocin A (Annonin – I), C & D. (b) Modeled structure of ND1. (c) Shows interactions between the best docked pose ND1 – Annonin – I complex. Circle emphasizes the region of hydrogen bond formation and the residues involved.

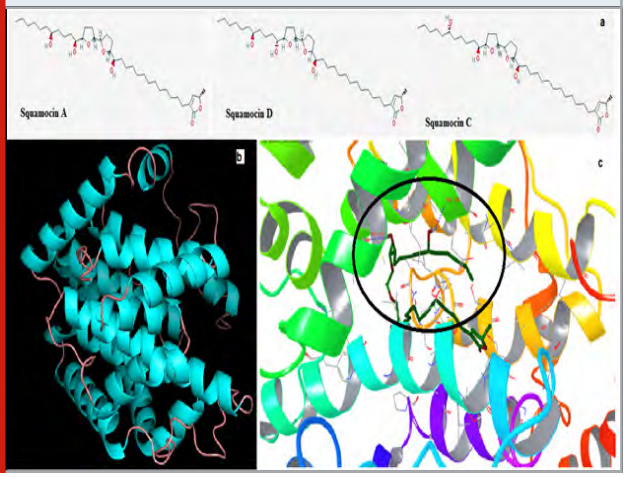


Figure 2: Position of residues and the interaction between the ND1-Annonin-I complex. (a) Before 30ns simulation of the docked complex (b) after 30ns simulation of the docked complex.

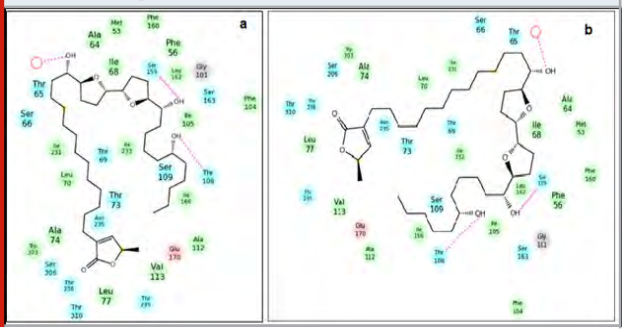


Figure 3: Trajectories of the 30ns simulation done for the ND1 alone (red) and ND1 – Annonin-I complex (red). (a) Shows the protein backbone RMSD plotted against the simulation duration. (b) Shows the C- alpha RMSD plotted against the time (ps). (c) Shows the behavior of ND1 & ND1-Annonin- I complex with respect to potential energy plotted against time (ps).

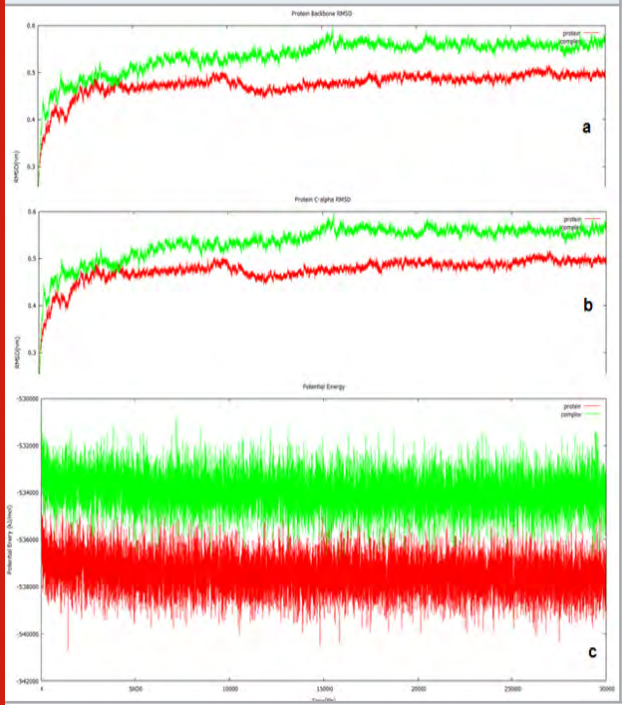
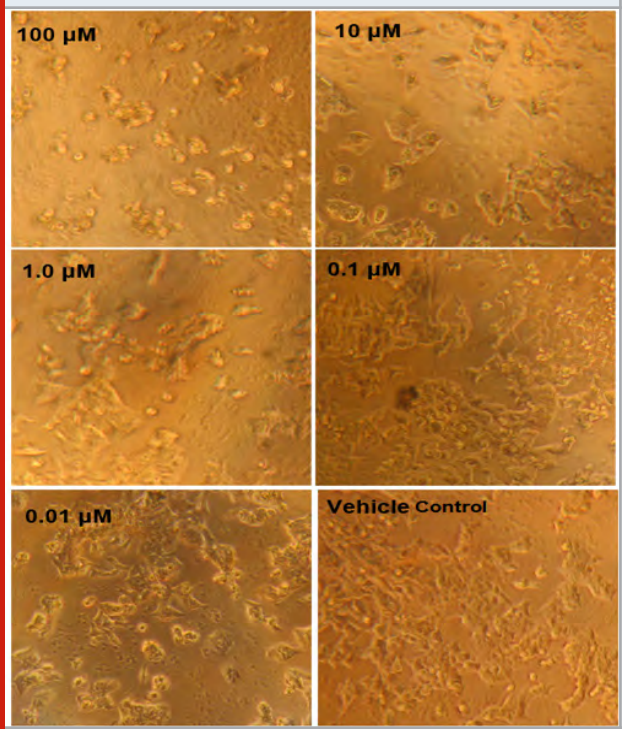


Figure 4: MTT assay results against HCT116 cells. Concentration dependent cell inhibition ranging from 0.01 to 10 µM



CONCLUSION

Annonaceous acetogenins are proven to have the potential to become anti-cancer drugs. The acotogenins selected in this study were positively tested to hold anticancer activity when tested upon brine shrimp cell line. The molecular mode of action of these acetogenins is known to be mitochondrial NADH dehydrogenase enzyme, playing a key role in the electron transport chain leading to energy synthesis. Molecular modeling results reported Annonin – I to have a favorable affinity towards ND1 and the molecular dynamics simulation studies also proved that ND1 upon binding to Annonin-I maintained its structural behavior as that of the ND1. From the in-silico studies it was decided to test annonin-I on cancer cell lines HCT-116. An MTT assay was performed on this cell line, at various concentrations and the growth inhibition was predicted to be ranging from 46% the least to 90% the most. The GI50 was predicted to be at 76.13µM, and these indicate that the compound to having a resounding activity against the HCT-116 cell lines. These findings could further be supported by more wet lab experiments and clinical assays to confirm the potential of annonin-I to be a candidate for cancer treatment. Due to financial limitations we could not perform further experiments where we could have tested the effect of annonin-I on different caspases which could be great idea to take this study forward in future.

DECLARATION

The corresponding author Habeeb Shaik Mohideen has been publishing articles earlier in the name of S. K. M. Habeeb.

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Stimulatory Effect of Probiotic Bacterium *Bacillus firmus* CAS 7 on Growth, Survival and Colour of Tomato Clown *Amphiprion frenatus* (Brevoort, 1856)

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ABSTRACT

In the present study, stimulatory effect through dietary administration of probiotic bacteria, *Bacillus firmus* CAS 7 on growth, survival and skin colour of the tomato clown *Amphiprion frenatus* was investigated. A total of four different experiments with different concentrations of probiotic *B. firmus* with with basal diet (25, 50, 100 and 150 mg kg^{-1}) and control (without probiotic) were planned and the fishes were fed for 120 days. The results obtained from the present study suggested that the fishes fed with the diets supplemented with probiotic at 100 and 150 mg kg^{-1} exhibited similar weight gain (21.06 ± 1.4 and 21.09 ± 1.5), specific growth (0.176), survival rate (100%) and feed conversion ratio (0.109), but higher than the control and other experiments. The carotenoid content in fish skin was comparatively higher in experiment III and IV (6.79 mg/g) than other experiments and control (4.48 mg/g). Although the stimulatory effect in terms of growth, survival and colour of fishes fed with diets containing 100 and 150 mg kg^{-1} of probiotic was higher than control and lower concentration, there was no significant difference between them and hence, it is recommended to use at a concentration of 100 mg kg–1 for the enhanced growth and color of the tomato clown *Amphiprion frenatus* which has significant importance in ornamental fish industry. Further study on immune stimulatory effect of this probiotic is under progress for its application in large scale to prevent any undesired effects.

KEY WORDS: *AMPHIPRION FRENATUS*, PROBIOTIC, *BACILLUS FIRMUS*, GROWTH, SKIN COLOR, CAROTENOIDS..

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INTRODUCTION

The demand for marine ornamental fishes has gained a thrust among aquarium hobbyists due to their multitudinal colour and beauty. The annual worldwide market for ornamental marine reef fish has shown a steady increase over the past few years. Therefore, research on the commercial rearing of these fishes is an imminent necessity to save this fragile ecosystem. However, till date efforts in this direction have been extremely limited to very few coral fishes like damsels, neon gobies etc. and that too only in temperate conditions (Hunziker, 1990; Danillowicz & Brown, 1992; Ignatius, 2001).

Probiotics in aquaculture have been reported to provide beneficial effects and the use of probiotics is an important management tool in ornamental fish culture (Balcazar et al., 2006). In general, probiotic administration during early developmental stages is most effective, usually resulting in greater than an order of magnitude increases in survivorship (Gatesoupe, 2007). It has also been reported that in captive rearing, higher mortality occurs frequently (Benetti et al., 2008), and growth abnormalities lead to higher incidence of skeletal deformities (Fernandez et al., 2008). Probiotics as feed supplements benefit the host by improving the feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, growth promoting factors and increasing immune response (Harikrishnan et al., 2010).

Several reports suggested that the probiotics supplementation can reduce the cost of culture by improving the growth and feed utilization efficiency of fish (Mazurkiewicz et al., 2007). The genus, *Bacillus* as putative probiotics has been used extensively as aquaculture feed additives, because of its resistance to high temperature and high pressure (Rengpipat et al., 2000). Dietary supplementation of *Bacillus spp*. improved the growth performance, immunity and disease resistance of fish fish (Ai et al., 2011; Geng et al., 2012). In rainbow trout (*Onchorhynchus mykiss*), significant improvement of feed conversation ration (FCR), specific growth rate (SGR) and protein efficiency ratio (PER) was observed when the fish was fed with diets containing Bacillus spp (Bagheri et al., 2008; Merrifield et al., 2010).

Carotenoids, which are lipid soluble pigments, are responsible for skin colour of ornamental fishes, and can determine their commercial value (Paripatanamont et al., 1999). The ornamental fishes are unable to perform de novo synthesis of carotenoids (CD) like other animals and therefore rely on dietary supply to achieve their natural pigmentation (Goodwin, 1984). Under intensive farming conditions and aquarium rearing, ornamental fish are fed exclusively with compound feeds, which must therefore be supplemented with carotenoids Tropical marine species are beautiful as some marine species, such as members of Pomacentridae, are important in the world trade for ornamental fish (Wilkerson, 2001), and a popular subject of research (Arvedlund et al., 2000). Of which, clown fishes are considered to be most attractions of aquarists, and they are important in the aquarium trade in view of their bright colour, interesting display behavior and their ability adopt in captive conditions (Wilkerson, 1998). The tomato clownfish or, after its scientific name, *Amphiprion frenatus* (Brevoort, 1856), also known as black back anemonefish, fire clown, one band anemonefish, or red clown are under Order Perciformes (Jung, 2006). The tomato clown fish is considered to be major candidate species in ornamental fish industry and it plays a major role in world aquarium trade. Although the utility of probiotics has been recognized in aquaculture by several researchers worldwide, no attempts were made to improve its growth, survival and color through probiotic administration till date. In the present study, an attempt was made to investigate stimulatory effect of probiotic bacteria *B. firmus* CAS 7 on growth, survival and skin color of tomato clown, *Amphiprion frenatus* (Brevoort, 1856).

Table 1. Ingredients used for feed formulation and proximate composition of the prepared basal diet

Ingredients	(g kg^{-1})	Proximate composition of the basal diet	(g kg^{-1})
Fish meal	600	Dry matter	941
Shrimp meal	160	Crude protein	491
Soybean meal	20	Crude lipids	98
Wheat flour	140	Ash	96
Fish oil	40		
Soybean phospholipids	20		
Vitamin mineral mix	10		

Vitamin mineral mix (EMIX PLUS, Mumbai, India) (Quantity per kg)

Vitamin A: 22 00 000 IU; Vitamin D3: 4 40 000 IU; Vitamin B2: 800 mg; Vitamin E: 300 mg; Vitamin K: 400 mg; Vitamin B6: 400 mg; Vitamin B12: 2.4 mg; Calcium Pantothenate: 1000 mg; Nicotinamide: 4 g; Choline Chloride: 60 g; Mn: 10 800 mg; I: 400 mg; Fe: 3000 mg; Zn: 2000 mg; Cu: 800 mg; Co: 180 mg; Ca: 200 g; P: 120 g; L L-lysine: 4 g; DL-Methionine: 4 g; Selenium: 20 ppm.

MATERIAL AND METHODS

Isolation and culture of probiotic bacteria B. firmus CAS 7: The probiotic bacteria, *B. firmus* CAS 7 was isolated from marine environment and identified by both conventional (morphology, physiology and biochemical) and molecular approaches (16S rRNA gene sequence). The 16S rRNA gene sequences of the probiotic strain

CAS 7 obtained from the present study was deposited in NCBI with accession number HQ116811. Further, the probiotic strain *B. firmus* CAS 7 was cultured and prepared as described by (Sun et al., 2010). 500 mL of fresh nutrient broth was seeded with 1% inoculum (1.50 x 106CFU mL-1) and kept in a shaker incubator (200 rpm) at pH 7.5, temperature 28 °C, and salinity 30 PSU for 48 h. After incubation period, the cells were harvested by centrifugation at 5000 xg for 10 min, washed twice with phosphate-buered saline (pH 7.5) and re-suspended in same PBS buffer. The growth of probiotic bacteria was estimated by measuring optical density at 600 nm from the aliquots withdrawn at every 6 h intervals.

Table 2. Morphological, physiological and biochemical characteristics of the probiotic strain CAS 7

Characteristics	Results
Shape	Rod
Gram stain	Positive
Spore formation	+
Motility	+
Glucose	+
Mannitol	+
Xylose	+
Starch Hydrolysis	+
Gelatin Hydrolysis	+
Fat Hydrolysis	+
Casein Hydrolysis	+
Catalase activity	-
Nitrate reduction	+
Indole	+
Citrate	+

Preparation of control and probiotic feed: The control basal diet was formulated using the ingredients such as fish meal, shrimp meal, soya bean meal, wheat flour, fish oil and vitamin mineral mix (Table1) (Sun, Y.Z., et al., 2010). All the ingredients were dried overnight at 80° C in a hot air oven and powdered. The powdered ingredients were sieved through a fine-meshed screen (0.5 mm diameter) and mixed well. The dough was prepared by adding required amount of water with the ingredients, sterilized (autoclave at 121° C for 15 min) and incorporated with 3% (v/w) commercial vitamin mineral mix (EMIX PLUS, Mumbai, India) and pelletized using hand pelletizer to obtain 1 mm pellets. The pellets were initially sun dried and then oven dried at 60 ± 5°C for 12 hours to get moisture content. Further, they were manually broken into smaller bits and stored at room temperature in an air tightened sterile polypropylene containers.

The test feeds for the experiments were prepared by gently spraying the required amount of bacterial suspension on the control diet and mixing it part-by-part in a drum mixer to obtain a final concentration of 25, 50, 100 and 150 mg kg-1 in experiments I, II, III and IV respectively. The probiotic cell suspensions were added to the control diet after the dosage had been autoclaved and subsequently cooled, before pelletizing. The proximate composition (moisture, protein, ash, lipid and fibre) of all probiotic feeds and control feed were determined by the standard procedures of AOAC (1990). The probiotic strain-incorporated feeds were packed in sterile polypropylene containers and stored at 4°C for viability studies.

Experimental setup: The juveniles of tomato clown Amphiprion frenatus (Brevoort, 1856) were obtained from MAV Breeders (Mandabam, Tamilnadu, India) and acclimatized for 4 weeks before the trial at Aquaculture breeding center, CAS in Marine Biology, Annamalai University, India. The feeding experiment was conducted in (20 L) rectangular fibreglass tanks, with temperature ranging from 26 - 30°C, salinity 28 -30 PSU, pH 7.4 - 7.8; and Dissolved oxygen 4.2 to 5.6 mg L^{-1}. A total of 30 fish seeds were maintained in each tank throughout the experiment and each treatment was conducted in triplicate. A total of five trials were made during the study (control and four as experiments).

The fishes were feed with prepared pellet feed alone in control and feed contains probiotic at a concentration 25, 50, 100 and 150 mg kg-1 in experiments I, II, III and IV respectively. The feeding rate was about 3% of biomass per day provided in equal rations at 8.00 AM, 1.00 PM, 6.00 PM for 120 days and the excess diet was collected and dried at 60 °C, put in room temperature for 3 days to restore the natural moisture and then weighed. Daily feed was adjusted every 30 days by batch weighing of fish in each tank after a 24 h period of starvation. Experimental tanks were cleaned and water exchange was done once a week.

Growth indices: The growth parameters such as weight gain, specific growth rate (SGR), survival rate and feed conversion ratio (FCR) were assessed at 30, 60, 90 and 120 days. The weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) was evaluated based on standard formula as follows.

Weight gain = (Final weight – Initial weight)
Specific Growth Rate (SGR) = 100 (ln W2 - lnW1)/T

where, W1 and W2 are the initial and final weight, respectively, and T is the number of days in the feeding.

Feed conversion ratio FCR= total feed quantity given (g)/total weight gain (g).

Colour enhancement and carotenoid content estimation: The color enhancement was monitored by visual examination and estimation of carotenoid content in the skin of experimental fishes. The carotenoid content of the experimental fish skin was extracted according to the method of Torrissen and Naevdal (1984). The fishes were randomly sampled from each experiment per sampling period (30, 60, 90 and 120 days) and used for carotenoid content analysis in triplicate. Briefly, 2 mg of skin were

collected from both sides between the abdominal and dorsal regions of the fish and then transferred to 10 mL of pre - weighed glass tubes after the fat layer had been removed from the skin and ground well with acetone containing anhydrous sodium sulphate and made up to 10 mL with acetone. The samples were stored for 3 days at 4oC in a refrigerator, and then extracted three times till no further colour could be obtained and centrifuged at 5000 xg for 5 min. The total carotenoid content of the samples was determined using spectrophotometer (Shimadzu, UV mini 1240) using extinction coefficients (E1%, 1 cm) of 2000 for astaxanthin (Hata & Hata, 1971) at 475 nm, and 2500 for carotenoids from alfalfa at 450 nm (Schiedt & Jensen, 1995).

Table 3. Growth and survival of tomato clown Amphiprion frenatus (Brevoort, 1856) fed with control (without probiotic) and experiments [basal diet supplemented with probiotic *B. firmus* CAS 7- 25 mg/kg (Experiment - I), 50 mg/kg (Experiment - II), 100 mg/kg (Experiment - III) and 150 mg/kg (Experiment - IV). The values are presented in mean ± SD, n = 3, FCR - feed conversion ratio; SGR - specific growth rate.

Days of culture	Growth Parameters	Control	Experiment I	Experiment II	Experiment III	Experiment IV
			25 mg kg–1	50 mg kg–1	100 mg kg–1	150 mg kg–1
0-30	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	10.27±1.6	11.89±1.1	13.49±1.2	15.53±1.4	15.94±1.1
	Weight gain (g)	2.94±1.6	4.28±1.3	5.92±0.29	7.9±0.30	8.05.24±1.2
	SGR	0.098	0.143	0.197	0.263	0.268
	FCR	0.061	0.089	0.123	0.164	0.167
0-60	Survival rate (%)	60	60	85	90	90
	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	12.83±1.5	15.13±1.3	16.92±1.6	20.57±1.1	21.56±1.3
	Weight gain (g)	5.5±1.5	7.52	9.35±1.2	12.94±1.4	13.67±1.4
	SGR	0.092	0.125	0.156	0.216	0.228
	FCR	0.057	0.078	0.097	0.134	0.142
0-90	Survival rate (%)	60	60	85	90	90
	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	15.24±0.8	17.78±1.3	19.32 ±1.5	24.02 ±1.3	24.56 ± 0.9
	Weight gain (g)	7.91±1.6	10.17	11.75 ±1.5	16.39±1.3	16.67±0.9
	SGR	0.008	0.113	0.131	0.182	0.185
	FCR	0.55	0.070	0.081	0.113	0.115
0-120	Survival rate (%)	60	60	85	100	100
	Initial weight (g)	7.33±0.38	7.61±0.31	7.57±0.29	7.63±0.32	7.89±0.41
	Final weight(g)	18.14±1.3	21.39±1.1	25.94±1.2	28.69±0.9	28.98±1.4
	Weight gain (g)	10.81±1.4	13.78±1.2	18.37±1.3	21.06±1.4	21.09±1.5
	SGR	0.090	0.115	0.153	0.176	0.176
	FCR	0.056	0.071	0.095	0.109	0.109
	Survival rate (%)	60	60	85	100	100

RESULTS

Isolation and growth of probiotic bacteria B. firmus CAS 7: The bacterial strain was isolated from marine sediments of Parangipettai, Tamil Nadu, India and identified as B. firmus based on the morphology, physiological and 16S rRNA analysis (GenBank accession no. HQ116805) and was designated as *B. firmus* CAS 7. The growth of the bacteria was started from the stationary phase itself and it reached the maximum during logarithmic phase (24 - 30 h) (Fig.1). After incubation, cells were harvested from the culture broth by centrifugation (6000 xg), washed and resuspended with PBS buffer after the incubation and added to the basal diet at desired concentration and used for further study.

Growth analysis: In the present study, the fishes were fed with basal diet supplemented with probiotic at four different experiment and control (without probiotic) for 120 days and the growth parameters such as weight gain, SGR and FCR were determined and the results are presented in Table 3. The results suggested that the weight of experimental fishes was increased with the increasing days of culture in control and experiments. It was reported that there was significant weight gained in all experiments where fishes were fed with probiotic supplemented basal feed than control. The weight gain was found to be higher in experiment IV (21.09±1.5) followed by III (21.06±1.4), II (18.37±1.3), I (13.78±1.2) and control (10.81±1.4). Similarly, the fishes in experiment IV and III had significantly higher SGR

(0.176) when compared to the fishes in experiment II (0.153), I (0.115) and control (0.090).

Figure 1: Time course and cell growth of probiotic bacteria B. firmus CAS 7.

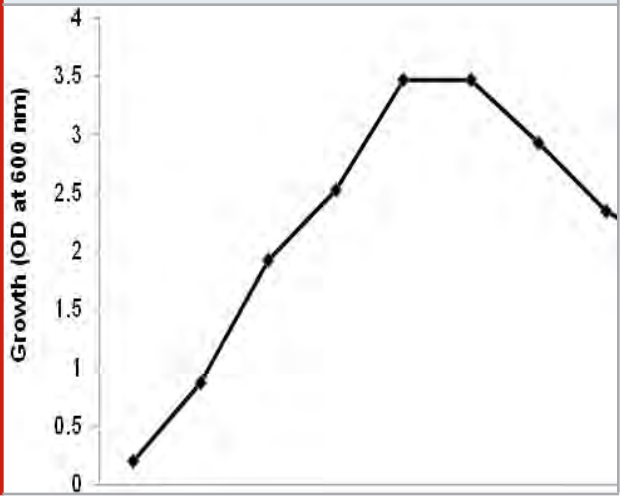
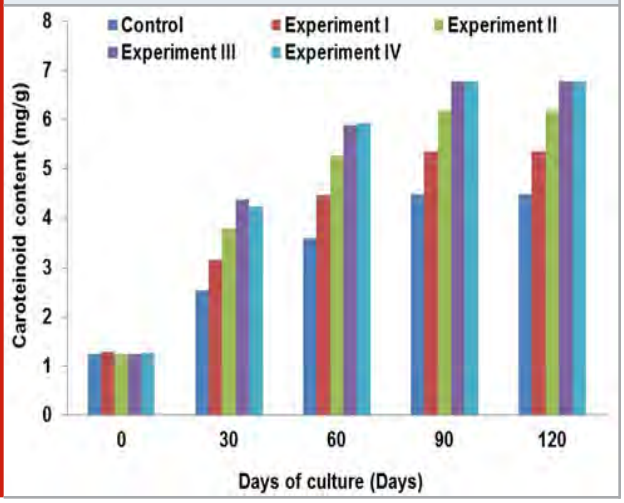


Figure 2: Carotenoid content of skin of tomato clown *Amphiprion frenatus* (Brevoort, 1856) in control and experiments.



Furthermore, feed conversion ratio (FCR) was similar in experiment IV and III (0.29) which comparatively lower than that of experiment II (0.33), I (0.44) and control (0.56). Likewise, survival rate in experiment IV, III, II, I and control was about 100, 100, 85,60 and 60% respectively. It seems that there was no mortality in experiment III and IV, whereas it was higher in experiment I, II and control setup. It seems that the probiotic supplemented in feed significantly stimulated the growth and survival of fishes in experiment III and IV. Although weight gain, SGR, FCR and survival rate was significantly higher in experiment III and IV than control and other experiments, no significant difference was reported when the feed supplemented with 100 and 150 mg kg-1 of probiotic.

Carotenoid content analysis: The results of the carotenoid content of skin suggested that the initial carotenoid content of the fish skin varied between 1.24 and 1.28 mg g-1 in experiments and control and it was increased gradually with increasing days of culture. Further, carotenoid content in experiments I, II, III, IV and control were about 5.3, 6.18, 6.79, 6.80 and 4.48 mg g-1 respectively (Fig. 2) at the end of experiment. It seems that the carotenoid content of fish skin in experiment III and IV where the feed was supplemented with 100 and 150 mg kg^{-1} of probiotic was comparatively higher than control and other experiments.

DISCUSSION

In the past decade of years, there are few reports on successful larval rearing of marine ornamental fishes such as *Amphiprion clarkii*, *A. percula* (Alava & Gomes, 1989; Malpass, 1996; Allen, 1998), *Dascyllus albisella* and *D. aruanus* (Danillowicz & Brown, 1992) in temperate waters. The benefits of probiotics in fish farming are improvements of growth performances, immunity and pathogen exclusions (Qi et al., 2009; Sun et al., 2010). *Bacillus* species significantly improved the growth in tilapia (Aly et al., 2008), *Catla catla* (Bandyopadhyay & Mohapatra, 2009), *Labeo rohita* (Ghosh et al., 2003), *Macrobrachium rosenbergii* (Keysami, M. A. et al., 2007) and *Penaeus monodon* (Rahiman, K. M. M. et al., 2010).

The probiotic bacterium, *B. firmus* CAS 7 isolated from marine environment was used as feed supplement to evaluate the stimulatory effect on growth, survival and skin colour of tomato clown *Amphiprion frenatus* (Brevoort, 1856). The growth studies suggested that the maximum cell growth was achieved at logarithmic phase (24th h). Similarly (Elayaraja, S. et al., 2011), studied the effect of B. cereus on the growth of polycheate and maximum cell growth as well as enzyme production at late logarithmic phase (36th h). Thus, maximum growth of this bacterium at shorter incubation period makes this a potential probiotic candidate species which could be used in the aquaculture industry.

In the present study, the fishes were fed basal diet supplemented with probiotic *B. firmus* CAS 7 at 25, 50, 100 and 150 mg kg^{-1} and the growth performance such as weight gain, survival rate and color enhancement were evaluated for 120 days. The weight of fishes in experiment III (28.98±1.4) and IV (28.69 ± 0.9 g) was significantly higher than experiment II (7.57 ± 0.29 to 25.94 ± 1.2 g), I (7.61 ± 0.31 to 21.39 ± 1.1 g) and control (7.33 ± 0.38 to 10.81 ± 1.4 g). Moreover, the weight gain (21.09 ± 1.5 g) and survival rate (100%) were also significantly higher in experiment III and IV where fishes were fed with 100 and 150 mg kg-1 of probiotic mixed with basal diet respectively when compared other experiments and control. The specific growth rate (SGR) were significantly higher in fishes reared in experiment III and IV (0.176) followed by II (0.153), I (0.115) and control (0.090).

Likewise, the FCR rate was in the range of 0.83 – 0.56, 0.57 -0.44, 0.41 – 0.33, 0.31-0.29 and 0.30 - 0.29 respectively in control, experiment I, II, III and IV. (Jafaryan et al., 2008), also reported that the probiotic (*Bacillus sp*.) supplemented diet significantly increased the weight, length and SGR of fish when compared to the control diet. (Giri, S.S. et al., 2013), suggested that the probiotic supplementation needs to be done for 60 days to obtain a significant improvement in SGR and FCR. Several studies suggested have that the probiotic *Bacillus sp*. supplementation has significantly increased the weight gain and SGR in *Rachycentron canadum* (Geng, X. et al., 2012), *Labeo rohita* (Giri, S.S. et al., 2013), *Oreochromis niloticus* (Aly et al., 2008), *Epinephelus coioides* (Sun, Y.Z. et al., 2010) and *Larimichthys crocea* (Ai et al., 2011). Thus, the reduction in FCR of fishes in experimental groups revealed dietary nutrients were utilized more efficiently when the diet was supplemented with probiotics.

Carotenoids are commonly found in pigmented bacteria which are known to have a positive role in the intermediary metabolism of fish that could enhance nutrient utilization and may ultimately result in improved growth (Steiger, S. et al., 2012). The results of stimulatory effect on skin color study suggested that the carotenoid content in the skin of fishes fed with probiotic maintained coloration during periods of social interaction, suggesting that the probiotics may play important roles in maintaining fish skin coloration. After 30 days, the carotenoid content in the skin of fishes fed with probiotic supplemented feed exhibited increasing pigment levels and started to differ from that of control diet. The carotenoid content of fish skin was about 6.80 mg g-1 in experiment III and IV, whereas it was 4.48 mg g-1 in control. Thus, the results confirmed that the color enhancement in terms of total carotenoid content in the fish skin increased significantly with increasing probiotic bacteria concentration in the basal diet. Support to the above fact, (Hong, 2008), reported that the yellow/orange carotenoids formed by *B. indicus* HU36 significantly increased the pigmentation of the experimental fishes.

The result obtained from the present study suggested that the fishes fed with basal diet supplemented 100 and 150 mg kg^{-1} of probiotic *B. firmus* exhibited better stimulatory effect on growth, survival and skin colour of *Amphiprion frenatus*. Although the fishes fed with diets containing 100 and 150 mg kg^{-1} of probiotic exhibited higher effect than control and lower dosage, no significant difference reported between 100 and 150 mg kg^{-1} and hence, it is recommended to use at a concentration of 100 mg kg^{-1} for the enhanced growth and color of the tomato clown *Amphiprion frenatus* which has significant importance in ornamental fish industry.

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Effect of Curcumin on Cell- ECM Interaction in Human Breast Cancer Cells MDA-MB-231

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ABSTRACT

Breast cancer is one of the most frequently diagnosed cancers and the leading cause of cancer deaths in females worldwide. Several matrix metalloproteinases (MMPs) were reported to show stronger expression in breast cancer tissue compared to normal breast tissue. Some breast cancer cell lines also constitutively express a wide variety of MMPs. Thus targeting those MMPs can be a possible route to combat breast cancer. In different cancer cells curcumin was reported to exert both anti-proliferative and pro-apoptotic effect. In the present study, human breast cancer cells, MDA-MB-231 was exposed to curcumin and change in expression, localization and activity of different procancerous factors were studied using zymography, RT-PCR, Western blot, immunocytochemistry. It was found that, curcumin affects several cell signalling molecules like FAK, ILK, PI3K, Akt, NF-κB, and reduces MMP-9 level, VEGF level, cell migration and increases E-cadherin level. Since most of the molecules mentioned are part of the FAK mediated signalling, curcumin might exhibit here appreciable effects on tumor cell- ECM interaction with potential antagonist role on cell metastatic properties involving increase in E-cadherin and decrease in VEGF level

KEY WORDS: BREAST CANCER , CELL SIGNALLING, ECM, INTEGRIN, MMP.

INTRODUCTION

Curcumin is the yellow pigment of turmeric, the dried rhizome of the plant, *Curcuma longa L.* This pigment, with the chemical structure of polyphenol with a diarylheptanoid, has been reported to have chemopreventive as well as chemotherapeutic activities (Lopez-Lazaro, 2008).

The anti-cancer activities of this compound are exerted through affecting several pro-cancerous factors (Tomeh et al., 2019). These include downregulation of transcription factors like NF-κB, AP-1; pro-metastatic factors like uPA, MMPs; growth factor receptors like EGFR, HER2; cell signaling components like protein tyrosine kinases, protein serine/threonine kinases (Aggarwal et al., 2003) including c-Jun N-terminal kinase (Chen and Tan, 1998). Apart from these, curcumin has been found to protect DNA damage by carcinogens, promote "mild but yet significant activation of apoptosis", and inhibit angiogenesis, invasion and metastasis.

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Breast cancer is one of the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide. Incidence rates are high in Australia/New Zealand, Northern and Western Europe, and North America; intermediate in South America, the Caribbean, and Northern Africa; and low in Asia and sub-Saharan Africa (Jemal et al., 2011; Bray et al 2018). Several matrix metalloproteinases (MMPs), which belongs to family of endopeptidases having the ability to degrade extracellular matrix (ECM) proteins and play a crucial role in tumor invasion and metastasis, were reported to show stronger expression in breast cancer tissue compared to normal breast tissue. Some breast cancer cell lines also constitutively express a wide variety of MMPs(Kohrmann et al., 2009; Katari et al, 2019). Thus targeting those MMPs can be a possible route to combat breast cancer.

Curcumin has been found to downregulate expression and activity of MMPs in different experimental models. Treatment of highly metastatic murine melanoma cells B16F10 with curcumin significantly inhibited matrix metalloproteinase-2 (MMP-2) activity, involving reduction of expression of membrane type-1 matrix metalloproteinase (MT1-MMP), an important component of MMP-2 activation, and focal adhesion kinase (FAK), an important component of the intracellular signalling pathway (Banerji et al., 2004). Curcumin can also reverse the effects of tumor promoting agent. Curcumin was found to significantly inhibit the MMP-9 expression and activity that was induced by phorbol ester PMA. Such inhibition of MMP-9 was attributed to curcumin mediated repression of the PMA-induced phosphorylation of ERK, JNK, p-38 MAP kinase and suppression of DNA binding activities of NF-κB and AP-1(Woo et al., 2005). Inhibitory effect of curcumin on expression of MMPs has been observed in mouse model also(Qi et al., 2009). Curcumin can enhance the expression of antimetastatic proteins, nonmetastatic gene 23 (Nm23), tissue inhibitor metalloproteinase (TIMP-2), and E-cadherin(Ray et al., 2003). In breast cancer cells curcumin was reported to exert both anti-proliferative (Liu et al., 2009) and pro-apoptotic effects (Choudhuri et al., 2002).

MATERIAL AND METHODS

Human breast cancer cell line MDA-MB-231 was procured from National Centre for Cell Sciences,(NCCS), Pune, India. Leibovitz's L-15 medium, Fetal Bovine Serum (FBS), Trypsin, Gentamycin were purchased from GIBCO™-Invitrogen. Curcumin was purchased from Sigma-Aldrich. Protease Inhibitor Cocktail Tablets were purchased from Roche, Germany. GelatinSepharose- 4B beads were purchased from GE Healthcare Biosciences AB, Uppsala, Sweden. All primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc, USA and chemiluminescent substrate SuperSignal West Femto was purchased from Thermo Fisher Scientific Inc. Primers (MMP-9, TIMP-1 and GAPDH) were synthesized by Operon, Germany. RNAqueous 4 PCR (Total RNA isolation kit) and Retroscript (RT-PCR Kit) were purchased from Ambion, Austin, TX, USA. DAB substrate and stable peroxidase substrate buffer were purchased from Pierce Biotechnology, USA. Avidin-biotinylated peroxidase complex reagent (Vectastain ABC kit) was purchased from vector laboratories, Burlingame, CA. MTT reagent, DMSO were purchased from Amresco. Coomassie brilliant blue was purchased from Merck. Immobilon-P Membrane (PVDF) was purchased from Millipore, USA.

MDA-MB-231, a highly metastatic human breast cancer cell line was grown and maintained in L15 medium, containing 10% FBS in a dry (CO_2 free incubator) at 37^{0}C, (ATCC, 2020). MDA-MB-231 cells were allowed to attach on 35 mm petridishes (300,000 cells/ml medium) and various concentrations of curcumin treatment were applied for different time periods. As cells remained attached upto 48 hrs in presence of 5, 10 µM curcumin treatment in L15 SFCM (serum free culture medium), this treatment condition was used for our experiments. As the curcumin stock solution was dissolved in DMSO, equal volume of this solvent was treated in control cells.

MDA-MB-231 cells were plated in 96-well plate and treated with curcumin of various conc. (0, 5, 10, 15, 20 µM) for 48 hrs. Wells with SFCM, but without cells were used as 'blank'. After the treatment, 50 µl MTT reagent (100 µg) was added to each well and incubated for 4 hrs. Culture supernatant was carefully discarded and formazan was dissolved in DMSO. Absorbance of the solution was measured at 570 nm against the blank wells and percentage of viability was calculated assuming that of 0 µM curcumin treated cells as 100%.

After the required treatments on MDA-MB-231 cells, the gelatinases were separated from the culture supernatants using gelatin sepharose 4B beads (shaking for 2 hrs at 4^{0}C). The beads were washed twice with Tris-buffered saline with 0.02% tween-20 (TBS-T) and eluted with 1X Laemli buffer (0.075 gm Tris, 0.2 gm SDS, in 10 ml water, pH 6.8) for 30 mins at 37^{0}C. The eluted supernatant (separated from bead by centrifugation) was subjected to zymography on 7.5% SDS-PAGE co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton-X-100 for 30 mins to remove SDS and was then incubated in reaction buffer (50 mM Tris-HCl pH 7.5, 4.5 mM $CaCl_2$, 0.2 M NaCl) for overnight. After incubation, the gel was stained with 0.25% Coomassie Blue in 40% methanol and 10% glacial acetic acid.The bands were visualized by washing the gel in water and photograph was taken (Sen et al., 2009).

RNA was extracted from 1x10^6 MDA-MB-231 cells treated with DMSO (Control) and 10 µM curcumin (Experimental) for 48 hrs. Total RNA was extracted (RNAqeous, Ambion, USA). 2 steps RT-PCR (Retroscript, Ambion, USA) was done with equal amounts of total RNA using specific primers for human MMP-9 and TIMP-1 genes. The steps followed have been previously described (Pal et al., 2013). The primer sequences are:-

hMMP-9: 5'-CGCTACCACCTCGAACTTTG-3' (forward), 5'GCCATTCACGTCGTCCTTAT-3'-(reverse);

hTIMP-1: 5'-CACCCACAGACGGCCTTCTGC-3'-(forward)
5'-AGTGTAGGTCTTGGTGAAGCC-3'-(reverse);
GAPDH: 5'-CGGAGTCAACGGATTTGGTCGTAT- 3' (forward)
5'-AGCCTTCTCCATGGTGGTGAAGAC- 3' (reverse).

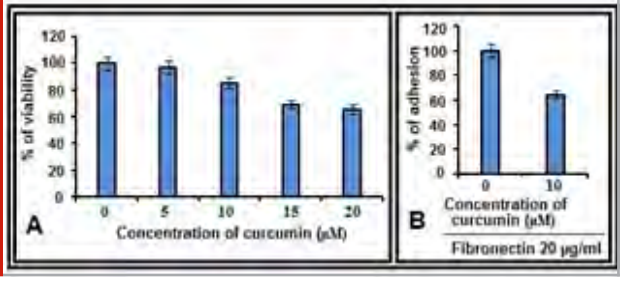
Conditions used for PCR consisted of 40 cycles for TIMP-1 and MMP-9 at 94°C for 30 sec, 58°C for 30 sec and 72°C for 90 sec in thermal cycler (Ganguly et al., 2012).

Whole Cell Extraction:- MDA-MB-231 cells were treated with DMSO (C) and 10 µM curcumin (E) for 48 hrs. The cells were trypsinized, washed with PBS (containing 10% NaF, 10% Na_3VO_4) and resuspended in RIPA extraction buffer. Extraction was done by snap-freeze method. Protein contents of the whole cell extracts were estimated by Lowry's method.

Western blot assay:- Equal amount (100 µg) of protein from whole cell extracts from control and experimental sets was loaded in each lane on the protein gel. After SDS-PAGE the proteins were electrophoretically transferred onto PVDF membrane (Millipore). Nonspecific binding sites on the membrane were blocked in 4% BSA. After treatment with required primary antibodies [anti-FAK, anti-PI3K (p85), anti-Akt, anti-MMP-9, anti-E-cadherin, anti-VEGF, anti-ILK; 1µg/2ml dilution for each] and their respective HRP- labeled secondary antibodies (1µg/200ml dilution), blots were developed by ECL method as previously described (Ganguly et al., 2012).

Immunocytochemistry:- The MDA-MB-231 cells were allowed to attach on coverslips (few thousand cells/coverslip) followed by curcumin treatment for 48 hrs. After PBS wash, cells on coverslips were fixed with 3.5% formaldehyde and treated with 0.5% Triton X-100. Non-specific sites were blocked using 1% BSA solution. Immunocytochemistry was done with anti-NF-κB p65 primary antibodies (1 µg/ml dilution), and respective biotin labeled secondary antibodies (1 µg/ml dilution) in a method described in earlier reports (Pal et al., 2013).

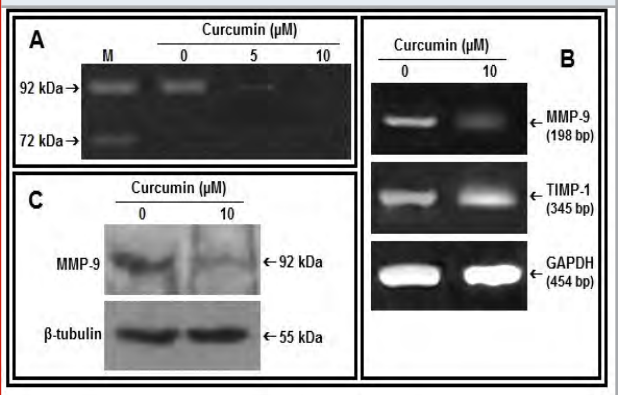
Figure 1: Cell adhesion and cell viability assays:A. MDA-MB-231 cells, treated with curcumin (0, 5, 10, 15, 20 µM for 48 hrs) were subjected to MTT assay and percentage of survival was calculated. B. MDA-MB-231 cells were treated with curcumin (0, 10 µM) for 48 hrs and percentage of adhesion on FN coated (20 µg/ml) wells were calculated



RESULTS AND DISCUSSION

MTT and cell adhesion assay results:- The effect of curcumin on cell viability was studied by MTT assay. Among the concentrations studied, the maximum concentration which resulted >80% cell survival was 10 µM (Fig 1A) and this concentration was used in the subsequent experiments. By the cell adhesion assay it was found that, MDA-MB-231 cells bind efficiently to fibronectin (FN), however this binding is reduced in cells treated with curcumin (Fig. 1B).

Figure 2: Effect of curcumin on MMP-9, TIMP-1:A. MDA-MB-231 cells were treated without (0) or with curcumin (5, 10 µM) in SFCM for 48 hrs. Lane M is the marker lane. The gelatinases in all the cases were separated from SFCM by mixing Gelatin Sepharose 4B beads and subjected to gelatin zymography. B. 2 steps RT-PCR was done with equal amounts of total RNA isolated from control and curcumin treated (10 µM, 48 hrs) cells and using MMP-9, TIMP-1 specific primers. G3PDH primers were used to check for equal loading. C. MDA-MB-231 cells were treated with curcumin (0, 10 µM for 48 hrs). The cells were collected, extracted and equal protein (100 µg) was subjected to western blot analysis with anti-MMP-9 antibody and respective HRP-coupled secondary antibody. The blots were developed by ECL method and β-tubulin was used as loading control.

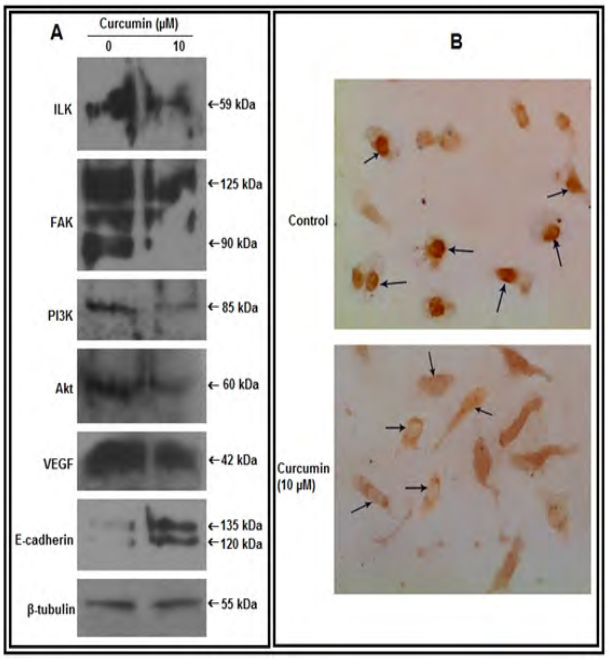


Curcumin reduces MMP-9 expression and secretion in MDA-MB-231 cells:- MDA-MB-231 cells treated with curcumin for 48 hrs showed decrease in MMP-9 gelatinolytic activity in the culture supernatant with appreciable downregulation at 10 µM concentration (Fig 2A). RT-PCR analysis of curcumin treated MDA-MB-231 cells (10 µM for 48 hrs) indicates decrease in expression of MMP-9 mRNA and increase in TIMP-1 mRNA (Fig 2B). Downregulation of MMP-9 protein expression under same condition was further confirmed by western blot assay (Fig 2C).

Effect of curcumin on cell signaling components:- Western blot analysis reveals that, curcumin treatment appreciably reduces expression of ILK, FAK, PI3K, Akt and VEGF and enhances expression of E-cadherin in MDA-MB-231 cells (Fig 3A). Immunocytochemical assay

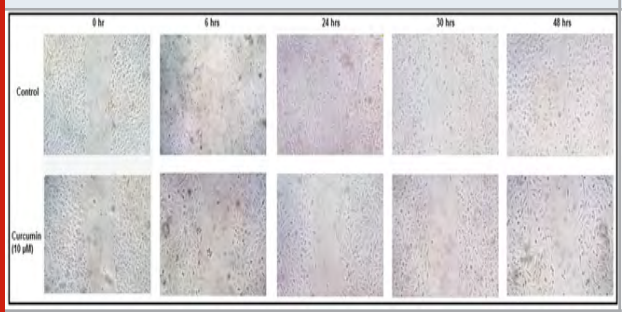
demonstrates that, curcumin treatment causes nuclear to cytosolic localization of NF-κB (Fig 3B).

Fig 3: Effect of curcumin on cell signaling components:A. Cell extracts from untreated and curcumin treated (10 µM for 48 hrs) MDA-MB-231 cells were subjected to western blot assay with anti-ILK, anti-FAK, anti-PI3K, anti-Akt, anti-VEGF, anti-E-cadherin antibodies (1 µg/2 ml dilution). β-tubulin was used as loading control. B. Cells were grown in absence (Control) and presence of curcumin (10 µM, 48 hrs) on coverslips. Immunocytochemistry was done using anti-NF-κB primary and respective biotin-labeled secondary antibody.



Effect of curcumin on cell migration:- Photographs taken at different time intervals indicate that, the ability of MDA-MB-231 cells to heal experimentally scratched wounds is reduced appreciably in presence of curcumin (Fig 4).

Figure 4: Effect of curcumin on cell migration: MDA-MB-231 cells were grown and treated without (control) or with 10 µM curcumin for 48 hrs before giving the scratch wound. Photographs were taken just after the scratch (0 hr), and then at 6, 24, 30, 48 hrs.



Signaling pathways leading to upregulation of MMPs have been studied within various cell lines in our laboratory. Among the breast cancer cell lines, MDA-MB-231 cells secrete MMP-9 and its upregulation at a certain ECM environment, i.e. in presence of fibronectin, require activation of ILK, FAK, PI3K and nuclear translocation of NF-κB(Maity et al., 2011). In our present study curcumin was found to reduce the ability of MDA-MB-231 cells to bind the same ECM ligand. Curcumin treatment also downregulated MMP-9 secretion in MDA-MB-231 cells. We further studied the effects of curcumin on the associated signaling molecules, specially those involved in regulation of MMP-9.

Several integrin receptors, specially the β1 integrins were found to play important role to initiate MMP-9 upregulating signal (Pal et al., 2012). ILK is a Ser/Thr kinase and can interact with the cytoplasmic domains of β1integrins to promote suppression of apoptosis, cell survival, cell migration and invasion (Yoganathan et al., 2002, Zheng et al, 2019). In our study, curcumin treated cells showed appreciable downregulation of ILK expression. FAK is another cell signalingmolecule, which is regulated by integrin mediated signaling and is a potent regulator of MMP-9 (Ganguly et al., 2012, Sein et al., 2000). Here FAK is found to be downregulated in MDA-MB-231 cells in presence of curcumin. FAK activity is coupled to assembly of focal adhesions and plays major role in cell attachment and migration (Gilmore and Romer, 1996, Mitra et al., 2005). In the present study, the natural ability of MDA-MB-231 cells to migrate and heal experimental scratch wounds was greatly reduced in curcumin treated cells.

PI3K, one of the downstream targets of FAK, was also found to be downregulated in curcumin treated cells. PI3K can exert its effects by activating Akt, followed by several transcription factors, including nuclear factor B (NF-κB) (Bader et al., 2005, Torrealba et al, 2019). In presence of curcumin, appreciable downregulation of PI3K, Akt and translocation of NF-κB out of the nucleus is observed in MDA-MB-231 cells. NF-κB was reported to modulate expression of MMP-9 gene in various cell lines (Tai et al., 2008; Guarneri et al, 2017). Reduced mRNA expression of MMP-9 (RT-PCR data) found in our study may be due to lack of availability of NF-κB as transcription factor within nucleus. In addition, enhanced mRNA expression of TIMP-1, a negative regulator of MMP-9, in presence of curcumin may affect cumulatively in downregulation of MMP-9 activity.

Among the other related signaling proteins studied, E-cadherin was found to be upregulated in curcumin treated cells. It is a cell adhesion molecule and known to form powerful invasion suppressor complex(Noe et al., 2001). Thus curcumin may lead to a situation that prevents invasion and metastasis. Successful metastasis require sufficient angiogenesis and VEGF is a potent inducer of angiogenesis. MMP-9 can release biologically active VEGF from the extracellular matrix of cancer cells(Hawinkels et al., 2008). Here downregulation of VEGF in curcumin treated cells may be due to the

reduced MMP-9 activity. Therefore it may be concluded that in MDA-MB-231 cells curcumin affects several cell signaling molecules like FAK, ILK, PI3K, Akt, NF-κB, the effect of which may be the reduction of MMP-9 level and cell migration. In addition, treatment with this natural drug enhances E-cadherin and reduces VEGF level. Curcumin exhibits here appreciable effect on tumor cell ECM interaction with potential antagonist role on cell metastatic properties.

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The State of Micro-Rheological Properties of Red Blood Cells in Rats on the Background of Physical Inactivity

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ABSTRACT

Objective: to establish changes in the micro-rheological properties of red blood cells in rats under conditions of inactivity. The experiment was undertaken for 34 healthy outbred male rats of six months age. Prior to inclusion in the study, all rats were healthy and did not participate in any earlier studies. Animals were placed in narrow cages for 1 month to minimize their movements. The control group consisted of 32 healthy outbred male rats of a similar age. The work was performed using biochemical, hematological and statistical research methods. In rats under hypodynamic conditions, an increase in the free radical oxidation of plasma lipids and red blood cells was noted. As the duration of hypodynamia in the blood of rats increased, the number of erythrocytes-discocytes decreased, which was most pronounced after a month of observation. This was accompanied by an increase in their blood levels of altered reversibly and irreversibly erythrocyte forms and an increase in spontaneous aggregation of red blood cells. In rats under hypodynamic conditions, a gradual decrease in the level of antioxidant protection of plasma develops. Aggregation readiness and the degree of change in the surface properties of red blood cells increased in these rats. These changes create a risk in animals of increasing morbid burden and weakening of the whole organism in relation to the negative environmental influences

KEY WORDS: RATS, PHYSICAL INACTIVITY, RED BLOOD CELLS, CYTOARCHITECTONICS, AGGREGATION.

INTRODUCTION

Systematic experiments in science allow us to solve various problems of the reaction of the mammals to the influence of the external environment, (Zavalishina, 2018a; Vatnikov et al., 2019). The implementation of any processes in the body is associated with the activation of biochemical and functional (Usha et al., 2019; Lenchenko et al., 2019) programs under the influence of external factors (Zavalishina, 2018b; Zavalishina, 2018c). This helps the body to adapt, while maintaining viability in any conditions (Skoryatina and Medvedev, 2019; Vorobyeva and Medvedev, 2019). The dynamics of the rheological parameters of blood and especially its shaped elements, which change under many functional conditions and effects on the body, is very important for maintaining the life support of the body (Zavalishina, 2018d; Bikbulatova, 2018a).Red blood cells are a particularly significant element of the microcirculation process, which, by changing their cytoarchitectonics and degree of aggregation, can regulate hemodynamics and metabolism in tissues and, thus, the course of all adaptive processes in the body (Stepanova et al., 2018; Medvedev, 2019).

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It was established that the rheological parameters of red blood cells change against the background of physiological and pathological processes (Zavalishina, 2018e). Moreover, strong effects on the body can worsen the properties of red blood cells and, thus, the microcirculation process in organs, exacerbating the course of pathology (Zavalishina et al., 2019). In the process of studying the body's reactions to various environmental effects on humans (Makhov, Medvedev, 2019), it is difficult to do without evaluating biological processes in an experiment on laboratory animals. Given the importance of erythrocyte rheological parameters for the development of many dysfunctions (Vorobyeva and Medvedev, 2020a) and diseases (Glagoleva and Medvedev, 2020), it is important to study the dynamics of aggregation and cytoarchitectonics of erythrocytes in rats exposed to adverse environmental conditions (Oshurkova and Medvedev, 2018a). This information can serve as a basis for a further search for experimental approaches to optimize the rheological characteristics of red blood cells in conditions of low physical activity of the body, including space flight (Oshurkov and Medvedev, 2018b; Vorobyeva and Medvedev, 2020b). The goal of the present study was set to establish changes in the micro-rheological properties of red blood cells in rats under conditions of inactivity.

MATERIAL AND METHODS

This study was carried out in full compliance with the ethical standards outlined by the European Convention for the Protection of Vertebrate Animals, which are used for experimental and other scientific purposes (adopted in Strasbourg on 03/18/1986 and confirmed in Strasbourg on 06/15/2006).The study took 34 healthy outbred male rats at the age of 6 months. They were placed in narrow individual cells, excluding free movement of animals for 1 month. Prior to inclusion in the study, all rats were healthy and did not participate in the studies.

The control group consisted of 32 healthy outbred male rats at six months of age. Rats were obtained at the age of two months from the laboratory animal nursery of the Branch of the Institute of Bioorganic Chemistry of the Russian Academy of Sciences. Prior to the experiment, the rats were under vivarium conditions in spacious cages (the cell area per rat was 200 cm2). Throughout the time, natural lighting was used, the temperature was maintained at 18-22 °C, the relative humidity was kept at the level of 50-65%. Prior to being taken into the experiment and in its process, all animals received a full-ration ration from compound feed for laboratory animals of the PK-120 brand (Laboratorykorm, Russia). Rats had free access to water throughout the experiment.

RESULTS AND DISCUSSION

The initial values in experimental rats and in the control group were comparable. Under conditions of physical inactivity in rats, an increase in the external manifestations of a deterioration in their general condition was noted—tarnishing and thinning of the coat, a decrease in interest in the environment, and a decrease in appetite. With an increase in the duration of inactivity in rats, an increase in body weight was found, which amounted to 269.5±4.86 g by the end of the observation. At the same time, a decrease in their endurance level was revealed in the test of forced swimming with weights -after a month of inactivity by 43.9% (table).

In rats in a state of physical inactivity, an increase in the activity level of free radical oxidation processes in plasma lipids was found (the level of acylhydroperoxides and thiobarbituric acid-active products increased by 29.4% and 25.1%, respectively) with a decrease in antioxidant activity by 27.6%. Comparable changes in lipid peroxidation in experimental rats were noted in red blood cells – the number of acyl hydroperoxides and malondialdehyde in them increased. After 4 weeks of inactivity, their number in rats prevailed over the outcome by 46.1% and 43.6%, respectively. The activity of erythrocyte catalase and superoxide dismutase in rats decreased by 24.6% and 20.4% in 4 weeks of inactivity, respectively (Table 1).

Under conditions of physical inactivity in the blood of experimental rats, a decrease in the level of erythrocyte-discocytes to 71.4±0.17% after a month of observation was observed, which led to a gradual increase in their level of changed reversibly and irreversibly erythrocyte forms by 57.9% and 2.1 times. In rats under conditions of physical inactivity, a gradual increase in erythrocyte aggregation was revealed with an increase in their number in the composition of the aggregates and the number of aggregates themselves with a decrease in the blood level of unaggregated red blood cells (229.2 ± 0.29), compared with the level of control and outcome (table).

Any indicators of the body that are significant for its viability respond to the influence of adverse factors from the external environment (Mal et al., 2018). Of great importance in this is the reaction to the action of the hemostatic and rheological characteristics of the blood (Vorobyeva, Medvedev, 2018). It is they who largely determine the volume of tissue perfusion, and, consequently, their trophic (Bikbulatova, 2018b; Boldov et al., 2018). For successful microcirculation, the parameters of the shaped elements, which are controlled by the vessel walls (Zavalishina, 2018f) and lipid peroxidation processes (Karpov et al., 2018), are very important.

It was found that in rats under conditions of physical inactivity, the antioxidant activity of plasma weakens, leading to an increase in the level of acyl hydroperoxides and thiobarbituric acid-active products in it. High lipid peroxidation in plasma damages the walls of blood vessels and the outer surfaces of erythrocyte membranes, negatively affecting their state (Vorobyeva et al., 2018). Moreover, in red blood cells of rats experiencing physical inactivity, the antioxidant defense weakens, which stimulates lipid peroxidation processes in them.High

lipid peroxidation in the plasma and in the erythrocytes violates the structural and functional characteristics of membranes and protein of the cytoskeleton of red blood cells. With increased peroxide oxidation of lipids in erythrocytes occurs the weakening of the synthesis of ATP, lowering the activity of ion pumps that in these circumstances, no longer cope with the removal of the erythrocyte cytoplasm of excess Ca2+ and Na+ and are unable to maintain an optimum level of K+ (Mal et al., 2018).

Table 1. Showing some haematological parameters of the rats under investigation compared with well Matched controls Legend: reliability of differences in indicators between control and aging rats - *<0.05; **- p<0.01

Indicators	Rats in conditions of physical inactivity, M±m			Control, n=32, M±m
	Exodus, n=34	2 weeks, n=34	4 weeks, n=34	
Body mass, g	232.4±3.26	248.5±4.27	269.5±4.86*	232.4±4.96
Swimming time, s	158.3±2.16*	136.2±3.32*	110.0±3.04**	161.2±3.62
Acyl hydroperoxides plasma, D_{233}/1 ml	1.46±0.016	1.71±0.012*	1.89±0.025**	1.45±0.009
Thiobarbituric acid l products, μmol/	3.38±0.018	3.97±0.019*	4.23±0.027**	3.42±0.014
Antioxidant activity, % Erythrocyte acyl	36.0±0.30	31.3±0.24	28.2±0.23*	35.1±0.07
hydroperoxides, $D_{233}/10^{12}$ erythrocyte Erythrocyte	2.82±0.017	3.52±0.012*	4.12±0.024**	2.82±0.015
malondialdehyde, nmol/10^{12} erythrocyte	1.10±0.013	1.37±0.011*	1.58±0.012**	1.11±0.010
Erythrocyte catalase, ME/10^{12} erythrocyte	8850.0±12.9	8000.0±13.6*	7100.0±19.8**	8960.0±16.2
Erythrocyte superoxide dismutase, IU/10^{12} erythrocyte	1590.0±7.16	1470.0±8.23*	1320.0±6.35**	1650.0±13.15
Discocytes, %	84.2±0.14	77.1±0.16*	71.4±0.17**	84.1±0.14
Reversibly altered red blood cells, %	9.5±0.12	11.8±0.07*	15.0±0.09**	9.5±0.11
Irreversibly altered red blood cells, %	6.3±0.07	11.1±0.13**	13.6±0.12**	6.4±0.12
The amount of red blood cells included in the aggregates	31.2±0.15	37.2±0.14*	42.6±0.08**	30.2±0.06
Number of units	6.3±0.09	7.0±0.12*	8.2±0.08**	6.1±0.05
The number of free red blood cells	292.3±0.18	257.0±0.26*	229.2±0.29**	292.8±0.22

In these circumstances, in the blood of rats develops a gradual increase in the number of red blood cells that do not have a biconcave shape. The resulting changes in the red blood cells ensures the growth of content in their blood reversible and irreversible changes of their species. So, the animals in the conditions of inactivity by increasing the level of red blood cells in a state of echinocytosis with very variable form and changed to stomatita having the form of a one-sided full drive. Further transformation of these red blood cells rapidly leads to the appearance of steroidica, serotoninocytes and in the end of spherocyte, which in a short time is lysed.

Found in rats in conditions of hypodynamy increased aggregation of red blood cells is provided by the emerging changes in the magnitude of the charge of their membranes due to active degradation on the surface part of glycoproteins as a result of the excess products of lipid peroxidation. Increased synthesis of reactive

oxygen species in rats in conditions of hypodynamia causes oxidative alteration of erythrocyte membranes and plasma damage to plasma proteins, having the ability to link with each other erythrocytes in the process of aggregation. In addition, the increasing lipid peroxidation in plasma and in red blood cells impairs the ability of erythrocytes to disaggregate due to the large force of red blood cells in aggregates and increase the speed of the process (Makhov, Medvedev, 2018).

The increase in the number of free aggregates in rat blood under conditions of physical inactivity contributes to damage to the vascular endothelium, which contributes to greater contact of the subendothelium and blood with activation of hemostasis due to this process, significantly worsening hemodynamics in the capillaries (Bikbulatova, 2018a). An increase in the number of aggregates in the blood of experimental rats can impede blood flow in parts of small vessels and in their vasa vasorum, which causes degeneration of the vessel walls and weakening of the synthesis of disaggregants in the vessels, providing control over the aggregation of red blood cells (Zavalishina, (2018e; Zavalishina et al., 2019).

CONCLUSION

In rats under hypodynamic conditions, a gradual weakening of the plasma antioxidant defense occurs. This is accompanied in rats by an increase in its levels of lipid peroxidation products. As a result, the alteration of the outer membranes of red blood cells is enhanced in animals, increasing the number of their altered forms. In rats under conditions of inactivity, the ability to aggregate also increased and the number of red blood cells in the blood increased. This is very important for reducing the resistance of animals to animals with low physical activity, which makes them very sensitive to the influence of any negative environmental factors.

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Nurses' Attitude Regarding Patient Safety in Primary Healthcare Centers of Saudi Arabia: A Cross-Sectional Study

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ABSTRACT

Patient safety is an essential component of healthcare quality. It is clear that there is a need to analyze patient safety issues primary healthcare centers (PHCC) as an important part of the healthcare system, since most of the research that deals with patient safety issues in Saudi Arabia have been applied in hospitals rather than primary healthcare centers. The aim of this study is to assess the attitude of nurses regarding patient safety in Riyadh PHCCs of Saudi Arabia. A descriptive cross-section design was used. A sample of 314 nurses working in primary healthcare centers in Riyadh were participated in the study. A self-administered questionnaire was used for data collection which consisted of two parts covered socio-demographic data and nurses' attitude regarding patients' safety using the Safety Attitudes Questionnaire (SAQ). The study showed that the mean age of the participants was 34.3. The participants had a positive attitude regarding teamwork climate, job satisfaction, and safety climate dimensions with weighted mean 3.64, 3.78, and 3.61 respectively while they had a negative attitude regarding perceptions of management, working conditions, and stress recognition dimensions with weighted mean 3.19, 3.12, and 2.41 respectively. The study concluded that nurses working in PHCCs of Riyadh city had a negative attitude regarding patient safety with a statistically significant difference between nurses' attitude and their years of experience and gender while there was no statistically significant difference between nurses' attitude and age, educational qualification, and staff position.

KEY WORDS: ATTITUDE; NURSES; PATIENT SAFETY; PRIMARY HEALTHCARE CENTER; SAUDI ARABIA.

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INTRODUCTION

Patient safety is considered a global health concern which affecting patients whether in developed or developing countries, World health organization (WHO, 2016) has recognized the importance of patient safety and focused on as a public health concern. The simplest definition of patient safety as mentioned by (WHO, 2018) is "the prevention of errors and adverse effects to patients associated with healthcare". According to the Saudi Patient Safety Taxonomy (S-PST), which is developed by Ministry of Health (MOH) to unify the conceptual framework related to patient safety in Saudi Arabia, has defined patient safety as "Freedom from accidental or preventable injuries produced by medical care" (MOH, 2017). The patient safety culture is the product of values, attitudes, skills, and standards of individuals and collective behaviors that determine the commitment, style, and proficiency of the administration of a safe organization (Fermo, et al., 2016). Thereby, a fulfilling culture of patient safety requires a grasp of these values and beliefs regarding what is important in an organization and which behavior and attitudes related to patient safety are supported and expected (Brasaite, et al., 2016).

Patient safety is the keystone of high-quality healthcare provided in all healthcare settings, and primary healthcare center is one of these settings which plays an important role in the healthcare system, as it is the first level of contact that individuals, families, and communities have with the healthcare system (Almalki, et al., 2011). So improving patient safety issues in primary healthcare centers (PHCCs) requires a new approach and thinking that including a greater emphasis on safety and prevention from harm. Because severe and complicated cases are treated in hospitals, most of the healthcare providers and the community underestimate the weight of primary healthcare services. This underestimation leads to errors in some fields as physician notification, communication, and staffing (Brotons, et al., 2012), add to that the most researches and activities concerned with patient safety issues are directed to hospitals, although it is well known that the majority of patients are examined first in primary healthcare centers by family doctors before they referred to hospitals (Webair, et al., 2015).

Community health nurse play a main role in maintaining and promoting patient safety in PHCCs, it not only just a part of what they do but it also committed through nurses' code of ethics to provide "safe, competent and ethical care" (Canadian Nurses Association, 2002). Patient safety is fundamental part of nursing care, nurses play a significant role in improving patient safety alongside with the roles of inter-professional teams, health-care organizations and the health-care system (Canadian Nurses Association, 2019).

It is important that community health nurse working in PHCCs should be more knowledgeable about patient safety issues in order to minimize the incidence of adverse events that may lead to serious disabilities to the patients (Ghobashi, et al., 2014). Therefore, to ensure patient safety in PHCCs, it is essential to promote and improve safety attitudes among healthcare professionals especially nurses (Geevarghese, 2012). Patient safety research in PHCCs gives out that millions of people around the world are suffering from disabilities, injuries, or death due to unsafe medical practices, and there has been little research on patient safety published from Gulf Cooperation Council (GCC) countries regarding patient safety in PHCCs (Al Lawati et al., 2019).

More researches are needed to know about patient safety in PHCCs.It is important to mention that the data published by WHO showed that patient harm was the 14th leading cause of the global disease burden, comparable to diseases such as tuberculosis and malaria (WHO, 2018). Recent literature reviews have revealed that as many as four out of 10 patients are harmed while receiving healthcare in PHCCs, with up to 80% of the harm considered to have been preventable. The most detrimental errors are related to diagnosis, prescription and the use of medicines (WHO, 2019). Administrative errors, those associated with the systems and processes of delivering care, were the most frequently reported type of errors in PHCCs (WHO, 2018). In the WHO Eastern Mediterranean Region, available data showed that healthcare-related harmful incidents affect 8 in 100 patients, and 4 out of 5 incidents were preventable (WHO, 2016). In Saudi Arabia; patient safety is considered as a top priority for the healthcare services provided by the Ministry of Health (MOH), which has been obvious in the quality improvement projects and initiatives carried out through MOH facilities (MOH, 2017). The aim of this study was to analyze the attitude of nurses regarding patient safety in PHCCs of Saudi Arabia.

Research questions:

The following research questions guided the study:

1. What is the attitude of nurses regarding patient safety in PHCCs of Riyadh city in Saudi Arabia?
2. Is there a statistical significant differences between nurses' attitude and their age, gender, qualification, years of experience and staff position?

MATERIAL AND METHODS

In this study a descriptive cross-sectional design was used, the data collection was carried out from the last of November 2018 until May 2019 after permission letter No. 1440-544543 was obtained from the Ministry of Health to the managers of the primary healthcare centers in order to facilitate collecting the data. For data collection; an online self-administered structured questionnaire was used; all items of the questionnaire were obligatory to answer so no data are missed. The pilot study was carried out on 10% of the study sample in order to assess validity and reliability, detect any ambiguity in the tools, clarity of the items, as well as, to determine the time consumed for data collection. Necessary modifications were carried out and the final form was developed, the reliability of the study tool measured using Cronbach's alpha; it was 0.896 for the

35 items. The nurses who participated in the pilot study were excluded from the study population. The tool of data collection consisted of the two parts first part; concerned with socio-demographic data of the nurses such as age, gender, qualification, and years of experience.

Second part; concerned with nurses' attitude regarding patient safety using the Safety Attitudes Questionnaire (SAQ), this questionnaire being one of the tools that most used to evaluate the safety attitudes among healthcare providers. The SAQ instrument was developed by the Center for Healthcare Quality and Safety, University of Texas. In order to use this questionnaire, permission was obtained from the University of Texas to do the needed modification to be suited to PHCCs. This questionnaire consists of six dimensions: teamwork climate, safety climate, perceptions of management, job satisfaction, working conditions, and stress recognition. The 5-points Likert response scale of the agreement was used, this Likert-type scale rates answers from 5=strongly agree to 1=strongly disagree; negative phrases are encoded inversely. The score for means interpretation reference were strongly disagree 1 - 1.79; disagree 1.80 - 2.59; neutral 2.60 - 3.39; agree 3.40 - 4.19; strongly agree 4.20-5. The cut-off point is equal to 3.39, so a positive attitude was acknowledged if the weighted mean is more than 3.39. In contrast, a negative attitude was acknowledged if the weighted mean is less than 3.39.

Regarding sampling strategy and setting; the study was conducted in PHCCs of Riyadh city, Saudi Arabia. According to MOH in Saudi Arabia, the number of PHCCs are 2325 which covered 20 regions and consists of 19863 nurses. In Riyadh city there are 96 PHCCs including 1697 nurses; spread over the five health regions of the city (North, South, East, West, and Middle). The services offered by them include general practitioner services, childcare services, family medicine, maternity care, care for chronic diseases, dentistry, preventive medical care, nursing care, and pharmaceuticals (MOH, 2016). The Steven K. Thompson equation (Thompson, 2012) was used to calculate the required sample size from the total population, and a sample of 314 participants was obtained with a 95% confidence level and 5% margin errors.

Multistage sampling was used which conducted through three stages; first stage Riyadh city was divided into 5 regions (East, West, Middle, North, and South); second stage simple random sample was used in order to select 35 PHCCs (7 centers from each region); third stage all nurses in each selected PHCC was included in the study. The inclusion criteria for the study was registered nurses who work in PHCCs with one year and more experience, while the exclusion criteria were nurses less than one year of experience and the nurses who participated in the pilot study. The data were analyzed using SPSS Version 20. For descriptive statistical analysis, frequencies and percentages were used for all variables included in the study to describe the demographic data and explore the attitude of nurses. Weighted arithmetic means, arithmetic averages and standard deviations are also used. For inferential analysis, The Chi-Square test was used to examine the association between variables. The statistical test of significance or p-value in this study was set at <0.05. The internal consistency of the tool was measured by calculating the Cronbach's alpha.

The ethical approval for this study was obtained from the King Fahad Medical City IRB committee with log number 18-597E. The explanatory covering letter was attached at the beginning of the survey indicating the aim of the study. Participating in the study is considered voluntary and participants have the right to refuse to participate in the study. They informed that " feedback would not affect their evaluation at work, work status, position or salary". Confidentiality and anonymity were maintained to protect the identity and position of the participants.

RESULTS AND DISCUSSION

Table 1. Demographic characteristics of nurses participated in the study (n=314)

Parameters	Frequency (%)	
Age in years :		
24 – 30	99	31.5
31 - 35	103	32.8
36 - 40	65	20.7
> 40	47	15
Mean (SD)	34.3 (6.14)	
Gender:		
Male	83	26.4
Female	231	73.6
Educational qualification:		
Diploma	183	58.3
Bachelor	131	41.7
Master	0	0
Staff position:		
Supervisor	44	14
Nurse practitioner	253	80.6
Assistant nurse	17	5.4
Direct contact with patients:		
Yes	294	93.6
No	20	6.4
Years of experience:		
1-5 years	66	21
6-10 years	101	32.2
> 10 years	147	46.8

Table 1 showed a demographic overview of the respondents. The mean age of the participants was 34.3 years. Regarding gender, about (73.6%) of the participants were females while only (26.4%) were males. In terms of educational qualification, around half of the participants (58.3%) had a diploma and (41.7%) had a

bachelor's degree while no one neither had a master degree nor a doctorate. Turning to a staff position, most of the participants were nurse practitioners (80.6%) and the majority of them (93.3%) had direct contact with patients, and about (46.8%) their years of experience were more than 10 years.

Figure 1: Percentage of nurses receiving training program regarding patient safety

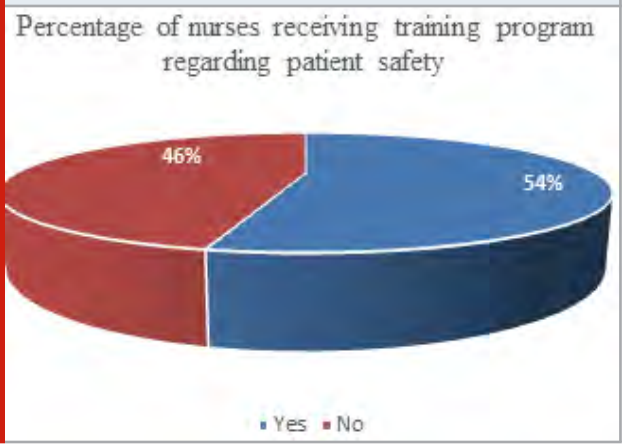

Figure (1) Presented that (54%) of participants received training program on patients' safety.

Figure 2: Nurses' source of information regarding patient safety

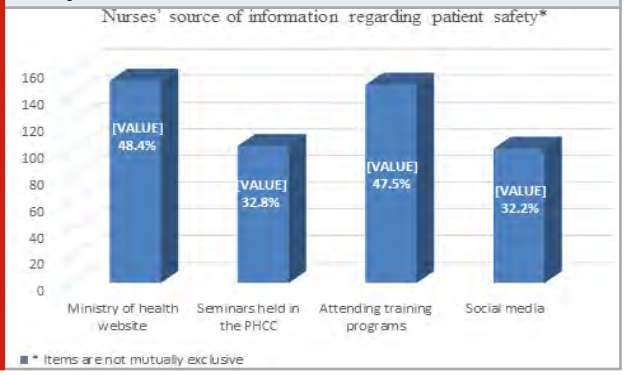

Figure (2) clarified that about (48.4%) received their information regarding patient safety from the website of Ministry of Health and about (47.5%) from attending training programs while (32.8%) and (32.2%) from seminars held in their PHCCs and social media respectively.

Table (2) Showed that the participants had a positive attitude regarding teamwork climate, job satisfaction, and safety climate dimensions with weighted mean 3.64, 3.78, and 3.61 respectively while they had a negative attitude regarding perceptions of management, working conditions, and stress recognition dimensions with weighted mean 3.19, 3.12, and 2.41 respectively. The overall attitude was negative with a weighted mean (3.29) which was less than the cut-off point (3.39).

Table 2. Attitude of nurses regarding patient safety in primary healthcare centers

Parameter	Mean	*SD	Attitude	Rank
Teamwork climate	3.64	1.15	Positive	2
Job satisfaction	3.78	1.02	Positive	1
Perceptions of management	3.19	.87	Negative	4
Safety climate	3.61	.83	Positive	3
Working conditions	3.12	.87	Negative	5
Stress recognition	2.41	.72	Negative	6
Overall attitude	3.29	.76	Negative	

*SD= standard deviation

Table (3) Clarified the statistical association between nurses' attitude and their demographic data, the table showed that there was a statistically significant difference between nurses' attitude and gender, also there was a statistically significant difference between nurses' attitude and years of experience. Meanwhile, there was no statistically significant difference between nurses' attitude and their age, educational qualifications, and staff position.

DISCUSSION

This study analyzed the attitude of nurses regarding patient safety in PHCCs using the SAQ questionnaire which is a useful tool for evaluating safety attitudes, some studies showed that the validity and reliability measures of the SAQ were acceptable and others showed that it has good internal consistency reliability (Gabrani, et al., 2015; Li, et al., 2017). This was evident in this study where the results showed that more than half of the nurses attended training programs on patient safety, which is beyond any doubt that receiving patient safety training programs considered one of the important factors influencing safety attitudes for nurses and other healthcare providers. Several studies proved the importance of providing patient safety training programs for healthcare professionals to create and maintain a culture concerning patient safety issues which are thought to have an important effect on ensuring positive safety attitudes (Onler, 2010; Amarapathy, et al., 2013; Sevdalis, 2012). Also nearly half of nurses got their information regarding patient safety from the website of the Ministry of Health which means that the Ministry of Health in Saudi Arabia doing its best to provide healthcare workers with all information related to patient safety.

However, the results of this study found that nurses

working in PHCCs generally had a negative attitude towards patient safety, this result may be attributed to the fact that more attention to patient safety measures are directed more to hospitals than to PHCCs. This result comes contrary to what was mentioned with (Brasaite, et al., 2016; Durgun & Kaya, 2018) who reported that attitudes related to patient safety issues were positive among healthcare professionals, furthermore it was reported also by (Al-Khaldi, 2013) that attitude of the physician in PHCCs of Asser region in Saudi Arabia was positive. While (Alzahrani, et al., 2018), on the other hand, found that nurses had a negative attitude toward patient safety which come in the line with the present study. Also other studies (Ongun & Intepeler, 2017; Saberi, et al., 2017) showed a moderate level of professional attitude regarding patient safety which is lower than the international standards attitude regarding patients' safety.

Table 3. Relationship between nurses' attitude regarding patient safety in PHCCs and years of experience, age, gender, qualification, and staff position

Overall attitude	Years of experience (%)				X^2	df	P
	1-5 years	6-10 years	> 10 years				
Positive	41 (13.1)	(17.2) 54	(20.7) 65		6.21	2	0.04*
Negative	(7.9) 25	(14.9) 47	82 (26.1)				
		(%)Age					
	30-24	35-31	40- 36	> 40	6.62	3	0.85
Positive	61 (19.4)	(15.3) 48	(9.6) 30	(6.9) 21			
Negative	(12.1) 38	(17.5) 55	(11.1) 35	(8.3) 26			
	Gender (%)						
	Male	Female					
Positive	(8.9) 28	(42.1) 132			13.3	1	0.000*
Negative	(17.5) 55	(31.5) 99					
	Education qualification (%)						
	Diploma	Bachelor			1.73	1	0.18
Positive	99 (31.5)	(19.4) 61					
Negative	(26.8) 84	(22.3) 70					
	Staff position (%)						
	Supervisor	Nurse practitioner	Assistant nurse		5.84	2	0.05
Positive	29 (9.2)	125 (39.8)	6 (1.9)				
Negative	15 (4.8)	128 (40.8)	11 (3.5)				

*P value is statistically significant < 0.05 level

The results of the present study have reported that the nurses had a positive attitude in teamwork climate and job satisfaction which take the high rank between other dimensions; patient safety can be maintained if the relationship between healthcare workers relate well with each other, as this is the only way to create a conducive working atmosphere where although errors are not condoned if they occur, measures are put in place to ensure that they do not recur in the future.

A recently carried out study by (Al Lawati et al., 2019) has described about patient safety culture in primary health care in Muscat, Oman, and has identified that the teamwork was one of the core areas of strength in PHCCS. Another study by (Ongun and Intepeler, 2017) asserted that high scores in teamwork and job satisfaction among nurses play an important role in ensuring patient safety and creating a positive atmosphere for patients. Moreover, job satisfaction enhances the development of nurse's collaboration with other healthcare professionals in order to achieve the goals of the healthcare organization. Thus, it is likely that nurses' satisfaction influences their level of engagement in patient safety activities as well as patient safety outcomes.

This result comes in concurrence with (Alzahrani, et al., 2018; Almutairi, et al., 2013; Algahtani, 2015) who found that more positive scores on job satisfaction are found between nurses. The results for this study also revealed that nurses had a positive attitude as regards to safety climate. It is important to ensure the safety climate in healthcare organizations to allow negotiation and mutual learning, as well as providing appropriate feedback in order to get rid of errors (Algahtani, 2015). A positive safety climate improves nurses' production and decreases exposure to environmental hazards which reduces the number of accidents.

For perceptions of management, the results showed that nurses had a negative attitude regarding this dimension,

and maybe this one of the important factors that affected the nurses' overall attitude. There is no doubt that healthcare managers play an important role in forming nurses' attitude toward patient safety, when managers openly display a positive attitude toward patient safety or manage the patient safety issues effectively, the general culture of patient safety within their organization is increased. Also, nurses tend to be more productive in the environment that stimulates a positive attitude, they feel more self-sufficient when their opinions heard and taken by their leaders in the context of improving patient safety and reduce the medical error, these are the main role of the managers.

According to (Institute for Healthcare Improvement, 2017) top management need to show commitment to patient safety through providing required resources, ensuring transparency; understanding and applying quality improvement science, reliability, safety and continuous learning, creating an inspiring environment for innovation and risk mitigation, fostering environment of mutual respect and trust were people feel their opinions are valued and trustworthy. The result reported by this study was quite different from what was mentioned by (Saberi, et al., 2017) who found that the highest rate of positive attitude toward patient safety belonged to the dimension of 'management perception', also the study was done by (Mahfoozpour, et al., 2012) who reported the highest percentage of the positive attitudes among healthcare providers was toward the perception of management.

Regarding the safety climate, the nurses were had a positive attitude regarding this dimension. It is worth mentioning that regularly reviewing safety climate processes lead to improve the nurses level of thinking, therefore bring new ideas and hypotheses, as well as enhancing team collaboration which, in the end, will improve overall nurses' performance resulting in improving the patient safety. Reporting systems which is one of the important part in assuring safety climate can be only effective when a strong feedback loops and evidence of action following reported harm is established. Feedback from incident reporting can optimize the learning from mistake, finding improvement opportunity, reduce the reoccurrence of errors, and enhance of overall patient safety (Panesar, et al., 2014)

Regarding the working conditions, the nurses were had a negative attitude, this maybe because of when nurses live in perpetual fear of retaliation from the immediate managers or supervisors, In the long run, patient safety further deteriorates since even opportunities that have been used to rectify the errors are squandered. This result was conflicting the results obtained by (Tabibi, et al., 2010) who demonstrated that the nurses' attitude was positive in the work conditions dimension. Moreover, this finding was in concurrence with the results obtained by (Gutiérrez-Cía, et al., 2010; Lee, et al., 2010; Marinho, et al., 2014) which indicated the lowest score to be in the SAQ dimension was in work conditions, also it congruence with (Saberi, et al., 2017) who found that the lowest mean score of attitude toward the patient safety culture belonged to the same dimension. Regarding the stress recognition, the study showed that nurses had a negative attitude, this result in agreement with (Ongun and Intepeler, 2017) who asserted that stress recognition had the lowest score among the sub-scales of the operating room professionals' SAQ. However, there are some studies in which the stress recognition score was found to be high (Onler, 2010; Kaya, et al., 2010).

Stress can trigger illness among healthcare workers especially nurses, in order to improve stress management, the healthcare professionals must acknowledge that stress caused by high work demands limits their activities and affects their health (Kogienl and Cedaro, 2014) which can lead to unsafe and low-quality of care. Moreover, the adequacy of the nurse-to-patient ratio and availability of the necessary information during the decision-making process affect positively on this dimension (Lee, et al., 2010). Many professionals encounter errors due to their heavy workload, which exhausts them and lowers their precision in the provision of care (Saberi, et al., 2017) all of these issues should be discussed with managers when addressing improvements to patient safety

Furthermore, the present study reported that about one-third of participants their age were ranged between 24 -30 years, more than half of them had a diploma as an educational qualification. Most of participants were nurse practitioners and less than half of them had more than 10 years' experience. It should be noted that the present study found that there was statistically significant difference between gender and nurses' overall attitude; also there was statistically significant difference between years of experience and nurses' overall attitude. These came in the same line with the study done by (Durgun & Kaya, 2018) who stated that years of experience and patients' safety issues were correlated, and nurses with experience showed expected results, used evidence more efficiently and developed critical thinking skills.

These results showed that nursing experience had a positive impression on patient safety. The present study also showed that there was no statistically significant difference between age and nurses' overall attitude, meanwhile, the study done by (Brasaite, et al., 2016) recognized that age seemed to be associated with many safety attitudes scales, which were found to be higher in older age groups. Also, the current study showed that there was no statistically significant difference between educational qualification and nurses' overall attitude, this may be because all participants had either diploma or bachelor no one had master or doctorate in nursing. The study was done by (Durgun & Kaya, 2018) also found that no noticeable difference between educational level and attitudes toward patient safety which supports the results of the current study.

CONCLUSION

As any healthcare organization; PHCCs continue to embrace the improvement of patient safety and quality,

they must recognize the importance of nurturing a patient safety culture as well as the attitude of their healthcare providers regarding it. To achieve this, PHCCs' management must understand how healthcare providers perceive their teamwork climate, safety climate, perceptions of management, job satisfaction, and working conditions. This study contributed to analyze nurses' attitude regarding patients' safety in primary healthcare centers of Saudi Arabia, which were found to be negative with a statistically significant difference between attitude and years of experience and gender while there was no statistically significant difference between nurses' attitude and age, educational qualification, and staff position.

The evidence-based about the nature of patient safety risks and patient safety solutions in primary healthcare centers still weak, so it's important to focus on patient safety and its adverse events in PHCCs, investigating attitude of all healthcare professionals working in PHCCs in relation to patient safety, in order to obtain a deeper understanding of the present situation. Attitude regarding patient safety in PHCCs is not only should be positive but also it should be effective. MOH can help shed light on the direction of the PHCCs as done in hospitals to instill a culture of patient safety within its workforce.

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Employing *Arabidopsis* Trichome Model in Studying Fiber Initiation in Improving Cotton Yield

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ABSTRACT

Cotton is valued for its fiber and requires yield improvement to compete with synthetic textiles and for sustainable cotton production. Cotton fiber production from seed coat epidermal cells can be categorized into two stages: initiation and development. While a great deal of research has been emphasized on the cotton fiber elongation and secondary cell wall biosynthesis, fiber initiation understanding is still in its infant stage due to the difficulty in studying the mechanism in cotton, namely lengthy transformation, lack of characterized mutants, and large and complex genomes. The *Arabidopsis* trichome, the most studied cell type, differentiates from the leaf epidermal cells and presents an excellent model system to elucidate the cotton fiber initiation mechanism. Knowledge gained from the initiation mechanism of *Arabidopsis* trichomes will facilitate, as a comparative model system, in understanding of the cotton fiber initiation mechanisms.

KEY WORDS: TRICHOME, FIBER INITIATION, TRIMERIC COMPLEX, DIPLOID, TETRAPLOID.

INTRODUCTION

Cotton, (*Gossypium spp*) fiber is the primary material for a textile industry, and currently, there is an immense interest in understanding the process of fiber initiation and development. With the recently published reference genome sequences of cotton species, the cotton fiber initiation and development has become an essential field of study. Cotton fibers are unicellular trichomes originating from seed coat epidermal cells. Of these cells, approximately 30% are differentiated into fiber cells resulting in the production of roughly 20,000 fibers/ovule (Berlin, 1986). Increasing the number of fiber initials will result in additional fiber yield, which will ultimately benefit the cotton producers and allied industries A mere 10% increase in initials results in about a 30% increase in the final fiber yield, (Chen et al., 2020, Patel et al., 2020).

Cotton fibers, which are highly elongated and thickened cells, are one of the few cells in the plant kingdom that can significantly expand in size (up to 6.0cm) or composition during growth and development. These fibers, also known as seed trichomes, will quasi-synchronously undergo four distinct yet overlapping stages of development (Guan & Chen, 2013). Fiber initiation stage begins at

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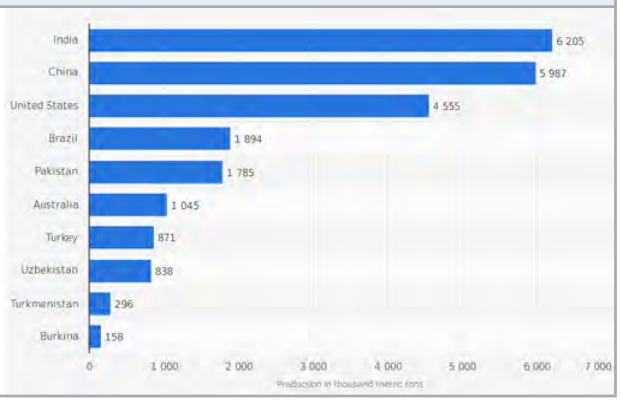
approximately -3 days post-anthesis (DPA) to 5 DPA, in which ovular epidermal cells emerge and differentiate into fiber initials. Subsequently, from 3 to 21 DPA, morphologically-distinct fiber cells continue to expand up to 6 cm in length without further cell division (Wilkins & Arpat, 2005). From 14 to 40 DPA, a massive amount of cellulose is deposited which is known as the secondary cell wall biosynthesis stage. Finally, fiber cells mature at 50 to 60 DPA, and at this stage, the cotton fibers and seeds are ready for harvesting and industrial applications (Basra & Malik, 1984). Elucidating the molecular mechanism of fiber initiation will provide specific Informationa on the genes involved in epidermal cell differentiation, and will facilitate the design of novel genetic and molecular strategies to improve the number of initials, thereby improving cotton fiber yield.

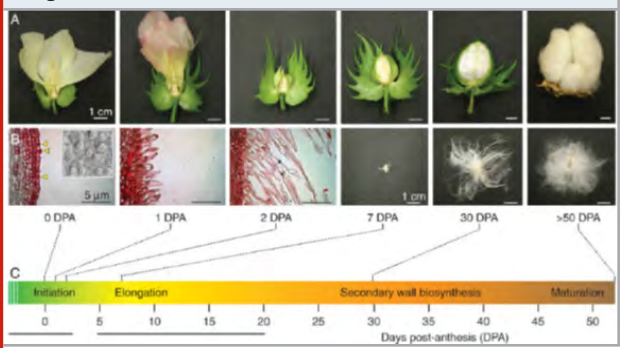
Figure 1: Leading cotton-producing countries worldwide in 2018/2019 (in 1,000 metric tons). Source: US Department of Agriculture 2019 (www.statista.com 2019)



Economic, Environmental and Scientific Importance of Cotton: Cotton is an essential raw material used to produce numerous commodities, including textile fabrics, medical applications, fine paper, computer screens and automobile brakes; it is also used for cooking oil, cattle feed, and biodiesel fuel. Although additional commercial value can be captured from cottonseed and its associated products, the fundamental economic value originates from cotton fiber (Campbell & Hinze, 2010). Of over fifty documented species in the *Gossypium genus* (Wendel, 1989), four species (*G. hirsutum, G. barbadense, G. arboreum, G. herbaceum*) are widely cultivated around the world. They have had a significant impact on global trade and economy (Zhang & Feng, 2000). China, the United States (US), and India produce most of the world's cotton comprising more than 15.9 million metric tons of cotton lint and 30.4 million metric tons of cottonseed, which was approximately equivalent to 22.8 billion and 6 billion dollars, respectively (Bowman et al., 2013). In 2019, these three countries contributed ¬about 13.5 metric tons of cotton corresponding to 40 to 100 million US dollars annually, (Figure 1). Globally, the economic impact of the cotton industry is estimated to be $500 billion (US) per year, with more than 100 million families from approximately 150 countries directly or indirectly dependent on cotton crop (Bowman et al., 2013, USDA 2019).

Currently, the cotton fiber industry is facing fierce competition from companies producing synthetic textile fibers such as polyester, nylon, and polypropylene. Compared to cotton, synthetic fibers are not environmentally friendly as they are made from fossil fuel sources, are non-biodegradable, hydrophobic, burn and melt quickly posing health risks (http://www.barnhardtcotton.net/blog/know-fibers-cotton-vs-synthetic-fibers/). Besides, a recent study showed the presence of synthetic textile fibers in sea fish sold for human consumption (Rochman et al., 2015), thus raising concerns about direct effect on human health. Cotton fiber is not only environmentally friendly, but it also helps to clean environmental pollutants such as oil spills and leaks; for example, 1 gram of raw cotton can absorb 30.5gram of crude oil (Singh et al., 2013). Cotton fiber is one of the most critical cell types on earth, which has scientific, economic, and environmental significance; hence, there is a need to make cotton cultivation more profitable for sustainability and meet the demand of the growing world population.

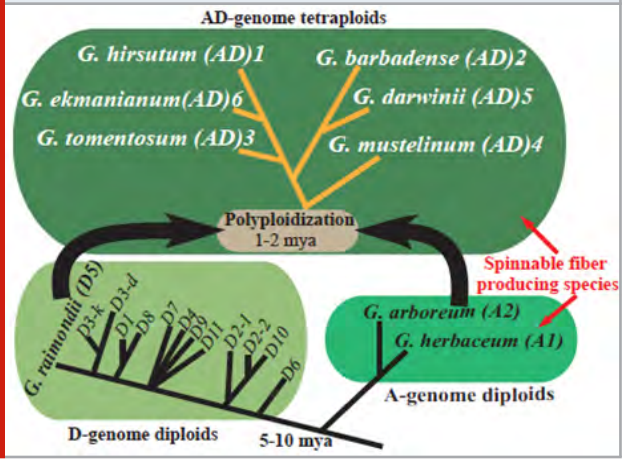
Figure 2: Cotton (*Gossypium*) fiber initiation and elongation stages (Lee et al., 2007)



Phylogeny, Genetics, and Genomics of Cotton: Approximately 1.5 million years ago, the spontaneous interspecific hybridization and genome duplication event of two formerly independent diploid genomes, (2n=2x=26): extant D- genome species closely related to *G. raimondii* (D5), and A- genome species related to *G. arboreum* (A2)/*G. herbaceum* (A1), resulted in allotetraploid species (2n=4x=52) (Wendel & Cronn, 2003). The ancestral A- species produce spinnable fibers while the D- species do not. The polyploidization and subsequent evolution resulted in the emergence of six tetraploid species (Figure 2): G. hirsutum (AD1), *G. barbadense* (AD2), *G. tomentosum* (AD3), *G. mustelinum* (AD4), *G. darwinii* (AD5) and recently described *G. ekmanianum* (AD6) (Grover et al., 2015). Among the six allotetraploids, Upland or American cotton, *G.hirsutum*, represents more than 95% of annual world cotton production, while the remaining 5% is primarily produced from Pima cotton, *G. barbadense*, known for its finer and longer fiber. Diploid cotton is cultivated in

South Asia; however, it contributes as little as 2% to total global cotton production (Wendel et al., 2003).

Figure 3: Phylogenetic framework of cotton (*Gossypium*) diploid and tetraploid species.



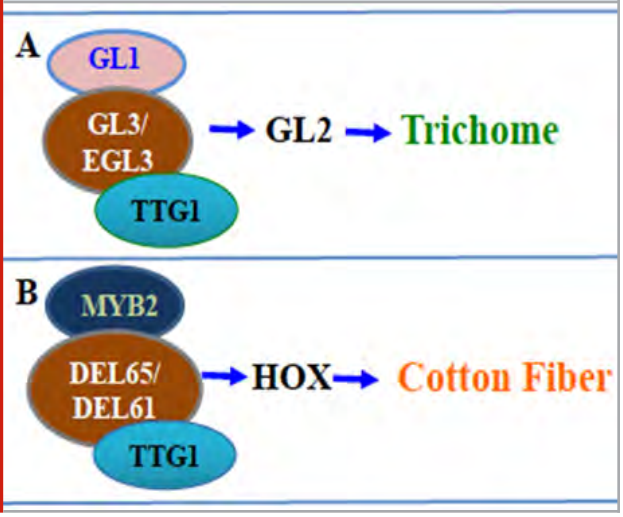
Progress in Understanding the Role of Subgenomes in Fiber Initiation and Development Using Tetraploid Cotton: Though the ancestral D-diploid genome progenitors do not produce spinnable fibers, the QTL (Quantitative Trait Loci) mapping studies have showed that most of the QTLs influencing fiber quality and yield are located on D^T subgenome of the tetraploid species. In contrast, differential expression of RNA transcripts during early stages of fiber development in tetraploid species show selective enrichment of A^T subgenome specific genes, which is consistent with production of spinnable fibers in ancestral A- diploid species (Samuel Yang et al., 2006). Following this result, systematic mapping of fiber developmental genes in tetraploid species have demonstrated that more genes associated with fiber development are located on A^T subgenome, while the D^T subgenome provides more transcription factors which regulate the expression of the fiber genes in the A^T subgenome (Xu et al., 2010).

In contrast, another study has showed that the ancestral D- genome provides many fiber genes after its hybridization with ancestral A- diploid species (Xu et al., 2015). Additionally, studies using mapped fiber gene-specific Simple Sequence Repeats (SSRs) indicate that both A^T and D^T subgenomes equally contribute to fiber traits (Han et al., 2006). Overall, the reviews are inconclusive on the subgenome contribution towards fiber development. Moreover, all these studies show expression bias or association of genes with fiber development but do not demonstrate the functional role of these genes. Hence, identification and characterization of individual genes involved in fiber development is essential to understand the specific contribution of different genes.

Since fiber initiation is a result of an interaction of different proteins to form active complex as well as activation of several downstream genes (Guan et al., 2007), a thorough understanding of this mechanism warrants a systematic and dedicated study. Current knowledge of the molecular mechanisms of cotton fiber initiation is in its infancy due to a complex and large genome, polyploidy, gene duplications, recalcitrance to genetic transformation (~1year), long growth cycles and lack of available (genetically characterized) mutants for functional studies (Pang et al., 2013). Of the total seed coat epidermal cells, approximately 30% are differentiated into fiber cells, which further complicate the isolation of pure fiber initial cells for the molecular analysis. To circumvent these complications, the *Arabidopsis* trichome has been successfully employed as a model system for functional characterization of cotton fiber initiation genes.

***Arabidopsis* Trichome Initiation is Regulated by Counteracting Positive and Negative Regulators:** The first step in the trichome initiation process is the formation of an active trimeric complex composed of an R2R3-MYB, a basic helix-loop-helix (bHLH), and a WD40 protein abbreviated as MBW complex. Because of its simplicity, flexibility and plasticity, the MBW regulatory complex has been utilized extensively by plants (Ramsay & Glover, 2005). The MBW complex plays diverse roles in *Arabidopsis* such as anthocyanin production, stomatal-cell identity and root-hair formation (Walker et al., 1999). Emerging evidence suggests that the same mechanism as in Arabidopsis may control trichome formation in other plant species. For example, MYB-like genes from *Mimulus guttatus* and peach mediate trichome formation (Scoville et al., 2011; Vendramin et al., 2014); ectopic expression of a R3 MYB gene from *Solanum lycopersicum* in *Arabidopsis* results in glabrous phenotypes (Tominaga-Wada et al., 2013).

Figure 4: Two MBW complexes is proposed to initiate *Arabidopsis* trichome and cotton fiber (by authors) A.Trimeric complex involved in the *Arabidopsis* trichome initiation. B. Proposed model of trimeric complex involved in cotton fiber initiation.



In *Arabidopsis*, the trichome initiation is positively mediated by a trimeric complex composed of GLABRA1 (GL1) (Oppenheimer et al., 1991), GLABRA3 (GL3), which acts redundantly with its close homolog ENHANCER OF GLABRA3 (EGL3) (Payne et al., 2000), and TRANSPARENT TESTA GLABRA1 (TTG1) (Serna & Martin, 2006). This trimeric activator complex up-regulates the expression of GLABROUS2 (GL2) (Hülskamp, 2004) and a small family of single-repeat MYB proteins lacking the typical transcriptional activation domains, including TRIPTYCHON (TRY) (Schellmann et al., 2002), CAPRICE (CPC), ENHANCER OF TRY & CPC1 (ETC1, 2 and 3) (Tominaga et al., 2008) and TRICHOME-LESS (TCL) (Wang et al., 2007). The GL2 initiates the trichome patterning and differentiation while TRY, CPC, ETC1, 2, 3, TCL are six small, R3-single repeat MYB transcriptional regulators that repress trichome initiation from adjacent cells in a redundant manner (Tominaga et al., 2008; Wester et al., 2009)

The regulation of trichome initiation is proposed to be mediated in a spatial and dose-dependent manner (Wester et al., 2009). Once a certain threshold level of activator MBW complex has been reached, expression of downstream regulators will be triggered, including positive regulators of trichome cell fate GL2 and inhibitory R3 MYB proteins (Zhao et al., 2008). The activation of GL2 mediates the trichome formation while the activated inhibitor proteins diffuse to adjacent cells and prevent them from becoming trichomes (lateral inhibition mechanism). The inhibitors, due to their smaller sizes and mobility, can laterally spread to neighboring cells to obstruct assembly of trimeric MBW activator complexes, thus preventing the adjacent cells from forming a trichome cell. The inhibitor proteins (TRY or CPC) prevent the formation of active MBW complex by competing with GL1, thus avoiding the trichome formation (Wester et al., 2009). Overall, Arabidopsis trichome is the most studied cell type with a wealth of information and resources that serves as an useful model system to study the cotton fiber initiation mechanism.

Composition of Cotton Fiber Initiation Protein Complex: The current method for rapid characterization of cotton fiber initiation genes is by complementation of cotton homologs in corresponding *Arabidopsis* mutants and by examining the trichome recovery phenotype (Guan et al., 2008; Guan et al., 2014; Li et al., 2017; Wang et al., 2013). Ectopic expression of MYB2 from *G. arboreum*, which is homologous to AtGL1, rescues trichomeless phenotype of the Arabidopsis gl1 mutant, confirming MYB2-A is a functional homolog of AtGL1 (Guan et al., 2014). Additionally, homologs of *Arabidopsis* GL3, TTG1, CPC, TRY, and GL2 have been isolated from *G. arboretum* (DEL65, TTG1, CPC, TRY, and HOX1, respectively) and functionally characterized using the Arabidopsis trichome model system (Guan et al., 2008; Wang et al., 2013). Formation of active trimeric complex is a prerequisite for the leaf epidermal cell differentiation into trichome in Arabidopsis (Li et al., 2017). Functional characterization and protein-protein interaction of the genes involved in cotton fiber initiation indicate a trimeric protein complex, similar to the Arabidopsis trimeric complex, is involved in cotton fiber initiation (Figure 2) (Guan et al., 2008; Guan et al., 2014; Wang et al., 2013).

Despite the characterization of individual genes, there is currently no comprehensive understanding of the nature of the cotton trimeric complex and its function. The trichomes initiation follows defined pattern on *Arabidopsis* leaves while the cotton fibers appear randomly, with no design on the seed coat. The fundamental difference in the patterning mechanism remains unanswered due to lack of suitable tools.

Future Research on Fiber Patterning on Cotton Fiber Initiation: To address this fundamental question, creating a trimeric cotton complex in *Arabidopsis* is proposed. Currently, *Arabidopsis* mutant defective for one or two gene(s) is replaced with a cotton homolog for functional characterization of cotton fiber initiation genes. It essentially replaces only one component of *Arabidopsis* trimeric complex with a cotton homolog while retaining the other parts of the *Arabidopsis* complex. This approach proved to be highly useful to study individual genes. However, it still reflects the *Arabidopsis* trimeric complex in its interactions, or complex formation, or activation of downstream genes, which is evident from the patterned trichomes in the *Arabidopsis* lines complemented with cotton genes. As a result, creating cotton fiber initiation complex in *Arabidopsis* without trichome initiation complex will be a novel tool to comprehensively understand the molecular basis for lack of fiber patterning on cotton seed. Comparative studies will be performed with leaf trichome and fuzz fiber systems to understand the intrinsic differences in these systems leading to differential pattern formation within cotton.

CONCLUSION

Cotton fiber, also known as seed trichome, is differentiated from the seed coat epidermal cells similar to *Arabidopsis* leaf trichome, which is differentiated from the leaf epidermal cells. Knowledge gained from the initiation mechanism of Arabidopsis trichomes will facilitate, as a comparative model system in understanding of the cotton fiber initiation mechanisms. Despite functional characterization of individual cotton fiber initiation genes, currently, there is not a comprehensive understanding of the mechanism behind cotton fiber initiation. Though there is a great of deal of resemblance in initiation mechanism, there is a fundamental difference in the pattern formation of *Arabidopsis* trichomes and cotton fibers. The trichomes are well patterned on *Arabidopsis* leaves due to the lateral inhibition mechanism, while there is no apparent pattern in fiber formation on cotton seed. We aim to address the fundamental differences in the pattern formation by developing a novel tool, cotton trimeric complex in *Arabidopsis*. The mechanistic studies will have broader implications in fiber production as they will have tremendous applications in improving the fiber yield.

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Analysis of Extra-Cellular Productions of *Bacillus subtilis* Sub-Merged Fermentation Cultures Supplemented with Rotten Potatoes and Sugar Beets

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ABSTRACT

The *Bacillus subtilis* (k1) is the SAFE microorganism with enourmous extracellular productions and it has ability to grow under large versatile nutrient conditions. Among the agriculture wastes, the rotten potatoes and sugar beets are being carbon-rich natural renewable agro-wastes. In compliance with current study, *B. subtilis* was fermented for 18 hrs on LB growth medium supplemented with 12.5 % extracts (v/v) of rotten potaoes and sugar beets i.e. LB_0 [1 % Bacto-trypton, 0.5 % NaCl, 0.5 % yeast extract in dH_2O, LB_1 (1/8 LB_0), LB_z (LB_1, peels of potatoes), LB_{za} (LB_1, peeled potatoes), LB_3 (LB_1, peels of sugar beet) and LB_{3a} (LB_1, peeled sugar beet). The significantly higher cell growth rate was observed in LB_3 and $LB_{3,a}$ cultures. Maximum reducing sugars observed in LB_α while fructose contents in LB_3 (3.214±0.077 mg ml^{-1}) and $LB_{3,a}$ (2.971±0.044 mg ml^{-1}) cultures. Simultaneously, higher enzymatic activities ($p \geq 0.05$) showed by amylases on LB_{za} and LB_{3a}, while *xylanases* on $LB_{z,a}$ and *pectinases* on LB_3 cultures. For instance, pectinases activities remarkably exceeded at 50°C in LB_3 cultures maintained for 30 min. Overall, *B. subtilis* (k1) can grow on both rotten potatoes and sugar beets with hug numbers extracellular productions, while sugar beets based cultures gave the best returns in bacterial sub-merged fermentation for the industrial enzymes productions.

KEY WORDS: *Bacillus subtilis*, POTATOES, SUGAR BEETS, GLUCOSE, ANTIOXIDANTS, FREE PROLINES, PECTINASE, PHYSICAL CONDITIONS.

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INTRODUCTION

The agricultures wastes are the unwanted materials produced from agro-based activities. It has been used as feed for the animals and fish. Even many are unsuitable for direct animal consumption so they are treated mechanically and chemically for their conversion into edible form (Jayathilakan et al., 2012). These costless waste materials are aggregated from the agriculture land to kitchen during its processing. With passage of time, these are converted to rotten form before its consumption or to get its end product (Obi et al., 2016). It depemds on the rate of production to its consumption over the time frame. The potato and sugar beets are the crops, which are observed into rotten form in the vegetable markete including the wastes excised during their processings (Wadhwa and Bakshi, 2013). The agriculture waste are comprised on mainly cellulose, hemicelluloses and lignin chemically. Utilization of these renewable carbon resources depends on their degratdation methods (Yahya et al., 2015; Emadian et al., 2017).

The narural degradation of agricultural wastes in the soil has been remained effecteive for the control of soil erosion and revegetation (Anastasi et al., 2005) and it is slower process. The chemo-mechanical degradation of the agriculture wastes is expensive and could be hazardous for human health, while biodegradation is cheap and manageable method. The soil born organisms are composting the organic wastes into organic fertilizers of soil through biological processes (Gautam et al., 2010). It means that a variety of microorganisms are able to grow on the agriculture wastes including *Bacillus subtilis* (An et al., 2018).

The agriculture wastes of crops contain sucrose (sugarcane, sugar beets), starch materials (corn, potatoes) and lignocellulosic materials (wood, grasses) are renewable lignocellulosic wastes available at lowest costs (Jenkins and Alles, 2011). However, their accumulation is causing various environmental problems, while these are potentially valuable sources to produce many value-added products like as fructose, glucose, ethanol, organic acids, food additives and enzymes (Pandey et al., 2016). Meanwhile, the type of production depends on the chemical composition of lignocellulosic source and the applied fermentation microorganism (Salazar et al., 2016; Tramontina et al., 2020).

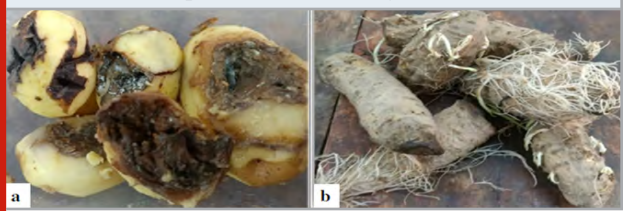
The value added-renewable lignocellulosic agro-industrial waste can be used as cellular growth energy source and other secondary metabolites productions (Rocha et al., 2014). Meanwhile, efficient bio-tools for the management of agro-wastes are the microbial enzymes. The microorganisms are producing a variety of cellulases, proteinases and pectinolytic enzymes (Jayani et al., 2010; Tripathi et al., 2014). These are significantly eco-friendly industrial enzymes (Singh et al., 2009). Especially *Bacillus subtilis* are potential fermentation microorganism have been using in the global food, textile industries to composting processes (Nawawi et al., 2017).

Table 1. Composition of different bacterial nutrient media used for *Bacillus subtilus* (k1) growth supplemented with potatoes and sugar beets as fermentation substrate

#s.	Medium	Composition of medium
01	LB_0	1 % Bacto-trypton, 0.5 % NaCl, 0.5 % yeast extract in dH_2O (w/v)
02	LB_1	$1/8LB_0$ in dH_2O (v/v)
03	LB_2	LB_1 +25 ml peels of potatoes(25 %, v/v)
04	$LB_{2,a}$	LB_1+25ml peeled potatoes (25 %, v/v)
05	LB_3	LB_1+25 ml peels of sweet-potatoes (25 %, v/v)
06	$LB_{3,a}$	LB_1+25 ml peeled sweet-potatoes (25 %, v/v)

Note: The final concentration of each extract adjusted to 12.5 %. Individual culture maintained in 4 replicates and volume of each replicate maintained 50 ml before its sterilization.

Figure 1: The agro-wastes used for *Bacillus subtilus* (k1) growth as carbon source in sub-merged fermentation cultures [rotten potatoes (a) and sugar beets (b)]



Perhaps the higher enzyme production costs are the major constraint for their commercialization. Though, selection of cheap carbon source, high yielding bacterial strains and optimal fermentation conditions significantly can reduce the enzyme production costs (Liu and Kokare, 2017; Tramontina et al., 2020). In present study is aimed to analyse the net production of reducing sugars especially fructose, glucose and extracellular enzymes. These productions with *Bacillus subtilis* might be helpful for the determination of its abilities and efficiencies for the saccharification of lignocellulosic rich agriculture wastes of rotten potatoes and sugar beets. This work could be useful for the production of stable commercial organic compounds and industrial enzymes able to withstand against the hazardous industrial conditions.

MATERIAL AND METHODS

Preparation of inoculum: The inoculum of *Bacillus subtillis* (k_1) prepared from its glycerol stock. For that it is mobilized in 2 ml LB_0 (TY) medium (Table 1), which was incubated at 37°C with constant shaking speed 250 rpm for overnight (De Vries et al., 2004). The 100 µl of above

B. subtilis cultre sub-cultured in 5 ml LB_0 medium. It was again incubated under same physical circumstances for 30 minutes (Table 1). Now this master culture is inoculated in the agro-based waste fermentation medium (Table 1) with final OD600 upto 0.02

Preparation of sugar beets and potatoes based agro-fermentation culture: The sugar beets and potatoes based fermentation cultures heightened in liquid nutrient LB_0 medium (5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 10 g l^{-1} Bacto-trypton, pH 7.0). Exact 12.5 % potatoes and sugar beets extracts (peels and peeled-potatoes and sugar beets) sustained in 1/8 strength of LB_0 medium, while the LB_1 medium itself was considered as standard positive control for the growth of fermentation organism as well as 1/8 LB_0 medium as essential nutrient deficit control medium (Table 1). All of these cultures sterilized at 121°C for 20 minutes than cool down before inoculation at the room temperature.

Table 2. Comparative biocomponents and enzyme productions in various nutrient cultures of *Bacillus subtilis* (k_1) supplemented with potatoes and sugar beets as carbon sources

#s.	Characters/ Parameters	LB_0	LB_1 (1/8LB_0)	LB_z	$LB_{2,a}$	LB_3	LB_{3a}	Data significance
01.	*Total sugars* (mg ml^{-1})	^{cd}3.664± 0.045	^{d}1.832± 0.034	^{bc}3.847± 0.055	^{c}3.752± 0.036	^{a}4.188± 0.045	^{b}3.949± 0.051	***
02.	*Reducing sugars* (mg ml^{-1})	^{c}2.387± 0.050	^{e}1.392± 0.077	^{bc}2.831± 0.058	^{d}1.758± 0.048	^{a}3.214± 0.077	^{b}2.971± 0.044	***
03.	*Total proteins* (mg ml^{-1})	^{b}8.743± 0.033	^{e}1.799± 0.056	^{c}8.221± 0.054	^{d}7.792± 0.052	^{a}9.684± 0.103	^{bc}8.630± 0.028	***
04.	*Fructose* (mg ml^{-1})	^{de}0.722± 0.003	^{e}0.147± 0.004	^{a}1.078± 0.003	^{d}0.789± 0.003	^{ab}1.058± 0.002	^{c}1.011± 0.002	***
05.	*Citric acid* (mg ml^{-1})	^{ab}0.086± 0.003	^{cd}0.050± 0.003	^{abc}0.087± 0.004	^{a}0.091± 0.002	^{bcd}0.063± 0.003	^{de}0.049± 0.003	**
06.	*Ascorbic acid* (mg ml^{-1})	^{d}9.44± 0.108	^{f}3.030± 0.105	^{c}12.49± 0.429	^{de}9.075± 0.108	^{a}14.67± 0.114	^{b}13.28± 0.130	***
07.	*Proline* (mg ml^{-1})	^{cd}1.007± 0.006	^{e}0.639± 0.005	^{ab}1.161± 0.011	^{a}1.338± 0.006	^{bc}1.122± 0.005	^{ab}1.260± 0.004	***
08.	*Glycinebetaine* (mg ml^{-1})	^{cde}0.131± 0.003	^{f}0.087± 0.004	^{def}0.129± 0.002	^{cd}0.140± 0.005	^{bc}0.169± 0.003	^{a}0.198± 0.004	**
09.	*Phenolics* (mg ml^{-1})	^{ab}7.264± 0.037	^{e}1.892± 0.040	^{a}7.450± 0.022	^{bc}6.403± 0.062	^{b}6.669± 0.092	^{cd}6.081± 0.023	***
10.	*Flavonoids* (mg ml^{-1})	^{bc}1.862± 0.060	^{cde}1.142± 0.025	^{def}1.273± 0.013	^{bcd}1.387± 0.032	^{a}4.799± 0.037	^{b}4.451± 0.040	***
11.	*Antioxidants* (mg ml^{-1})	^{a}0.865± 0.013	^{d}0.242± 0.002	^{ab}0.671± 0.008	^{bcd}0.575± 0.005	^{a}0.673± 0.003	^{bc}0.591± 0.006	***
12.	*Phosphates* (mg ml^{-1})	^{a}0.984± 0.003	^{d}0.345± 0.007	^{ab}0.964± 0.005	^{ab}0.967± 0.004	^{abc}0.958± 0.006	^{bcd}0.939± 0.002	**
13.	*Methionine* (mg ml^{-1})	^{c}0.181± 0.005	^{e}0.090± 0.002	^{cd}0.123± 0.003	^{de}0.115± 0.006	^{b}0.223± 0.003	^{a}0.240± 0.004	***

Note: Each parameter is presented with mean values of 4-replicates with their standard error and significances at 0.05 level (5%) with a,b,c,d ... letters for DMR test.

Preparation of agro-fermentation substrate: The old and rotten potatoes and sugar beets collected from the nearby local vegetable market (Fig 1). These rotten potatoes and sugar beets were washed with running tap-water to clean dust or soil propely. Their skin or peels were separated with fine knife than peels and peeled stuff of both potatoes and sugar beets weighted exactly 50 g. These weighed material crushed with grinder grinder in 50 ml sterilized dH_2O (w/v). After that the grinded mixture centrifuged at 4,000 rpm at room temperature for 10 min. The supernatant preserved at 4°C for next use in the agro-fermentation medium preparation while its pallet was discarded .

Harvesting of Bacillus fermentation culture: The inculated cultures incubated for 18 hours at 37°C with constant shaking at 250 rpm. After 18 hrs of incubation of the fermentation cultures, they were harvested. Before the collection of culture for harvest, their OD600 was measured. These cultures were centrifuged for 10 minutes at 7,000 rpm. The supernatants of the cultures transferred to the clean dark-colored glass-bottles, while

its pallet was discarded. The supernatant of each culture stored at 4°C for next it was used as a sample for the measurements of different biochemical and activities of enzyme outcome.

Figure 2: Comparative cell growth rates of *Bacillus subtilis* (k1) on various nutrient cultures supplemented with potatoes and sugar beets as carbon sources (Each graph is presented with mean values and their standard error from 4-replicates with significances at 0.05 level (5%) and with a,b,c,d ... letters for DMR test).

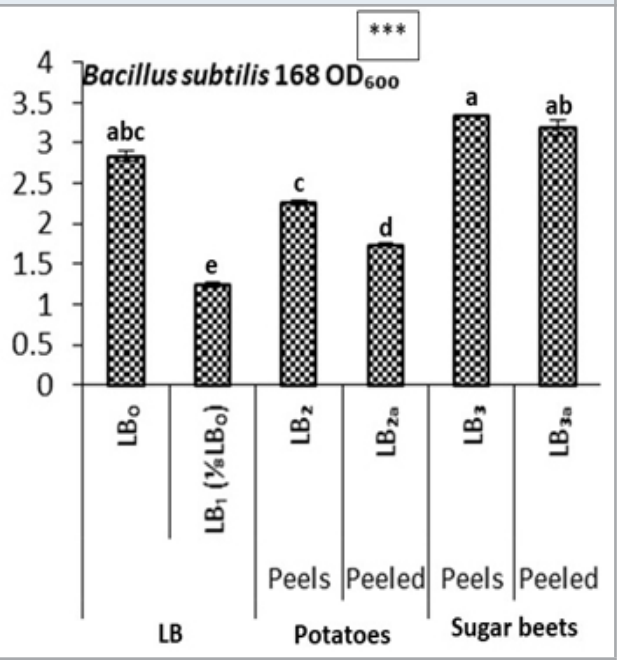
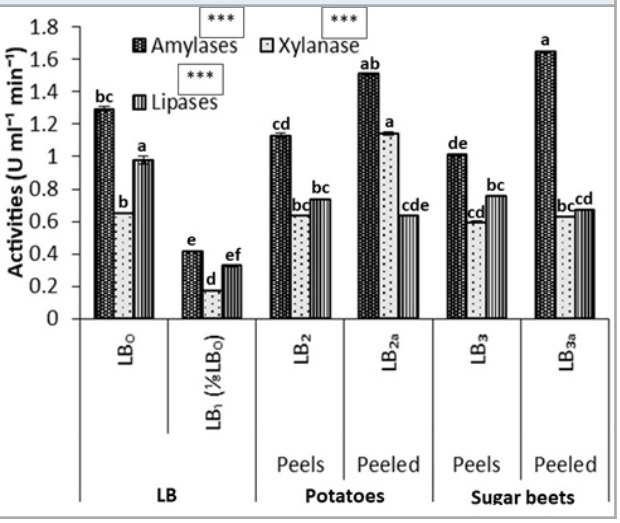


Figure 3: Comparative crude enzyme activities in the supernatant of *Bacillus subtilis* (k1) culture supplemented with potatoes and sugar beets as carbon sources. Each graph is presented with mean values and their standard error from 4-replicates with significances at 0.05 level (5%) and with a,b,c,d ... letters for DMR test.



Biochemical analysis of fermented supernatant: A number of biochemical testes performed on the fermented supernatant of *Bacillus subtilis*. Like as the total sugars anlysed by mixing 1 ml supernatant with 2.50 ml concentrated H_zSO_4 and 5 µl 80 % phenol in a clean and dried glass test tube. This mixture was allowed to stand at room temperature for minimum 10 min than its absorbance was read at OD485 (Dubois et al., 1956). Similarly, reducing sugars were also measured by mixing 1 ml sample with 2.0 ml DNS (3, 5-Dinitrosalicylic acid) reagent than mixture was heated for 15 min in boiling water-bath. After that its OD540 was read (Aakanchha and Richa, 2020). Furthermore, total protiens were also determined with following Lovrien and Matulis (2004) method. Exact 2.5 ml alkaline copper reagents mixed with 1 ml supernatant. The mixture was mixed thoroughly and incubated at room temperature for 10 min than 0.25 ml folin reagents (1:1, w/v) poured into the wall of the test tube and than its OD750 was read. Meanwhile the free prolines (Schweet, 1954), glycinebetaine (Grieve and Grattan, 1983), total flavonoids (Woisky and Salatino, 1998), total phenolics (John et al., 2014), ascorbic acid (Tabata and Morita, 1997) and antioxidants (Prieto et al., 1999) were also analyszed in the same *Bacillus* culture supernatants by following the respective reported methods.

The phosphate contents analyzed by mixing the 0.5 ml ammonium molybdate with 3 ml concentrated H_2SO_4 then 4 ml test sample added. After that 1 ml 0.05 M sodium sulphate also added. Its OD was read at 715 nm (Mahadevaiah et al., 2007). The methionines were also analyzed by mixing 0.2 ml 5 N NaOH with 1 ml test samples than 0.02 ml 10% Nitro-Prusside was added. After 10 min, 0.4 ml 3% aqeous glycine poured to the reaction mixture. It was incubated for 10 min at room temperature than finally 0.4 ml ortho-phosphoric acid added. Absorbance was read against blank at 540 nm after 5 min of incubation at room temperature (Gensch and Higuchi, 1967; Lavine, 1943). The citric acid quantified with titration method (Aguiar et al., 2005) and its quantity was calculated by applying this formula; Aceti acid (g 100 ml^{-1}) = Volume of NaOH used*(0.03)*20.

The total fructose contents were quantified in the supernatant by mixing 3.5 ml 30 % HCl. The mixture was incubated in ice-bath for 10 min than 1 ml sample added. After 5 min of incubation in ice-bath 0.5 ml resorcinol thiourea was added. After that the reaction mixture was incubated at 80°C for 10 min than it was cool down under running tap water and its absorbance was read at 520 nm (Arsenault and Yaphe, 1966).

Measurments of enzyme activity: The supernatant of agro-fermented cultures of *Bacillus subtilis* was used as a crude enzyme mixture for the analysis of different enzymes activities. For the analysis of the *amylases* activities, its 1 ml used crude enzyme mixture mixed with 1 ml 0.5% starch fresh solution. The reaction mixture was incubated for 15 min at 37°C and than 2 ml DNS added to develop product complex and to stop

the enzyme reaction. The absorbance of the reaction mixture was read at OD540 against blank (Mulimani and Lalitha, 1996). For determination of *xylanases* activity, 1 ml supernatant mixed with its 1 ml of xylanase substrate (0.5 % xylose) and reaction mixture incubated at 60°C. After 15 min, 2 ml DNS was added to stop the reaction and OD540 was noted (Bailey et al., 1992). Similary, other activities including the lipases (Espinosa-Ramírez et al., 2014), proteases (Anson, 1938) and pectinases (Miller, 1959) activities also measured. Alongwith the pectinases activities their thermostability also determined at different teparatures. Exact 1 ml 0.5 % pectin substrate mixed with 1 ml *B. subtilis* culture supernatant than incubated for 30 min at different temperatures (i.e 37°C, 25°C and 50°C). The 2 ml DNS reagent added in reaction and than its was kept in boiling water bath for 5 min. When it was cool down to room temperature, its OD540 was read.

Statistical analysis of data: The collected data of the studty was subjected to ANOVA (analysis of variance) and DMR (Duncan's multiple range) tests at 5 % ($p \geq 0.05$) for the data significance analysis (Snedecor and Cochran, 1983). These statistical analysis were analysed with computer based software "COSTAT" package (CoHort Software, Berkeley, USA).

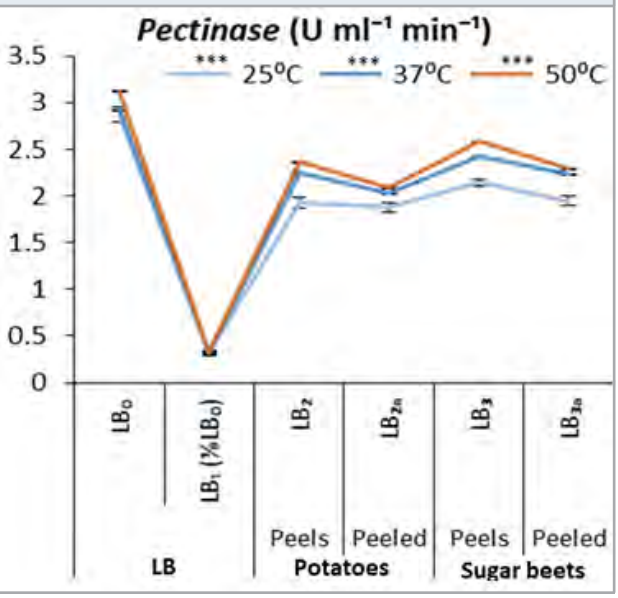
RESULTS AND DISCUSSION

In the world, ligno-cellulosic plant biomass is an abundant renewable carbon complex. It is available as the great potential natural resource for bioconversion into various value-added bioproducts. These deposited agricultural residues (lignocellulosic) wastes are not properly disposed by the farmers that's why it is being hazardous for environment and well as human health. These natural substrates with immense free energy potential are used to nurture the growth of fermentation organism in this study. Earlier, it has been demonstrated successfully about the implementation of these agro-renewable cost effective wastes for the screening of different hydrolytic fermentation microorganisms (Nagar et al., 2012; Varghese et al., 2017). Furthermore, it can also provide the plant organ based selective microbial growth for optimization of fermentation conditions and its respective extracellular products (Haq et al., 2017).

In this experiment, different LB-medium based cultures supplemented with lignocellulosic wastes of potatoes and sugar beets prepared to study the growth and extra-cellular productions of *Bacillus subtilis* (k1) (Table 1). There LB-medium contained valued nutrion to boost the optimal *B. subtilis* growth, which was compared with LB-deficit ($1/8LB_0$) to its different forms supplemented maintained with different regions of rotten potato and sugar-beet tubers. The comparative cell growth rates of *B. subtilis* (LB_0 to LB_{3a}) drastically increased in LB_3 and LB_{3a} cultures and seems to be comparable with LB_3 standard medium. Meanwhile, the lowest cell multiplication rate observed in LB_1 (nutrient deficit) medium (Fig 1), while overall these raised cultures (LB_2, LB_{2a}, LB_3 and LB_{3a}) showed higher cell multiplication. Along the series of cultures from LB_0 to LB_2, relatively it was higher than LB_1. It means that the cultures supplemented with peels and peeled-off potatotes and sugar beets are growth supportive carbon containing agriculture wastes, which could serve as good nutrient-medium for the growth of *B. subtilis* and also for other fermentation organisms (Tin Lee, 2016). The *Bacillus subtilis* has already been identified as the faster organic food-waste hydrolyzer (Ale et al., 2015), it remains most effected if the wastes are treated with high temperatures (Kwon et al., 2014; Lim et al., 2017).

The capabilities of the *Bacillus subtilis* to reduce the agriculture wastes remains differential from medium to medium. It is known to be attractive *Bacillus* to show up higher growth rate in agro-wastes, reductions to reducing sugars, biosynthesis of different metabolites and secretion of many hydrolyzing enzymes with GRAS status (Table 2, Figure 2). These properties are most attractive for industrial point of view for including its abilities for differential and stable multi-enzymes production, which can degrade diversed forms of substrates under diversed environmental conditions (Parrado et al., 2014). The *B. subtilis* is a soil growing microorganism able to solubilize phosphate (Chen et al., 2006; Chatli et al., 2008). It means that they are well adapted for colonization with plants (Reva et al., 2004; Allard-Massicotte et al., 2016). So it means that *B. subtilis* is being worth to grow on plant in narure and also useful for various industrial productions with agricultural wastes utilization.

Figure 4: Comparative pectinases stability at different temperatures produced by *Bacillus subtilis* (k1) on different nutrient cultures supplemented with potatoes and sugar beets as carbon sources (Each graph is presented with mean values and their standard error from 4-replicates with significances at 0.05 level (5%) and with a,b,c,d ... letters for DMR test)



Among the other metabolites, production of total sugars sharply high in LB_3 , LB_z and $LB_{2,a}$ while it is declined in LB_0. The reducing sugars and total proteins observed higher in LB_3 culture. The production of fructose noted higher in LB_z and LB_3, citric acid in LB_z, ascorbic acid LB_2 and LB_3 (Table 2) cultures. The free prolines and glycinebetain were observed higher in LB_z and $LB_{2,a}$ while phenolics in LB_3 and LB_0 cultures. Production of flavonoids increased in LB_2, antioxidants and methionine in LB_3 cultures.

The *B. subtilis* has showed the production of various extra-cellular enzymes, which can lead to degrade the agro-based substrates (Meng et al., 2014). Similarly, amylases, xylanases, lipases *and* pectinases are also produced among other extra-cellular productions in the cultures when rotten rotten potatoes and sugar beets are used as carbon source. Both are rich in starch, which may be the basic source for the production of fructose/glucose syrup. Meanwhile, carbohydrates based agricultural products like starch from potato as well as sugar beets occur abundantly. The amylases have shown very drastic activities among the peeled-potatoes and sugar beets, while xylanases and pectinases are vey well performing in the cultures supplemented with peels of both agro-wastes as the carbon source. This differential formate of activities of various enzymes is due to the presence of their respective substrate in the plant orgn (Haq et al., 2017 and 2018).

From the results as shown in figure 3 about the pectinases production on various cultures and its stability at under different temrature conditions for 30 min. Maximum activities of *pectinases* observed at 50°C in LB_0, which is declined in LB_z (nutrient deficit medium) then slightly increased in LB_1 and LB_3, while it was relatively lower in LB_z and in LB_3 cultures. Almost similar but directly proportional pattern of activies observed among the cultures at 37°C and 25°C (Fig 3). Overall, pectinases showed best activities at 50°C in all cultures and it could be suggested that the production of pectinases are higher where its substrate is present in the medium (Amin et al., 2013). Similarly, the pectinases of *A. fumigates* and *P. italicum* remain stable even upto 60°C while remained highest active at 50°C (Phutela et al., 2005).

CONCLUSION

The rotten vegetables especially potatoes and sugar beets are rich with starch and other carbohydrates i.e. cellulose and lignin. These renewable agriculture wastes are available as the cheapest carbon source for the growth of fermentation microorganisms for the production of industrial hydrolytic enzymes. The *Bacillus subtilis* (k1) is one safe fermentation organism can grow on these substrates with aboundent prosuction of industrial enzymes especially amylases, pectinases and xylanases. The same setup could be best for the production of glucose syrup as the potatoes and sugar beets are rich in starch, which is major key source of sucrose.

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Agro-Industrial Residues as Solid Substrate for α-Amylase Production Using Solid State Fermentation by Filamentous Fungi: A Review

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ABSTRACT

Now a days, many industries like textile, food and pharmaceuticals use solid dry wastes as substrate for fermentation. In the solid-state fermentation (SSF), solid surface matrix in the absence or nearly in the absence of available free water used to carry out fermentation process. In this, microorganisms are grown in the absence of free flowing aqueous phase. A traditional way for the production of α-amylase in the past was the use of submerged fermentation (SmF) which is now replaced with SSF. The alternative of SmF is SSF in a variety of industrial production like enzymes, biofuels, single cell proteins, organic acids, antibiotics, aroma, biopestistdes etc. Earlier, SSF was the choice of fermentation process only where the choice of microorganisms is fungi, but now variety of bacterial strains are also being used in such processes. SSF facilitates the natural habitat of microorganisms and has a better choice due to its simplicity, relatively low capital investment and operating cost and less water output, etc. SSF with an agro-industrial residues have also replaced the high cost media used in submerged fermentation for α-amylase production. Agro-industrial residue utilization by filamentous fungi provides an alternative source for utilization of these substrates as a solid substrate for production of amylase and other useful industrial products. In this review we tried to study various solid substrates used for fungal amylase production, their potential, and optimization strategies

KEY WORDS: SOLID SUBSTRATE, SSF, BIO-PROCESSING, AGRICULTURAL WASTE, α-AMYLASES.

ARTICLE INFORMATION

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INTRODUCTION

Among many industrially important enzymes, one of the most commonly used enzymes is α-amylases, which is required for the production of many fermented foods. Apart from food and starch industries α-amylases are also having a wide application in other industries such as paper pulp and textile, etc. (Gupta et al., 2003). Amylase enzymes having a wide variety of industrial applications ranging from the conversion of starch to sugar, corn and maltose syrup for food and productions of cyclodextrins. (Kunamneni et al., 2005). Demand of amylases is also increasing in paper and pulp industry, textile industry These enzymes account for about 30% of the world's total enzyme productions. Due to increasing demand in various industries, there is enormous interest in developing amylase with better properties, thermal stability and suitability for industrial application by using cost effective production techniques (Pandey et al., 2000). α-amylase are produced by microorganisms plants and animals, where they play a key role in carbohydrate metabolism. From centuries amylases from plant and microbial origin contatntly being used as food additives. Amylases isolated from barley have been used in brewery industries and fungal amylases have been widely used in the preparation and processing of oriental foods (Reddy et al., 2003).

Barley amylases have been used in brewery industries and fungal amylases have been widely used in the preparation and processing of oriental foods (Reddy et al., 2003). Amylases from microbial sources are used for the industrial production of various useful products due to the cost effectiveness, consistency, thermal stability and ease of process modifications and optimization. Amylases play key role in the carbohydrate metabolism and are produced by microbe, plants as well as animals also. From centuries amylases from plant and fungal origin are being constantly used as food additives. Bacteria and filamentous fungi such as *Aspergillus* are used for the production of α-amylase.(Ajayi et al., 2003). However the amylase of fungal origin has been found to be most stable than the bacterial enzyme. Filamentous fungi are attractive host organism for the production of commercially useful enzymes. Coproduction of two industrially important enzyme amylase and protease through SSF by *Rhizopus oryzae* using bread waste as a solid substrate have been studies and effect of various physical and chemical parameters were studied (Benabda et al., 2019).

Amylase production using *Bacillus subtilis* as a bacterial strain using solid residues such as wheat bran, rice bran, banana peel and other fruit peel for optimize the production of enzyme with minimal amount of supplementary carbon and nitrogen source (Almanaa et al., 2020). Combined effect of solid state fermentation to submerged fermentation was studied using the edible fungus *Neurospora intermedia* to biotransformation of ethanol and this study may be useful for the production of other industrially important products (Gmoser et al,2019).

All these types of fermentation processes agro-industrial solid wastes can be efficiently used in the form of solid substrates for SSF. The purpose of this review article is to summarize the information related to SSF for α-amylase production, which is distributed across various literatures to help scientific community. Various different types of solid substrates are used in this purpose and each substrate used to have their own merits and demerits. Hence this article will felicitate the researchers with the information regarding various solid substrates used for fungal amylase production, their potential, and optimization strategies.

2.Solid State Fermentation strategies for amylase production: In solid state fermentation solid matrix is used in presence of minimal water content and sometimes in absence of water to support growth and metabolic activity of microorganism. Solid matrix could be either used as support material or simply a source of nutrient content allows the growth and metabolism of microorganism. SSF provides natural habitats to the microorganisms therefore considered as a preferred choice over submerged fermentation. SmF is considered as a violation to natural living of microorganism, especially unfavorable for the growth of fungi. Scientific data reveals that more than 98% of microbial isolates from marine habitat have been obtained from the underwater surfaces of solid substrates (Kelecom et al., 2002, Holker et al., 2004).

This indicates that SSF is well adapted suited for the growth and metabolism of fungi. At laboratory level studies many research article have been published for observing various physical and chemical factors that affects on fungal metabolism. SSF provides better economic feasibility for production of amylase and other high end products by using low cost agricultural residues and resulting in solving the problem of waste disposal. Solid substrate not only supply nutrient for fungal growth it also play a key role as a support material which helps in effective growth of fungus due to its porosity, moisture holding capacity etc. These agricultural byproducts are rich in starch and lignocellulosic compound based on their origin in agricultural practices or agro-industrial sources (Pandey et al., 1992).

In present time enzyme production strategies are centered in the selection and screening of suitable solid substrate for maximum enzyme production and optimization of various physical and chemical parameters process parameters that affects the growth of fungus and enzyme production. Industrial production of amylase using SSF having advantages for growth of fungus which have capability to penetrate these solid substrate effectively. (Ramachandran et al., 2004). In addition to the utilization of these agro-industrial wastes as solid substrate in amylase production as solid substrate as well as in solving the waste disposal problem. With the improved production of lignocellulose degrading enzyme such as xylanase and cellulase SSF becoming most favorable process for cost effective production

of industrially important enzymes and biofuels etc (Taherzadeh et al., 2019).

With the increasing harmful effect of traditional chemical pesticides now researchers are working on the production of biological control agents through SSF by using agro-industrial waste as substrates. Production of entomopathgens of fungal origin is being produced by SSF for adopting sustainable production method and for effective biological control method to reduce the pest eradication in the agricultural practices (Sala et al,. 2019). Now novel approaches are developing for agricultural waste treatment and further being used for up scaling of SSF processes using bacterial culture for the production of poly γ glutamic acid in continuously stirred solid state bioreactors (Fang et al,. 2020)

3. Role of filamentous fungi for amylase production: However many fungal strains are commercially used for production of amylase but researchers are constantly trying to isolate novel fungal strains for amylase production. A team of researchers of Brazil isolated new fungus from the Atlantis forest region having ability to produce higher glucoamylase using agro-industrial waste as solid substrate under standardized conditions. The isolated fungi *A. carbonarius* showed maximum production of amylase at pH 6.0, temperature 30°C and incubation time 96 hours when supplemented with 1% starch in the media (Pasin et al., 2020).

Since fungi have very efficient enzymatic system therefore fungi having ability to degrade lignocellulosic waste resulting in the formation of many valuable compounds that may be useful for production vast variety of useful products. Two types of extacellular enzymatic system are recognizable in fungi i.e. hydrolytic and ligninolytic system. Lignocellulosic residues from agricultural and municipal solid wastes are particularly abundant in nature and have a potential for bioconversion (Abdel-Azeem et al., 2020).

Filamentous fungi have unique and excellent property to degrade cellulosic waste and resulting in the production of α-amylases and other industrially important enzymes, amino acids, antibiotics, organic acids etc. using SSF for past several decades. As these moulds are known for the production of extracellular products such as enzymes. Therefore filamentous fungi are widely exploited for various SSF processes for the production of a variety of industrial products including α-amylase. Production of enzymes as well as other industrially important products by solid-state fermentation (SSF) using agricultural wastes through moulds turned to be a cost-effective production technique and surprisingly most of these processes are optimized. Detailed literature is available on various fungal sources for the productions of amylases are given in the table.

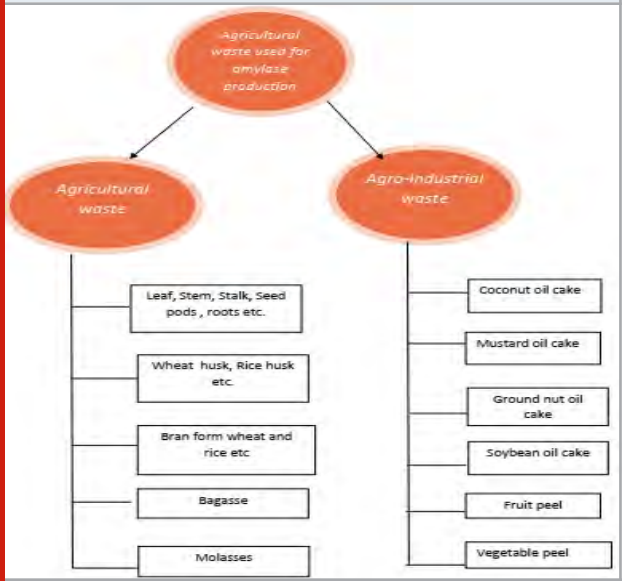
Table 1. Different types of agro-substrate used for α-amylase production using SSF by Filamentous fungus

S. No	Organism	Substrate	Activity(U/g)	References
1	*Aspergillus oryzae*	Spent brewing grain	6583	Francis et al., (2003)
2	*Aspergillus oryzae*	Coconut oil cake	3388	Vishwanathan et al., (2001)
3	*Aspergillus flavus*	Amaranthus grains	1920	Ramachandran et al., (2004)
4	*Thermomyces lanuginosus*	Wheat Bran	534	Kunamneni et al., (2005)
6	*Aspergillus oryzae*	Ground nut oil cake	9196	Ramachandran et al., (2004)
7	*Aspergillus oryzae*	Wheat Bran	15095	Gangadharan et al., (2007)
8	*Aspergillus awamori*	Corn streep liquor	738	Prakasham et al., (2006)
9	*Penicillium fellutanum*	Soluble starch with sea water	157	Kathiresan & Manivannan 2006
10	*Penicillium expansum*	Loquat kernel flour	1012	Erdal & Taskin (2010)
11	*Aspergillus sp.*	wheat bran	164	Chimata et al., (2010)
12	*Penicillium brevicompactum*	Wheat bran	666.6	Balkan & Ertan (2010)
13	*Trichothecium roseum*	Wheat bran	1048	Balkan et al., (2011)
	Aspergillus niger	Ipomoea batatas	450	Sunder et al., (2012)
15	*Aspergillus niger*	Rice Bran	334.51	Rajasekar et al., (2013)
16	*Aspergillus fumigatus*	Pomegranate peel	341.7	Singh et al., (2014)
	Aspergillus awamori	Casava Peel	31.64	Kalaiarasi & Pavatham (2015)
17	*Aspergillus oryzae*	Agro-waste	750	Naili et al., (2016)
18	*Aspergillus terreus*	pearl millet	19.19	Sethi et al., (2016)
19	*Pleurotus ostreatus*	Potato peel waste	2503.6	Ergun et al., (2017)
20	*Rhizopus delemar*	Apple pomace	21.03	Pathania et al., (2018)
21	*Rhizopus oryzae*	Bread waste	100	O Benabda et al., (2019)
22	*Aspergillus carbonarius*	Wheat and brewing residues	404	Pasin et al,.(2019)
23	*Aspergillus oryzae*	soybean husk and flour mill	47000	Melnichuk et al.,(2020)

4. Agricultural by products in amylase production: Microbial enzymes are versatile in nature and relatively more stable compared to plant and animal enzymes and having wide range of industrial applications. Since these agricultural residues are rich in cellulosic content along with other biologically active compounds. Therefore these agricultural wastes can be used as alternative solid substrate in SSF for the production of industrially important compounds like biofuel production, animal feed, mushroom cultivation, as well as for the production of industrial products such as enzymes, organic acids, amino acids etc.

These agro industrial wastes can be used in industrial processes for the production of high value industrial products to reduce the production cost which also reduces the pollution load from the environment these agro industrial residues consist of molasses, rice husks, rice bran and wheat bran, bagasse, leaves, straw, stalk, shell, pulp, peel, roots, etc. These substrate traditionally used for animal feed, soil quality improvement, biofertilizers etc. from ancient times, But due to scientific innovations and technology development in agricultural practices huge amount of field residues are generated. Disposal of underutilized agricultural waste is problematic and non eco friendly practice. In oil exploration industries, huge amount of processed residues in the form of oil cake are produced. These residues contain high concentration of fat, carbohydrate suspended solids and as well as dissolved solids are also present. Oil cake is of different types like canola oil cake, sunflower oil cake, coconut oil cake, sesame oil cake, mustard oil 0cake, soy bean cake, groundnut oil cake etc. (Ramachandran et al., 2007). Different types of agricultural by products which have been used as solid substrate for production of various industrial products including enzymes in fermentation process are summarized in Fig-1

Figure 1: Different types of agricultural waste used in SSF as solid substrate for amylase production



These agricultural wastes are generated throughout the year through various agricultural practices and having various limiting nutrients for the growth of microorganism. Therefore, these substrates having an unlimited prospective to be used as alternative solid substrates in SSF. Researchers are constantly using these agro industrial residues for α-amylase production by using various microbial strains to optimize fermentation process parameters for enhanced production. A similar type of study was carried out by busing wheat bran as solid substrate for single cell protein and amylase production by using *A. Niger* as microbial strain. Maximum biomass was produced when media was supplemented with NH_4Cl, $NaNO_3$, KNO_3 and $(NH_4)2SO_4$ as additional nitrogen source for enhanced growth of fungal mycelium in submerged fermentation. However media supplemented with $(NH_4)2SO_4$ showed maximum production of biomass and amylase activity i.e. 326 U/L with 6 days of fermentation (Oshoma et al., 2019).

In many countries bread waste is easily available and disposal of these wastes is a problematic affair. To solve these issues researchers are trying to use such waste for production of enzymes i.e. amylase and protease. *Rhizopus oryzae* was used to degrade humidified bread waste by SSF with minimal salt solution for production of amylase and protease. Highest amylase activity was observed 100U/g and for protease 2400U/g (Benabda et al., 2019). Since fungi have very efficient enzymatic system therefore fungi having ability to degrade lignocellulosic waste resulting in the formation of many valuable compounds that may be useful for production vast variety of useful products. Two types of extracellular enzymatic system are recognizable in fungi i.e. hydrolytic and ligninolytic system. Lignocellulosic residues from agricultural and municipal solid wastes are particularly abundant in nature and have a potential for bioconversion (Abdel-Azeem et al., 2020).

Brazil is the major producer of citrus low cost source of hesperidin a polyphenolic compound having potential to restrict anti-glycation effects (AGEs) which constitute a vast variety of compounds synthesized due to interaction between amino acids of proteins and reducing sugars which induces chronic diseases through pathogenesis. This research work concludes that inhibition of AGEs results in the reduction of amylase activity 50% proving that there is strong correlation between anti-glycation with polyphenolic content and antioxidant capacity (Fernandes et al., 2020).

CONCLUSION

Agro-industrial waste are rich in various nutrient that contain bioactive compounds makes them favorable for the growth of filamentous fungi in solid state fermentation. Composition of these products ranges from sugars minerals proteins and amino acids etc. therefore these agro-industrial residues are considered as raw material in bio-conversion processes for the production of high value industrial products. The microorganisms having unique properties to use these

solid substrate as raw materials for their growth and nutrition during fermentation processes and results in the formation a variety of biochemical of industrial application. SSF is relatively simpler but cost effective process for the industrial scale optimization and bio-processing. Alternatively, use of agro-industrial wastes as raw materials can help to reduce the production cost as well as recycling of waste make the environment more eco-friendly.

Amylase is the most widely used enzymes among different enzymes and being constantly used in different industrial and medicinal purposes. With the development of new and novel techniques in bioprocess engineering such as Response Surface Methodology (RSM), Artificial Neural Network) ANN based optimization and Bioreactor designing for SSF leads the production of amylase at large scale for reducing the cost and efficient utilization of agro-waste. This review will facilitate for establishing the large scale fermentation process with the use of appropriate fungal strain.

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Analyzing Students' Perceptions of Educational Environment in New Dental Colleges, Turkey using DREEM Inventory

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ABSTRACT

Aims of the study were to assess dental students' perceptions of educational environment and identify differences in terms of gender, age, type of high school, level of education, total monthly family income, and type of housing. A cross-sectional, descriptive questionnaire was administered in a dental school in Turkey. A total of 185 undergraduate dental students from the third-, fourth-, and fifth-year were personally approached by the investigator to complete the questionnaire. Data were analyzed using the IBM SPSS-22 Türkiye program. Comparisons were performed via ANOVA and t-test to detect the significance between and within the groups. This study gotten a response rate of 96.67%, and the total Dundee ready education environment measure score was more positive than negative (60% and 48%). No significant difference existed between the gender scores at 100.97 ± 22.18 and 100.19 ± 16.77. Other parameters showed no influences the findings. A significant difference was observed in SASP, with a p 0.042 in the graduated school type. The students in the third-, fourth-, and fifth-year levels obtained DREEM scores of 95, 36 ± 18, 67 103, 02 ± 20, 75, and 103, 31 ± 19.31, respectively. Significant differences were observed in SPT and SPA, with p values of 0.029 and 0.035. We concluded that the EE perceptions of students were at the margin of the positive side, with no significant difference in terms of gender, age, monthly family income, and housing type. However, certain weaknesses were identified, particularly in students' perception of learning and atmosphere.

KEY WORDS: DENTAL STUDENTS; DREEM; EDUCATIONAL ENVIRONMENT; PERCEPTION, GENDER .

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INTRODUCTION

Educational environment (EE) is a broad concept. Here, education encompasses teaching and learning, whereas the environment encompasses everything that surrounds. EE can be described as anything involved with educational institutions (Salam et al, 2014). In 1998, the World Federation of Medical Education highlighted EE as a target for the evaluation of health/dental education programs (WFME 1998). The effects of academic and clinical learning environments on dental students' attitudes, knowledge, skills, progression, and behaviors are important determinants of education (Roff and McAleer, 2001). Evaluating EE in clinical and academic sites is important for providing of high-quality education and curriculum. The gap between student expectations and their actual experiences must be identified. However, differences exist between students' experiences at the various levels of dental education (Chandran and Ranjan, 2014 Idon et al, 2015; Jnaneswar et al, 2016; Kang et al, 2017, Batra et al, 2018; Al-Saleh et al, 2018).

Roff et al., (1997) proposed the Dundee ready education environment measure (DREEM), a multidimensional and multicultural instrument that can measure the five separate fundamentals of EE, namely, students' perceptions of learning (SPL), students' perceptions of teachers (SPT), students' perceptions of atmosphere (SPA), students' academic self-perception (SASP), and students' social self-perception (SSSP) (Roff et al, 1997). DREEM can be used to highlight the weaknesses and strengths of an educational institution, compare the performance and success of dental schools, and contrast the different levels of study and gender among students (WFME 1998; Roff et al, 2001). In addition, this tool can be used to help amend the curriculum, compare present and past programs, and evaluate the effectiveness of college curriculums (Al-Hazimi et al, 2004a; Bassaw et al, 2008. DREEM can also help health and dental schools distinguish their priorities (Roff et al, 2001; Al-Hazimi et al, 2004a; (Al-Hazimi et al, 2004b; Hammond et al, 2012; Denz-Penhey and Murdoch, 2009), while comparing their performance and productivities against their peers. The results of this comparison can be educationally insightful ((Al-Hazimi et al, 2004b). The use of DREEM is important in providing a consistent method for global comparisons among dental schools, thereby leading to the standardization of educational environments (Hammond et al, 2012). DREEM is successfully used in studies carried out in North America, Africa, Europe, South America, the Middle East, and Asia (Hammond et al, 2012).

The perceptions of students should be monitored in the continuous improvement of EE by defining its strengths and weaknesses (Hammond et al, 2012). Excellent learning is positively correlated with educators' perceptions of EEs. It influences how, why, and what the students learn (Roff and McAleer, 2001; Mayya and Roff, 2004). Our private dental college was established 10 years ago. Since then, three groups have graduated, and we have plans of modifying the curriculum and studying plan. Thus, this cross-sectional study aimed to assess EE and the climate of undergraduate dental students' perceptions at the different studying levels of a dental college in Turkey. It also plans to detect the gender differences among students' perceptions and evaluate the association between EE and age, type of graduated school, level of education, total monthly family income, and type of housing.

MATERIAL AND METHODS

Design and Study Population: A cross-sectional questionnaire-based survey was administered to investigate the perception of students toward the EE of a dental school in Turkey. DREEM is a widely used instrument for collecting evidence about EE in dental schools in developed and developing countries. This tool was first developed at the University of Dundee and is now a global and generic "diagnostic inventory for measuring the quality of educational environment."

Questionnaire Validation and Pilot Study: Questionnaire validation was performed on 20 students to review and determine if the questionnaire successfully measured as per the method of Bhosale (2015). The answers to each item were reviewed by the authors, and the requisite modifications and deletions were applied to validate the 50 items in the questionnaire. The validation criteria, such as time required to complete the questionnaire (20–30 minutes), appropriateness of questionnaire in collecting data, repetition or inappropriate questions, logical order of questions, clarity of questions, conciseness of questions, easy and meaningful instructions, and specified comments and suggestions on the application guidelines, were evaluated as well (Bhosale 2015; Wilson et al, 2012).

Data Collection: Ethical permission was obtained from the Faculty of Dentistry Board. All dental students from this college enrolled in Years III–V of the BDS program (185 in total) were the target participants. Therefore, universal sampling was used. The subjects were given the DREEM questionnaires and given a period of 20–30 minutes to retrieve and complete the questionnaire. Data were collected between December 2018 and February 2019. The aim of this study was explained, and consent was obtained from each participant.

Students were asked to read each statement carefully and respond using a five-point Likert scale as follows: 4 for strongly agree (SA), 3 for agree (A), 2 for uncertain (U), 1 for disagree (D), and 0 for strongly disagree (SD). Each student regardless of gender must apply his or her own current learning situation in answering the questions. However, 9 out of 50 items (numbers 4, 8, 9, 17, 25, 35, 39, 48, and 50) are negative statements and should be scored as follows: (0 for SA), (1 for A), (2 for U), (3 for D), and (4 for SD). The 50-item DREEM questionnaire has a maximum score of 200, which is the ideal EE, as perceived by the registrar. The minimum score of 0 will cause concern on any dental educator. The approximate guide to interpreting the overall score is presented as

follows: (0–50) Very Poor, (51–100) Many Problems (This environment is viewed with considerable ambivalence by the students and thus needs improvement.), (101–150) More Positive than Negative, and (151–200) Excellent (Roff et al., 1997; Roff et al, 2001; Mayya and Roff, 2004; Al-Saleh et al, 2018; Idon et al, 2015; Jnaneswar et al, 2016; Batra et al, 2018; Kang et al, 2017; Ostapczuk et al, 2012).

The DREEM subscales are presented as follows: 1) SPL: 12 items, maximum score of 48; 2) SPT: 11 items, maximum score of 44; 3) SASP: 8 items, maximum score of 32; 4) SPA: 12 items, maximum score of 48; and 5) SSSP: 7 items, maximum score of 28 (Roff et al., 1997; Roff et al, 2001; Al-Saleh et al, 2018; Idon et al, 2015; Jnaneswar et al, 2016; Batra et al, 2018; Kang et al, 2017; Ostapczuk et al, 2012).

Data Statistical Evaluations: We used the IBM SPSS Statistics 22 (IBM SPSS, Türkiye) program for statistical analysis. The parameters were assessed via the Shapiro–Wilks test, and the results showed that the parameters conformed to the normal distribution. During the evaluation of the study data, the comparisons of quantitative data, descriptive statistical methods (mean, standard deviation), and categorical variables were presented in frequencies and percentages. One-way ANOVA was used in the intergroup comparisons of parameters, and Tukey HDS test was used to determine the differences among the group parameters (gender, age, type of graduated secondary school, level of education, monthly income, and type of housing). Student t-test was used in the intergroup comparisons of parameters. The Fisher–Freeman–Halton test was used to compare the qualitative data, and the statistical significance was evaluated at the level of $p < 0.05$.

RESULTS

A total of 171 completed questionnaires were collected from the students, reflecting a response rate of 96.67%. Demographic data are presented in Table 1. A total of 171 students responded to the questionnaire. The age of participants ranged from 21 to 26 years. As for gender, 92 (53.8%) were male, and 79 (46.2%) were female. The average age is 23.0 ± 1.4. (66.1%) between 21–23 years of age with 33.9% between 24–26 years of age. On the basis of the type of high school, 81.3% and 18.7% of the students graduated from government and private high schools, respectively. The response rates based on their year level in school were 56, 32.7%; 55, 32.2%; and 60, 35.1% for third, fourth, and fifth year students, respectively. On the basis of monthly family income, 20.5%, 56.1%, and 23.4% of the participants were below 3000 Turkish Lira, between 3000–6000 Turkish Lira, and over 6000 Turkish Lira, respectively. The majority of our participants at 74.3% lived with their families, whereas 25.7% resided in dormitories.

Table 2 shows the mean and standard deviation (SD) of the DREEM items and subscales. The highest recorded value was 2.91 ± 1.04 for question number 28 ("I seldom feel lonely."), whereas the minimum registered value was 1.2 ± 1.23 for question number 3 (a good support system was available for registrars who get stressed). Both questions are found in the SSSP subscale. The total mean and SD of the DREEM items was 100.61 ± 19.81, whereas the mean and SD of subscales based on the original values of the DREEM subscales SPL, SPT, SASP, SPA, and SSSP were 23.44 ± 5.57/48; 22.71 ± 4.56/44; 17.49 ± 4.09/32; 22.04 ± 6.67/48; and 14.94 ± 3.56/28, respectively (Table 3).

The results of the Student t-test showed that the total mean of DREEM items and subscales SPL, SPT, SASP, SPA, and SSSP scores were insignificantly different in terms of gender (male and female), age groups (21–23 and 24–26), and accommodation types (with family and student housing), and the p-values were greater than 0.05. Moreover, no statistically significant difference was observed between the total mean of DREEM items and subscale scores of SPL, SPT, SPA, and SSSP in relation to the type of graduated high school with $p > 0.05$). The SASP average scores of private high school students are significantly higher than those of government high school students ($p = 0.042$).

Table 1. Demographic profiles of respondents (n = 171)

		N	%
Gender	Male	92	53,8
	Female	79	46,2
Age	21–23	113	66,1
	24–26	58	33,9
Type of school	Government	139	81,3
	Private	32	18,7
Educational level	3	56	32,7
	4	55	32,2
	5	60	35,1
Monthly Family Income	Up to 3000	35	20,5
	Between 3000–6000	96	56,1
	More than 6000	40	23,4
Housing	With family	127	74,3
	Student housing	44	25,7

According to the level of education, the results of the ANOVA test showed that the total mean score of DREEM items was nearly significant at $p = 0.053$. No statistically significant difference existed among the total scores of subscales SPL, SASP, and SSSP in all the student levels with $p > 0.05$. However, a significant difference was observed between the classes in terms of SPT scores with $p = 0.029$. Post hoc comparisons were conducted to determine the origin of significance. The SPT scores of fourth level students were significantly higher than those of third level students with a p-value of 0.035.

Table 2. Dental students' mean item DREEM scores (n = 171)

			Mean ± SD	Median
SPL	1	I am encouraged to participate in teaching sessions.	2,07 ± 1,09	2
	7	The teaching is often stimulating.	2,34 ± 1,02	3
	13	The teaching is registrar-centered.	1,78 ± 1,01	2
	16	The teaching helps develop my competence.	2,21 ± 1,06	2
	20	The teaching is focused well.	1,81 ± 1,01	2
	22	The teaching helps develop my confidence.	2,11 ± 1,03	2
	24	The teaching time is put to good use.	1,73 ± 0,94	2
	25	The teaching overemphasizes factual learning.*	1,67 ± 1,01	1
	38	The learning objectives of the course are clear to me.	1,97 ± 1,01	2
	44	The teaching encourages me to be an active learner.	1,83 ± 1,1	2
	47	Long-term learning is emphasized over short-term learning.	2,5 ± 0,98	3
	48	The teaching is too teacher-centered.*	1,42 ± 0,88	1
SPT	2	The teachers are knowledgeable.	2,35 ± 0,96	3
	6	The teachers espouse a patient cantered approach to consulting.	2,35 ± 1,19	3
	8	The teachers ridicule their registrars.*	1,5 ± 1,01	1
	9	The teachers are authoritarian.*	1,19 ± 0,96	1
	18	The teachers can effectively communicate with their patients.	1,99 ± 1,09	2
	29	The teachers regularly provide feedback to their registrars.	2,43 ± 1,02	2
	32	The teachers provide constructive criticism.	1,94 ± 1,08	2
	37	The teachers give clear examples.	2,06 ± 1,01	2
	39	The teachers get angry during teaching sessions.*	2,09 ± 0,95	2
	40	The teachers come to class prepared.	2,14 ± 0,99	2
	50	The registrars irritate the course organizers.*	2,66 ± 1,11	3
SASP	5	The learning strategies that used to work for me are still effective until now.	2,32 ± 0,92	2
	10	I am confident about passing this year.	2,08 ± 1,08	2
	21	I feel that I am being well prepared for my profession.	2,15 ± 0,98	2
	26	Last year's work was good preparation for this year's work.	2,05 ± 0,97	2
	27	I can memorize the important facts.	1,7 ± 1,07	2
	31	I learned a lot about empathy in my profession.	2,5 ± 1,05	3
	41	My problem-solving skills are developed well in this school.	2,26 ± 0,91	2
	45	The majority of my lessons are relevant to a career in healthcare.	2,43 ± 0,99	3
SPA	11	The atmosphere is relaxed during consultations.	1,98 ± 1,07	2
	12	The course is well timetabled.	1,57 ± 1,16	1
	17	Cheating is a problem in this course.*	2,06 ± 1,19	2
	23	The atmosphere is relaxed during lectures.	1,84 ± 1,06	2
	30	There are opportunities for me to develop interpersonal skills.	1,68 ± 1,1	2
	33	I feel socially comfortable during teaching sessions.	1,85 ± 1,02	2
	34	The atmosphere is relaxed during seminars/tutorials.	1,9 ± 1,04	2
	35	I find the experience disappointing.*	1,63 ± 0,99	1
	36	I can concentrate well.	2,2 ± 1,06	2
	42	The enjoyment outweighs the stress of studying medicine.	1,59 ± 1,17	2
	43	The atmosphere motivates me as a learner.	1,87 ± 1,06	2
	49	I feel that I can ask all the questions I want.	1,88 ± 1,1	2
SSSP	3	There is a good support system for registrars who get stressed.	1,2 ± 1,23	1
	4	I am too tired to enjoy this course.	1,51 ± 1,07	1
	14	I am rarely bored with this course.*	2,05 ± 1,17	2
	15	I have good friends in this course/school.	2,61 ± 1,1	3
	19	I have a good social life.	2,16 ± 1,17	2
	28	I seldom feel lonely.	2,91 ± 1,04	3
	46	I have a pleasant accommodation.	2,51 ± 1	3

*Negative statements are scored in reverse.

Furthermore, a statistically significant difference existed between the classes in terms of SPA scores ($p = 0.035$; $p < 0.05$). The SPA scores of fourth level students were significantly higher than those of third level students with a p-value of 0.038 (Table 4). Finally, the results of the ANOVA test showed that no significant difference was observed among the monthly income groups in terms of the DREEM total score and subscales SPL, SPT, SASP, SPA, and SSSP scores ($p > 0.05$). No statistically significant difference was observed between genders in terms of total DREEM score and all the subscale scores of the SPL, SPT, SASP, SPA, and SSSP distributions ($p > 0.05$) (Table 5 and Figure 1).

Table 3. Mean scores of the total DREEM and its subscales

	Min–Max	Mean ± SD	Percentage of the maximum score
Total DREEM	43–153	100,61 ± 19,81	65,76%
SPL	5–38	23,44 ± 5,57	61,68%
SPT	11–34	22,71 ± 4,56	66,79%
SASP	6–27	17,49 ± 4,09	64,78%
SPA	6–39	22,04 ± 6,67	56,51%
SSSP	6–23	14,94 ± 3,56	64,96%

DISCUSSION

Roff and McAleer, 2001; Roff et al, 1997; Roff et al, 2001), developed and validated the DREEM scales using the standard methodologies of ground theory and Delphi panel of nearly 110 educators in the medical heath profession from different countries worldwide. Owing to the limited studies on dental colleges in Turkey, we conducted this study to assess gender differences in dental students' perceptions of EE by using DREEM and investigate the relation among EE and age, type of graduated school, level of education, total monthly family income, and type of housing among the participants.

A traditional curriculum is teacher centered and discipline based without optional modules or electives. Teaching is primarily dependent on gathering information, with the teacher as the main source of information. Teaching methods consist of lectures, preclinical classes, and clinical sessions without or with limited problem-based sessions. In general, students view learning as something "done to them" by the teacher and the curriculum as an aggregate of separate subjects (Al-Hazimi et al., 2004a ; Al-Hazimi et al., 2004b). Previous dental studies used the DREEM items and showed that EE is positively correlated with academic success and satisfaction with the educational curriculum (Thomas et al., 2009; Ostapczuk et al, 2012; Kossioni et al., 2012; Babar et al., 2015 Idon et al, 2015; Jnaneswar et al, 2016 Kang et al, 2017; Al-Saleh et al, 2018 Batra et al, 2018).

In the current study, the response rate was 96.67%, which was significantly higher than some of the earlier studies conducted in different dental schools worldwide in (Saudi Arabia, Riyadh 60.73%; Croatia, Nibal, & India 26.9%; and Greece 64%) (Al-Saleh et al, 2018; Batra et al, 2018; Kossioni et al., 2012). However, similar results were obtained in the studies from Bhubaneswar City (88.14%) and Odisha (92.68%) in India, Nigeria (95%), and New Zealand (82%–94%) (Idon et al, 2015; Jnaneswar et al, 2017; Kang et al, 2017). The percentage of male to female participants in this study was 53,8%/46,2%, which was similar to that in Al Saleh et al., 2018 in Riyadh (44%/56%) and Kang et al., 2017 in New Zeland (50%/50%) but higher than that in Idon et al., 2015 (38%/62%) and Jnaneswar et al., (2017) (66.4%/33.6%) in India.

In the overall DREEM score (Table 3), the recorded/obtained score was 100,61 ± 19,81, which indicates a "more positive than negative perception" and "many problems." These findings were partially consistent with the scores obtained in Germany for the first level (81) and among interns in SA (105 ± 21.3), Al-Saleh et al, 2018; Ostapczuk et al., 2012, but inconsistent with those in India (119.65 ± 19.68, 124) and New Zealand (127.7/200, 145) for the fifth year, Nigeria (145.6/200) for the second year, Germany (122.95 ± 15.52), and Riyadh (118.36 ± 15.8) for SA (Jnaneswar et al., 2016; Kang et al., 2017; Idon et al., 2015; Ostapczuk et al., 2012; Al-Saleh et al., 2018).

The recorded scores for the DREEM subscales are presented in Table 2. SPL, SPT, SASP, SPA, and SSSP obtained the scores of 23,44 ± 5,57/48; 22,71 ± 4,56/44; 17,49 ± 4,09/32; 22,04 ± 6,67/48; and 14,94 ± 3,56/28, respectively. The majority of the scores clearly obtained at least 50% of the original score in the DREEM subscales, except SPL and SPA. This finding shows students' perception of learning and atmosphere. These scores are significantly less than those of subscales of dental studies conducted in each of the following countries such as India (Chandran and Ranjan., 2014; Jnaneswar et al., 2016), in Nigeria, Germany, Greece (Idon et al., 2015; Ostapczuk et al., 2012; Kossioni et al., 2012), and a single study conducted in India, Croatia, and Nepal (Batra et al., 2018). Our results were similar to the scores in (Al-Saleh et al., 2018), in Riyadh SA, which recorded the scores of 25.30/48, 24.42/44, 19.80/32, 25.16/48, and 14.47/28, for SPL, SPT, SASP, SPA, and SSSP, respectively. In addition, no significant difference was detected among the five DREEM subscales, and this finding is consistent with those studies Thomas et al., 2009,Chandran and Ranjan., 2014; Al-Saleh et al., 2018; Jnaneswar et al., 2016).

The different demographic parameters of our participants and their relation to the total DREEM items and subscales are presented in Tables 1 and 4. No significant difference existed in terms of gender, age, monthly family income, and housing. This finding is consistent with those of previous studies. However, significant differences ($p = 0.042$) were observed between high school-graduate students in SASP likely because

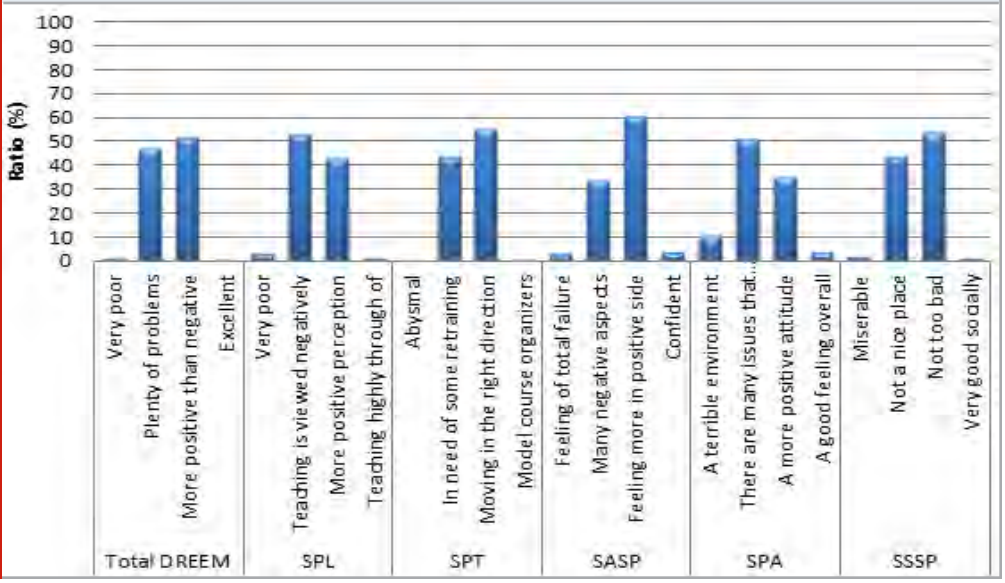
most of the students graduated from the government schools (81.3%). Moreover, significant differences were detected between the different student levels in the overall total DREEM and SPT and SPA. A similar finding was detected in Jnaneswar et al., (2015) in the SPT subscale. Gender did not significantly affect the EE of students' self-perceptions in this Turkish college. Similar findings were obtained among dental students in SA (Al-Saleh et al., 2018), in Greece (Kossioni et al., 2012), and New Zealand (Kang et al., 2017). However, gender differences were recorded in other DREEM studies conducted in India (Chandran and Ranjan., 2014 and Jnaneswar et al., 2015).

Table 4. Mean Score of DREEM based on the Demographic and Education characteristics of Dental Students (n = 171)

		Total DREEM	SPL	SPT	SASP	SPA	SSSP
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Gender	Male	100,97 ± 22,18	23,52 ± 5,85	22,78 ± 4,67	17,73 ± 4,43	22,14 ± 7,51	14,79 ± 3,72
	Female	100,19 ± 16,77	23,34 ± 5,25	22,63 ± 4,46	17,2 ± 3,67	21,91 ± 5,59	15,1 ± 3,39
	1p	0,795	0,834	0,831	0,404	0,819	0,575
Age	21–23	99,64 ± 20,27	23,24 ± 5,71	22,58 ± 4,43	17,31 ± 4,22	21,55 ± 6,56	14,96 ± 3,7
	24–26	102,5 ± 18,93	23,83 ± 5,31	22,97 ± 4,84	17,83 ± 3,85	22,98 ± 6,84	14,9 ± 3,31
	1p	0,373	0,514	0,606	0,435	0,184	0,918
Type of school	Government	99,37 ± 20,05	23,14 ± 5,54	22,6 ± 4,67	17,18 ± 4,21	21,68 ± 6,66	14,77 ± 3,52
	Private	105,97 ± 18,08	24,75 ± 5,57	23,19 ± 4,08	18,81 ± 3,28	23,56 ± 6,61	15,66 ± 3,7
	1p	0,090	0,140	0,516	0,042*	0,151	0,206
Educational	3	95,36 ± 18,67	22,05 ± 5,82	21,41 ± 4,22	16,79 ± 3,98	20,2 ± 5,9	14,91 ± 3,44
level	4	103,02 ± 20,75	23,91 ± 5,53	23,55 ± 4,65	17,82 ± 4,3	23,29 ± 6,7	14,45 ± 3,91
	5	103,3 ± 19,31	24,3 ± 5,2	23,17 ± 4,59	17,83 ± 4	22,6 ± 7,05	15,4 ± 3,34
	2p	0,053	0,070	0,029*	0,298	0,035*	0,366
Monthly	Below 3000	99,26 ± 17,64	23,6 ± 4,49	22,2 ± 4,93	17,2 ± 3,72	21,83 ± 5,96	14,43 ± 3,14
Family Income							
	Between 3-6000	101,7 ± 20,45	23,36 ± 5,71	23,13 ± 4,54	17,72 ± 4,36	22,64 ± 6,68	14,85 ± 3,66
	More than 6000	99,18 ± 20,35	23,48 ± 6,17	22,18 ± 4,29	17,18 ± 3,79	20,78 ± 7,19	15,58 ± 3,69
	2p	0,720	0,977	0,412	0,703	0,328	0,362
Housing	With family	100,94 ± 19,27	23,39 ± 5,38	22,8 ± 4,59	17,58 ± 3,91	22,2 ± 6,5	14,98 ± 3,49
	Student housing	99,66 ± 21,52	23,59 ± 6,14	22,48 ± 4,51	17,2 ± 4,62	21,57 ± 7,21	14,82 ± 3,8
	1p	0,714	0,834	0,691	0,599	0,592	0,801

1Student t test
2One-way ANOVA Test *p < 0.05

Figure 1: Percentages of the total score of DREEM and subscales



Regarding the registered DREEM score in the different levels, fourth- and fifth-year students recorded higher scores (103,02 ± 20,75 and 103,3 ± 19,31) than third-year students (95,36 ± 18,67). These values were entirely different from the DREEM score obtained by the second-year students in studies by Chandran and Ranjan., 2014; Bhosale U., 2015; Al-Saleh et al., 2018. These studies determined that second year students obtained the highest DREEM scores due to the total number of participants in their studies.

More than a quarter of the students who spent almost 2–3 years in this college scored in the problem areas. Hence, additional investigations must be conducted. The subscales were mainly observed in the learning and atmosphere subscales (SPL and SPA), whereas the lowest scores were obtained in 50 DREEM items, which indicated that the teaching was excessively teacher-centered, a good support system was available for stressed students, and students who were too tired to enjoy the course (Table 2). In this study, many DREEM items obtained scores of less than 2, but question numbers 3, 4, and 48 achieved the lowest scores. This finding is consistent with Idon et al., 2015 and Thomas et al., 2009, but inconsistent with those of Ostapczuk et al., 2012 and Kossioni et al., 2012, likely due to the number of changes and improvements in their study plans and the number of service years in the institute, which is only 10 years in our institute compared with other studies that have more years in academic service (Table 1).

Table 5. Summary of association between gender and educational characteristics [DOMINE] with the mean score of DREEM and subscale of dental students (n = 171)

Level of score based on domain		Gender n (%)		P value
	Male	Female	Overall	
Total DREEM				
Very poor	2 (2,2%)	0 (0%)	2 (1,2%)	0,585
Many problems	44 (47,8%)	36 (45,6%)	80 (46,8%)	
More positive than negative	45 (48,9%)	43 (54,4%)	88 (51,5%)	
Excellent	1 (1,1%)	0 (0%)	1 (0,6%)	
SPL				
Very poor	4 (4,3%)	1 (1,3%)	5 (2,9%)	0,358
Teaching is viewed negatively	50 (54,3%)	41 (51,9%)	91 (53,2%)	
More positive perception	36 (39,1%)	37 (46,8%)	73 (42,7%)	
Teaching highly through of	2 (2,2%)	0 (0%)	2 (1,2%)	
SPT				
Abysmal	1 (1,1%)	0 (0%)	1 (0,6%)	0,745
In need of some retraining	42 (45,7%)	33 (41,8%)	75 (43,9%)	
Moving in the right direction	48 (52,2%)	46 (58,2%)	94 (55%)	
Model course organizers	1 (1,1%)	0 (0%)	1 (0,6%)	
SASP				
Feeling of total failure	3 (3,3%)	2 (2,5%)	5 (2,9%)	0,466
Many negative aspects	26 28,3%)	31 (39,2%)	57 (33,3%)	
Feeling more in positive side	59 (64,1%)	44 (55,7%)	103 (60,2%)	
Confident	4 (4,3%)	2 (2,5%)	6 (3,5%)	
SPA				
A terrible environment	11 (12%)	7 (8,9%)	18 (10,5%)	0,463
There are many issues	45 (48,9%)	42 (53,2%)	87 (50,9%)	
that need changing				
A more positive attitude	31 (33,7%)	29 (36,7%)	60 (35,1%)	
A good feeling overall	5 (5,4%)	1 (1,3%)	6 (3,5%)	
SSSP				
Miserable	3 (3,3%)	0 (0%)	3 (1,8%)	0,531
Not a nice place	39 (42,4%)	35 (44,3%)	74 (43,3%)	
Not too bad	49 (53,3%)	43 (54,4%)	92 (53,8%)	
Very good socially	1 (1,1%)	1 (1,3%)	2 (1,2%)	

Fishe– Freeman–Halton test

The overall results of this study were consistent with those conducted in India (Chandran and Ranjan., 2014; Jnaneswar et al., 2016; Thomas et al., 2009), SA (Riyadh), Nigeria, New Zealand, and Greece (Al-Saleh et al., 2018; Idon et al., 2015; Kang et al., 2017; Kossioni et al., 2012), but inconsistent with those in Babar et al., 2015 in Malaysia, which concluded that stress was a major factor affecting their dental students because the items in their DREEM scale failed to monitor if the students were feeling stressed in relation to the different levels of studying, gender, monthly family income, housing, or graduated high school types.

The limitation of the DREEM scale and its subscales in relation to dental EEs is the exclusion of questions related to the dental educational program, including clinical requirements of students, such as filling of carious teeth, removable and fixed prostheses, extraction of badly broken down teeth, and root canal treatments, and a community program of services, including preventive programs of oral hygiene. These factors were ignored in the design of DREEM items and subscales.

CONCLUSION

The total DREEM mean score indicated a more positive than negative perception (50%) and many problems (48%), and the DREEM scores for the third, fourth, and fifth year students were 95,36 ± 18,67, 103,02 ± 20,75, and 103,31 ± 19.31, respectively. Gender, age, monthly family income, and housing types had no significant differences and did not influence the findings. However, a significant difference existed in SASP at $p = 0.042$ in the graduated school type and the different levels of education in SPT and SPA. Among the DREEM items, question number 28 ("I seldom feel lonely.") obtained the highest recorded value at 2.91 ± 1,04, whereas question number 3 ("A good support system is available for registrars who feel stressed.") obtained the minimum value at 1.2 ± 1,23. Both questions were found in the SSSP subscale.

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Antiseptic Efficacy of Combination of Certain Essential Oils Against Multi Drug Resistant Pathogens

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ABSTRACT

Incorporation of chemicals in the antiseptics and cosmetic products has increased. Natural antiseptics are non-toxic and ideal for human skin; they are eco-friendly and biodegradable. Numerous researches are focusing on herbal plant-based studies for biomedical therapeutic application. This study was designed to examine antiseptic potential of certain essential oils (Lemon grass oil, Cinnamon oil, Citronella, Cedar wood oil, Lemon oil, Lavender oil, Tea tree oil, Sandal wood oil, Basil oil. Essential oils (n = 9) were selected based on the previous studies, the selected essential oils were purchased from Naturoman Pvt. Ltd., Antimicrobial testing of the essential oils was done by CLSI standard, further the antiseptic application of essential oil was tested by American Society for Testing and Materials (ASTM) standards. In addition, Drug resistant clinical pathogens were used to screen antiseptic activity. The following drug resistant pathogens were isolated from our previous studies, Such as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Candida albicans*. Antioxidant property of essential oil by 2,2-diphenyl-1-picrylhydrazyl (DPPH). 9 essential oils were subjected to antimicrobial testing with drug resistant isolates from DFU, Cinnamon oil, Lavender oil, and Tea tree oil have broad-spectrum activity of 0.125-2µg/ml against drug-resistant *S. aureus E. coli, and C. albicans*. And it has 0.6-0.8µg/ml of antioxidant property done by DPPH method. ASTM standard such as time-kill assay, Anti-biofilm, and Real-life disinfectant efficacy testing showed promising activity for Tea tree oil and Cinnamon amalgamation. The present study showed the importance of essential oil alone and mixture in antiseptic and cosmetic applications. Thus, essential oil blend might be a prospective source of alternative antimicrobial agents and may play a vital role in antiseptic and skin care product development.

KEY WORDS: ANTISEPTIC, ESSENTIAL OILS, MULTIDRUG RESISTANT PATHOGENS.

ARTICLE INFORMATION

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INTRODUCTION

Antibiotic resistance is documented to be a serious problem that affects the choice of appropriate antibiotic therapy and increases the probability of unfavorable infection outcome. One of the proposed methods to cope with multidrug-resistant (MDR) bacteria is the use of alternative antibacterial treatments, which include natural antimicrobial substances such as plant essential oils (EOs) (Kon et al., 2012). In addition to dramatic events of this type is the ongoing struggle to identify new and effective antibiotics, especially against Gram-negative bacteria; new classes of effective antibiotics have not yet emerged and the prospects of this happening in the near future are low (Piddock et al.,). Clinicians frequently reach a therapeutic limit when treating patients with serious bacterial infections and efforts are being made worldwide to find new treatments for resistant bacteria. New approaches include educating the public, improving sanitation infrastructures, imposing strict regulations on antibiotic prescription, developing new antibiotics, reevaluating old and rejected antibiotics, abolishing the nontherapeutic use of antibiotics (e.g., in agriculture and animal feed), and developing nonantibiotic approaches that can successfully prevent and protect against infectious diseases, (Carlet et al., 2011; Elcocks (2020).

Herbal plants are considered as good antioxidant since ancient times. Aromatic and medicinal plants, through their secondary metabolism, provide a complex mixture of volatile molecules known as essential oils. These volatile molecules exert antibacterial activity that has been used in folk medicine for centuries. During the last few decades, the emergence of antibacterial resistance has forced us to search for new and efficient antimicrobial agents. Moreover, the use of essential oils and their components in combination with antibiotics may increase bacterial susceptibility, thus limiting resistance (Faleiro and Miguel 2013). Essential oils are known to possess potential as natural agents for food preservation. Many of them recently have been qualified as natural antioxidants and proposed as potential substitutes for synthetic antioxidants in specific sectors of food preservation where their use is not in contrast with their aroma (Ruberto & Baratta, 2000). An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule (Yamagishi et al., 2011). The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases, (Wu et al., 2011 Elcocks (2020).

The different antioxidant assays is available and, because results rely on different mechanisms, they strictly depend on the antioxidant models employed and on lipophilic/hydrophilic balance. A single-substance/ single-assay produces relative results, and it is perceived as a reductive approach whenever a phytocomplex is involved. Therefore, a multiple-test and a simultaneous chemical characterization must be taken into account whenever assays of essential oils are performed to allow a balance between the sensory acceptability and functional properties (Saccheti et al., 2005).

Hence this present study was undertaken to evaluate the antiseptic efficacy of essential oils

MATERIAL AND METHODS

Essential oils: The following nine essential oils (Lemon grass oil, Cinnamon oil, Citronella, Cedar wood oil, Lemon oil, Lavender oil, Tea tree oil, Sandal wood oil, Basil oil) Purchased from Naturoman Pvt. Ltd(table-1).

Table 1. Selected Essential oil for antimicrobial studies

s.no.	Common name	Botanical Name	Part used
1	Lemon	*Citrus limonum*	Fruit skin
2	Basil	*Ocimum basilicum*	Leaf
3	Cinnamon Bark	*Cinnamomum zeylanicum*	Bark
4	Lavender	*Lavandula officinalis*	Flower
5	Citronella	*Cymbopogon nardus*	Grass
6	Pepper Mint	*Mentha piperita*	Leaf
7	Lemon grass	*Cymbopogon citratus*	Grass
8	Cedar wood	*Cedrus libani*	Wood
9	Eucalyptus	*Eucalyptus polybractea*	Leaf

Antimicrobial Susceptibility test: Preliminary Screening of essential oils for antimicrobial characterization was done by the agar well diffusion method. All the test pathogen with appropriate standard cultures (Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli*, ATCC 35218, *Klebsiella pneumoniae* ATCC 700603 and *Candida albicans* ATCC 90028), were prepared according to McFarland standard. The 18 h bacterial cultures were adjusted to approximately 105CFU/ml with sterile saline solution. Lawn culture were made on Mueller-Hinton agar using a sterile cotton swab for uniform microbial growth. The essential oils were diluted in DMSO with 0.5% Tween 80. Well were made at 6mm diameter in inoculated MHA agar plate. A standard mineral oil and 5µg concentration of Ciprofloxacin was used as positive control. Then the plates were incubated at 37°C for overnight. After incubation, the zone of inhibition was measured with standard procedures.

Minimum Inhibitory concentration were performed 9 oils based on preliminary antimicrobial results such as Cinnamon, lemon, lemon grass, and tea tree oil were identified to have potent antimicrobial activity and their Minimum Inhibitory Concentrations (MIC) were determined. The standard broth dilution method followed by the Clinical Laboratory standard institute, 2018. Standard two fold dilution method followed for

each oil, ranging from 1 to 512 µg/ml, was prepared in Muellur Hinton broth. 0.5 McFarland standard broth culture suspension were prepared and inoculated in individual MIC tube, Inoculated tubes were incubated at 37°C for overnight for bacteria for fungi 24-72 hrs based. The MIC were determined as the lowest concentration of oil inhibiting visible growth of each organism on the tube.

Organisms used: The bacterial test strains used in this study were Staphylococcus aureus ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Candida krusei* ATCC 6258, *Candida albicans* ATTC 10231. clinical pathogens were used to screen antiseptic efficacy, the pathogens were isolated from my previous studies, it was stored and maintained, Drug resistant *S. aureus* (31), *E. coli* (11), *K. pneumoniae* (07) and *C. albicans* (08). The bacterial strains were maintained on 60% glycerol BHI broth at -200C in the Lingam Microbiological Laboratory, Kanchipuram.

Synergistic effect of Essential oil: Effect of Tea tree oil and cinnamon oil; Tea tree oil and Lemon grass oil mixture were tested for synergistic activity against routine bacterial, viral and fungal pathogens. This study could serve as a baseline data to investigate new combination of essential oil for antiseptics.

Antioxidant activity of essential oil by DPPH method: The hydrogen atoms or electrons donation ability of the corresponding extracts and some pure compounds were measured by bleaching the purple colored methanolic solution of DPPH. The effects of methanolic extract and essential oil on DPPH radicals were evaluated according to a method described elsewhere (21). 4 mL samples of various concentrations of the extracts in methanol were separately added to a 1 mL solution of DPPH radical in methanol (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allowed to stand for 30 min after which the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer.

Inhibition of free radical DPPH as percentage [I(%)] was calculated as follows:

$$I\ (\%) = 100 \times (A\text{blank} - A\text{ sample}) / A\text{blank}$$

where A blank is the absorbance of the control (containing all reagents except the test compound) and A sample is the absorbance of the test compound. EC50 value (µg /mL) is the effective concentration at which DPPH radicals are scavenged by 50%. This was obtained by interpolation and using linear regression analysis.

Time kill assay for the essential oil blend: Sterile 96 well microtitre plates were used test with Muller Hinton broth for the determination of the time kill assay. Briefly, 180 µl of sterile freshly prepared MH broth was added in each well. 10µl of essential oil were added to the media. 10 µl of the standard bacterial strains was inoculated in test medium. Every 30mins, killing activity was measured by spot culture in new plate MHA, this procedure repated every 20mins for 6 hrs.

Anti-biofilm assays: Antibiofilm activity was carried out in polystyrene microtitre plate method. An overnight culture of standard strains was diluted to the McFarland standard 0.5. After the addition of different concentrations of Essential oil1-128 µl/ml of Muller Hinton broth, 10-µl broth culture was inoculated in all tube except negative control. incubated for 48 to 72 h at 37^0C. Thereafter Planktonic cells of Standard strains is discarded, 100µl of 1% (w/v) aqueous solution of crystal violet will be added to microtitre plate and incubated at room temperature for 20 minutes. Then crystal violet solution is removed from the microtitre plate and it will be washed thoroughly with sterile water. For quantification of attached cells, the bound crystal violet solubilized in dimethyl sulfoxide (DMSO) and spot culture method.

RESULTS AND DISCUSSION

Antimicrobial assay: Agar well Diffusion method: Lemon grass oil showed highest antimicrobial activities against *C. krusei*_ATCC 6258 (19 mm) followed by *E. faecalis*_ATCC 29212 (18mm), *E. coli*_ATCC 25922 and *P. aeruginosa*_ATCC 27853 (17mm), *S. aureus*_ATCC 29213 (13mm) and minimum inhibition against *C. albicans*_ATTC 10231 (12mm).*Cinnamon oil* showed highest antimicrobial activities against *S. aureus*_ATCC 29213 (30mm), *E. coli*_ATCC 25922 and *E. faecalis*_ATCC 29212 (25mm), *C. albicans*_ATTC 10231 (20mm), *C. krusei*_ATCC 6258 (18 mm) and minimum inhibition against *P. aeruginosa*_ATCC 27853 (16mm).*Citronella oil* showed highest antimicrobial activities against *E. faecalis*_ATCC 29212 (12mm) followed by *P. aeruginosa*_ ATCC 27853 (11mm) and minimum inhibition against *S. aureus*_ATCC 29213 (7mm). other organisms not shown any activities against other organisms .Cedar wood oil showed highest antimicrobial activities against *E. coli*_ATCC 25922 (11mm) and minimum activities against *S. aureus*_ATCC 29213 (10mm).

Lemon oil showed highest antimicrobial activities against *S. aureus*_ATCC 29213 (24mm), *P. aeruginosa*_ ATCC 27853 (21mm) *E. coli*_ATCC 25922 (20mm), *E. faecalis*_ATCC 29212 (17mm), *C. albicans*_ATTC 10231 (15mm) and minimum inhibition activities against *C. krusei*_ATCC 6258 (13 mm).Lavender showed highest antimicrobial activities against *E. faecalis*_ATCC 29212 (12mm), *P. aeruginosa*_ATCC 27853 and *C. krusei*_ATCC 6258 (11 mm), *E. coli*_ATCC 25922 and *C. albicans*_ATTC 10231 (10mm) and minimum inhibition activities against *S. aureus*_ATCC 29213 (9mm).Tea tree oil showed highest antimicrobial activities against *S. aureus*_ATCC 29213 (35mm), followed by *C. albicans*_ATTC 10231 (32mm), *C. krusei*_ATCC 6258 and *E. coli*_ATCC 25922 (28mm) *E. faecalis*_ATCC 29212 (26mm) and minimum inhibition activities against *P. aeruginosa*_ATCC 27853 (25mm). Sandal wood oil showed highest antimicrobial activities against *E. coli*_ATCC 25922 (11mm), *E. faecalis*_ATCC 29212 (10mm) and minimum inhibition activities against

*S. aureus*_ATCC 29213 (7mm).Basil oil showed highest antimicrobial activities against *S. aureus*_ATCC 29213 (11mm), *C. krusei*_ATCC 6258 (10 mm) and minimum inhibition activities against *P. aeruginosa*_ATCC 27853 (9mm) (table-2)

Table 2. Antimicrobial property of essential oils

Microbes	Agar well diffusion assay									Minimum Inhibitory Concentration(µL)			
	Lemon grass	Cinnamon oil	Citronella oil	Cedar wood	Lemon oil oil	Lavender	Tea tree	Sandal wood oil	Basil oil oil	Lemon grass	Cinnamon oil	Lemon oil	Tea tree oil
S. aureus ATCC 29213	13	30	7	10	24	9	35	7	11	64	1	32	0.5
E. coli ATCC 25922	17	25	-	11	20	10	28	11	-	16	1	64	1
P. aeruginosa ATCC 27853	17	16	11	-	21	11	25	-	9	64	2	128	1
E. faecalis ATCC 29212	18	25	12	-	17	12	26	10	-	32	1	64	0.12
C. krusei ATCC 6258	19	18	-	-	13	11	28	-	10	-	-	-	-
C. albicans ATTC 10231 Multi drug Resistant diabetic foot ulcer isolates	12	20	-	-	15	10	32	-	-	16	4	128	1
MRSA	11	32	-	-	18	7	28	-	10	128	0.5	64	0.5
P. aeruginosa	14	30	6	-	21	8	28	15	-	64	1	64	2
E. coli	13	20	11	-	20	10	25	-	9	32	0.5	128	0.5
K. pneumoniae	12	22	-	8	14	8	26	-	-	64	1	128	0.25
C. albicans	14	19	-	-	13	9	28	9	10	32	8	64	1

Effect of essential oil with Multi drug Resistant isolates : Lemon grass oil showed highest antimicrobial activities against *P. aeruginosa* and *C. albicans* (14mm) followed by *E. coli* showed (13mm), *K. pneumonia* (12mm) and minimum inhibition activities against MRSA (11mm). Cinnamon oil showed highest antimicrobial activities MRSA (32m), followed by *P. aeruginosa* (30mm), *K. pneumonia* (22mm), *E. coli* (20mm) and minimum inhibition activities against *C. albicans* (19mm). *Citronella oil* showed highest antimicrobial activities *E. coli* (11mm) and minimum inhibition activities against *P. aeruginosa* (6mm).Cedar wood oil showed highest antimicrobial activities *K. pneumonia* (8mm). Other organisms not shown any activities. Lemon oil showed highest antimicrobial activities *P. aeruginosa* (21mm) followed by *E. coli* (20mm), MRSA (18mm), *K. pneumonia* (14mm) and minimum inhibition activities against *C. albicans* (13mm).Lavender oil showed highest antimicrobial activities *E. coli* (10mm) followed by *C. albicans* (9mm), *P. aeruginosa* and *K. pneumonia* (8mm) and minimum inhibition activities against MRSA (7mm).Tea tree oil showed highest antimicrobial activities MRSA, *P. aeruginosa* and *C. albicans* (28mm), followed by *K. pneumonia* (26mm), minimum inhibition activities against *E. coli* (25mm).Sandal wood oil showed highest antimicrobial activities *P. aeruginosa* (15mm) and minimum inhibition activities against *C. albicans* (9mm).Basil oil showed highest antimicrobial activities MRSA and *C. albicans* (10mm) and minimum inhibition activities against *E. coli* (9mm)

Table-3. Anti-biofilm activity of essential oil by viability

Microbes	Essential oil (µL/mL)			
	Lemon grass oil	Cinnamon oil	Lemon oil	Tea tree oil
S. aureus ATCC 29213	16	2	128	4
E. coli ATCC 25922	16	2	128	2
P. aeruginosa ATCC 27853	128	2	128	4
E. faecalis ATCC 29212	64	2	32	2
C. krusei ATCC 6258	-	-	-	-
C. albicans ATTC 10231	64	8	128	4

Oil Blend: Cinnamon oil: Tea tree oil=1:0.25; 1:0.5; 1:1; 0.5:1; and 1:0.25

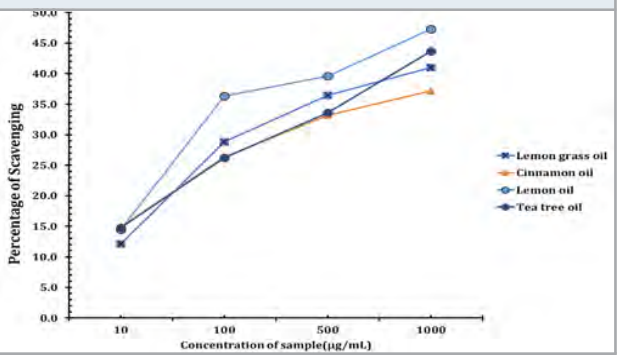
Minimum Inhibitory Concentration: Based on the antimicrobial activities Lemon grass oil, Cinnamon oil, Lemon oil and Tea tree oil taken for the Minimum

Inhibitory Concentration. Lemon grass oil showed maximum Minimum Inhibitory Concentration values against *S. aureus*_ATCC 29213 and *P. aeruginosa*_ATCC 27853 (64 µL) followed by *E. faecalis*_ATCC 29212 (32 µL), *E. coli*_ATCC 25922 and *C. albicans*_ATTC 10231 (16 µL).Cinnamon oil showed maximum Minimum Inhibitory Concentration values against *C. albicans*_ATTC 10231 (4µL), *P. aeruginosa*_ATCC 27853 (2µL), *S. aureus*_ATCC 29213, *E. coli*_ATCC 25922 and *E. faecalis*_ATCC 29212 (1µL),Lemon oil showed maximum Minimum Inhibitory Concentration values against *P. aeruginosa*_ATCC 27853 and *C. albicans*_ATTC 10231 (128 µL), *E. coli*_ATCC 25922 and *E. faecalis*_ATCC 29212 (64µL), *S. aureus*_ATCC 29213 (32 µL).Tea tree oil showed maximum Minimum Inhibitory Concentration values *E. coli*_ATCC 25922, *P. aeruginosa*_ATCC 27853 and *C. albicans*_ATTC 10231 (1 µL), *S. aureus*_ATCC 29213 (0.5 µL), *E. faecalis*_ATCC 29212 (0.12µL) (table-2)

Table 4. Minimum Inhibitory concentration of essential oil Blend-

	Tea tree oil:	cinnamon oil	Essential oil	blend	(µL/mL)
Microbes	1:0.25	1:0.5	1:1	0.5:1	0.25:1
S. aureus ATCC 29213	16	1	1	2	2
E. coli ATCC 25922	16	0.5	0.5	1	0.5
P. aeruginosa ATCC 27853	128	1	0.5	4	2
E. faecalis ATCC 29212	64	2	0.12	0.5	1
C. krusei ATCC 6258	-	-	0.5	2	2
C. albicans ATTC 10231	64	4	0.25	2	4

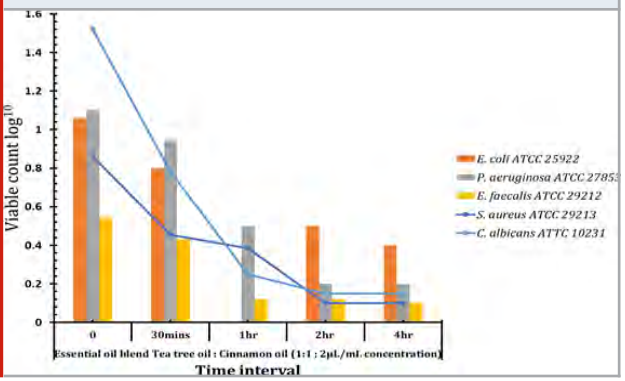
Figure 1: Antioxidant activity of essential oil by DPPH method



Effect of essential oil with Multidrug resistant isolates Based on the antimicrobial activities of Multi drug Resistant diabetic foot ulcer isolates.

Lemon grass oil, MRSA (128 µL) *P. aeruginosa* and *K. pneumonia* (64 µL) , *E. coli* and *C. albicans* (32 µL)) Cinnamon oil *C. albicans* (8µL), *P. aeruginosa* and *K. pneumonia* (1µL), MRSA and *E. coli* (0.5 µL) Lemon oil- *E. coli* and *K. pneumonia* (128µL), MRSA, *C. albicans* and *P. aeruginosa* (64 µL) Tea tree oil- *P. aeruginosa* (2 µL), *C. albicans* (1 µL) MRSA and *E. coli* (0.5 µL), *K. pneumonia* (0.25 µL)

Figure 2: Time kill assay for the essential oil blend



Antioxidant and Time kill assay: Antioxidant of essential oil was shown in figure 1. Lemon oil showed Highest percentage of anti-oxidant activities when compare with other oil. Time kill assay for the essential oil blend show in figure 2. Better growth suppression or killing assay was started on 30 mins above at the concentration of 2 µL of 1:1 Cinnamon oil and Tea tree oils. Recent study found cinnamon oil alone exhibited a broad-spectrum activity against Gram-negative and Gram-positive bacteria and showed bacteriostatic and bactericidal effects against *P. aeruginosa* PAO1 at 2%, including known multidrug resistant species. The number of scientific studies on the antimicrobial activity of plant essential oils has strongly grown over the last three decades. Due to the use of many different microbiological methods for susceptibility testing and different definitions of antimicrobial activity, the comparability of studies on essential oils is often critical. Many studies focus on selected EOs, providing insight into their activity against one or more microorganisms, but only few publications compress information by testing multiplicities of essential oils with a defined single method. Therefore, this study was conducted to investigate as many EOs as possible with a single quantitative microbiological method, to achieve maximum comparability.

CONCLUSION

The present study showed the importance of essential oil alone and mixture in antiseptic and cosmetic applications. Thus, essential oil blend might be a prospective source of alternative antimicrobial agents and may play a vital role in antiseptic and skin care product development.

Authors Contributions: All authors have equal contribution in bringing out this research work.

Conflict of Interest: None.

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Adventurism in Herbal Medicine the Only Option for Economically Challenged Indian Cancer Patients: Perspective Based on Case Histories

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ABSTRACT

Due to lack of awareness about cancer, shortage of oncologists & infrastructure, and above all, financial crunch compels many Indian patients to avoid conventional treatment or report to a clinic with advanced malignant disease. As a result, curative treatment is not an option in most of the cases. Thus, many patients have to depend on some form of alternative therapy for the treatment of cancer. One of the most sought after therapy is polyherbal medicine. We follow-up two suspected cancer patients trying an alternative polyherbal therapy HUMA. This polyherbal therapy comprises of various important anti-cancer herbs described in Ayurvedic literature viz. *Azadirachta indica, Curcuma longa, Embelica officinalis, Ocimum sanctum, Semecarpus anacardium*, and *Tinospora cordifolia*, among others. Both the patients were immensely benefited by this polyherbal therapy. Complete regression of the disease/ tumour was observed in both the patients. In the absence of any conventional therapy, the disease regression observed could be attributed to alternative polyherbal therapy. As a large number of terminal cancer patients try various herbal therapies all over the globe; hence, proper research attention should be given in this area.

KEY WORDS: ALTERNATIVE CANCER, HUMA, POLYHERBAL THERAPY, CAM.

INTRODUCTION

India faces a major disease burden from cancer (Dhillon et al., 2018). This non-communicable disease carries high levels of mortality and disability and requires expensive treatments. The cancer scenario in India is quite alarming. India has some of the highest cancer rates in the world (Mudur, 2005). Due to lack of awareness about cancer, shortage of oncologists & infrastructure (Khan, 2018), and above all, financial crunch (Mahal et al., 2013) compels many Indian patients either to avoid conventional treatment or report to a clinic with advanced malignant disease (Pal, 2002). As a result, curative treatment is not an option in most of the cases. Moreover, the expense of conventional treatment is not always within the reach of many patients compelling them to abandon it and venture elsewhere in search of treatment. The financial toxicity of cancer treatment is now a well-recognized problem in cancer (Gyawali, 2017). Continued advances in fundamental immunology, genetic engineering, gene editing, and synthetic biology exponentially expand opportunities to enhance the sophistication of immune

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cell therapies, increasing potency and safety and broadening their potential for the treatment of disease, (Weber et al., 2020). However, these advances in molecular medicine will come at a price that is far from the reach of the common people of our country. Be it India or Africa cancer patients across the globe depend on some form of herbal therapy (Omara et al., 2020).

Medicinal plants are considered to be one of the most popular alternative therapeutic modalities for cancer patients (Gratus et al., 2009) and may also be cost-effective (Chaudhary et al., 2015). India has a very long, safe, and continuous usage of many herbal drugs in the officially recognized alternative systems of health (Vaidya and Devasagayam, 2007).

Medicinal plants hold a special place in India. Herbal medicine in India has been refined by thousands of years of practical application and experience. In India, a substantial proportion of the population uses herbal drugs for their primary health care needs (WHO, 2003). After conventional treatment fails to cure chronic malignancy the biggest predicament of cancer patients and their caregivers is the acceptance of the grim reality of death. Most of the patients and their caregivers are not likely to give up easily and may end up trying some alternative herbal therapy. In some instances, the patients often do not disclose the use of alternative therapy (Sanford et al., 2019). However, for many Indian patients, conventional therapy is a luxury which they just cannot afford. In this article, the follow up of two suspected cancer patients who tried a polyherbal therapy 'HUMA' for treatment is described.

HUMA Therapy: This polyherbal formulation for the treatment of cancer was first advocated by Dr. S M Atiq in Lucknow in the late 80s and now over 500 cancer patients try this therapy every year (Pal, 2013). The polyherbal formulation comprising of various anti-cancer herbs viz. *Azadirachta indica, Curcuma longa, Embelica officinalis, Ocimum sanctum*, *Semecarpus anacardium*, and *Tinospora cordifolia*, among others. This polyherbal medicine is orally administered and well tolerated by patients. Instances of adverse side effects are less due to individual dosing of the medicine. The dose of the medicine is not fixed; rather it is titrated according to the patient's condition and maybe one of the main reasons for the lack of adverse effects. Though most of the patients try this polyherbal therapy mainly for palliation; however, there are some reports of complete regression of cancer/tumour, especially oral tumours with HUMA (Pal and Fatima, 2014; Pal and Fatima, 2017a).

Case 1: A 74 year old lady presented with allergic bronchitis on April 15, 2003. She had an earlier history of Chronic Obstructive Pulmonary Disease (COPD); however, her abdominal USG and chest X-ray were normal. She was advised and continued allopathic medication for the next 4 months. Two months later she started having difficulty swallowing solid as well as liquid food. The patient then underwent a barium swallow test on May 23, 2003. The report indicated irregularity in the lateral wall of thoracic esophagus with proximal dilation of cervical esophagus suggestive of esophageal carcinoma. In view of old age and financial problems the caregivers of the patient did not consent for any curative therapy. Hence, a biopsy was not done. She was on palliative therapy till October 2003, then her health condition deteriorated drastically and she became very sick. She was then admitted to Lucknow Cancer Institute on October 05, 2003, for supportive and palliative treatment. On October 10, 2003, the patient again underwent a barium swallow test.

The study revealed a tracheoesophageal fistula at D1 vertebral level with the opacification of tracheo bronchial tree. Barium study also revealed the growth of tumor extended to the C6 vertebral level. Covered metallic stents were placed from C7 vertebral (Lower end) and extended below to cover the fistula. The patient was advised radiotherapy; however, the patient was not in a position to undergo any conventional therapy. She opted for the polyherbal therapy mainly for palliation from October 15, 2003. When the therapy started the patient was anorexic and cachexic. Remarkable clinical improvement was noticed in the patient after the start of therapy. Within 15 days the patient regained her normal appetite. The next 6 months were relative event free. In the first week of April 04 the patent started having breathing problems. She underwent a CT scan of the chest and upper abdomen on May 10, 2004. The study revealed no mass/lesion around the two metallic stents placed in the esophagus (Figure 1). Her problem was managed with allopathic medicines. Two months after this incidence the patient expired due to cardiorespiratory arrest.

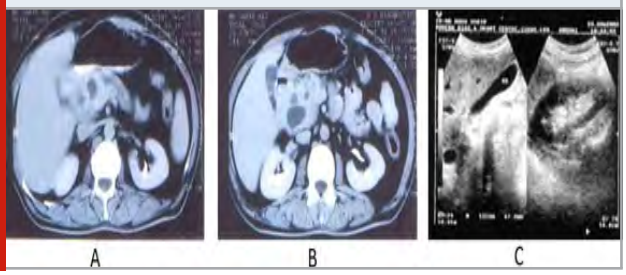
Case 2: A 43 year old male who presented with acute epigastric pain radiating to his back on February 26, 1998. An abdominal ultrasound dated March 1, 1998 revealed marked dilatation of pancreatic duct and findings suggestive of an acute exacerbation of chronic pancreatitis. The patient was offered and received conventional therapy for 2 months; however, his condition did not improve. An abdominal ultrasound dated May 2, 1998 revealed a slightly bulky pancreas with an irregularly dilated pancreatic duct with bulky head of pancreas suggested of either chronic pancreatitis or carcinoma at the head of pancreas. He was advised to undergo an ERCP and was given an enzyme supplement for the treatment of chronic pancreatitis. Despite medication his upper abdominal pain continued to increase. An abdominal CT scan dated October 7, 1998 indicated a pancreatic head neoplasm with multiple cystic components, dense calcification, dilated pancreatic duct and atrophic pancreatic body and tail regions. A CT guided biopsy of the pancreatic tumor was suggested.

The suggested treatment plan involved surgery; however, the patient declined because of his financial constraints. He was a motorcycle repair mechanic and the money required for conventional treatment was beyond his reach. Instead he opted for the polyherbal HUMA therapy from November 10, 1998. A gradual improvement in

his condition was noted after the start of polyherbal therapy. After about 3-4 months of therapy, his appetite improved, the abdominal pain was gone, and he resumed normal activities. He continued the herbal therapy for 11 months and then stopped the therapy. An abdominal ultrasound dated September 20, 2003 was reported as normal aside of some fatty changes in the liver (Figure 2). We followed the patient for the next 5 years and he was leading a normal life.

Figure 1: Treatment of esophageal tumor with polyherbal therdapy HUMA A- Barium swallow test; B- Esophageal stenting; C- CT scan after 6 months

Figure 2: Treatment of pancreatic tumor with polyherbal therapy HUMA A & B- CT scan at presentation; C- USG after 5 years



DISCUSSION

In the absence of any conventional therapy, the disease regression observed in both the patients could solely be attributed to the alternative polyherbal therapy. Though the biopsy of these patients could not be performed; however, complete regression of cancer was observed with HUMA in many other patients (Pal and Fatima, 2017a; Pal & Fatima, 2014; Pal, 2013; Pal et al., 2013). In few terminal cancer patients prolongation of survival time was also note with HUMA (Pal & Fatima 2017b).

In India, not all cancer patients have the luxury to undergo conventional therapy or allopathic follow-up. When cancer struck, many have to search for their own treatment options, be it herbal, traditional, local, or black magic. Many patients try all these therapies out of financial compulsion and in some when conventional therapy fails to cure their cancer. In the first case the caregivers of the patient did want conventional therapy due to the advanced age of the patient and in the second case finance was again the main constrain. Accessing good cancer care is a big issue for many living in the developing world (Jack, 2014). The cost of treatment for advanced cancer may cost millions of rupees a year. Only a few patients are able to bear the cost (Goyal, 2014). Government health schemes like Yashaswini, Arogyashri, and Vajpayee Arogya Yojana did not cover cancer (Anonymous, 2014). However, the recently introduced 'Ayushman Bharat' the National Health Protection Scheme (NHPS), will now cover the cancer treatment cost including radiation and chemotherapy which do not need hospitalization (Sharma, 2018).

People have used herbal medicines throughout our human history and they are currently the most commonly used medicines worldwide. Herbal medicines are in great demand in the developed world for primary health care due to their efficacy, safety, and lesser side effects. According to WHO about 70 – 80 % of the people in African and Asian countries are primarily dependent on the herbals of treatment of various diseases (Vijayaalakshmi, 2015). Medicinal plants maintain the health and vitality of individuals and also cure various diseases, including cancer without causing toxicity. These medicinal plants possess good immunomodulatory and antioxidant properties, leading to anticancer activities. The antioxidant phytochemicals protect the cells from oxidative damage (Patil et al., 2013).

The herbals that are presently used for treatment are also known for their anti-cancer activity viz *Azadirachta indica* (Al Saiqali et al., 2018), *Curcuma longa* (Willenbacher et al., 2019), *Embelica officinali*, (Baliga and Dsouza, 2011), *Ocimum sanctum* (Baruah & Kma, 2019) *Semecarpus anacardium* (Joseph et al., 2013), and *Tinospora cordifolia* (Ansari et al., 2017). As compared to conventional chemotherapy the polyherbal therapy HUMA is not only less expensive but also produced lesser side effects (Pal, 2013).

Cancer arises from various genetic defects, either in isolation or familial (Hannah-Shmouni and Stratakis, 2020). Increased levels of telomerase are found in the vast majority of human cancers (Roake and Artandi, 2020). For the successful treatment of cancer, there is a need for an altogether different approach that can target the individual cancer cells at a molecular level. Cell therapies present an entirely new paradigm in drug development. Within this class, immune cell therapies are among the most advanced, having already demonstrated definitive evidence of clinical benefits in cancer and infectious disease. Continued advances in fundamental immunology, genetic engineering, gene editing, and synthetic biology exponentially expand opportunities to enhance the sophistication of immune cell therapies, increasing potency and safety and broadening their potential for the treatment of disease (Weber et al., 2020). However, these advances in molecular medicine will come at a price that is far from the reach of the common people of our country.

Be it India or Africa (Omara et al., 2020) cancer patients across the globe depend on some form of herbal therapy. Two important commercial anti-cancer drugs vincristine

and vinblastine are derived from a plant *Catharanthus roseus* (L.) G. Don (Nobili et al., 2009). Anti-cancer activity is reported in many plants; however, the proper clinical trial is lacking in herbal medicine due to a lack of standardization of the medicine. The anti-cancer herbals that are used by patients are often very toxic in nature. Hence, small doses and frequent titration of the medicines are required which makes them unsuitable for the clinical trial. Many herbal therapy created tremendous interest in the past. Essiac is one of the most popular herbal cancer alternatives in North America. PC-SPES is one of the most studied herbal therapies in prostate cancer. It is comprised of a combination of eight herbal compounds (Pal and Fatima, 2014). Mistletoe (*Viscum album* L.) still continues to be the medical herb prescribed most frequently for cancer patients in German-speaking countries (Rostock, 2020).

In India, Carctol an alternative herbal cancer therapy developed by Dr. Nandal Tiwari is quite popular. This herbal therapy is composed of 8 different herbs viz. *Hemidesmus Indicus, Tribulus Terrestris, Piper Cubeba* Linn, *Ammani Vesicatoria, Lepidium Sativum* Linn, *Blepharis Edulis, Smilax China* Linn and Rheum emodi Wall. (Pal, 2013). A dietary regimen 'Sarvapasti' developed by the scientist of D S Research, Varanasi has also become a very popular alternative therapy for cancer patients. Sarvapasti contains many important medicinal herbs (Ghosh, 2002). Maharishi Amrit Kalash [MAK] produced by the 'Maharishi Ayurvedic Products' is a polyherbal preparation of many important Ayurvedic herbs. This formulation was shown to be effective in patients undergoing chemotherapy (Maharishi AyurVeda).

CONCLUSION

Due to ignorance and lack of effective cancer screening process many treatable cancer patients land in the cancer clinics with advanced disease when virtually not curative treatment is possible (Broom et al., 2009). Waiting time for cancer treatment is more in India due to a lack of infrastructure and treatment facilities. Above all, financial problems will push many cancer patients to alternative practitioners with no allopathic qualifications and having limited knowledge about oncology. From the present observation, it can be concluded that not all alternative herbal cancer therapies may be evil. Some like HUMA may show some beneficial effect whereas others may be completely bogus. The need of the hour is a comprehensive dialogue between practitioners of different systems of medicine so that the good therapies are identified and the bad ones are weeded out. The overall aim should be to offer affordable evidence-based best cancer treatment and palliation to economically challenged patients in a developing country like India.

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Conflict of interest: Dr. S Hina Fatima is the practicing clinician and President of the Huma Cancer Society, Lucknow.

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Identification of Parkinson's Disease Using Machine Learning Algorithms

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ABSTRACT

Parkinson's disease is Progressive nervous system disorder. It affects movement of the human beings. Symptoms starts gradually. The result of syndrome is the patient is not able to do the activities like talking, strolling, and tremor during motion. Normally the physicist identified this disease using two scales are Hoehn and Yahr scale and Unified Parkinson's Disease Rating Scale. There are so many features in the dataset. Audio signal is one of features taken in the dataset from UCI dataset repository. Parkinson's disease patient has a low-volume noise with a monotone quality. In This system different audio signals like jitter, simmer, New Human Revolution (NHR), Multidimensional Voice Program (MDVP) are given as a train and test data. MinmaxScale method is used for preprocessing the data. Threshold value and correction coefficient of audio data are played as a parameters of feature selection. The Machine Learning classifiers are utilized to identify the disease. In our model we employed Logistic regression and eXtreme Gradient Boosting (XGBoost) classifiers for classification. Among twenty one features only twelve played as an important role for predicting the disease. The system has achieved result in predicting whether the Parkinson's disease patient is healthy or not. The performance of machine learning classifier XGBoost provided the accuracy of 96% and the Matthews Correlation Coefficient (MCC) of 89%.

KEY WORDS: MULTIDIMENSIONAL VOICE PROGRAM MATTHEWS CORRELATION COEFFICIENT PARKINSON 'S DISEASE, XGBOOST.

INTRODUCTION

Parkinson's disease is described as a neuro degeneration disorder which is death of dopamine generating cells (Jankovic et al 2008). The loss of dopaminergic neurons in the mid brain decrease the achievable rate of communication. Parkinson disease affects central nervous system which leads to the effect in motor system, the main PD symptoms are tremor, rigidity and movement disorders, (Ramezani et al 2017).

The people who are having Parkinson's Disease mostly 90% of them have a speech impairment, only 3% to 4% of PD patient receives speech therapy and also only one of the most important factor for PD is age, the patient of PD are most of them are aged between 45-60, (Levine et al 2003). The speech of PD patient have change in the frequency specter in their voice because they loss the control of the limb, which decrease the frequency of the audio. So, the low frequency region gives important data to differentiate the speech impairments in PD. Unified

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Parkinson disease rating scale (UPDRS) is used to find the severity of the PD by help of clinical expertise and experience (Dobson et al 2008).

Centre for Machine Learning and Intelligent system (2009) given that we perform a feature selection for the audio features dataset created by Max Little of the University of Oxford, high prediction has been achieved with classification accuracy, algorithm predict various accuracy for various variables that are relevant on the other attributed present in the feature dataset, as feature plays important role the dataset which we taken from UCI repository contains 21 features and applied a Pearson's correlation coefficient on feature to determine the coefficient correlation among features.

Neharika et al (2020) have given the Multi-Dimensional Voice Program (MDVP) is a computer program that can calculate as many as 33 acoustic parameters from a voice sample. It is standard. Dobson et al (2008) presented this section presents the comparative determination endeavors, here both model-based and model-free techniques algorithms are used for predicting Parkinson's disease. Rätsch et al (2001) have presented that most commonly used model-based tool is Logistic regression which it measures the outcome on a binary scale (e.g. healthy/not), here classification process carried out based on the estimation probabilities. Whereas model-free methods like XGBoost adapt to the intrinsic.

Fietzek et al (2020) given the high dataset size requirements are met through a supervised data collection approach by which we were able to generate informative annotations in one-minute intervals. To our knowledge, collecting expert annotations on a one-minute basis has not been reported to date at such a large scale. Abós et al (2017) described that data characteristic without any priori model. We used XGBoost algorithm for classification, XGBoost algorithm benefit from constant learning or retraining, they don't guarantee optimized classification/regression. However, when trained and maintained, XGBoost learning method have great potential than Logistic regression in solving real world problems. The prior report of using XGBoost technique to diagnose Parkinson's disease are determines according to their cognitive status.

XGBoost provided an accuracy of 96% for classification the dataset and logistic regression provided an accuracy of 79%, this system that predict PD has been formulated which compares the accuracy of LR and XGBoost on the train and test dataset. It utilized co-efficient correlation to find the correlation among features, on comparison it provided that XGBoost performed better than LR with accuracy of 96%. Mohammad et al (2014) performed a comparative analysis to detect Parkinson Disease using various classifiers like Support vector Machine (SVM), Random Tree (RT), feed forward back-propagation Artificial Neural Network (FBANN) classifiers are utilized in this system. Geetha et al (2011) presented a comparison was made between the classifiers to differentiate between PD and Healthy persons and the study has the dataset contains 195 voice samples and consist of both male and female. The dataset has 23 PD patient and healthy, by comparing all the classifiers, FBANN classifier has achieved 97.37% accuracy.

Srilatha et al (2019) have presented Classification is an important task within the field of computer vision. Image classification refers to the labelling of images into one of a number of predefined categories that includes image sensors, image pre-processing, object detection, object segmentation, feature extraction and object classification. Many classification techniques have been developed for image classification. The highest concentration is on using various classifiers combined with several segmentation algorithms for detection of tumor using image processing. Shraddha et al (2019) have proposed as Performance parameters used by authors are true positive, true negative and accuracy. Authors make use of various semi-supervised classifiers for intrusion detection. All classifiers used NSL KDD dataset for intrusion detection.

Ramani et al (2011) discussed a system to classify PD and Non-PD patient was proposed by utilized Binary Logistic Regression, Linear Discriminant Analysis LDA, Random tree and SVM. The dataset used in this system are from UCI repository of PD, the training dataset consist of 195 samples with 21 features, here the LDA and random tree achieved an accuracy greater than 90%. Resul et al (2010) used various classification models to identify PD. Classification techniques were implemented and analyzed, they are neural network, regression and decision tree. For classification various evaluation methods were used, the performance of the classifiers were evaluated from the results, only Neural network classifier yield the good result among other, here the input dataset was randomly inserted into train and test dataset.Paul et al (2019) have used a machine learning techniques for predicating student dropout using data mining.

In this model decision tree was used to predict the dropout in student and they obtained an accuracy with 97.69% and the prediction was done by using various parapets, which are considered for every student. Mallikarjuna et al (2020) presented the feedback-based approach comparison of the normality and abnormality with the back propagation approach. In the training phase, the extracted feature sequence of a normal walking and abnormal walking, the three classes A, B, C, D normal, Parkinson gait, Hemiplegic gait, Neuropathic gait data sets compared with the normal data set.

MATERIAL AND METHODS

In this system, we applied two machine learning algorithms which are Logistic Regression and XGBoost. We implemented this model to find the best model among them for the datasets Logistic regression: Mohammad et al (2013) given as Logistic Regression is the appropriate regression analysis to conduct when the dependent variable is dichotomous. It is used to explain the

relationship between one or more independent variables and one dependent binary variables, the dependent variable must be binary in nature, e.g. 0 or 1. They shouldn't be high correlation among the prediction, this can be assessed by a correlation matrix. Here the outcome has two classes, Logistic regression starts with different model setup than linear regression instead of modeling Y as a function of X directly, we model the probability that Y is equal to class 1, gives X. First, abbreviation P(X)=P(Y=1/X).

A. XGBoost: Zhang et al (2019) presented XGBoost is a boosting algorithm, it is statistical learning method and derived from gradient boosting decision tree, it has better performance and optimization. The reason why we used XGBoost is it has good efficiency and feasibility, XGBoost allows dense and sparse matrix as the input and a numeric vector uses integer starting from 0 for classification, we can add number of iteration to the model A dataset with of n samples and d features of every sample then s_k is the prediction from decision tree.

The prediction score of each individual are summed up to get the final score. Mathematically, our model in the form

$$\hat{y} = \phi(x_i) = \sum_1^k s_k(x_i), s_k \in s \quad (1)$$

Where k is number of trees, s is function in function space s.

B. Data preprocessing: MinMaxScaler, Normalizer are method in scikit-learn are preprocessing methods, based on our features values we select the method, as we know machine learning algorithm will perform better and faster when features are relative or similar scale, we suggest MinMaxScale () for preprocessing, as it subtracts the minimum value in feature and divide with its range, difference of maximum and minimum is range MinMaxScale () return the default range 0 to1.

C. Feature selection: Arefi et al (2011) prescribed as we know, features play important role in classification, there are different approach in feature selection and based on the threshold value and benchmark algorithm we determine the optimality of feature in the dataset, Correlation coefficient features selection is the most widely used parameter, because feature selection is based on their correlation factor among the features (Shahbakhti et al 2013).

Let suppose f1 and f2 are two correlated features then to find Pearson's correlation coefficient (ρ)

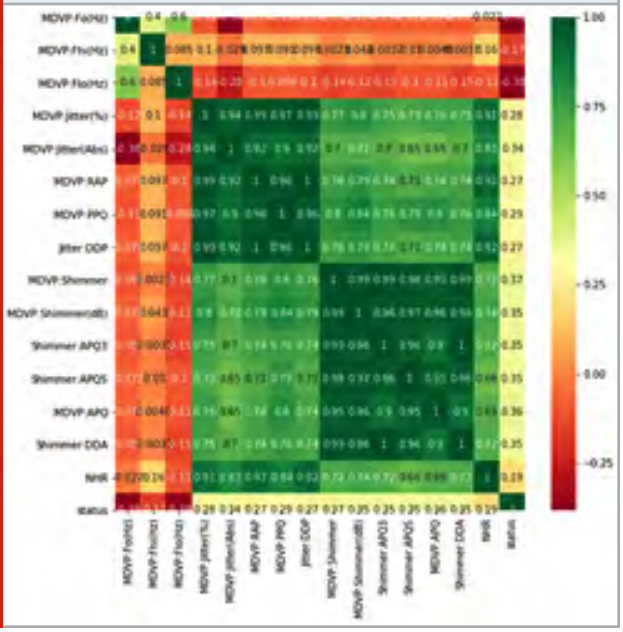
$$\rho xy = \frac{\text{cov}(x,y)}{xy} \quad (2)$$

Where, cov(x, y) are covariance of variable x and y

$$R = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]}} \quad (3)$$

Figure 1 shows the correlation between features, it differentiate the strong positive and negative correlation among features

Figure 1: Cross correlation among features



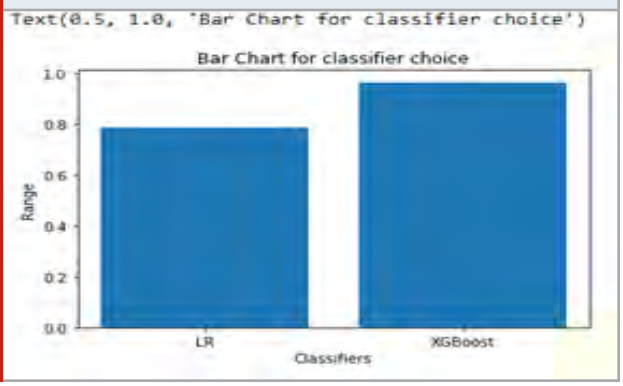
RESULTS AND DISCUSSION

By comparing the result of the system, the maximum classification rate is achieved by XGBoost than LR with an accuracy 96%, whereas LR achieved only 79% accuracy. Figure 2 shows the bar chart of the classifiers accuracy and correlation coefficient of the XBoost and Linear Regression. Figure 1 shows that only 12 features are the most important and characteristic among other features present in the dataset.

Table 1. Classification Accuracy of both model

Algorithm	Accuracy	MCC
Logistic Regression	0.79	0.42
XGBoost	0.96	0.89

Figure 2: Bar chart of classifiers accuracy



The entitled technology will permit us to compare two or more new algorithms with this XBoost and show the performance of the XBoost classifier.

CONCLUSION

The aim of the study is to analyze which algorithm provide the high accuracy of prediction for the Parkinson's disease dataset, here the classification accuracy was studied and compared, with good performance and fast implementation XGBoost achieved a high accuracy with 96%. This system provides the comparison between machine learning classifiers of LR and XGBoost in PD disease diagnosis with high dimensional data.

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Dielectric and Electrical Properties of Water-Soluble Bacterial Melanin Extracted from *Pannonibacter phragmitetus* [EP83] at Optimum Temperature Range

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ABSTRACT

Melanins are natural pigments distributed in living organisms and they are responsible for pigmentation of surface structure. The present work emphasis the complex relative dielectric function $\varepsilon^*(\omega) = \varepsilon'-j(\omega)$" of bacterial *Pannonibacter phragmitetus* [EP83], water soluble melanin and 80 (Ω) milli Q water at varying concentrations viz. 0, 25, 50, 75, 100 µg/mL have been measured using LCR meter in the frequency range 20 Hz to 2MHz at various temperature ranges of 95, 98.6, 102.2, 105.8, 109.4 and 113 K i.e. a temperature range between 35 to 45 ˚C with a difference of 2˚C. The electric/dielectric properties of the melanin sample in milli Q water was represented in terms of intensive quantities namely, complex relative dielectric function $\varepsilon^*(\omega)$, electric modulus $M^*(\omega)$, electrical conductivity $\sigma^*(\omega)$ and extensive quantities like complex admittance $Y^*(\omega)$ and complex impedance $Z^*(\omega)$. All of these presentations are used to explore various processes contributed in the electrical/dielectric properties of the melanin in pure state of water so that it may give a direct idea and influence of melanin in cellular system when incorporated or induced with. Result so obtained in the selected temperature ranges makes the base line for the influence of the extracted *Pannonibacter phragmitetus* [EP83] melanin in the cellular system. This may shed a new horizon in the pharmaceutical sciences for the cosmetic allegiance of a skin and also would be made employed for a treatment of leucoderma or for the development of the cosmetic product with dielectric/electric based mode of actions. Further aspects of this implication are under study and focused to increase the application part of *Pannonibacter phragmitetus* [EP83] water soluble melanin.

KEY WORDS: COMPLEX PERMITTIVITY, ELECTRIC MODULUS, COMPLEX IMPEDANCE, PRECISION LCR METER, WATER SOLUBLE MELANIN, PANNONIBACTER PHRAGMITETUS [EP83].

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INTRODUCTION

Melanins are amorphous, irregular polymeric pigments distributed in living organisms and they are responsible for the pigmentation of surface structure (Crippa and Michelini, 1999; Mosca et al., 1998; Riley, 1997; Bilinsk, 1996; Rosei and Mosca, 1996; Prota, 1992; Crippa et al., 1989; Miyake et al., 1986). The basic chemical structure of melanin is not well defined but it is usually represented by covalently linked models. Melanin synthesis is produced either by auto-oxidation of catechola or by the tyrosin action on the enzyme tyrosinase. Melanins are synthesized from tyrosine by hydroxylation to dihydroxyphenylalanine (DOPA) and subsequent oxidation to dopaquinone, both reactions are catalyzed by the enzyme tyrosinase, (Rile, 1997; Rosei and Mosca, 1995; Rosei and Mosca, 1996; Blarzino et al., 1999,Hutchinson et al., 2019).

Several investigations have been devoted to characterize their physical and chemical properties, such as its higher molecular weight, insolubility in water and common organic solvents, specific heat, carrier mobility, electrical conductivity, redox, chelating, and photo protective action (Crippa and Michelini, 1999; Mosca et al., 1998; Riley, 1997; Bilinsk, 1996; Rosei and Mosca, 1996; Prota, 1992; Crippa et al., 1989; Miyake et al., 1986; Harki et al., 1997; Blarzino et al., 1999; Rosei and Mosca, 1995; Kollias et al., 1991; Saran, 1992; Krol and Liebler, 1998; Yong-gang et al., 2009; Hengshan et al., 2006; Mejia-Caballero et al., 2016 Maranduca et al., 2019).

However, the molecular mechanism of melanin at cellular and sub-cellular level is not yet fully explained by the biophysical and biochemical studies (Crippa and Michelini, 1999; kollias et al., 1991; Saran, 1992). Dielectric properties of biological materials have been previously investigated (Ghannam et al., 2002; Foster and Schwan, 1995; Subrata, 1992; Grant et al., 1978). It has been established that the direct current (DC) conductivity of 3, 4-dihydroxyphenylalanine (DOPA) melanin is strongly dependent on the water content in the polymer structure (Jastrzebsk et al., 2002, 1995; D'Alba and Shawkey 2019).

The melanin under study from *Pannonibacter phragmitetus* [EP83] bacterial origin is a water soluble melanin. This enchants the study in a more urge and a mandatory need to understand its dielectric properties. Dielectric measurements are very important to study the molecular, solute-solute, solute-solvent interactions and dynamics of polar liquids, (Sengwa and Sankhla, 2007 Qashou et al., 2017).

This paper presents the result of measured parameters over the frequency range of 20 Hz to 2MHz on mixture of water-soluble melanin with 80 Ω milli Q water. The dielectrics have been studied at various temperatures viz. 95, 98.6, 102.2, 105.8, 109.4 and 113 K i.e. a temperature range between 35 to 45 ˚C with a difference of 2˚C under various concentration ranges as 0, 25, 50, 75, 100 µg/mL of water soluble melanin extracted from *Pannonibacter phragmitetus* [EP83]. The confirmation of the primary melanin structure under varying environmental conditions and interaction amongst the molecular position is one of the most interesting and challenging subjects in the scientific field like medicine, physics, biology, electronics engineering etc.

MATERIAL AND METHODS

Sample preparation: Melanin extract was obtained from the bacterial source, *Pannonibacter phragmitetus* [EP83] GenBank: AJ400704.1 (Pithawala and Jain, 2013) as a water soluble black-brown pigment. The pigment was extracted and made purified using the previously standardized method (Wei and Chen, 2005). The culture broth was mixed with a four volume of methanol and mixed vigorously. The resulting solution was centrifuged at 10,000 rpm for 10 min. The upper layer was collected and filtered through a 0.22 µpore sized filter paper. The filtrate was concentrated using a rotary evaporator and subsequently extracted with 3.0 M chloroform. The chloroform phase was collected and concentrated to obtain the resultant product, the black-brown pigment. The melanin as produced by *Pannonibacter phragmitetus* [EP83] is also known to solubilize in a mixture of aqueous chloroform. The mixtures of melanin extracted with milli Q water were prepared at different concentrations by volume as 0, 25, 50, 75 and 100µg/mL.

Measurements: An Agilent E 4980A precision LCR meter with a four terminal liquid dielectric test fixture (Agilent 16452A) were used for the capacitance and resistance measurement in the frequency range 20 Hz to 2MHz. The capacitance and resistance of the liquid dielectric test fixture, with and without sample, were measured in order to compensate for a short. The test fixture correction co-efficient was also considered to cancel the effect of the stray capacitance during the evaluation of the value of the complex dielectric function. The measurements were carried out at constant temperature by thermostat with an accuracy of ± 0.1 K.

Evaluation of Different Parameters: The complex dielectric constant ε*(ω) of the material is determined from the Eq. 1 (Agilent 16452A manual 2000).

$$\varepsilon^*(\omega) = \varepsilon' - j\varepsilon'' = \alpha\left(\frac{C_p}{C_0} - j\frac{1}{\omega C_0 R_p}\right) \quad (1)$$

where ω=2πf is the angular frequency, C0 is the capacitance in free space, Cp is the capacitance with sample, Rp is the equivalent parallel resistance with sample and α is the corrective co-efficient of the cell. The charges considering as an independent variable, conductivity relaxation effects can be suitably analyzed within the modulus formation in terms of a dimensionless quantity, called electric modulus M*(ω) is obtained from the below relation (Shinyashiki et al., 1998).

$$M^*(\omega) = \frac{1}{\varepsilon^*(\omega)} = M' + jM = \frac{\varepsilon'}{\varepsilon'^2+\varepsilon''^2} + j\frac{\varepsilon''}{\varepsilon'^2+\varepsilon''^2} \quad (2)$$

The main advantage of this formalism is that the space charge effects often do not mask the features of the

spectra. The complex impedance formalism Z*=1/Y*, where Y* is the complex admittance which is commonly used to separate the bulk and the surface phenomena (Sengwa and Sankhla 2007). The electrode polarization (EP) is a highly capacitive phenomenon and therefore it characterized by large relaxation times than the polarization mechanisms in bulk. A common feature of dielectric with σ_{dc} is a discontinuity at electrode/dielectric interface, which has different polarization properties than the bulk of the dielectric. The frequency dependent value of the complex impedance Z*(ω) of the material was evaluated by the relation

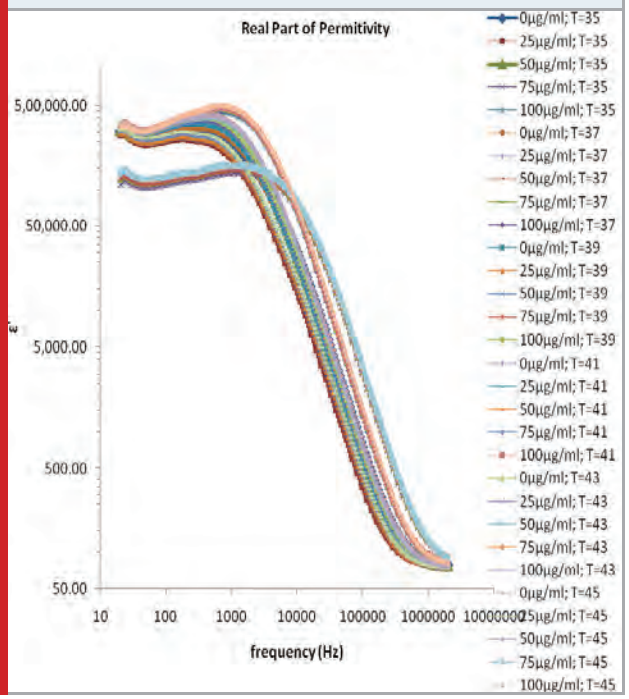
$$Z^*(\omega) = Z' + jZ'' = \frac{R_p}{1+(\omega C_p R_p)^2} - j\frac{\omega C_p R_p^{\ 2}}{1+(\omega C_p R_p)^2} \quad (3)$$

All dielectric parameters were carried out at various temperatures between 35˚C to 45˚C with a temperature difference of 2˚C.

RESULTS AND DISCUSSION

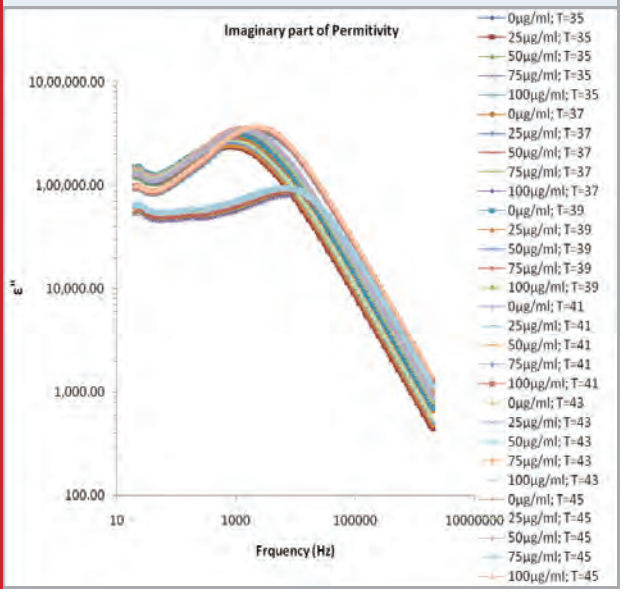
The frequency dependence of the real part of relative dielectric function ε' of various concentrations of melanin at various temperature ranges are shown in Fig. 1, in which the value of ε' is high in the lower frequency region corresponding to the electrode polarization effect. Here time consistency between hydration and temperature effects for the conductivity model utilizing dielectric spectroscopy, heat capacity measurements, frequency scaling phenomena. Level of hydration is sufficient for melanin to be in the conductive regime in which the reaction is strongly exhibited. (Motovilov et al., 2019). The electrode polarization (EP) phenomena occur due to formation of electric double layer (EDL) on the electrode surfaces. The magnitude of EP depends on the fractal structure of the electrode surface and the material used for fabrication of the electrodes. The large value of ε' (Fig.1) and ε'' (Fig.2) at lower frequency make it easy to identify the effect of blocking electrodes which corresponds to free charge motion within the material. These values are rather corresponding to free charge build up at the interface between the dielectric materials and the electrodes. The values of ε' in lower frequency region also change with concentration of mixture constituents.

Figure 1: Frequency dependence of the real part of relative dielectric function ε' for various concentrations of melanin in 80 Ω milli Q water at various temperature ranges



In Fig. 1 the values of ε' are almost independent of frequency at higher frequency range these values of ε' represents the static dielectric constant ε0 of the melanin samples. The values of ε0 in the higher frequency range symmetrically increase with the decreased concentration of melanin (Fig. 1). This happens with a general rule with all selected temperature range between 35˚C to 45˚C with a difference of every 2˚C temperature. Fig. 3 shows the frequency dependent tanδ spectra of the mixture of water-soluble bacterial melanin with 80 Ω milli Q water. The tanδ spectra have the peak value corresponding to the electrode polarization (EP) relaxation frequency fEP, which is used to separate the bulk material property and EP phenomena's (Zhang, et al. 2005; Motovilov et al., 2019).

Figure 2: Frequency dependence of the dielectric loss ε' various concentrations of melanin in 80 Ω milli Q water at various temperature ranges.

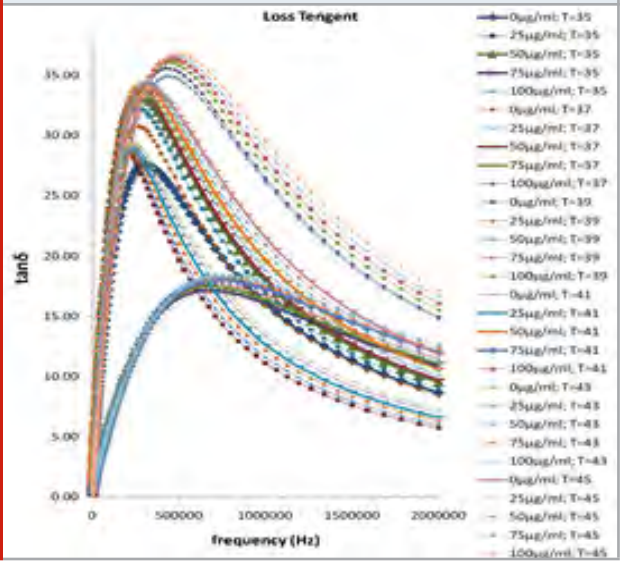


The values of fEP where used to evaluate the electrode polarization relaxation time (Sengwa et al., 2007 a; b; c and Shinyashiki et al., 1998). The τEP values representing the mean time for an ion to travel from one electrode to another and involves charging/discharging time of electric double layer capacitance, which is associated with the overall dynamics of the absorbed ions on the electrode surface in the alternating electric field. Such behavior is accompanied by moderate increase of the

frequency dependent conductivity (Sengwa et al., 2007d, Shinyashiki et al., 1998; Motovilov et al., 2019).

In Fig. 3 the values of the tanδ peak frequency changes with the concentration of the *Pannonibacter phragmitetus* [EP83] melanin, whereas the magnitude of the tanδ peak values are in between 15 to 38. The frequency of electrode polarization relaxation increases with increases of EP83 melanin concentration up to around 80% and then it starts to decrease. The value of electrode polarization relaxation time τEP with volume fraction of melanin concentration is reported in Table 1. The variation of the electrode polarization relaxation time τEP versus volume fraction of *Pannonibacter phragmitetus* [EP83] melanin in milli Q water is shown in Fig. 4, which clearly indicates that the values of τEP increases with increase of the concentration of *Pannonibacter phragmitetus* [EP83] melanin in milli Q water up to certain concentration and later decreases

Figure 3: Frequency dependence of the loss tanδ for pure milli Q water and mixtures of mili Q water and EP83 melanin at various temperature ranges.



The dielectric spectroscopic study provides insights into the structure of compounds, grain boundary, grain, transport properties and charge storage capabilities of dielectric material. The dielectric properties depend on several factors, including the chemical composition and the method of preparation, etc.

Fig. 5 and Fig. 6 show the variation of M' and M" versus frequency of varying concentration of *Pannonibacter phragmitetus* [EP83] melanin. At lower frequency the charges has to build up at the electrodes and material interface before change of the field direction, giving a high effective value of ε' (electrode polarization phenomenon). With increasing frequency there is less time for the buildup of the charges at the boundaries of conducting species in the material and at the ends of conducting path. (Uchino and Nomura 2017).

This phenomenon leads to the so-called conductivity relaxation and in fact reflects the distribution of the conductivity relaxation time (Kyritsis et al., 1995), which is the time of electrical stress associated with ion mobility (Zhang et al., 2005; Uchino and Nomura 2017). There is no peak found within the selected frequency range and hence the values and graph of τEP and thus the ionic conductivity relaxation time τ_σ is not feasible.

Figure 4: Plots of the electrode polarization relaxation time FEP versus concentration of EP83 melanin for the binary mixture of milli Q Water and melanin at various temperatures.

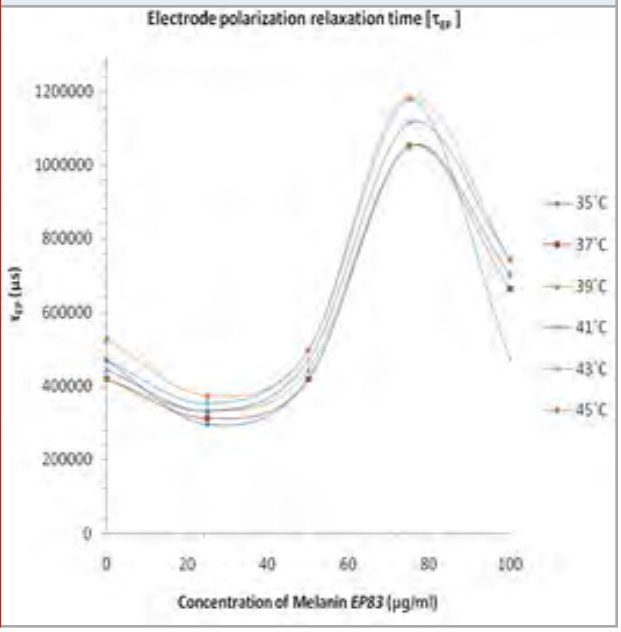


Figure 5: Frequency dependence of the real part of electric modulus M' for pure milli Q water and mixtures of miliQ water and EP83 melanin at various temperature ranges.

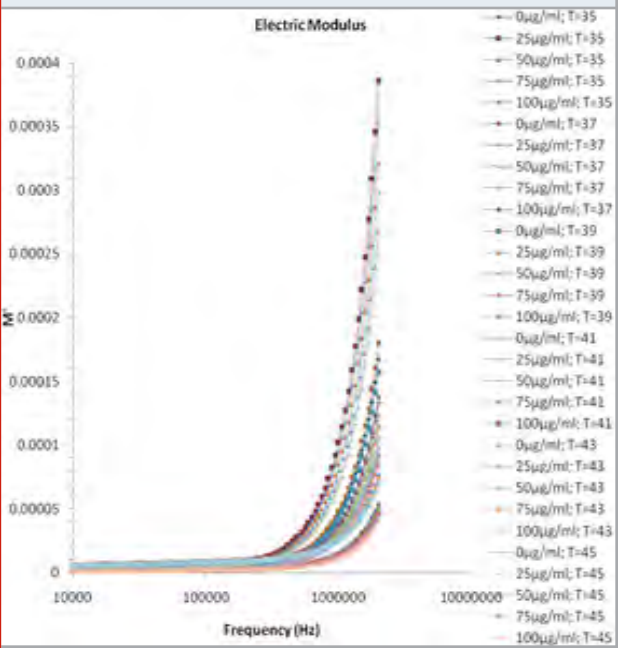
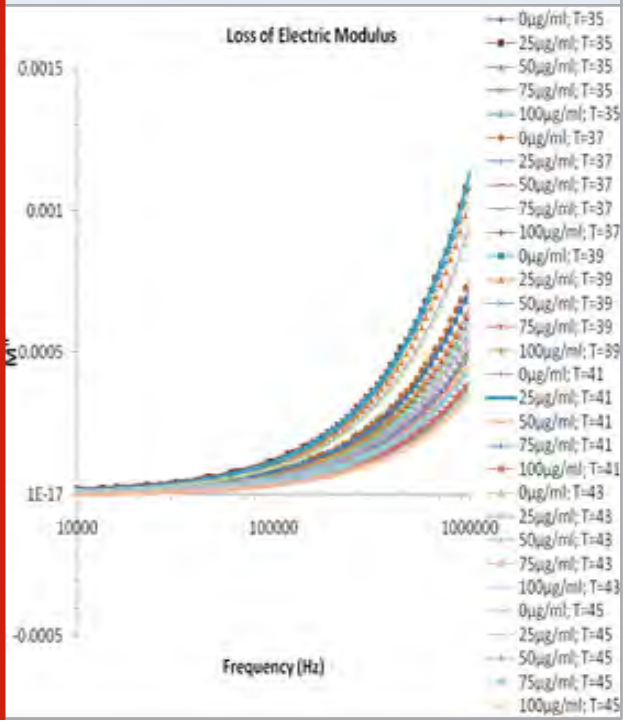


Figure 6: Frequency dependence of the electric modulus loss M' for pure milli Q water and mixtures of miliQ water and EP83 melanin at various temperature ranges.



The alternating current (ac) complex conductivity σ*(ω) has the frequency dependent real part σ' and the imaginary part σ". The σ*(ω) of the liquid sample were obtained from the below equation

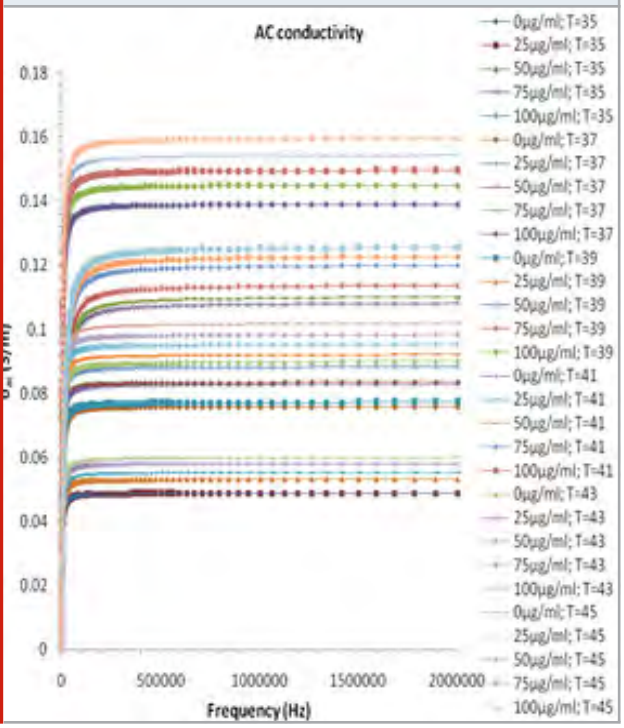
$$\sigma^*(\omega) = \sigma' + j\sigma^{''} = \omega\varepsilon_0\varepsilon^{''} + j\omega\varepsilon \quad (4)$$

Where εo(8.854×10-12 N/m) is dielectric constant of the vacuum. The ac conductivity plot of the different volume fraction mixture of the system is shown in Fig. 7. The σ' spectra of melanin and milli Q water have frequency dependent plateau (Fig. 7), which corresponds to ionic or ac electric conductivity σ_{ac} and exhibits dispersion at lower frequency region. Usual increase of ionic conductivity of a mixture is due to the increment of the number of mobile charge carrier produced in the liquid system with change in concentration at the constituent.

The real part of ac conductivity σac of the milli Q and *Pannonibacter phragmitetus* [EP83] melanin mixture increases with the increase up to 80% of the melanin concentration and then for higher concentration of melanin in the mixture shows the anomalous behavior in concentration dependant conductivity values, which is due to the significant change of the hydrogen bond interactions and molecular dynamics of the binary mixture of milli Q Water and *Pannonibacter phragmitetus* [EP83] melanin.

The complex impedance plane plot for the mixture of milli Q and *Pannonibacter phragmitetus* [EP83] melanin is shown in Fig. 8. From this plot it can be seen no separate arcs are observed corresponding to the bulk material effect (the high frequency arc) as well as electrode surface polarization effect (the low frequency arc). For mixture of milli Q and *Pannonibacter phragmitetus* [EP83] melanin, single arc is observed which representing the bulk material effect. Usualy the frequency values corresponding to Z" minimum values in the Z" versus Z' plot separates the bulk effect and the surface effect (Pissis and Kyritsis, 1997). This frequency is found to vary with the concentration of melanin in milli Q.

Figure 7: Frequency dependence of the real part of ac conductivity (σ_{ac}) for pure milli Q water and mixtures of miliQ water and EP83 melanin at various temperature range.



Electrical behavior of synthetic melanin films has been investigated in vacuum, to prevent any possible effect from the ambient humidity, as it has been reported for thick melanin samples (pellets) prepared from powders (Goncalves et al. 2006). The explanation of the electrical properties could be done assuming the existence of two types of water in the melanin structure. The electrical conduction, observed in all samples, could be related to an irreversible mechanism of easy water desorption with a consequent change of the structure affecting the electrical behavior. In fact, from RT to 380–400K the resistance will decrease due to the raise of the carrier density, while water desorption will have minor effects on the electrical conduction.

At higher temperatures the electron density will further increase together with a higher rate of easy water desorption from the melanin molecules. These water molecules are permanently lost by the melanin, since the electrical characterization is carried out in

high conditions. This result acts as an independent confirmation that significant amounts of liquid like water is present in the wet melanin sample at high temperatures. Results also demonstrate that bimolecular conductivity models should account for temperature and hydration effects coherently. Such a behavior persists, even if reducing its amplitude, as long as water is bound to the melanin molecule. The present study will be great application potentials in cosmetics, and pharmaceutical industries.

Figure 8: Plots of Z' versus Z' for the mixture of EP83 melanin and milli Q water at various temperature range.

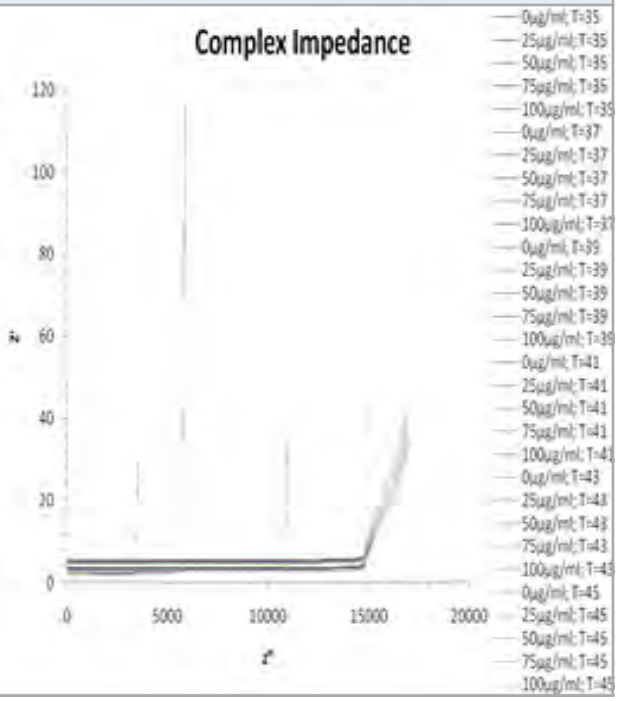


Table 1: Calculated values of electrode polarization relaxation time τ_{EP} with change in volume fraction of *Pannonibacter phragmitetus* [EP83] melanin concentration at various temperature ranges

Volume Fraction	35 ˚C		37 ˚C		39 ˚C		41 ˚C		43˚C		45 ˚C	
of Melanin (µg/ml)	fEP (Hz)	τEP (µs)	fEP (Hz)	τEP (µs)	fEP (Hz)	τEP (µs)	fEP (Hz)	τEP (µs)	fEP (Hz)	τEP (µs)	fEP (Hz)	τEP (µs)
0	299247	469817.79	266704	418725.3	266704	418725.3	282508	443537.6	299247	469817.8	335761	527144.8
25.00	188812	296434.84	200000	314000	211851	332606.1	211851	332606.1	224404	352314.3	237700	373189
50.00	266704	418725.28	266704	418725.3	282508	443537.6	299247	469817.8	316979	497657	316979	497657
75.00	669931	1051791.67	669931	1051792	669931	1051792	709627	1114114	751675	1180130	751675	1180130
100.00	447744	702958.08	422698	663635.9	447744	702958.1	474275	744611.8	474275	474275	474275	744611.8

CONCLUSION

]The dielectric and electrical properties of the binary mixtures of *Pannonibacter phragmitetus* [EP83] melanin in milli Q water were investigated at various temperatures viz. 95, 98.6, 102.2, 105.8, 109.4 and 113 K i.e. a temperature range between 35 ˚C to 45 ˚C with a difference of 2˚C under various concentration ranges as 0, 25, 50, 75, 100 µg/mL in the frequency range of 20 Hz to 2 MHz. The complex dielectric function of these binary mixtures in lower frequency region is governed by ionic conduction and electrode polarization phenomenon. The comparative analysis of the various dielectric and electrical quantity spectra conforms that the behavior of ionic conduction and EDL dynamics is dependent on the concentration of the mixture constituents.

The ionic conduction and surface effect in the mixture systems are confirmed by the electric modulus and the impedance formalism. The electrode polarization relaxation time τEP depend on the concentration of melanin in the mixture. Melanin possesses physicochemical properties and biological activities that make it a suitable biomaterial for a wide range of

applications in cosmetic, pharmaceutical, electronic, and other processing industries. In addition, this pigment has a considerable biotechnological interest because it can be produced on a large scale with low cost, making its use for future practical economically advantageous applications.

However, it is necessary to expand the knowledge about the dielectric properties of water-soluble melanin from bacterial origin. The present research article emphasis on *Pannonibacter phragmetetus* [EP83]; GenBank: AJ400704.1 for production of water-soluble melanin. However, there are very few known bacterial species that produces water soluble pigment especially melanin. In this context, the information will be useful and will encourage a greater number of researches on melanin, which might be useful to deploy innovative and sustainable solutions for human health and the environment.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evaluation of Fusion Tags for Recombinant Protein Expression in Bacterial System

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ABSTRACT

The primary objective of this work has been to evaluate the expression level of recombinant protein in *E.coli* in the presence of different fusion tags. One of the major challenges in using bacterial host in overexpression of recombinant protein is the formation of inclusion bodies, therefore, resulting in biologically inactive protein. Tedious procedures of denaturation and refolding of inclusion bodies are required to obtain functional protein. In this work, a human gene is fused with different fusion tags such as nusA, endoxylanase signal, pelB leader and asparginase signal sequence and expressed in *E.coli* BL21(DE3) host cells. Results showed that nusA fusion showed highest expression level of total protein (approx. 80 mg g^{-1} DCW) while endoxylanase signal sequence demonstrated a high expression level of soluble protein (~40% of total protein). In the case of pelB leader fusion, the overall productivity was low with the insoluble fraction comprising the majority of the total target protein and ~ 16 % in soluble fraction. Also, in the case of asparaginase signal fusion, major part of the expressed target protein was in the insoluble fraction with approximately 12 % in soluble form. These results demonstrated that recombinant production in the presence of fusion tags leads to variation in the level of soluble and insoluble forms. There seemed to be a stringent regulation for the expression of soluble protein suggesting that the host machinery may favor the inclusion body formation possibly due to the toxicity of the recombinant product. This study would be helpful in optimization of genetic parameters for the selection of most suitable vector-host combinations, as well as further understanding of bacterial strategies in adaptation and survival to stress.

KEY WORDS: RECOMBINANT, FUSION TAGS, INCLUSION BODIES, E.COLI.

INTRODUCTION

Approximately 70% of recombinant proteins are produced as inclusion bodies in *E. coli* over-expression system (Yang et al., 2011). In spite of having many advantages, inclusion body formation offers serious disadvantages such as the need for strong denaturants for solubilization and a subsequent refolding process to regain protein activity (Makrides, 1996; Singh et al., 2015, 2020). Moreover, refolded protein may not regain its biological activity and often resulted in reduction of protein yield even under optimized conditions of buffer composition, protein concentration, temperature, pH or ionic strength (Makrides, 1996, Lilie et al., 1998). It has been therefore, desirable to over-express recombinant protein in its soluble or active form, and thereby avoid the trial-and-error procedures required to develop an efficient refolding process (Kim et al., 2005). However, optimized solubilization method of inclusion bodies has

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also been reported for the retention of native secondary structure (Nekoufar et al., 2020)

Interferon-γ (IFN-γ) also known as a Type II interferon is secreted by lymphocytes against mitogenic stimulation and is involved in differentiation, proliferation and maturation of hematopoeitic cells. It also enhances non-specific immunity to tumors, as well as to microbial, viral and parasitic infections (Mamame et al., 1999, Sen and Lengyel, 1992). There have been several reports regarding the production of recombinant human interferon-gamma (rhIFN-γ) in *E.coli* (Kumar et al., 2014). Large-scale production of rhIFN-γ has been recently reported by using prokaryotic *E.coli* expression system in fed-batch culture (Babaeipour et al 2007). In most cases, over-expression of rhIFN-γ resulted in the formation of inclusion body in the cytoplasm (Kumar et al., 2014). The importance of IFN-γ in antiviral response against NDV in chicken fibroblasts has been demonstrated recently (Yang et al., 2020).

Among the most potent solubility enhancing proteins characterized to date are the *E. coli* maltose binding protein, MBP (40 kDa) and N-utilizing substance A, NusA (54.8 kDa). MBP and NusA act as solubility enhancing partners and are especially suited for the expression of proteins prone to form inclusion bodies (Sorensen and Mortensen, 2005). MBP fused with mouse leukemia inhibitory factor (mLIF) was used for its soluble expression in the cytoplasm of *E.coli* (Guo et al., 2020).

Soluble human fibroblast growth factor 21 (hFGF21), and human oncostatin M(OSM) were also expressed in *E. coli* by MBP-tagging (Nguyen et al., 2016, 2019a). Both MBP and NusA have been used for the solubilization of highly insoluble ScFv antibodies in the cytoplasm of *E. coli* (Bach et al., 2001). Recently, a comparative expression study showed that solubility was enhanced by using MBP and NusA fusion constructs at lower temperature of 18 °C (Nguyen et al., 2017).

The export of hGM-CSF to the periplasm using the pelB leader sequence marginally increased hGM-CSF expression; however fusion with a MBP-tag resulted in the maximum expression of ~70µg/ml (Bhattacharya et al., 2005). It has also been reported that the native endoxylanase signal sequence is efficient in secreting recombinant proteins to the culture supernatant (Srivastava and Mukherjee, 2001) or to the periplasm (Jeong and Lee, 2001). The pelB signal sequence of pectate lyase B from *Erwinia carotovora* is commercially available and commonly used for periplasmic export of recombinant proteins. Several studies have employed the pelB signal sequence for secretion of recombinant product in bacterial host (Cho et al., 2018; Santos et al., 2019; Zhou et al., 2019; Perez-Perez et al., 2020).

Asparaginase and endoxylanse signals have been shown to improve hGM-CSF expression in *E.coli* (Khasa et al., 2011). The asparaginase signal sequence also helped in efficient secretion of soluble asparaginase to the extracellular space (Khushoo et al., 2005). Human Interferon-gamma has been chosen mainly due to its intrinsic nature to easily form inclusion bodies and also high level of expression. The main objective of this study is to compare the expression level of different rhIFN-γ constructs as fusion proteins with nusA, endoxylanase signal, pelB leader and asparginase signal sequences The expression level of target protein may reveal the regulatory role and influence of different fusion partners in host cells. This will be useful for selection of best host-vector combinations and other optimization studies in recombinant protein expression using *E.coli*.

MATERIAL AND METHODS

Bacterial strains and Plasmids: *E.coli* BL21(DE3) (Stratagene, USA) strain was used as host for expression of rhIFN-γ and fusion rhIFN-γ genes. *E.coli* DH5α (Amersham, USA) was used for cloning purposes and maintenance of plasmids. The plasmids employed were pRSET-A (Invitrogen), pET22b, pET14b (Novagen, USA) and pET 43a (+) (Novagen, USA).

Cloning of rhIFNγ gene: Primers bearing NdeI and BamHI restriction sites were used to amplify rhIFN-γ cDNA by RT-PCR method from total RNA isolated from peripheral blood mononuclear cells as described previously (Vaiphei et al., 2009). The amplified cDNA fragment was cloned between the NdeI and BamHI sites of plasmid pET14b (Novagen) and pRSET-A (Invitrogen) to obtain the plasmid pET14-IFNγ and pRSET-IFNγ respectively.

Construction of fusion plasmids: The plasmid pETnusAIFNγ (hIFN-γ gene fused with the nusA gene) was constructed by amplifying the hIFN-γ fragment from the plasmid pRSET-IFNγ and then introducing it into another plasmid pET-43a (Figure 1). For this, two primers were used in which the forward primer 5'CGC GGATCC CAG GAC CCA TAT GTA 3' was designed to contain a BamHI restriction site and the reverse 5' CCC AAGCTT TTA CTG GGA TGC TCT 3' have Hind III restriction enzyme site. This allowed the fusion of rhIFN-γ gene at its N-terminal with the nusA gene (Figure1d). The plasmid pETxyl-IFNγ (rhIFN-γ gene cloned under the endoxylanase signal sequence) was constructed by inserting the rhIFN-γ gene fragment in plasmid PET-xyl (Srivastava and Mukherjee, 2001).

Two restriction enzymes NcoI and BamHI were used to double digest the pET14-IFNγ to retrive the rhIFN-γgene fragment and ligated into pET-xyl vector digested with the same restriction enzymes (Figure1c). Expression vector for rhIFN-γ fused with pelB leader sequence γ was constructed by inserting the NcoI and BamHI digested rhIFN-γ fragment in pET22b vector also digested with the same restriction enzymes resulting in pET22-IFNγ (Figure 1e). Also, for the construction of expression vector pETasp-IFNγ (rhIFN-γgene cloned under the asparginase signal sequence), NcoI and BamHI fragment of rhIFN-γ from pET14IFNγ was ligated to NcoI/B*am*HI digested pET22asp vector as shown in Figure 1f (Khushoo et al., 2005).

Expression and Quantification of rhIFN-γ as fusion protein: BL-21(DE3) cells containing rhIFN-γ plasmid constructs from Figure 1 were grown in 5 ml LB media containing ampicillin (100 µg/ml) and grown overnight at 37 °C shaker. About 1-2% of the overnight culture was transferred to 50 ml LB in 500 ml flask and grown at 37°C until OD600 reached 0.6. Then, the cells were induced with 1 mM IPTG and allowed to grow for 4 hours for expression of the recombinant proteins. Samples were collected at regular intervals for SDS-PAGE analysis and rhIFN-γ production was quantified as described earlier (Vaiphei et al., 2009). In order to distinguish between soluble and insoluble protein fractions, the pellets were resuspended in phosphate buffer and incubated at 37 °C for 15 minutes in the presence of 200 µ g/ml lysozyme. The samples were then lysed by sonication. This was followed by centrifugation at 14,000 rpm for 20 minutes, which pelleted the cell debris and any insoluble protein fraction. The supernatant consisting of the soluble cytoplasmic fraction was analysed. The insoluble pellets were resuspended in PBS containing 2% SDS in order to solubilize the inclusion bodies with appropriate dilution. The specific product yield was calculated for determining the rhIFN-γ production as described earlier (Vaiphei et al., 2009).

RESULTS AND DISCUSSION

Expression of IFNγ nusA fusion protein: Expression of rhIFN-γ and nusA fusion was checked by inducing cells containing pETnusIFNγ for 5 hours. The SDS-PAGE profile shows an induced band slightly above 70 kD which corresponds to rhIFN-γ and nusA fusion protein which comprised about 30 % of the total cellular protein (Figure 2) and more than 85 % of the fusion protein was in the form of inclusion bodies (Table 1). On the other hand, the expression level did not increase significantly even after the 5 hour post induction (Figure 2). Besides, there was no significant improvement of soluble protein expression at 30 °C (data not shown).

Expression of IFN-γ-xylanase signal fusion: For the expression of rhIFN-γ-xylanase signal fusion, pETxyl-IFNγ containing cells were induced with IPTG for several hours. In this case, the recombinant protein mostly occurred in the form of immature precursor about the size of 21 kD and a very low level of the processed form of 19 kD (Figure 3A). The mature form constitutes the soluble fraction whereas the insoluble fraction comprised of the immature protein (Figure 3B). The maximum specific product yield of soluble rhIFN-γ was found to be approximately 30 mg g^{-1} DCW that is about 41 % of the total cellular fraction (Table 1).

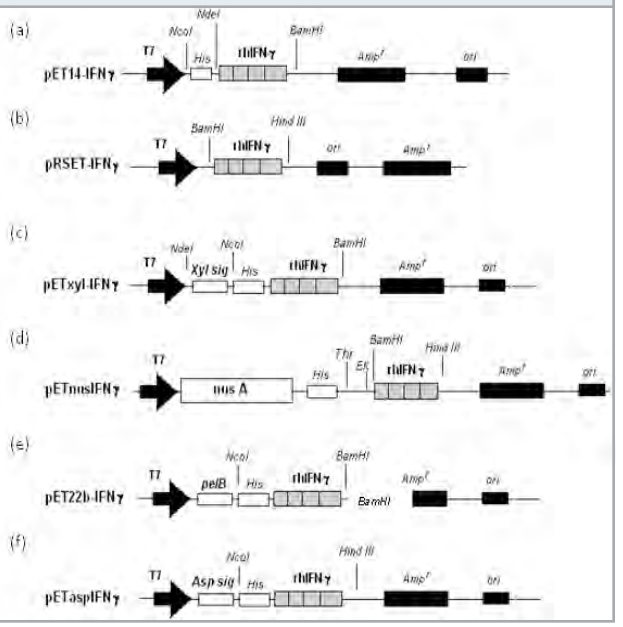
Expression of IFN-γ-pelB leader fusion: The plasmid pET22b-IFNγ was used for the expression of rhIFN-γ and pelB leader sequence in BL-21 (DE3) cells. The expression level of the fusion protein was also found to be considerably low as seen from the SDS-PAGE and the pattern of expression suggested that the level of productivity remained the same till 4 hour after induction (data not shown). The major part of the insoluble fraction constitutes the recombinant protein being produced as fusion partner. The specific product yield of the soluble form was estimated to be about 16 % of the total fraction (Table 1).

Expression of IFNγ asparginase signal fusion: The fusion rhIFN-γ with the asparginase signal showed that majority of the recombinant fusion protein was synthesized in insoluble form (Table 1). The maximum specific product yield estimated being from total cell fraction was approximately 58 mg g^{-1} DCW from (Table 1). The expression level at 2 hour and 3 hour post induction time did not showed comparable difference (Data not shown).

Table 1. Comparison of different IFNγ fusion proteins expression

Fusion Plasmid	Product concentration	
	Total cell	Soluble
pETnusIFNγ	80 ± 4 mg g^{-1} DCW	12.5 %
pETxyl-IFNγ	72 ± 3 mg g^{-1} DCW	41 %
pET22b-IFNγ	60 ± 3 mg g^{-1} DCW	16 %
pETaspIFNγ	58 ± 3 mg g^{-1} DCW	12 %

Figure 1: Construction of different fusion constructs for this study



The solubility of the recombinant protein rely on several factors such as lowering the rate of protein synthesis by using moderate or weak promoters, substitution of the amino acid residues (Dale et al., 1994) or employing low temperature (Schein, 1993; Nguyen et al., 2017).

However, the strategy involving the substitution of amino acid residues is limited to applications where these substitutions do not affect the desired function or stability of the target protein. Another well-known approach is fusion of the target proteins to highly soluble partners to increase the overall solubility of the fusion protein (LaVallie et al., 1993; Wilkinson et al., 1995), ubiquitin (Baker, 1996; Pilon et al., 1996); NusA (Davis et al., 1999). Unlike the affinity tags, solubility tags can differentially affect the target protein expression and therefore, heterologous expression systems involving fusion tags for improving solubility have continued to be tested (Costa et al., 2013; Nguyen et al., 2019b; Ki and Pack, 2020).

Figure 2: Expression of IFNγ and nusA fusion protein (pETnusIFNγ) in E.coli. (A) Comparison of expression level at different post-induction time intervals. (B) Expression profiles showing total cell lysate (T), soluble fraction (S) and insoluble fraction (I).

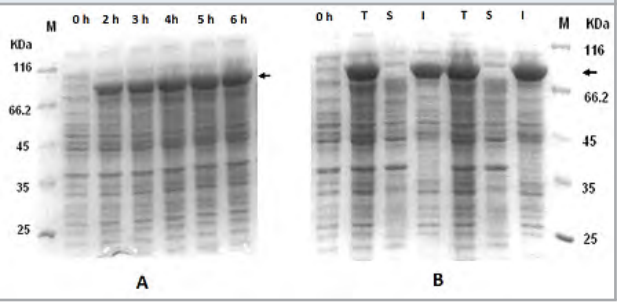
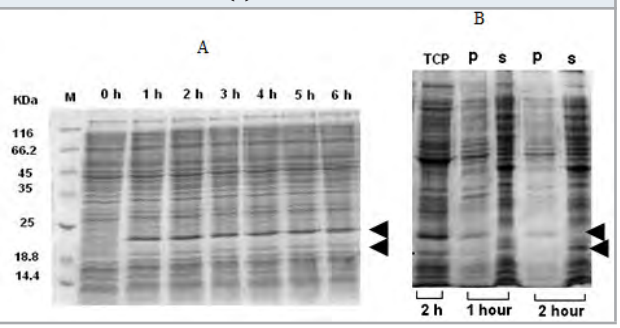


Figure 3: Expression of IFNγ and endoxylanase signal sequence fusion protein (pETxyl-IFNγ) at different post-induction time intervals (A). Expression profile at 2h post-induction of total cell-lysate , insoluble fraction (p) and soluble fraction (s).



The propensity for rhIFN-γ like proteins to form insoluble aggregates in *E.coli* has been well known. In this study, rhIFNγ-nusA fusion showed highest total protein expression level as compared to the other fusion partners. There was no significant difference in the expression level at 30 °C and 37 °C. Large fusion tags like NusA exhibit chaperon-like activities that slow down translation process providing more time for protein folding and stabilization of target protein (Costa et al., 2014). The rhIFNγ-xyl fusion showed both soluble and insoluble in the form of matured and immatured respectively (Figure 3B). This also indicates that the processed or matured form were soluble whereas the unprocessed ones are insoluble. However, this signal peptide was by far the most effective for expression of the fusion protein in soluble form.

Similarly, previous report also showed that native endoxylanase signal sequence fusion helped in efficient secretion of recombinant proteins to the culture supernatant (Srivastava and Mukherjee, 2001). In this work, pelB leader and asparaginase fusion partners have no significant increased in the level of soluble rhIFN-γ (Table 1). The use of pelB leader sequence was shown earlier to have only marginal increase of hGM-CSF in the periplasmic space (Bhattacharya et al., 2005). In spite of using genetic strategy of fusion with different soluble partners, rhIFN-γ was produced primarily as inclusion bodies demonstrating the propensity of the protein to aggregate upon over-expression in *E.coli* as host. This could possibly prevent efficient export to the periplasm and/or extracellular space which led to a drastic reduction in product yields. Since different fusion tags can have variable affect on the target protein expression, optimization with different combinations may be required on trial-and-error basis for best results (Costa et al., 2013, 2014; Paraskevopoulou and Falcone, 2018; Nguyen et al., 2019b; Ki and Pack, 2020).

Moreover, there is no direct correlation between the propensity of inclusion body formation of a certain protein and its intrinsic properties, such as molecular weight, hydrophobicity, folding pathways, and so on (Villaverde and Carrio, 2003). The reducing environment provided by the cytosol is suitable for the formation of inclusion body only in the case of disulfide bonded proteins, however, rhIFN-γ do not contain cysteine residues for such bonding. Moreover, if the expressed protein is toxic to the host, the formation of inactive inclusion bodies might increase the viability of the cells and the yield of the target protein (Seo et al., 2005; Haught et al., 1998). It is obvious that the cellular pathway would prefer the inclusion bodies or non-production of the recombinant protein as seen from this study, to maintain cellular viability. Low or non expression of rhIFN-γ with certain fusion partners could be the result of transcriptional or translational regulation of the bacterial genome. The cellular machinery might have adopted an alternative means to degrade or cleave the newly synthesized polypeptides to avoid formation of a soluble protein that could be lethal for the cells.

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Personalized Perceptions and Attitudes Towards Healthy Weight Management in Ha'il Region, Northern Saudi Arabia

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ABSTRACT

In recent years, obesity/overweight has represented a major health issue in Saudi Arabia, particularly among the younger population. Consequently, the current investigation is aimed to characterize the personalized perceptions and attitudes towards healthy weight management in Ha'il Region, Northern Saudi Arabia. In the present study 317 Saudi participants living in the city of Hail, Northern Saudi Arabia were recruited to find out the personalized perceptions and attitudes towards healthy weight management in Ha'il Region, Northern Saudi Arabia. The prevalence of obesity/overweight was 53%. The majority of the participants believe that "Calories' contents knowledge", can maintain a healthy body weight followed by "Reducing meal quantity", "Reducing meals frequencies", and "Reducing meals frequencies and Reducing meal quantity", constituting 223/315(70.8%), 210/313(67%), 161/313(51.4%), and 127/313(40.6%), respectively. Obesity/overweight is prevalent in Northern Saudi Arabia, which is behavioral and habitual determinant in most occasions. Personalized bodyweight determinant factors, including food intake habits, have a strong impact on Saudi weight management.

KEY WORDS: OBESITY, OVERWEIGHT, WEIGHT LOSS, BMI, SAUDI ARABIA.

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INTRODUCTION

Obesity/overweight (classified by Body Mass Index (BMI)) is a growing health issue worldwide associated with increased mortality due to its strong link to diverse comorbidities. Several diseases well documented to be resulting from obesity/overweight conditions including cardiovascular diseases, type 2 diabetes mellitus, hypertension, fatty liver disease, dyslipidemia, sleep apnoea, cancers, and some mental disorders (Fava et al 2019; Sung et al 2019; Mouton et al 2020).Many dietary and food habits have been linked to the increasing burden of obesity/overweight due to the imbalance of energy consumed within the food. Diets rich in processed food, trans/saturated fatty acids, sugars rich diets are the most weight (wt.) gain factors. Conversely, physical inactivity, low intake of whole grains, vegetables, fruits, and legumes can also elevate the risk of obesity (Legaand Lipscombe 2020).The etiology of obesity/overweight is extremely complicated, comprising an interaction of biological, psychological, social, and environmental factors. As a result, maintaining or achieving a healthy weight is challenging, and several weight loss accomplishments soon initiated with weight regain (Ataey et al 2020).

In recent years, an increase in self-attempts of weight loss worldwide. Maintaining continued weight loss requires continued management of dietary, food intake habits/behaviors, and other strategies (Evans et al 2020). However, the self-insight of weight loss and weight maintenance may promote long-term healthy weight maintenance (Han et al 2019; Dhurandhar et al 2019). The highest reported obesity/overweight prevalence rates (63.6%) (Ahmed et al 2014) in Saudi Arabia were from Ha'il region, Northern Saudi Arabia. Consequently, the current study aimed to characterize the personalized perceptions and attitudes towards healthy weight management in Ha'il Region, Northern Saudi Arabia.

MATERIAL AND METHODS

In the present study 317 Saudi participants living in the city of Hail, Northern Saudi Arabia were recruited to find out the personalized perceptions and attitudes towards healthy weight management in Ha'il Region, Northern Saudi Arabia. Data were obtained during a cross-sectional survey conducted in the period from October 2019 to Feb 2020. The study population was randomly selected regardless of age or sex. A purposeful questionnaire was designed and filled during a personal interview. Besides demographical data, the questionnaire included inquiries such, Attractants of maintaining normal BMI, attitude towards food intake, and maintaining healthy body weight, the claimed causes of weight gain, and regular meal habits (Pardo et al 2004).

Data analysis: Statistical analysis was performed using SPSS V22.0 SPSS. Frequencies and percentages and cross-tabulation of variables were obtained.

Ethical consent: Each participant has consented before the personal interview. The proposal of the study was approved by the Ethical Committee at the College of Medicine, University of Ha'il, Hail, Saudi Arabia. Ethical Approval Number: EC-00140/CM/UOH.01/19

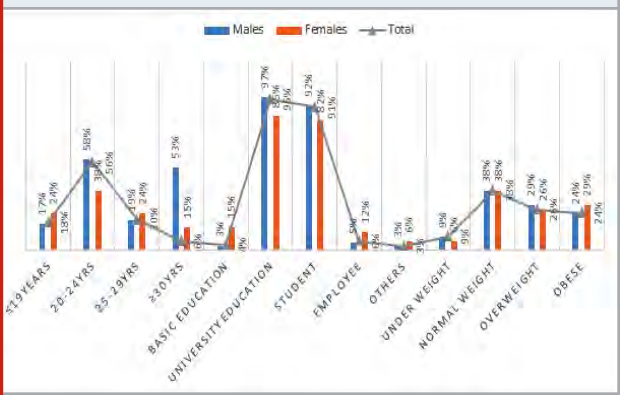
RESULTS AND DISCUSSION

This study investigated 315 volunteers, aged 17 to 56 years with a mean age of 21 years. The majority of respondents were males 281(89%) compared to 34(11%) females. The great majority of the study subjects were aged 20 to 24 years, representing 176/315(56%), followed by age range 25-29 and ≤19 years constituting 62(20%) and 57(18%), respectively. The great majority of the participants were with the university level of education constituting 272 (86%). The great majority of the participants were students constituting 287 (91%), as shown in Table 1, Fig 1. Out of the 315 participants, 90(29%) were over wt. and 77(24%) were obese, as shown in Table 1, Fig 1.

Table 1. Distribution of the study population by demographical features

Variable	Males	Females	Total
Age			
≤19 years	49	8	57
20-24	163	13	176
25-29	54	8	62
≥30	15	5	20
Total	281	34	315
Education			
Basic education	9	5	14
University education	272	29	301
Occupation			
Student	259	28	287
Employee	15	4	19
Others	7	2	9
BMI			
Underweight	26	2	28
Normal weight	107	13	120
Overweight	81	9	90
obese	67	10	77

Figure 1: Demographical features and BMI



The most common attractant for maintaining normal body weight among the study subjects was "having a nice look" followed by "disease prevention", "improve mood", and "spend time", representing 146/306(47.7%), 110/306(36%), 45/306(14.7%), and 15/306(4.9%), in that order. Out of 146 requiring "nice look", 36/146(25%) were obese and 59/146(40%) were over wt. Out of 110 demanding "disease prevention", 42/110(38%) were obese and 33/110(30%) were over wt. Out of 45 demanding "improve mood", 11/45(24%) were obese and 24/45(53.3%) were over wt. The majority of those demanding "spend time" were over wt. 8/15(53.3%), as indicated in Table 2, Fig 2.

Table 2. Distribution of the study population by attractants of maintaining normal BMI

Category	Variable	Under wt.	Normal wt.	Over wt.	Obese	Total
Disease prevention						
	Yes	1	34	33	42	110
	No	24	81	56	35	196
	Response	25	115	89	77	306
Look Nice						
	Yes	4	47	59	36	146
	No	21	67	30	41	160
	Response	25	115	89	77	306
Improve mood						
	Yes	0	10	24	11	45
	No	25	105	65	66	261
Spend Time						
	Yes	1	3	8	3	15
	No	24	112	81	74	291

Figure 2: Proportions of attractants of maintaining normal BMI

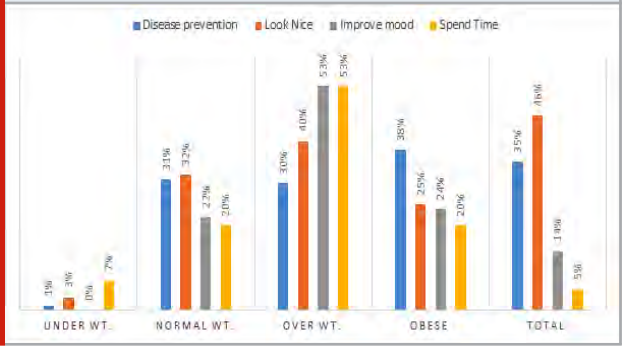


Table 3, summarized the distribution of the study population by BMI and attitude towards food intake and maintaining healthy body weight. The majority of the participants believe that "Calories' contents knowledge", can maintain a healthy body weight followed by "Reducing meal quantity", "Reducing meals frequencies", and "Reducing meals frequencies and Reducing meal quantity", constituting 223/315(70.8%), 210/313(67%), 161/313(51.4%), and 127/313(40.6%), respectively. Out of the 223, 210, 161, and 127, over wt./obese represented 120/223(53.8%), 120/210(57%), 94/161(58.4%), and 68/127(53.5%), correspondingly. About 153/313(49%) of participants "Did efforts to maintain healthy weight" of whom 86/153(56.2%) were over wt./obese. Around 71/313(22.7%) of participants "Did you regularly measure your wt" of whom 47/71(66.2%) were over wt./obese, as indicated in Table 3.

Table 4, summarized the distribution of the study population by BMI and the claimed causes of weight gain. About 49/308(16%), 112/314(35.7%), and 168/303(55.4%), believed that "Living alone", "Increased appetite", and "Food habits" are the main causes of weight gain. Out of the 49, 112, and 168 participants, 25/49(51%), 78/112(69.6%), and 122/168(72.6%) were over wt./obese.

For "meals frequencies per day", 9/21(43%), 56/104(53.8%), 86/149(57.7%), and 16/41(39%) of over wt./obese individuals were found to take meals, once, twice, 3times and more than 3 times, respectively. For "Snacks frequencies per day", 73/139(52.5%), 59/114(51.8%), 25/43(58%), and 6/14(43%) of over wt./obese individuals were found to take meals, once, twice, 3times and more than 3 times, respectively, see Table 4.For "Fast food frequencies", 34/68(50%), 73/138(53%), 33/60(55%), 13/25(52%), 7/10(70%), and 7/12(58.3%) of over wt./obese individuals were found to take meals, every day, twice/week, once/week, Once/month, rare and never, one-to-one, see Table 4.For "Vegetables and fruit intake", 23/51(45%), 52/98(53%), 40/78(51.3%), 17/25(68%), 17/49(34.7%), and 4/5(80%) of over wt./obese individuals were found to take meals, every day, twice/week, once/week, Once/month, rare and never, one-to-one, see Table 4.

The distribution of the study population by BMI and regular meal habits was summarized in Table 5, Fig 3. About 181/301(60%) didn't use to take their breakfast (BF) regularly, of whom 98/181(54%) were over wt./obese, compared to 63/120(52.5%) over wt./obese persons among those taking their breakfast regularly. The risk of over wt./obese associated with irregular breakfast, the relative risk (RR) and 95% confidence interval (95% CI) was 1.0041 (0.8099 to 1.2449), P = 0.9703, as indicated in Table 5, Fig 3. About 196/311(63%) use to take their dinner meal regularly, of whom 103/196(52.6%) were over wt./obese, compared to 64/115(55.7%) over wt./obese persons among those taking their dinner irregularly. The risk of over wt./obese associated with regular dinner, the RR(95%CI) = 0.9443(0.7650 to 1.1655), P = 0.5935, as indicated in Table 5, Fig 3.Over wt./obese individuals represented 21/47(44.7%), 92/167(55%), 53/95(55.8%) of those used to take little, normal, and heavy dinner meal quantity, in that order, as indicated in Table 5, Fig 3.

In recent years, Obesity/overweight represented a major health issue in Saudi Arabia, particularly among the younger population. Consequently, multifarious efforts

are ongoing to find out suitable strategies to tackle the burden of obesity-related health consequences. Obesity/overweight prevention and healthy weight management are from the top recommendations. The present study attempted to explore personalized perceptions and attitudes towards healthy weight management in order to assist in the establishment of future plans and strategies to reduce the burden of obesity in the Saudi community.

In the present study, the prevalence of obesity/overweight was 53%. These findings support a previous similar study from Saudi Arabia reporting obesity/overweight prevalence of 55.1% among adults population aged 18 to 60 years old (Azzeh et al 2017). A more recent study from Saudi Arabia reported a prevalence of 65.9% for obesity/overweight (Al-Qahtani 2019).

Table 3. Distribution of the study population by BMI and attitude towards food intake and maintaining a healthy body weight

Category	Variable	Under wt.	Normal wt.	Over wt.	Obese	Total
Reducing meals frequencies						
	Yes	12	55	51	43	161
	Not sure	10	44	29	24	107
	No	6	20	9	10	45
	Response	28	119	89	77	313
Reducing meal quantity						
	Yes	14	76	58	62	210
	Not sure	10	31	23	9	73
	No	4	12	8	6	30
	Response	28	119	89	77	313
Reducing meals frequencies and Reducing meal quantity						
	Yes	14	45	42	26	127
	Not sure	12	48	32	27	119
	No	2	26	15	24	67
Calories' contents knowledge						
	Yes	16	87	67	53	223
	Not sure	11	20	15	19	65
	No	1	13	8	5	27
	Response	28	120	90	77	315
Did efforts to maintain a healthy weight						
	Yes	6	61	53	33	153
	No	22	58	36	44	160
	Response	28	119	89	77	313
Did you regularly measure your wt						
	Yes	3	21	34	13	71
	No	25	97	56	64	242
	Response	28	118	90	77	313

Figure 3: BMI and regular meals habits

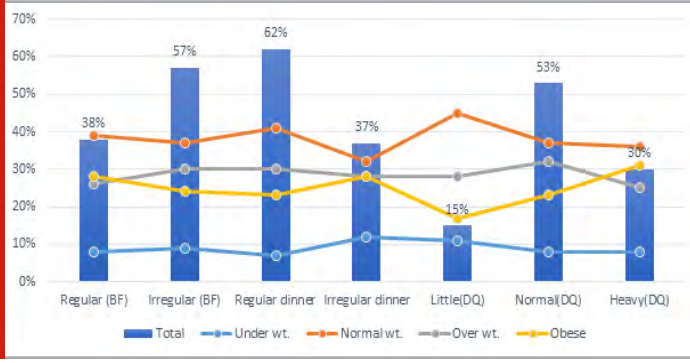


Table 4. Distribution of the study population by BMI and the claimed causes of weight gain

Category	Variable	Under wt.	Normal wt.	Over wt.	Obese	Total
Living alone						
	Yes	2	22	19	6	49
	No	25	94	69	71	259
	Response	27	116	88	77	308
Increased appetite						
	Yes	5	29	43	35	112
	No	23	91	47	41	202
	Response	28	120	90	76	314
Food habits are the main						
	Yes	7	39	62	60	168
	No	17	75	26	17	135
	Response	24	114	88	77	303
Meals frequencies per day						
causes of weight gain	Once	2	10	6	3	21
	Twice	10	38	33	23	104
	3times	11	52	41	45	149
	More than 3	5	20	10	6	41
	Response	28	120	90	77	315
Snacks frequencies per day						
	Once	12	54	35	38	139
	Twice	13	42	35	24	114
	3times	3	15	13	12	43
	More than 3	0	8	5	1	14
	Response	28	119	88	75	310
Fast food frequencies						
	Every day	8	26	20	14	68
	Twice/week	14	51	38	35	138
	Once/week	4	23	19	14	60
	Once/month	2	10	5	8	25
	Rare	0	3	2	5	10
	Never	0	5	6	1	12
	Response	28	118	90	77	313
Vegetables and fruit intake						
	Every day	4	24	11	12	51
	Twice/week	6	40	34	18	98
	Once/week	9	29	18	22	78
	Once/month	0	8	7	10	25
	Rare	7	15	16	11	49
	Never	0	1	2	2	5
	Response	26	117	88	75	306

In the present study, the most common attractant for maintaining normal body weight among the study subjects was "having a nice look" followed by "disease prevention", "improve mood", and "spend time", representing 47.7%, 36%, 14.7%, and 4.9%, in that order. Although aiming at all of these attractants has some beneficial effects on an individual's quality of life, "disease prevention" is the most important factor. Several studies have documented the role of maintaining healthy body weight in preventing diverse diseases including type 2 diabetes, cardiovascular disease, and other miscellaneous diseases (Muralidharan et al 2019; Pallazola et al 2019).To achieve successful weight loss outcomes, adherence to multifarious approaches may increase the chance of maintaining long-term optimum weight (Coupe et al 2019). In this study, many participants believe that "Calories' contents knowledge", "Reducing meal quantity", "Reducing meal frequencies", and "Reducing meal frequencies and reducing meal quantity", have strong impacts on determining individual weight. However, meal quantity, frequencies, and calories contents are well-documented as body weight

determinant (Lopes et al 2019). Besides, the food caloric contents and quantity, the balance between consumed energy and energy expenditure is another important factor determining an individual's weight management (Lu et al 2019). Increasing meal frequencies with low caloric contents may lead to weight loss, due to the energy consumed in the metabolic process.

Approximately 56% of the participants claimed planning for weight loss. Moreover, about 66% claimed regular weight measurement. These measures indicating low awareness of maintaining a healthy weight. In the present study, many participants were accustomed to high frequencies of snacks and fast food with relatively low intake of vegetables and fruits. Such behaviors and dietary habits can increase the possibility of weight gain (Ahmed et al 2019). In the present study meals, regularity status and quantity didn't reveal significant differences, though the risk of weight gain was elevated with breakfast irregularity and heavy dinner.The limitation of the present study includes its cross-sectional setting, younger population, and qualitative assessment.

Table 5. Distribution of the study population by BMI and regular meals habits

Category	Variable	Under wt.	Normal wt.	Over wt.	Obese	Total
Taking Breakfast(BF) regularly						
	Yes	9	47	31	33	120
	No	16	67	55	43	181
	Response	25	114	86	76	301
Taking dinner regularly						
	Yes	13	80	58	45	196
	No	14	37	32	32	115
	Response	27	117	90	77	311
Dinner quantity(DQ)						
	Little	5	21	13	8	47
	Normal	14	61	53	39	167
	Heavy	8	34	24	29	95
	Response	27	116	90	76	309

CONCLUSION

Obesity/overweight is prevalent in Northern Saudi Arabia, which is behavioral and habitual determinant on most occasions. Personalized bodyweight determinant factors, including food intake habits, have a strong impact on Saudi weight management.

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Phytomedicinal Potential of Ethanolic Extracts of Some Trees and Herbs from Thal Desert: *In vitro* Assessment of Plant Antioxidants Effects on Human Haematological Attributes

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ABSTRACT

Secondary metabolites synthesized in desert plants have medicinal properties on one hand and toxicity nature on the other hand. The use of such plants as food or drugs by human being needs to explore their toxic or friendly nature. The effects of secondary metabolites on hematological parameters can provide an insight for finding out their nature. Ethanolic extracts sourced from a variety of plant species are considered to have the potential for inducing changes in human haemotological indices which, when altered, can have pivotal role in determining human health conditions. Aiming to explore this, the ethanolic extracts of some plants from Thal desert of Pakistan were used to determine their effects on some human hematological attributes like counts for granulocyte; leukocytet; eosinophils; monocyte; lymphocyte; granuloytes and lymphocytes. Haemoglobin (Hb), Red Blood Cell (RBC), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Packed Cell Volume (PCV), Hematocrit (HCT) and Red Cell Distribution (RDW) were also studied. Eethanolic extracts of some trees and herbs collected from Thal desert were mixed with human blood in 1:4 ratio and tested for complete blood count tests (CBC) using Automated Hematology Analyzer machine. The blood without addition of extract was treated as control. Data were statistically analyzed by using one way ANOVA (Analysis Of Variance). The level of statistical significance was $P < 0.05$. Mean values were differentiated by Duncan's multiple range tests. Among the studied blood characteristics, eosinophils perctage and count, granulocytes percentage, RBC, MCHC, MPV were decreased by all plant extracts. RDW were decreased by extracts of *Tamarix aphylla* stem *Capparis aphylla* stem and root, Lymphocytes were lowered by *Orobanche* stem extract. All other extracts increased the components of blood as comared to control except platelets decline by *Capparis decidua* stem extract.

KEY WORDS: PHYTOMEDICINAL POTENTIAL, ETHANOLIC EXTRACTS,THAL DESERT, IN VITRO, ANTIOXIDANTS, HUMAN BLOOD.

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INTRODUCTION

In whole history of humanity, in his struggle against diseases, man has seeked help from plants which are the sources of biologically-active nature of health-promoting constituents. The use of plants in treating ailments is too old to date back 1550 BC (Petrovska, 2012, Kumar et al. 2019). Almost every medical systems, may it be Traditional Medicine, Ayurvedic medicine, Kampo medicine, European medicine, is based on valuable plant derived medicinal substances (Lu et al. 2016). Currently, medicine is faced with a growing demand for a wide range of biologically-active compounds of natural origin that can demonstrate preventive or therapeutic. A large proportion of the phytochemicals used for effects against causes of death, such as cardiovascular disease, cancer, diabetes or respiratory disease are secondary metabolites. These compounds have many different functions in plant itself such as protecting plants from pathogens (Zaynab et al. 2018: Bednarek et al. 2018), herbivores (Huang et al.2019; Huber et al. 2016) and ultraviolet light (Köhler et al. 2017; Takshak and Agrawal 2019).

They also provide color and fragrances to facilitate seed dispersal and pollination by animals. These play an important role as signals and regulatory molecules in primary metabolism. In modern medicine, plant secondary metabolites play a vital role. Secondary metabolites have antioxidant (Zlatic et al. 2019; Gonçalves 2019), antibacterial (Barbieri et al. 2017), anti-inflammatory (Bernstein et al. 2018, Flores-Sánchez et al. 2019), antifungal (Reichling 2010Lagrouh et al. 2017), hepatoprotective (Pereira et al. 2016) and neurological (Epifano et al.2008) effects. The increasing uses of these plant secondary metabolites demands the use of new biotechnological tools to create new and productive transgenic plant cultures. (Kowalczyk etal. 2020).

Plants products have been used as medicine since long time owing to their availability, no side effects and easy usage. In developing countries including like Pakistan, even today, the practice of medicine relies on plants and their products. Plant extract based medicine is culturally well accepted by peoples of urban and rural areas. Many plants contain number of secondary metabolites such as saponins, phenols, necessary oils, and many other phytochemicals. These metabolites have medicinal properties on one hand (Aggarwal et al. 2003) and have been reported to be toxic on the other hand (Jennifer et al. 2005). These secondary metabolites have been well documented to be present in plants of the adverse environmental conditions such as deserts and act as antioxidants for scavenging reactive oxygen species (ROS).

Deserts are arid and semi-arid lands which comprise approximately one third of the world's land surface and are under constant threat of drought (Wickens, 1998). In arid regions, drought occurs regularly and annual evaporation frequently more than total amount of annual precipitation. Due to these environmental conditions, the vegetation of the desert comprises xerophytic species which are adapted to these various environmental stresses, like extreme aridity, high salinity, high temperature and low nutrient availability (Naz et al, 2010). Water deficiency also affects many aspects of the plant from cell function to mechanistic properties stress and it will have great importance in worldwide both economically and environmentally (Lambers, 1998 and Rachmilevitch et al. 2006) Plants are adapted to these adverse environmental conditions by synthesizing secondary metabolites. Some of the plant secondary metabolites show negative effects on health, survival and behaviour of consumer especially herbivores because of the harmful toxins (Jennifer et al. 2005).

Concentration of these metabolites in plants and nature of their toxicity for consumer vary with stress severity and type (Close and McArthur, 2002). Some common and the most important bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. Moreover, a number of medicinal plants containing flavonoids and alkaloids are used in natural medicine and are known to contain important therapeutic agents. Their identification needs to be interpreted in the light of traditional use and preparation of plant drugs (Taylor et al. 2001). Plants need to be well diagnosed for their medicinal potential, antioxidant status and toxicity nature of secondary metabolites prior to its use for human health. The use of herbal preparations, without any proper scientific studies on their safety, can raise concerns on their toxicity (Saad et al. 2006).

Furthermore, blood parameters reveal the health status of an individual. This is because blood plays a vital role in physiological, nutritional and pathological status of organism. Of these, Physiological parameter could be a valuable means of diagnosing health of an individual (Ganong, 2005). Hence, the exploration of various blood parameters might be useful criteria that can be used to assess the toxic or medicinal potentials of plant extracts (Sunmonu, 2010). Effects of herbal extract on animals are commonly used to deduce potential health risk for humans indirectly (Ashafa, 2011). However, direct evaluation of in vitro toxicity effects on human hematological parameters, can have more predictive value for human health. If no ethnomedicinal knowledge available, plant can be selected randomly and screened for medicinal constituents derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc. After a careful experimental testing for its toxicity or medicinal potential, a plant can be used for medicinal purposes.

MATERIAL AND METHODS

Our experiment is aimed to explore the toxic or medicinal nature of ethanol soluble secondary metabolites (Hemwimon et al. 2007) of desert plants. Toxic or medicinal effects of herbal extract are commonly used in animals to deduce their potential health effects on humans indirectly (Ashafa, 2011). However, in vitro evaluation of toxicity effects on human hematological parameters directly, can have predictive value for human

health. To assess the ethno-medicinal or toxicological validation of plant secondary metabolites, the desert plants were selected. The selection of desert plants is based on arid environmental conditions which enforce plants to synthesize secondary metabolites for adaptation to stressful environment (Naz et al. 2010). Desert plants have traditional utilization in the treatment of some diseases and free radicals related disorders by local peoples with no proper documentation of their side effects (Taylor et al. 2001). Moreover, the choice of in vitro utilization of human blood is based on the role blood plays in determining the physiological, nutritional and pathological status of organism. Among these, physiological parameters are dependent on blood attributes and could be a valuable means of diagnosing a disease (Ganong, 2005). Hence, the investigation of various hematological parameters might be a useful index that can be employed to assess the toxic or useful potentials of plant extracts containing secondary metabolites (Sunmonu and Oloyede, 2010).

Experimental design: Using ethanol as solvent, plant extract was prepared (Kinuthia et al 2014, and Saha, 2008). Human blood from a healthy volunteer was analysed for haemotological indices after mixing with plant extract (Ughachukwu et al. 2013 and Sayeed et al. 2014). Three samples of each extract were pooled to get means to reduce the error. A comparison with normal blood characteristics was statistically calculated.

Field survey and trees sample collections: Thal desert is located in Punjab provice of Pakistan lying between Indus and Jehlum rivers. It extends over an area of 23000 square kilometers between 70.8°- 72°E longitude and 30° – 32.5° N latitude. (Mares, 2017). In a preliminary survey of Thal desert, meetings with local peoples were arranged to know the geographical area and local plant names. Intact specimens were collected and herborized from the study area for each new plant species present and mentioned by local people, Herborized specimens were identified specialists, by matching them with the labelled herbarium exsiccates lying in the departmental herbarium (Dr.Mumtaz Bukhari herbarium) of Botany Department Bahauddine Zakarya University, Multan Pakistan and/or the literature (Ali,1993). Data and specimens were collected according to an appropriate methodology (Jain, 1995; Khan 1993) keeping uniformity among age of plants, size of plants and size of sand dunes. Further processing of collected specimens was carried out in laboratory of the department.

Table 1. In vitro effect of ethanolic extract of some trees and herbs of Thal on human hematology[values represent mean±standard daviation; n=3]

Name of species	leukocyte count (10×3/u L)	Granulocyte (%)	Lymphocyte (%)	Monocyte (%)	Eosinophils (%)	Granulocyte (10×3/L)
Normal blood	5.63 ± 0.05 k	0.13 ± 0.05 o	47.93 ± 0.05 i	1.96 ± 0.05 m	2.76 ± 0.05 a	0 ± 0 d
Tamarix aphylla (r)	7.26 ± 0.65 j (+28.95)	32.53±1.05 e (+24923.08)	58.53 ± 1.106 bc (+22.11)	6.43 ± 1.105 k (+228.06)	0.20± 0.1 cd (-92.75)	2.56 ± 1.22 c (-256)
Capparis decidua (s)	10.13 ± 0.60 efg (+79.92)	23.13± 1.305 i (+17692.31)	55 ± 0.9bcd (+14.75)	20.56 ± 0.95 fg (+948)	0.03 ± 0.05 e (-98.91)	2.5 ± 1.25 c (-250)
Capparis decidua (r)	8.20± 0.55 hij (+45.64)	21.43± 0.83 ij (+16384.62)	54.43± 1.02 bcd (+13.56)	19.6± 0.75 g (+900)	0.03 ± 0.05 e (-98.91)	2.56± 1.22 c (-256)
Fagonia arabica (s)	9.20± 0.55 gh (+63.41)	31.36 ± 0.8 ef (+24023.08)	53.1 ± 0.95 bcde (+10.85)	11.53 ± 1.05 j (+488.26)	0.40± 0.1 b (-85.71)	2.9 ± 1 c (-290)
Orobanche aegyptica (f)	16.56 ± 0.87 c (+194.13)	31.83 ± 1.05 ef (+24384.62)	54.5 ± 0.91 bcd (+13.77)	13.5 ± 1.1 i (+588.77)	0 ± 0 e (-100)	5.46 ± 0.96 b (-546)
Orobanche aegyptica (s)	17.13 ± 1.15 bc (+204.26)	43.3 ± 1.6c (+33207.69)	40.3 ± 0.90 fgh (-15.86)	16.56 ± 1.2 h (+744.89)	0 ± 0 e (-100)	7.6 ± 1.08 a (-760)
Citrullus colocynthis (r)	10.66 ± 0.90 ef (+89.34)	20.76 ± 1.10 j (+15869.23)	57.36 ± 0.80 bc (+11.32)	19.36 ± 0.86 g (+887.75)	0.3 ± 0.1 bc (-89.28)	2.66 ± 1.07 c (-266)

Values sharing the different letters represent significance difference in respective row; values in parenthesis represents percentage difference over control group; LSD= least standard deviation; r=root; s=stem; f=flower

Crude ethanolic extract preparation:The collected specimens were first washed with water and later on with 2% ethanol to remove dust and other surface contaminants, dried at room temperature, and were grounded to fine powder using pestle and mortar. Following the procedure adopted by Afolayan et al. (2010), crude ethanolic extract was prepared from finely grounded 100.0 g plant material in 200ml ethanol by shaking at room temperature for 3h. The ethanol used was of highest purity. The extract was filtered and residue was re-processed for extraction. Solvent was evaporated by rotory evaporator at and material was stored at -4°C.

Blood sampling and in vitro analysis: After ensuring the confidentiality and anonymity to a blood donor and approval from local ethical committee, human blood was obtained from a healthy volunteer of 25 years age having O+ blood group. The volunteer was selected after a questionnaire of not taking any medications or addictive substances (including tobacco, alcohol and aspirin or any other anti-platelet drugs) and keeping a balanced diet (meat and vegetables); using no antioxidant supplementation. By adding ethanol, plant extract was diluted up to 5ml. After consulting literature, the ratio of mixing blood to plant extract was determined by trial method to find appropriate dose when no coagulation occurred. Finally, plant extract was added into 4ml blood (1:4) and was shaked smoothly. Blood sample without addition of extract was considered as control for comparison. Complete blood count tests (CBC) by using Automated Hematology Analyzer machine was performed for hematological indices.

Statistical analysis of data: Data obtained for blood test were analyzed by using one way ANOVA (Analysis Of Variance) at 5% level of statistical significance. Means were compared by Duncan's multiple range test (Duncan, 1955).

Table 2. Table In vitro effect of ethanolic extract of some trees and herbs of Thal on human heamatology[values represent mean±standard daviation; n=3]

Name of species	Lymphocyte count (10×3/L)	Monocyte count (10×3/L)	Eosinophils count (10×3/L)	RBC (10×6/ uL)	HGB (g/dL)	HCT (%)
Normal blood	2.68 ± 0.02 j	0.13 ± 0.05g	50.33 ± 0.57 h	5.14 ± 0.005 c	8.33 ±0.05 d	22.73 ± 0.05h
Tamarix aphylla (r)	4.56 ± 1.19 efgh (+70.14)	0.5 ± 0.1fg (+284.61)	2.73 ± 0.90 cde (-94.57)	4.28 ± 0.95 bc (-16.73)	8.66 ± 1.02 bcd (+3.96)	24.66 ± 1.05e (+71.84)
Capparis decidua (s)	5.7 ± 1.15 cde (+112.68)	2.6 ± 1.04bcd (+1900)	1.6 ± 0.2efg (-96.82)	3.7 ± 1.113 bc (-28.01)	8.63 ± 1.05 bcd (+3.60)	20.83 ± 1.006h (+54.993)
Capparis decidua (r)	5.73 ± 1.06 cde (+113.80)	2.63± 1.15bcd (+1923.07)	1.46 ± 0.15 fg (-97.09)	4.45± 1.19 bc (-13.42)	8.43 ± 1.00 bcd (+1.20)	24.7± 1.15e (+72.01)
Fagonia arabica (s)	4.8 ± 1.1 defgh (+79.10)	1 ± 1 efg (+669.23)	1.46 ± 0.15 fg (-97.09)	4.56 ± 0.92 bc (-11.45)	8.63±1.02bcd (+3.60)	27.63 ± 1.05c (+21.7)
Orobanche aegyptica (f)	9.4 ± 0.62 a (+250.74)	2.66 ± 1.00bcd (+1946.15)	4.53 ± 1.11 b (-90.99)	3.78 ± 1.095 bc (-26.60)	8.36±1.00bcd (+0.36)	25 ± 1 de (+9.98)
Orobanche) aegyptica (s	7.63 ± 1.13 ab (+184.70)	3 ± 1 bc (+2207.69)	0 ± 0 h (-100)	4.28 ± 0.92 bc (-16.89)	9.46±1.15abc (+13.56)	24.7 ± 1.05e (+8.66)
Citrullus colocynthis (r)	6.5 ± 0.79 bcde (+142.53)	2.53 ± 1.1 bcd (+1846.15)	0± 0 h (-100)	4.38 ± 1.30 bc (-14.95)	9.63±1.12abc (+15.60)	24.63 ± 1.10 e (+8.35)

Values sharing the different letters represent significance difference in respective row; values in parenthesis represents percentage difference over control

Values sharing the different letters represent significance difference in respective row; values in parenthesis represents percentage difference over control group; LSD= least standard deviation; r=root; s=stem; f=flower

RESULTS AND DISCUSSION

Tamarix aphylla root: Ethanolic extract of *Tamarix aphylla* root substantially altered blood parameters. The extract had increased Leukocyte (28.95%), granulocyte count (24923.08%), lymphocyte count (22.11%), Monocyte count (228.06%), lymphocyte (70.14%), Monocyte (284.61%), HGB (3.60%), HCT (71.84%), MCV (32.33%), MCH (40.91%), RDW (12.37%) and platelets (42.96%) respectively. Ethanolic extract had affected Eosinophils count, granulocyte, RBC, Eosinophils, MCHC, and MPV with different degrees there by lowering values from control as Eosinophils count (98.91%), granulocyte (256%), RBC (16.73%), Eosinophils (94.57%), MCHC (7.45%), and MPV (28.86%) .

***Capparis decidua stem*:** Ethanolic extract of *Capparis* decidua stem proved its significant influence . Mean values revealed that an increse was noted for leukocyte (79.92%), granulocyte count (17692.31%), lymphocyte count (14.75%), Monocyte count (948%), lymphocyte (112.68%), Monocyte (1900%), HGB (3.60%), HCT (54.993%), MCV (21.75%), MCH (23.99%), MCHC (16.29%) and RDW (11.34%). An exception in this correlation was found MPV, RBC, platelets, Eosinophils, granulocyte, Eosinophils count parameters. Blood parameters MPV (28.42%), platelets (25.48%), RBC (28.01%), Eosinophils (96.82%), Granulocyte (250%) and Eosinophils count (98.91%) revealed a significant decreased from control.

***Capparis decidua* root:** Ethanolic extract of *Capparis* decidua root change in blood parameters. The most marked increase was in leukocyte (45.64%), Granulocyte count (16384.62%), lymphocyte count (13.56%), Monocyte count (900%), lymphocyte (113.80%), Monocyte (1923.07%), HGB (1.20%), HCT (72.01%), MCV (31.42%), MCH (40.73%) and RDW (18.63%). The application of extract seems to decreased significantly Eosinophils count (98.91%), Granulocyte (256%), Eosinophils count (97.09%), RBC (13.42%), MCHC (8.81%) and MPV (33.62%).

***Fogonia arabica* stem:** Different sensitivity range was found in response of blood parameters treated with ethanolic extract of Fogonia arabica stem. The application of extract seems in enhancing the leukocyte (63.41%), granulocyte count (24023.08%), lymphocyte count (15.68%), Monocyte count (488.26%), lymphocyte count (10.85%), lymphocyte (79.10%), Monocyte (669.23%), HGB (3.60%) HCT (21.7%), MCV (31.92%), MCH (29.57%), RDW (49.34%) and platelets (18.69%) from control. But the extract decreased MPV, MCHC, granulocyte, Eosinophils, Eosinophils count and RBC parameters. The maximum decrease was for MPV (21.21%), MCHC (11.39%), granulocyte (290%), Eosinophils count (85.71%), RBC (11.45%) and Eosinophils (97.09%) from control.

***Orobanche aegyptica* flower:** Eethanolic extract of *Orobanki aegyptica* flower. increased the leukocytes (194.13%), granulocytes count (24023.08%), lymphocyte count (13.77%), Monocytes count (588.77%), lymphocyte (250.74%), Monocytes (1946.15%), HGB (0.36%), HCT (9.98%), MCV (48.14%), MCH (9.29%), RDW (90.21%) and platelets (82.55%) from control. But the extract decreased the values for MPV, RBC, MCHC, Eosinophils count, Eosinophils and granulocytes parameters. The maximum decrease was for MPV (6.78%), MCHC (9.39%), granulocyte (290%), Eosinophils count (100%), RBC (26.60%) and Eosinophils (90.99%) from control.

***Orobanche aegyptica* stem:** The extract of *Orobanki aegyptica* stem changed blood parameter A significant increase in leukocytes (204.26%), granulocytes count (33207.69%), Monocyte count (744.89%), lymphocyte (184.70%), Monocyte (2207.69%), HGB (13.56%), HCT (8.66%), MCV (37.25%), MCH (39.05%), MCHC (2.45%), RDW (74.76%) and platelets (100.26%) was noted. Ethanolic extract rdecreased MPV, Eosinophils count granulocyte lymphocyte count parameters by values of MPV (23.52%), Eosinophils count (100%), granulocytes (760%) and lymphocyte count (15.86%).

***Citrollus colocynthis* root:** *Citrollus colocynthus* root Ethanolic extract also changed the blood parameters. The significant increase was in leukocyte count (89.34%), granulocyte count (15869.23%), lymphocyte count (11.32%), monocyte count (887.75%), lymphocyte (142.53%), HGB (15.60%), HCT (8.35%), MCV (40.13%), MCHC (1.17%) and RDW (94.54%). The application of extract decreased significantly MPV (44.73%), granulocyte (266%), Eosinophils count (89.28%), RBC (14.95%), Eosinophils (100%) from control.

The hematological parameters assessment acts as diagnostic criteria for effects of any foreign compounds on the blood components (Ashafa et al. 2012). Addition of chemical compounds at toxic level changes the blood parameters indicating hematological disorders like anemia which is characterized by low hemoglobin content (Price and Schrier 2008)); decline in platelet results in lymphoma and myeloma (Izak and Bussel 2014; Bradbury and Murray 2013). Blood parameters are used for diagnosing the physiological status of an organism (Pankaj and Varma 2013). Low levels of hemoglobin and RBC is due to iron deficiency or blood cell destruction which to anemia (Junqueira et al. 2006).

The increased levels of free hemoglobin in blood (hemoglobinemia) might be as a result of massive hemolysis Ugwu et al. 2013). Hematocrit (PCV) (called Packed Cell Volume (PCV) is clinically used to signal known or suspected anemia (Wintrobe and Greer 2009). Mean cell/corpuscular volume (MCV) is volume or size of a red blood cell. High MCV mean larger RBC size). Low MCV is due to iron deficiency (Aslinia et al. 2006). Mean corpuscular hemoglobin (MCH) is average amount of hemoglobin in single red blood cell. Invrease in MCH is due to anemias (Kasper et al. 2005). Mean corpuscular hemoglobin concentration (MCHC) is also average concentration of hemoglobin inside a single red blood cell. A low MCHC is iron deficiency orindication of abnormal hemoglobin synthesis MCV is size of red blood cells while MCH and MCHC are for concentration of hemoglobin. White blood cells (WBC) count and its indices play a vital role in immune function. A high number of eosinophils are due to a variety of disorders (Wintrobe and Greer 2009).

Our results revealed a diversified action of ethanolic extracts sourced from different plants parts on various hematological attributes. The results contradict to the findings of Lohar et al. (2009) while are in accordance to the findings of Straus, 1998 regarding effects on RBC and Hb concentration (MCH, MCHC). It has been reported that plants extracts vary in their actions and are often non-specific in their actions (Treasure, 2000). Many plants have been known to produce biologically active substances which are related to their special flavours, taste and antioxidant status. Among the phenolic compounds, antioxidants and secondary metabolites, the most abundant are natural antioxidants (Fiorentino et al. 2006). Antioxidants can act as cell saviors, as reducing agents, free radical scavengers, singlet oxygen quenchers or hydrogen donors (Fattouch et al. 2007).

Antioxidants are synthesized in plants under environmental stresses to neutralize the reactive oxygen species (ROS). Damage to cells and biomolecules caused by reactive oxygen/nitrogen species (ROS/RNS) is nullified by antioxidant (Fiorentino et al. 2006).The

concentration ratio of antioxidant to ROS determines not only the potential of plants to withstand adverse environmental conditions but also when present in plant extract or its products, these might play important role against the hemolytic activity of ROS by stabilizing blood cells and molecules or by their direct action on ROS. The plant organic extracts have high amount of antioxidants such as flavonoids and phenolic. Flavonoids are especially important for protection against human diseases. (Tiwari, 2001).

Erythrocytes are considered as major target of free radicals (ROS) due to the presence of high concentrations of polyunsaturated fatty acids in cell membrane (Ebrahimzadeh et al. 2009). ROS may cause oxidative damage to the erythrocyte membrane due to hemolytic activity. Red Blood Cells (Erythrocytes) are the most abundant cells in the human blood. Medicines can have more effect on Erythrocytes than any other blood cells (Hamidi and Tajerzadeh, 2003). Results of present studies revealed that ethanolic extracts of desert plants specimens reduced the RBC. The hemolysis of red blood cells by ROS, produced in desert plants parallel to stress, damages the cell membrane with release of hemoglobin from these cells. All these factors, in union, cause deterioration of cell membrane, which may, perhaps, be the key step for lysis of cell.

Results revealed that ethanolic extracts of desert plant specimens decreased MCHC and increased MCH and MCV (Table.1). The MCHC and MCH are indices of haemoglobin concentration in blood and in its each cell respectively (Wickramasingh, 1991). Mean cell volume (MCV) is the volume of red cells. An increase in MCV accompanied with a decrease in MCHC could accounts for the reduction in osmotic fragility of cell membrane (Olaleye, 1999). MCH, MCV and MCHC relates to individual red blood cells while Hb and RBC are concerned with the total numbers of red blood cells (Ashafa, 2011). The MCV determines the size of the RBCs. RBCs of normal size are termed normocytic. When the MCV is high, the RBCs will be larger and are termed as macrocytic. When the MCV is below normal, the RBCs are smaller which are called as microcytic. These categories of size are used to classify anemias. A significant reduction in MCV, if observed, accounts for interference in iron uptake by hemoglobin. Furthermore, it is reported that decrease in the blood cells may be due to the increased glycosylation (non enzymatic) of membrane proteins which can cause hyperglycemia.

Table 3. In vitro effect of ethanolic extract of some trees and herbs of Thal on human heamatology[values represent mean±standard daviation; n=3]

Name of species	Lymphocyte count (10×3/L)	Monocyte count (10×3/L)	Eosinophils count (10×3/L)	RBC (10×6/ uL)	HGB (g/dL)	HCT (%)
Normal blood	2.68 ± 0.02 j	0.13 ± 0.05 g	50.33 ± 0.57 h	5.14 ± 0.005 c	8.33 ±0.05 d	22.73 ± 0.05 h
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Capparis decidua (s)	5.7 ± 1.15 cde (+112.68)	2.6 ± 1.04 bcd (+1900)	1.6 ± 0.2efg (-96.82)	3.7 ± 1.113 bc (-28.01)	8.63 ± 1.05 bcd (+3.60)	20.83 ± 1.006 h (+54.993)
Capparis decidua(r)	5.73 ± 1.06 cde (+113.80)	2.63± 1.15 bcd (+1923.07)	1.46 ± 0.15 fg (-97.09)	4.45± 1.19 bc (-13.42)	8.43 ± 1.00 bcd (+1.20)	24.7± 1.15 e (+72.01)
Fogoniaarabica (s)	4.8 ± 1.1 defgh (+79.10)	1 ± 1 efg (+669.23)	1.46 ± 0.15 fg (-97.09)	4.56 ± 0.92 bc (-11.45)	8.63±1.02bcd (+3.60)	27.63 ± 1.05c (+21.7)
Orobankiaegyptica (f)	9.4 ± 0.62 a (+250.74)	2.66 ± 1.00 bcd (+1946.15)	4.53 ± 1.11 b (-90.99)	3.78 ± 1.095 bc (-26.60)	8.36±1.00bcd (+0.36)	25 ± 1 de (+9.98)
Orobankiaegyptica (s)	7.63 ± 1.13 ab (+184.70)	3 ± 1 bc (+2207.69)	0 ± 0 h (-100)	4.28 ± 0.92 bc (-16.89)	9.46±1.15abc (+13.56)	24.7 ± 1.05 e (+8.66)
Citrolluscolocynthus (r)	6.5 ± 0.79 bcde (+142.53)	2.53 ± 1.1 bcd (+1846.15)	0± 0 h (-100)	4.38 ± 1.30 bc (-14.95)	9.63±1.12abc (+15.60)	24.63 ± 1.10 e (+8.35)

Values sharing the different letters represent significance difference in respective row; values in parenthesis represents percentage difference over control group; LSD= least standard deviation; r=root; s=stem; f=flower

Ethanolic extracts of some specimens reduced the haemoglobin concentration while others induced an increase. This change in haemoglobin quantity in blood might be due to Iron deficiency as Iron is a component of heme group in hemoglobin. This Iron deficiency could be due to interference of plant extract biomolecules with iron directly or interference during its biosynthesis metabolism. A failure in production of hemoglobin results in cells smaller than normal cells. This occurs in many diseases, including iron deficiency anemia,

thalassemia, and anemias associated with chronic infection. Iron deficiency might be due to desert plant ROS which cause mobilization of Fe^{2+} by Ca^{2+} via Fenton reaction (Kupier-Goodman and Scott, 1989). The increase in Hb (MCH and MCHC) facilitates oxygen transport to the tissues. An increase in Hb concentration (MCH, MCHC) may be due to the presence of active gradients that stimulate haemopoiesis, or support in availability of iron for haemopoiesis, or agents for chelating iron may be absent or weakly present in the plant extract which change the extent of hemolysis of RBC (Lohar et al, 2009).

An increase or decrease in blood attributes might be owed to free radical scavenging activity of extract (Saha et al. 2008); antiuglycosylation (Nair et.al.,2013); thrombolytic potential (Sayeed et.al. 2014) , anticoagulation by plant extract (Ughachukwu et.al. 2013) or by genotoxicity (Pereira et.al. 2014). Reduction in platelets by ethanolic extracts was observed (Table.1). Blood platelets reduction may be beneficial at some level because platelets decrease the blood viscosity which positively adds to blood pressure and may be beneficial by view of the clinical haematology. Change in Blood platelet might be by their adhesion to collagen and platelet aggregation by ROS species of desert plants (Olas, 2008). Plant antioxidants might change blood platelet by antiplatelet activity (Dutta-Roy, 2002).

Conclusively, based upon the criteria of importance of blood attributes,the extract of Orobanche plant was the most promising in enhancing haemoglobin concentration than other plants. The Capparis decidua stem extract proved to be worst for decreasing platelets of blood. Different blood parameters were influenced to varying extent by these plants antioxidant extracts. Hence, the practical application of the plant antioxidant used should be based upon their careful and extensive study regarding their pharmaceutical or toxicological nature.

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Methylene Tetrahydrofolate Reductase Gene Polymorphisms and Risk of Myocardial Infarction

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ABSTRACT

Methylenetetrahydrofolate reductase (*MTHFR*) enzyme is one of the chief players in metabolism of circulating forms of homocysteine (Hcy). Any variation in the *MTHFR* gene can affect *MTHFR* enzymatic activity and is associated with serious cardiovascular profile like Myocardial Infarction (MI). The present study is designed to evaluate the association of *MTHFR* (C677T and G1793A) gene polymorphisms with MI in Jammu region. For the study purpose a total of 109 individuals were recruited (49 cases with MI and 60 unrelated healthy controls). Genotyping was done by PCR-RFLP technique and further data was statistically analysed. The frequencies of variant *MTHFR* alleles were higher in patient group than in controls (677T=12.24% vs 1.67% and 1793A=16.33% vs 15%). Logistic regression analyses have shown that *MTHFR* C677T polymorphism was significantly associated with the development of MI whereas *MTHFR* G1793A polymorphism was not associated with the disease in our study population. The haplotype T-G was giving approximately 8.23-fold risk [OR=8.23 (1.79-37.74), p=0.001] and C-G is conferring 2 folds protection [OR=0.5 (0.26-0.96), p=0.03] towards MI outcome. Based on measure of linkage disequilibrium (LD), the two *MTHFR* variants (C677T & G1793A) were in complete LD (D'=1, r2=0.02) in patients and in controls (D'=0.99, r2=0). The obtained data proved the association of *MTHFR* polymorphisms with the progression of MI severity in the population of Jammu region of the Union territory of Jammu and Kashmir State. Although a study comprising of large sample size is required to reach on a final conclusion.

KEY WORDS: C677T, G1793A, MI, *MTHFR*, POLYMORPHISM.

INTRODUCTION

Recent progresses in the field of genetics and genomics have provided substantial benefits to clinical medicine including facilitation of characterization of disease pathogenesis and diagnosis. Genetics has offered effective approaches to the identification of complex diseases like Myocardial Infarction (MI). It is an important clinical problem because of its large contribution to mortality. MI is multifactorial in origin and involves interplay of various environmental exposures viz. smoking, sedentary lifestyle, altered blood lipid levels, obesity, family history, hypertension and genetic profile. All these exposures in concert are responsible for the commencement of immuno-inflammatory cascade leading to endothelial damage, atherosclerosis, thrombus formation, blockage of arteries and finally event of MI. Elevated circulating levels of homocysteine (Hcy) is a well known predictor for complex cardiovascular phenotypes such as MI (Kaur et al., 2016; Raina et al., 2016a).

Homocysteine [COOH-CH(CH2CH2SH)-NH3] is a homologue of the amino acid cysteine and is

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synthesised in the body from dietary methionine. Methylenetetrahydrofolate reductase (*MTHFR*) is a folate and vitamin B12-dependent enzyme which is involved in conversion of Hcy back to methionine. Thus, maintaining plasma Hcy levels (Iqbal et al., 2011). There are two well characterised single nucleotide polymorphisms (SNP) at *MTHFR* locus viz. C to T transition at nucleotide position 677 in exon 4(rs1801133) and G to A transition at nucleotide position 1793 in exon 11 (rs2274976) which have been identified for bringing up alteration in *MTHFR* enzyme activity and disturbance in Hcy metabolism (Frosst et al., 1995; Raddy et al., 2002). These polymorphisms are associated with reduced *MTHFR* enzyme activity by about 70% and 40% in homozygotes and heterogzygotes, respectively (Mao et al., 2008; Kour et al. 2016b Jakó and Sinkó (2017).

Previous reports on *MTHFR* gene polymorphisms have shown that the said gene is significantly associated with CVD in Jammu region (Raina et al., 2016a; Raina et al., 2016b) but the polymorphisms have not been studied in MI so far. Hence, in the present investigation we have evaluated the association of *MTHFR* C677T and *MTHFR* G1793A gene polymorphisms with MI in population of Jammu region (J&K).

MATERIAL AND METHODS

Study population and Ethical Approval: A total of 109 individuals, 49 patients with MI and 60 healthy unrelated, control individuals were recruited for the present study. The patients were enrolled from the Department of Cardiology, ASCOMS, Sidhara, Jammu (J&K) and from private clinics. Sampling for controls was done from University premises. Physically healthy individuals with no history of CAD, hypertension, diabetes, thyroid problem, any form of cancer and other major medical conditions were included as controls. The present study design was approved by institutional Ethical Committee, University of Jammu. A brief health questionnaire covering different parameters such as gender, height, weight, smoking and physical inactivity, along with a consent form was duly filled by every subject enrolled for study. Diagnosis of MI was based on WHO criteria which included clinical history, ECG changes indicating myocardial damage and elevation of biochemical markers (Report of the Joint International Society and Federation of Cardiology, 1979).

Genotyping of MTHFR gene polymorphisms: Genotyping of *MTHFR* C677T and *MTHFR* G1793A polymorphisms was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described previously (Raina et al., 2016b; Raddy et al., 2002). Statistical analysis: Statistical analysis was carried out by using SPSS (Chicago, IL, USA) software version 21. Clinical characteristics of all the subjects were expressed as mean ± SD. Allelic frequencies were calculated by the gene-counting method. Hardy-Weinberg equilibrium (HWE) and the genotypic as well as allelic distribution of both polymorphisms of MTHFR gene were analyzed using Pearson's goodness of fit chi-square ($\chi 2$) test. The odd-ratios (OR) for *MTHFR* polymorphisms were calculated with 95% confidence interval (CI) under different genetic models. SHEsis software (Shi and He, 2005) was used to calculate the haplotype frequencies and Linkage disequilibrium (LD) pattern. A p-value of <0.05 was considered as statistically significant.

Table 1. Showing genotypic and allelic distribution of MTHFR gene polymorphisms in study participants.

Genotypes/ alleles/	MI Cases					Controls (N=60)
MTHFR C677T	IWMI (n=21)	AWMI (n=10)	STEMI (n=13)	NSTEMI (n=5)	Total (N=49)	
CC	16 (76.19%)	7 (70%)	11 (84.62%)	4 (80%)	38 (77.55%)	58 (96.67%)
CT	4 (19.05%)	3 (30%)	2 (15.38%)	1 (20%)	10 (20.41%)	2 (3.33%)
TT	1 (4.76%)	0	0	0	1 (2.04%)	0
C	36 (85.71%)	17 (85%)	24 (92.31%)	9 (90%)	86 (87.76%)	118 (98.33%)
T	6 (14.29%)	3 (15%)	2 (7.69%)	1 (10%)	12 (12.24%)	2 (1.67%)
MTHFR G1793A						
GG	15 (71.43%)	7 (70%)	9 (69.23%)	4 (80%)	35 (71.43%)	42 (70%)
GA	5 (23.81%)	2 (20%)	4 (30.77%)	1 (20%)	12 (24.49%)	18 (30%)
AA	1 (4.76%)	1 (10%)	0	0	2 (4.08%)	0
G	35 (83.33%)	16 (80%)	22 (84.62%)	9 (90%)	82 (83.67%)	102 (85%)
A	7 (16.67%)	4 (20%)	4 (15.38%)	1 (10%)	16 (16.33%)	18 (15%)

RESULTS AND DISCUSSION

These 49 cases of MI were further categorised into different subtypes according to ECG patterns and location of infarct. It was found that the inferior wall myocardial infarction (IWMI) was prominent type of heart attack occurring in 21 patients followed by ST- Elevation Myocardial Infarction (STEMI) in 13 patients, Anterior Wall Myocardial Infarction (AWMI) in 10 patients and Non-ST Elevation Myocardial Infarction (NSTEMI) in 5 patients only. The percentage distribution of both *MTHFR* C677T and *MTHFR* G1793A polymorphisms are summarised in Table 1. It was observed from the data that the wild CC genotype was the most prevalent one followed by heterozygous CT genotype and then the variant homozygous TT genotype. There was a considerable increase in CT genotype in patient group in judgement to controls (20.41% vs 3.33%). The prevalence of TT-genotype was higher in MI patients (2.04%) whereas there was complete absence of variant genotype in healthy controls. Overall, the frequency of variant T-allele was significantly higher in patients i.e. 12.24% than in controls i.e. 1.67%.

The prevalence of genotypes/ alleles were also studied in different MI categories and it was observed that risk allele (T) was highly prevalent in IWMI patients (14.49%) followed by AWMI (15%), NSTEMI (10%) and then, STEMI (7.69%). As regards of *MTHFR* G1793A polymorphism, the genotype distribution pattern in both MI patients and control individuals showed that the percentage of wild GG- genotype was higher (71.43% vs 70% respectively) in comparison to heterozygous GA-genotype (24.49% vs 30%% respectively) and risk AA-genotype (4.08% vs 0% respectively). Additionally, the allele percentage of the variant A- allele in MI patients was almost comparable with the control group (16.33% vs 15% respectively). The order of prevalence of A-allele in different MI subtypes was as follows: AWMI (20%), IWMI (16.67%), STEMI (15.38%) and NSTEMI (10%). The observed frequencies of *MTHFR* polymorphisms were in concordance with HWE in both study groups.

Table 2: Showing Logistic regression analysis for *MTHFR* (C677T) polymorphism

Genetic Model	Genotypes/ Alleles	MI (N=49)	Controls (N=60)	OR (95% CI)	p-value
Co-dominant	CC	38	58	1 (Reference)	
	CT	10	2	7.63 (1.58-36.76)	0.004
	TT	1	0	Not possible†	-
Dominant	CT+TT	11	2	Not possible†	-
	CC	38	58	1 (Reference)	
Recessive	TT	1	0	Not possible†	-
	CT+CC	48	60	1 (Reference)	-
Allelic	C	86	118	1 (Reference)	
	T	12	2	8.23 (1.79-37.73)	0.001

†Some genotype combinations were not observed, so it was not possible to calculate odds ratio.

Table 3. Showing Logistic regression analysis for *MTHFR* (G1793A) polymorphism

Genetic Model	Genotypes/ Alleles	MI cases (n=49)	Controls (n=60)	OR (95% CI)	p-value
Co-dominant	GG	35	42	1 (Reference)	
	GA	12	18	0.80 (0.34-1.89)	0.6
	AA	2	0	Not possible†	-
Dominant	GA+AA	14	18	Not possible†	-
	GG	35	42	1 (Reference)	
Recessive	AA	2	0	Not possible†	-
	GA+GG	47	60	1 (Reference)	
Allelic	G	82	102	1 (Reference)	
	A	16	18	1.11 (0.53-2.30)	0.8

†Some genotype combinations were not observed, so it was not possible to calculate odds ratio.

The association of *MTHFR* gene polymorphisms with risk of MI can be obtained by calculating Odds Ratio (OR) [Table-2 and 3]. For *MTHFR* C677T polymorphism, there was a significant difference in frequencies of CC vs. CT genotypes between the MI patients and healthy controls (OR=7.63 (1.58-36.76); p=0.004). OR for C vs. T allele showed that the 'T' allele was conferring approximately 8.23-folds risk, which was statistically significant for the development of MI in our study population. In context to *MTHFR* G1793A SNP, none of the applied genetic models were associated with risk of MI occurrence. Allocation of haplotype frequencies of *MTHFR* C677T and G1793A polymorphism among MI cases and healthy controls is given in Table 4. The haplotype T-G was giving approximately 8.23-fold risk [OR=8.23 (1.79-37.74), p=0.001] and C-G is conferring 2-folds protection [OR=0.5 (0.26-0.96), p=0.03] towards MI outcome. Based on measure of linkage disequilibrium (LD), the two *MTHFR* variants (C677T & G1793A) were in complete LD (D'=1, r^2=0.02) in patients and in controls (D'=0.99, r^2=0) (figure 1).

Table 4. Association of *MTHFR* haplotypes with risk of MI.

Variant MTHFR C677T/ G1793A	MI Cases (N=49)	Controls (n=60)	OR (95% CI)	p-value
C-A	0.163	0.15	1.10 [0.53-2.30]	0.8
C-G	0.714	0.833	0.5 [0.26-0.96]	0.03
T-G	0.122	0.016	8.23 [1.79-37.74]	0.001

Table 5. Showing general characteristics of the study participants

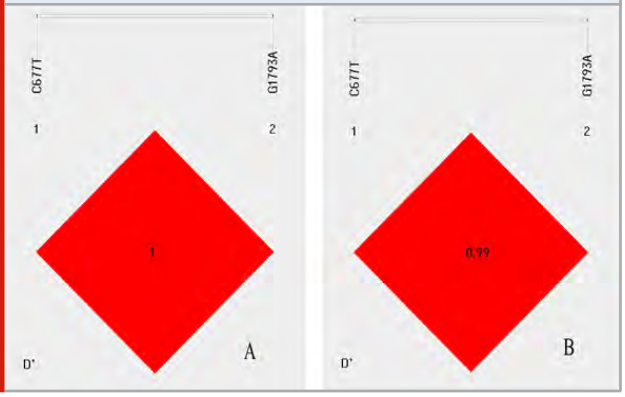
Variables	MI cases (N=49)	Controls (N=60)	OR (95% CI)	p-value
Age (yrs.)	46.75±6.7	48.91±7.4	-	0.1
Sex				
Males	40 (81.63%)	36 (60%)	-	-
Females	9 (18.37%)	24 (40%)	-	-
Blood pressure (mmHg)				
SBP	143.33±20.81	123.05±8.23	-	<0.0001
DBP	87.51±10.44	80.55±4.65	-	<0.0001
Pulse pressure	55.82±16.74	42.50±5.74	-	<0.0001
BMI	23.65±4.58	22.93±4.09	-	0.4
Smoking				
Y	26 (53.06%)	15 (25%)	3.39 (1.51-7.62)	
N	23 (46.94%)	45 (75%)	Ref. (1)	0.003
Sedentary lifestyle				
Y	23 (46.94%)	35 (58.33%)	0.64 (0.30-1.35)	
N	26 (53.06%)	25 (41.67%)	Ref. (1)	0.2

The general characteristics of the study participants are presented in Table-5. The controls were slightly elder than patients, with a mean age of 48.91 years compared to 46.75 years in the patient group. BMI was significantly higher in patients with a mean value of 23.65±4.58 than in controls with mean value of 22.93±4.09, howsoever the values did not reach a statistical significance (p=0.4). The physiometric characteristics viz. systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP) were higher in patients in comparison to controls in a significant manner (p<0.0001). In patient group, 53.06% cases were smokers whereas; among controls only 25% were involved in habit of tobacco smoking. OR analysis revealed that smoking was adding nearly 3.39 folds risk to the progression of MI in our population [OR = 3.39 (1.51–7.62); p = 0.003]. Majority of patients as well as controls were living a sedentary lifestyle and hence, lack of association was reported with this parameter.

The incidence of MI in India is relatively higher being 64.37/1000 people (Rao et al., 2014). The disease etiology involves dual interaction of environmental and genetic factors. Homocysteine is a sulphur containing amino acid made from a common dietary amino acid, methionine that inflicts damage to the inner arterial lining and contributes to coronary heart disease and event of MI in longer duration. Henceforth, genes controlling Hcy metabolism are considered as an emerging candidates for progression of MI. With this background the present

research work was aimed to study *MTHFR* (C677T and G1793A) gene polymorphisms with the risk of MI in population of Jammu region. A high prevalence of CC genotype was reported in the present study in comparison to CT and TT genotypes. In fact, a complete absence of TT genotype was observed in MI subtypes including AWMI, STEMI and NSTEMI as well as in healthy controls. Raina and researchers (2106a and 2016b) have also reported extremely higher frequency of CC genotype and lower prevalence of TT genotype in cardiovascular diseases in Jammu region. The results are consistent with other studies done by Markan et al., 2007; Lakshmi et al., 2011; Iqbal et al. 2011; Matam et al., 2014; Ezzat et al. (2014).

Figure 1: Linkage Disequilibrium (LD plot for MTHFR gene polymorphisms (A) patients (B) Controls.



The *MTHFR* 677T-allele was having a significant role in the aetiology of MI in our population (C vs T: OR=8. 23, p= 0.001). Gülec et al. (2001); Ezzat et al. (2014), Shaker et al. (2014) and Grek et al. (2015) were also in agreement of association of this polymorphism in onset of MI. Contrary to these observations, there were reports on lack of association of *MTHFR* C677T polymorphism with increased risk for MI in different population groups (Angeline et al., 2007; Iqbal et al., 2011; Verdoia et al., 2014; Iqbal et al. 2016).

Rady et al. (2002) reported a functional polymorphism (G1793A) in exon 11 of *MTHFR* gene that results in an arginine to glutamine substitution at codon 594 (R594Q) and is associated with coronary heart diseases. There is scarcity of data establishing a connection of *MTHFR* (G1793A) genotypes and MI. In the present study it was observed that the prevalence of wild (GG) genotype was higher in MI patients than in controls whereas heterozygous (GA) genotype frequency was lower in patient group when compared to healthy controls. The prevalence of variant (AA) genotype was also low in study population and controls were found to be devoid of AA genotype completely. Among different MI subtypes, variant genotype was present only in IWMI and AWMI.

In view of GG-genotype, Rady and co-associates (2002) also reported a lower prevalence of GA and AA-genotypes in their study populations. The results of our study demonstrated that the *MTHFR* (G1793A) was not in association with the development of MI. This is consistent with previous studies which have shown lack of *MTHFR* G1793A polymorphism with cardiovascular diseases (Kebert et al., 2006; Trifonova et al., 2012; Neto et al., 2013).Haplotype analysis was also performed and it has been observed that the haplotype combination T-G was giving approximately 8.23-fold risk whereas C-G was providing 2 folds protection towards MI outcome in population of Jammu region.

CONCLUSION

The results suggested that *MTHFR* gene is an informative candidate which can be used as a potential diagnostic marker for MI. Thus, further studies are necessary to ascertain the relationship between *MTHFR* variants and MI.

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Nutritional Communication

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BBRC Bioscience Biotechnology Research Communications

Conformation and Nutritional Evaluation of Gummy Candy Complemented with *Annona muricata*

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ABSTRACT

Candies are the most favoured eats by people of all age group. At present, due to increasing nutritional awareness candies are restricted from consumption as they provide empty calories and also cause dental caries. In order to make the common man enjoy the flavour and savour of candies, it becomes vital to nutritionally enhance the sugar candies by value addition so that the candies are no more in the block list of purchase. As a primitive measure, the study aims to incorporate *Annona muricata*, commonly known as graviola or soursop a fruit which is cultivated in the tropical areas, known for its antioxidant, anti-carcinogenic properties and immune enhancing properties as a major ingredient for the preparation of gummy candy. The mishmash of sour and sweet taste of the fruit and wellness of the whole fruit has immensely contributed for the mouth-watering taste of the gummy candy and nutritional factors as well. This study is outlined with the objective of developing gummy candy by incorporating soursop pulp, puree and extract to the regular ingredients of gummy candy respectively. The developed soursop gummy candies were subjected to sensory analysis and the accepted variation was subjected to nutritional analysis. It was observed that the soursop puree incorporated gummy candy was significantly rich in micronutrients and polyphenols compared to plain gummy candy which provided only calories.

KEY WORDS: ANTI-CARCINOGENIC, GUMMY CANDY, IMMUNE ENHANCEMENT, SOURSOP, VALUE ADDITION

INTRODUCTION

Annona muricata, Soursop fruits possess a strong smell and flavor, because of the high content of Vitamin C, Vitamin A and B complex. Mineral content quite complete couple with amino acids such as lysine, methionine and tryptophan (Rady et al.,2018). The specific bioactive constituents responsible for the major anticancer, antioxidant, anti-inflammatory, antimicrobial, (Sun etal., 2014, Prabhakaran etal.,2016) and other health benefits of soursop include different classes of alkaloids, flavonoids, sterols, and others (Chamcehyu and Rady, 2018).

Soursop acknowledged as a potent immune booster (Sun et al.,2017) and has proven effective against cancer, haemorrhoids, renal disorders, hepatic-biliary disorders, urinary tract infection, osteoporosis and even delays ageing.Ripe soursop fruit with short shelf life, will deteriorate and price of soursop fruit in the market by the farmers is not fetched (Amusa et al.,2003, Abbo et al.,2006). Therefore, the addition of the soursop fruit to gummy or jelly candy not only improve the taste, but also acts as a source of vitamins, minerals and amino acids as well expected to improve the utilization of soursop fruits (Moghadamtousi et.al., 2015, Daddiouaissa and Amid, 2019).

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As the jelly candies prepared incorporating fruits or vegetables along with gelatine and natural sweeteners has the advantage of nutritional value compared with those prepared only from essence and simple sugars. The texture and consistency of gummy candy allows the flexibility, to mold into different shapes, size making the product a highly acceptable one.

The present study is outlined with the objective of developing gummy candy by incorporating soursop pulp, puree and extract to the regular ingredients of gummy candy respectively. The developed soursop gummy candies were subjected to sensory analysis and the accepted variation was subjected to nutritional analysis. It was observed that the soursop puree incorporated gummy candy was significantly rich in micronutrients and polyphenols compared to plain gummy candy which provided only calories.

MATERIAL AND METHODS

All the ingredients required for the preparation of soursop gummy candy viz. soursop fruit, gelatin and jaggery were procured from Salem and Coimbatore, Tamilnadu India. The undamaged, healthy, mature soursop fruits were used for this study. The outer cover and seeds were removed. The flesh was cut in to small pieces and the fresh fruit pulp stored in refrigerator to prepare candy. In a stainless steel pan add soursop flesh and add ½ cup of drinking water and allow it to boil for few minutes. Once it reached room temperature put it into a blender to obtain soursop puree. The flesh was cut in to small pieces and it was put in stainless steel pan. Add drinking water 1½ times of fruit pulp. Boiled fruit sample was filtered through the muslin cloth to get soursop extract. After the preparation of pulp, puree and extract they were posed to physiochemical analysis to determine the suitability of incorporation to prepare gummy candy.

Table 1. Physio-chemical Properties of Soursop Pulp, puree and extract

Physicochemical Analysis	Pulp	Puree	Extract
Titratable acidity (g/100g)	1.02±0.43	0.61±0.02	0.81±0.04
Ascorbic acid (mg/100 g)	20.9±1.84	9.83±0.26	8.72±0.28
Cloud stability (at 660nm) Total	0.94±0.06	0.65±0.00	0.42±0.01
Total Soluble solids (^{0}Brix)	11.00±0.4	8.00±0.00	1.00±0.00
pH	3.70±0.06	3.70±0.04	3.70±0.08
Viscosity (cp)	25.60±0.14	19.20±0.16	8.02±0.22
Fructose, g/100 g	3.60±0.27	3.09±0.22	3.27±0.23
Glucose, g/100 g	2.97±0.24	2.90±0.22	2.87±0.20
Sucrose, g/100g	1.02±0.28	0.99±0.05	0.87±0.02

RESULTS AND DISCUSSION

The results of physio-chemical analysis are presented in table-1.

Titratable acidity deals with measurement of the total acid concentration contained within a food was determined by exhaustive titration of intrinsic acids with a standard base. Titratable acidity is a better predictor of acid's impact on flavor than pH, while Cloud Stability is linked to electrostatic repulsion between these particles, where negatively charged pectins surrounds protein nucleus. The solution viscosity gives an indication of the average degree of polymerization of the cellulose and gives a relative indication of the degradation (decrease in cellulose molecular weight) resulting from the pulping and/or bleaching process. To Measure the sugar content TSS content was used.

This was measured using a refractometer, and is referred to as the degrees Brix. The results depicted that all the criterions were similar in all three formulations viz. pulp, puree and extract except TSS values in extract, which was less may be because of processing method that was used. It was also observed that the values obtained were complimentary for the formulation of gummy candy. Hence, it was decided to prepare three formulations of soursop gummy candy (SGC) by using pulp, puree and extract as well. The soursop gummy candy was prepared by using soursop pulp, puree, extract. The ingredients required for the formulation is listed in Table 2.

Table 2. Composition of Ingredients for Formulation of Soursop Gummy Candy

Ingredients	Sample		
	F-1(Pulp)	F-2(Puree)	F-3(Extract)
Soursop pulp (g/100 g)	50	-	-
Soursop puree (g/100 g)	-	50	-
Soursop extract (ml/100 g)	-	-	50
Jaggery (g/100 g)	50	50	50
Gelatin(g/100 g)	2	2	2
Water(ml)	50	50	50

The steps in preparation of soursop gummy candy were as follows: Hot water was added to gelatin and was rested for 10 minutes. Water was boiled and to it was added portioned amount of jaggery to prepare syrup. Once the syrup reached one – thread stage, other ingredients were added except soursop preparation and stired well. After this soursop pulp/puree/extract was added and stirred for 2 mins without any lumps, in different ampoules. It was moulded into required shapes and refrigerated overnight, being ready to serve.

The different formulations of gummy candies prepared from soursop pulp, puree and extract were subjected to organoleptic evaluation to assess the maximum acceptability of the products. The quality attributes in terms of colour, appearance, flavor, texture, taste and over all acceptability were evaluated by untrained judges using score card with 5 point Hedonic scale.

Figure 1: Mean Organoleptic Scores of Different Formulations of Soursop Gummy Candies

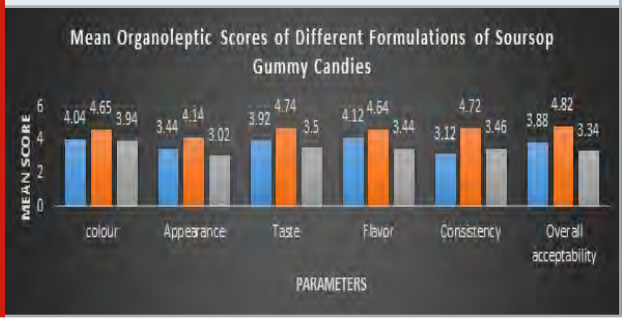

Figure 1 depicts that the overall acceptability, and other parameters of assessment like colour, appearance, taste, flavour and consistency was on the higher side for the gummy candy prepared from soursop puree. Hence, the accepted formulation was subjected to nutrient evaluation.

It is evident from the above table that the gummy candy prepared from soursop puree is superior in nutritional aspects in all means. The energy and carbohydrate values are more in SGC which may be attributed to combined calories and carbohydrates obtained from SF and jaggery. The protein available from gelatine and soursop has increased the protein content in SGC. The presence of fibre in candies is astounding and solely the inclusion of soursop fruit has proved the possibility (Qazi et al.,2018).

Fibre has sufficient supporting factors to improve the gut health, provide satiety and prevent obesity. The presence of B complex vitamins aid to boost metabolism. Vitamin C present in SGC (20.2mg/ 100gms) is a welcoming parameter to promote the production and use of SGC. Minerals like calcium, magnesium and iron known to play a vital role in boosting immunity is present in sufficient quantity in SGC. The presence of phytonutrients like alkaloids, flavonoids and polyphenols was also observed after qualitative estimation of phytochemicals in SGC.

Table 3. Comparison of Nutritive Value of *Annona muricata* Fruit, Plain Gummy Candy and Soursop Gummy Candy (Puree)

Nutrients	Nutritive Value of Soursop Fruit (SF)	Nutritive Value of Plain Gummy Candy (PGC)	Nutritive Value of Soursop Gummy Candy (Puree) (SGC)
Energy (kcal)	66	262	302.4
Protein(g/100g)	1	0.2	1.03
Fat (g/100g)	0.3	0	0.32
Cho (g/100g)	16.84	70	73.85
Fibre (g/100g)	3.3	0.1	3.11
Niacin(mg/100g)	0.9	0.01	0.3
Riboflavin(mg)	0.05	0	0.01
Thiamin(mg/100g)	0.07	0	0.03
Ascorbic acid (mg/100g)	20.6	2.2	20.23
Calcium(mg)	14	10	71.36
Potassium(mg)	60	54	63.76
Sodium(mg)	14	30	231.87
Magnesium(mg)	21	5	25
Iron(mg)	0.6	0.1	3

CONCLUSION

The gummy candy prepared using soursop puree will be ready to lend a hand for patients with cancer undergoing chemotherapy, as they experience nausea and throat or mouth dryness for which slurping of soursop gummy candies will prove effective. Also, renal patients for whom water intake is restricted, soursop gummy candy consumption may relieve thirst, along with the nutritional wellness of soursop. It is recommended that more awareness and promotion is required, regarding the pervasiveness and importance of soursop as it is rarely used as a whole fruit or processed. The food industries can also be encouraged to use soursop fruit in various recipes or menus, as a means of value addition. More supplementation studies are required to study the complete health potential of soursop.

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Microbiological Communication

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Antimicrobial and Antioxidant Potential of Endophytic Bacteria Isolated from *Emilia sonchifolia* (Linn.)DC.

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ABSTRACT

Endophytes are symbiotic bacteria that inhabit plant tissues and they were recognised as beneficial microorganisms which does not cause any disease symptoms or adverse effect on the host plant. They may be associated with the production of metabolites that either directly or indirectly influence the medicinal properties of plants. The antimicrobial and antioxidant property of the endophytic bacterial species associated with *E. sonchifolia* can be beneficial for the identification and isolation of valuable bioactive compounds. Six endophytic bacteria were isolated from *Emilia sonchifolia* (Linn.)DC. and their ethyl acetate extract was prepared. This extract was used for the study of antimicrobial and antioxidant properties. The total phenol and flavanoid contents of the bacterial extracts were estimated and antioxidant activity by DPPH, ferric ion reducing, nitric oxide scavenging and cupric ion reducing assays were done. Antioxidant analysis revealed the potential antioxidant property of endophytic bacterial isolates. The Isolate ES1 indicated the highly efficient antioxidant property. GC-MS and LC-MS analyses were employed for the identification of compounds which imparts antimicrobial and antioxidant property of the endophytic bacterial isolate ES1 this pointed the presence of bioactive compounds like surfactin, fengycin, iturin from the bacterial extract.

KEY WORDS: *EMILIA SONCHIFOLIA*, ANTIMICROBIAL, ANTIOXIDANT, ENDOPHYTE, SURFACTIN, ITURIN.

INTRODUCTION

The rapid emergence of multiple drug resistant strains of pathogens demanded the need for the development of plant based antibiotics. Extensive screening of medicinal plants for unexplored metabolites is fast progressing in this area. Many of them harbour bacterial or fungal endophytes that support the production of metabolites. Endophytes are mutualistic organisms residing inside the plant and produce compounds of pharmacological importance (Pezzuto, 1996). In the recent years, studies on these endophytic microorganisms as an alternative source of various bioactive metabolites resulted in the development of many probiotic and antibiotic compounds. The interest on endophytes associated with medicinal plants led to the identification of different antimicrobial compounds producing strains of microbes. There were many reports on natural products like alkaloids, flavanoids, phenolic compounds, peptides and steroids obtained from medicinal plants that harboured endophytes. In most of the cases the endophytes may either participate or gain some genetic information that led to the production of such metabolites, (Shukla et al., 2015, Ahemad and Kibret, 2014, Gond et al., 2015, Sulistyani et al., 2016, Mohamad et al., 2018, Hazarika et al., 2019).

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Along with the production of antimicrobial compounds endophytes are reported to produce potent antioxidants (Zheng et al., 2016, Bintang et al., 2015). Health care and food processing industries demand novel antioxidant compounds that rapidly reduce oxidation and manage reactive oxygen species causing damage to cells. Endophytes play a major role either directly or indirectly by enhancing the growth of the host plant and thus the plant growth promotion. Plants harbouring endophytes shows increased growth rate due to the production of various phytohormones, ameliorating the stress tolerance or by checking pathological conditions (Swarnalatha et al., 2015). Therefore metabolites produced by endophytes gained attention as future prospective antibiotics and antioxidants, (Hazarika et al., 2019, Hanif et al 2019).

The present investigation was carried out in *Emilia sonchifolia* (Linn.)DC., a member of Asteraceae family of Dicots. In Ayurveda the plant is highly reputed for its medicinal uses and is an important member in Dasapushpa (Raj et al., 2013). This medicinal plant was reported to produce a broad spectrum of therapeutically active metabolites possessing antimicrobial (Latha et al., 2009, Thenmozhi et al., 2013), antioxidant (Sophia et al., 2011) and anti-inflammatory (Essien et al., 2009) activities. It has been used in the treatment of asthma, inflammatory disorders, cuts and wounds. In ethnomedicine, the plant reported for sore throats, eye, ear ailments, malaria, and measles (Kumar et al., 2015). In this regard enquiry of bacterial endophytes in *Emilia sonchifolia* (Linn.)DC. has been undertaken along with their antioxidant and antimicrobial studies.

MATERIAL AND METHODS

Endophytic bacteria were isolated and identified by 16S rDNA sequencing from *Emilia sonchifolia* (Linn.) DC. (Urumbil and Anilkumar, 2019). The sequence data were submitted to NCBI Gen Bank. The endophytic bacteria isolated were *Bacillus subtilis* strain UCCBOT-ES1 (MG692780), *Paenibacillus sp* strain UCCBOT-ES2 (MG692781), *Microbacterium sp*. strain UCCBOT-ES3 (MG692782), *Bacillus cereus* strain UCCBOT-ES4 (MG692783), *Bacillus aryabhattai* strain UCCBOT-ES5 (MG692784) and *Micrococcus sp*. strain UCCBOT-ES6 (MH027648).

Preparation of extract: The endophytic bacterial isolates were inoculated into 500ml nutrient broth and incubated at 25±2°C for 5 days. The cultures were centrifuged at 8000 rpm for 10 minutes and the supernatant was extracted with double the volume of ethyl acetate and concentrated to a powder form. It was further dissolved in methanol and used for the antimicrobial and antioxidant analysis.

Antimicrobial analysis: The antimicrobial activity of endophytic bacterial extracts were tested against human pathogenic bacteria such as *Escherichia coli* (MTCC 40), *Salmonella typhi* (MTCC 426), *Klebsiella pneumonia* (MTCC 109) and *Proteus vulgaris* (MTCC 3220), and fungi such as *Candida albicans* (NCIM 3102) and *Aspergillus niger* (NCIM619) by co culture and disc diffusion methods. The plates were incubated overnight at 37°C and observed for the growth of pathogenic bacteria on either side of the isolates. Further antimicrobial activity of the extracts was studied by disc diffusion method. Streptomycin (10µg/ml) and itraconazole (50µg/ml) was used as antibacterial and antifungal drugs respectively.

Antioxidant analysis: Estimation of total phenol: The total phenol content of the endophytic bacterial extract was calculated by Folin and Ciocalteu method (Swarnalatha et al., 2015). The experiments were repeated thrice and mean value was calculated. The phenolic content was expressed as Gallic acid equivalents in µg/ml.

Estimation of total flavonoid: The total flavanoid content was measured by aluminium chloride colorimetric assay (Kamtekar et al., 2014). The total flavanoid content was expressed as µg/ml of quercetin equivalent.

DPPH free radical scavenging Assay: 50µl of extract in methanol was added to 100µl of DPPH solution and 850µl methanol, so that the final volume was 1ml. Nutrient broth was used as the control and methanol as blank. Percentage of scavenged DPPH radical was calculated using following formula

$$\% \text{ Scavenging} = \frac{Ac - A1}{Ac} \times 100$$

Where, Ac is the absorbance of control and A1 is the absorbance of sample. Ascorbic acid was used as standard, (Sulistiyani et al., 2016).

Ferric ion reducing assay: Extracts prepared from the endophytic bacterial isolates were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml potassium ferricyanide (1%w/v). Method reported by Jayanthi and Lalitha, 2011 using trichoro acetic acid and ferric chloride solution was employed for the ferric iron reducing assay. Ascorbic acid at various concentrations was used as standard.

Nitric oxide radical scavenging assay: 1ml of different concentrations of the extract was mixed with 0.5ml of 10mM sodium nitropruside in phosphate buffered saline and incubated at 25°C for 180 minutes. After incubation the extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extract but with an equal volume of buffer were prepared similar to test sample (Boora et al., 2014). Ascorbic acid was used as positive control. The percentage of nitric oxide radical scavenging activity of the endophytic bacterial extract was calculated.

Cupric iron reducing antioxidant capacity assay (CUPRAC Assay): Cupric iron reducing capacity was measured in accordance to the method of Apak et al., (2008). 1ml of crude extract in ethanol was added with 1ml of 7.5x10-3M Neocuproine (Nc) solution, 1ml of 1x10-2M CuCl2 Solution, 1ml of ammonium acetate buffer (pH=7) and

1ml water. Incubate for 30minute at 25°C and measure the absorbance at 450nm and ascorbic acid was used as standard.

Statistical analysis: All data were expressed as mean ±SD. The mean values were statistically analysed using One-way analysis of variance (ANOVA) using the graph pad instat software package.

GC-MSAnalysis and identification: Ethyl acetate fraction of the endophytic bacterial extract with greater antioxidant property was used for GC-MS analysis (Model Number: QP2010S), Column (Rxi-5Sil MS,30 meter length,0.25 mm ID, 0.25 µm thickness), GCMS Software (GCMS Solutions) and Libraries (NIST 11 & WILEY 8).

LCMS/MS-Q-TOF analysis: The crude ethyl acetate fraction of bacterial extract was subjected to liquid chromatography coupled with mass analyser (Waters Xevo G2 QTOF-MS/MS). The separation was carried out in a Acquity BEH C18 column and the two component solvent system contained 90% water (Acidified with 1% formic acid) and 10% acetonitrile with a flow rate of 0.3 ml/min.

RESULTS AND DISCUSSION

Endophytic bacteria are one of the unexplored, promising and relevant producers of metabolites useful in pharmaceutical, health care and agricultural industries. Endophytic bacteria especially many members from the *Bacillus* species, are reported to possess antimicrobial activity as an indirect mechanism for plant growth promotion by secretion of compounds to check phytopathological conditions (Ahemad and Kibret, 2014, Gond et al., 2015, Mohamad et al., 2018). In the present study, among the different isolates, ES4, ES5 and ES6 exhibited clear growth inhibition against all tested bacterial strains. At the same time ES1, ES2 and ES3 showed growth inhibition against *P.vulgaris* and *S.typhi* only (Fig.1, 2). When disc diffusion method was performed to assess the antibacterial activity, the strain ES4 (*Bacillus cereus* strain), ES5 (*Bacillus aryabhattai* strain) and ES6 (*Micrococcus sp*.strain) produced zone of inhibition against all bacterial strains and maximum zone of inhibition was reported against *P.vulgaris* and *S.typhi* (Table 1, Fig.3, 4). On the contrary strain ES1, ES2 and ES3 showed negligible zone of inhibition against the tested pathogens. Sunkar and Nachiyar (2012) reported the use of *Bacillus cereus* isolated from *Garcinia xanthochymus* for the synthesis of antibacterial silver nanoparticle indicated its significance as an antibacterial agent.

Of the six isolates tested for antifungal activity, ES2 (*Paenibacillus sp*.) produced a zone of inhibition of 15mm which is greater than the standard drug(itraconazole,11mm) (Table 1,Fig.5) Sulistiyani et al., (2016) reported an endophytic *Paenibacillus sp*. with antifungal property from *Curcuma longa* rhizome. A particular peptide showing antifungal properties was isolated from *Paenibacillus sp*. (Alkotaini et al., 2014). Anandaraj et al., (2009) isolated two antibacterial peptides Paenibacillin P and Paenibacillin N from Paenibacillus sp.

Table 1. Antimicrobial activity of the isolates

	Inhibitory activity as per co-culture method						Zone of inhibition(in mm) in disc diffusion method						
Pathogenic strains	ES1	ES2	ES3	ES4	ES5	ES6	ES1	ES2	ES3	ES4	ES5	ES6	Std.
E.coli	-	-	-	-	+	-	0	0	0	0	9	0	25
S.typhi	+	+	+	+	+	+	0	0	0	24	19	17	21
K.pneumoniae	-	-	+	+	+	+	9	8	0	9	9	9	22
P.vulgaris	+	+	+	+	+	+	8	9	8	20	15	12	20
C.albicans	-	-	-	-	-	-	0	0	0	0	0	0	13
A.niger	-		-	-	-	-	0	15	0	0	0	0	11

Figure 1: Total phenol content of the isolates at different concentrations of extract

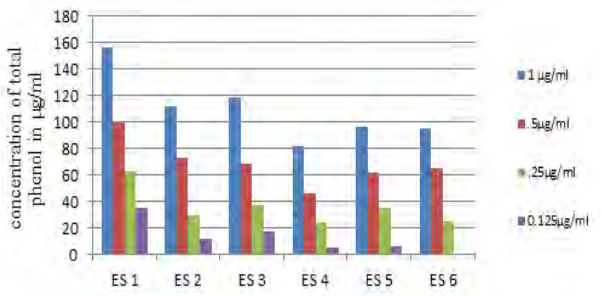


Figure 2: Total flavanoid content of the isolates at different concentrations of extract.

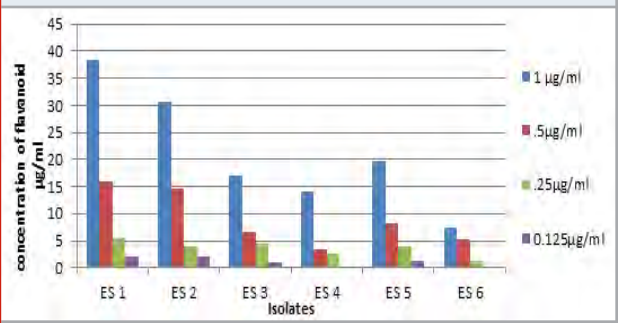
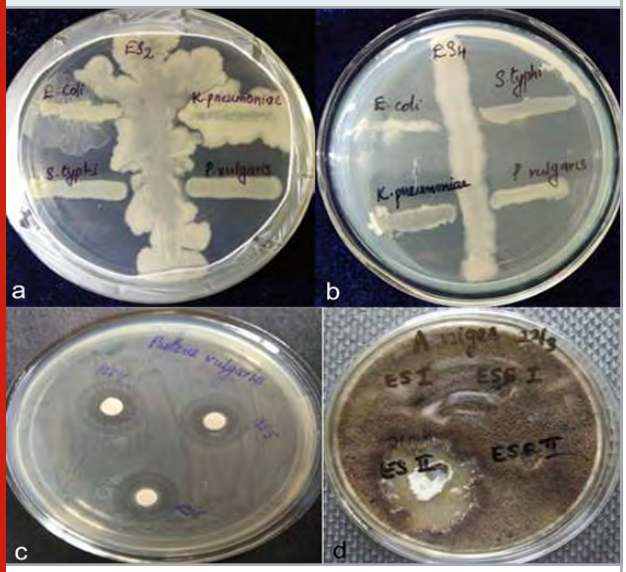


Figure 4: Antifungal activity of isolate ES2 against *A.niger*



Antimicrobial activity of endophytic bacterial extract-Fig.1 &t2-growth inhibition activity of endophytic bacterial isolate in co culture,Fig.3-Antibacterial activity of isolates against *Proteus vulgaris*, Fig.4-Antifungal activity of isolate ES2 against *A.niger*

Sophia et al., (2012) reported the antioxidant property of *E.sonchifolia* the medicinal plant used for the isolation of endophytes in the present study. In the present attempt the antioxidant avtivity of the endophytic bacterial extracts showed positive results indicating that the isolate as well as host plant synthesized some metabolites with common functions that might be due to the plant microbe interactions. Estimation of phenolic content revealed that ES1 produced maximum amount (156µg/ml), while ES4 produced the lowest (Graph 1). Total flavanoid content of the extracts showed that the isolate ES1 contained 37.3µg/ml quercetin equivalent of flavanoid and ES2 and ES5 have comparable flavanoid contents of 29.6µg/ml,18.6µg/ml of quercetin equivalent respectively (Graph 2). *Bacillus sp.* have been preferred as a probiotic bacteria in the feed industry because of its antioxidant property (Wang et al., 2017).

The most accurate method used for antioxidant analysis was found to be the DPPH assay. Total phenol and flavanoid estimation showed higher amount of these components in *Bacillus subtilis* strain. Free radical scavenging activity was measured in terms of IC50 value and lowest IC50 indicates a highest antioxidant activity. The highest antioxidant activity by DPPH assay was observed in ES1 (IC50 0.825µg/ml), followed by ES5 (IC50 1.19µg/ml), and ES6 (IC50 1.044µg/ml). Reports by Nongkhlaw and Joshi (2015) specified that L-Asparginase production was directly linked with antioxidant property and the *Bacillus subtilis* strain cenB associated with *Centella asiatica* showed positive results for L-Asparginase production and antioxidant property.

The results of each antioxidant assay may be different among the isolates based on the presence of various metabolites produced by the isolates and it cannot be comparable among the assays (Rafat et al., 2012). When ferric ion reducing power analysis was conducted, isolate ES1 (IC_{50} 0.33µg/ml) showed highest reducing power. The extracts of isolates, ES5 (IC_{50} 0.43µg/ml) and ES6 (IC_{50} 0.68µg/ml) also showed higher antioxidant property in accordance with the principles of Ferric reducing power assay. The IC_{50} value of Nitric oxide radical scavenging assay showed that isolates ES6 (IC_{50} 2.79µg/ml), ES3 (IC_{50} 3.14µg/ml), ES4 (IC_{50} 3.12 µg/ml) had significant antioxidant activity. Among the isolates ES5 showed highest cupric ion reducing property with lowest IC_{50} value (0.103µg/ml). The statistical analysis was carried out by one way ANOVA followed by Tukey test (Table 2).

Table 2. IC50 values in antioxidant assays

	DPPH Assay	Ferric ion reducing power	CUPRAC Assay	Nitric oxide radical Scavenging assay
Std	1.654	0.84	0.138	3.68
ES1	0.825***	0.33***	0.66ns	3.49ns
ES2	2.23***	1.55*	0.147***	5.6 ns
ES3	2.17***	1.25**	0.266**	3.14 ns
ES4	2.9***	2.21ns	0.254**	3.12 ns
ES5	1.19***	0.43**	0.103***	3.47 ns
ES6	1.044***	0.68**	0.423ns	2.79**

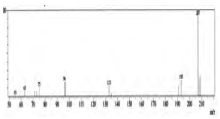
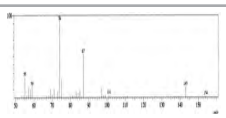
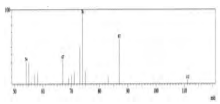
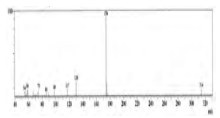
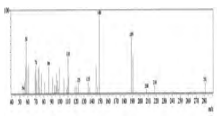
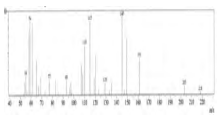
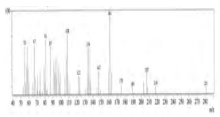
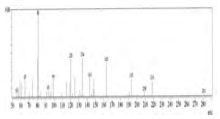
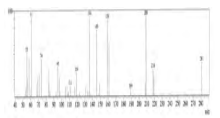
***p < 0.001- highly significant, **p < 0.05-significant, nsp > 0.05-not significant

Antioxidant and antimicrobial studies revealed that the isolate ES1 has significant activity and hence GC-MS Analysis of the ES1 extract was carried out. It revealed nine detectable compounds (Table-3). The major compound detected in the GCMS analysis in accordance with area percentage is Hexadecanoic Acid, 15-Methyl-, Methyl Ester (Common name-Methyl isoheptadecanoate) and it was reported to have antioxidant, nematicide (Imran et al, 2007, Zayed et al., 2014), antifungal and antibacterial properties (Ali et al., 2017). It was for first time we report the presence of this compound from the extract of endophytic bacteria that may contribute in the bioactivity. We conclude that the endophytic bacteria isolated from *E. Sonchifolia* possess significant antimicrobial and antioxidant properties.

Bioactivity studies revealed that *Bacillus subtilis* strain UCC BOT ES1 was the most promising among all isolates. LCMS/MS analysis was employed to screen the bioactive compounds produced by this isolate. *Bacillus subtilis* was already reported as one of the frequently identified bacterial endophytes known to be efficient in plant growth enhancement (Malfanova et al., 2011) and biocontrol activities (Luo et al., 2015, Kim et al., 2017).

Liquid chromatography with mass spectral analysis of different *Bacillus* members revealed high potential for the production of natural bioactive compounds (Hazarika et al., 2019).

Table 3. GC-MS identification of compounds in extract of isolate ES1

Peak#	R.Time	Area	Area%	Height	Height%	Name	Base m/z	
1	7.618	116466	18.15	33533	17.29	Cyclotrisiloxane, Hexamethyl-	207.00	
2	28.493	162474	25.32	50188	25.87	Hexadecanoic Acid, 15-Methyl-, Methyl Ester	74.10	
3	32.317	26412	4.12	11411	5.88	Hexanoic Acid, 2-Methyl-	74.05	
4	32.403	137413	21.41	45488	23.45	2,2',2''-Nitrilotriethanol, triethyl ether	174.10	
5	44.641	55758	8.69	8570	4.42	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-,methyl ester	149.15	
6	45.232	6727	1.05	8144	4.20	Thiofanox	145.15	
7	46.539	108149	16.85	18678	9.63	1-Heptatriacotanol	161.10	
8	48.131	20271	3.16	9335	4.81	Propenone, 1-(1-hydroxycyclohexyl)-3-(4-hydroxyphenyl)-	81.10	
9	49.166	8002 641672	1.25 100.00	8651 193998	4.46 100.00	15-(4-HYDROXYANILINO) RETINAL #	209.05	

A cyclic lipopeptide (cLP) biosurfactant 'surfactin' was reported from *Bacillus* species (Haddad et al., 2008). Apparent surfactin peaks were noticed in the current study and were comparable with the earlier reports (Sarwar et al., 2018). Peaks corresponding to the molecular mass 992, 1007, 1022 and 1035 therefore indicated the surfactin biosynthetic potential of *Bacillus subtilis* strain UCC BOT ES1. Peaks observed at 1083.58 [M+H+] was noted to be that of another non-ribosomal peptide iturin as per the previous reports. Iturin category of compounds was generally considered as antifungal compounds (Arrebola et al., 2010). Fengycin is another category of lipopeptide with wide range of applications as antifungal compounds and they were efficient in preventing the growth of filamentous fungi (Malfamova et al.,, 2011). Presence of fengycin was reported from different members of endophytic *Bacillus* (Hanif et al., 2019).The molecular weight observed in the present study [M+H+] 1417.78 and 1415.82 are in accordance with fengycin A (Table 4). Non-ribosomally synthesised lipopetides showed greater degrees of structural similarity and the difference among them was confined to the fatty acid chain (Roongsawang et al., 2010). However efficient purification and screening methods were required to separate and analyse the different class of lipopetide bioactive compounds from the bacterial extract.

Table 4. LC-MS/MS Analysis – Compounds identified in extract of isolate ES1

Lipopeptide	Molecular Mass	[M+H+]	[M+NH4]	[M+2K+H]	[M+Cl]	[M+CH3OH+H+]	[M+Na-H+]	[M-H+]
Surfactin	992.51	993.51	1011.53	1069.56	-	-	-	-
	1007.52		1025.53	1083.58	1041.5469	-	-	-
	1022.52	1023.53	1041.54	-	-	-	-	-
	1035.56	1036.56	1053.56	-	-	1069.56	-	-
Iturin	1082.57	1083.58	1101.57	-	-	-	1129.56	-
Fengycin	1414.41	1415.82	1432.03	-	-	-	1058.59	-
	1416.77	1417.78					1461.77	1415.76

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Conflict of Interest: The authors has no conflict of interest

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Prevalence and Antimicrobial Resistance of Pathogenic Bacteria Isolated from Cuticles of *Blattella germanica* and *Periplaneta americana* in Gizan City, Saudi Arabia

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ABSTRACT

Cockroaches are major microbial vectors around human dwellings that cause serious public health threats. They harbor a number of pathogenic bacteria on their cuticles with antimicrobial resistance. This study aims at investigating the bacterial carriage of *Blattella germanica,* and *Periplaneta americana* and their antimicrobial resistance in Gizan, Saudi Arabia. 152 cockroaches were trapped in houses in Gizan City during January - July 2018. Standard methods were followed in all the microbiological investigations and antibiotic susceptibility tests using Vitek2 Automated Microbiology System, Biomerieux®. All of the 152 cockroaches were found with bacteria load on their cuticles. Twenty tow species of bacteria belonging to ten genera were identified. However, *Klebsiella pneumoniae* 33 (21.7%), *Serratia marcescens* 26 (17.1%), and *Pantoea agglomerans* 20 (13.1%) were the predominant isolates. Half of the isolates 11 (50. %) were multidrug-resistant strains. High resistance percentages were noted to Ampicillin and Amoxicillin clavulanate (41%), Cefoxitin (36%) and Cefazolin and Fosfomycin (27%). Cockroaches are potential source of pathogenic bacteria with multidrug resistant strains. This fact implies the epidemiological risks, complicating therapeutics, and leads to more medical costs in urban environments. Preventive and control measures are highly needed to minimize cockroach related food-borne diseases and other infections.

KEY WORDS: PATHOGENIC BACTERIA, BLATTELLA GERMANICA, PERIPLANETA AMERICANA, ANTIMICROBIAL RESISTANCE, JAZAN REGION, SAUDI ARABIA.

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INTRODUCTION

Cockroaches are among the most common insects that exist nearly around the world. Some biologists consider them one of the most adaptable and successful animal groups (Bennett et al., 1997). Cockroaches are one of the most serious food and residential pests around the world and invade places where food is stored, prepared or served. They are known to be notoriously resilient and difficult to control (Service 2004). There are about 4,400 species of Cockroaches (Blattaria or Blattodea). They are considered to be the oldest and most primitive of insects, dating to the Permian, about 275 million years ago (termite ancestry) (Brenner and Kramer, 2019).

The filthy behavior of cockroaches along with their nocturnal lifestyle enables them to contribute to food-borne diseases and to transmit many pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella spp., Shigella dysenteriae, Bacillus cereus,* and *Entamoeba histolytica* (Fotedar et al., 1989; Blazar et al., 2011; Burgess and Chetwyn 1981; Tachbele et al., 2006). In addition, cockroaches not only spoil food, but also cause allergic reactions and psychological disorders (Brenner, 1995). Control strategies should therefore be reoriented to emphasize the biology and ecology of target cockroaches, as well as the use of insecticides, if any, and should be more selective and environmentally friendly (WHO, 1996). Boric acid powder and cockroach gels were proved very effective against cockroaches and thus are highly recommended to be used in households and health care settings to control cockroaches (Noureldin and Farrag, 2008). The use of insecticidal baits had also resulted in a sustained cockroach elimination over a year in New Orleans and led to improve in asthma outcomes (Rabito et al., 2017).

Antimicrobial resistance (AMR) is one of the public health problems that threatens the prevention and effective treatment of a growing group of infections caused by bacteria. The problem of antimicrobial resistance is especially urgent when it comes to antibiotic resistance in bacteria. Bacteria that cause common or severe infections have developed over many decades, and to varying degrees, resistance to each new antibiotic. This necessitates the need for action to avert a growing global crisis in health care facilities (Prestinaci et al., 2015). The multiple resistance of the bacteria isolates from residential areas in Nigeria revealed the importance of surveillance on pattern and origin of antimicrobial drug resistance, as well as, the awareness of people at the resident areas about the danger of cockroaches in residential areas and vicinities (Adeleke et al., 2019).

Multidrug resistance of many bacterial species is considered a serious threat for patients in a sense that it restricts and limits therapeutic options. Cockroaches are becoming medically important as external vectors for many infectious diseases. Nonetheless, the public health significance of this vector so far has not been well documented, and to the best of our knowledge, no data has been published on this issue in Saudi Arabia and Jazan region in particular. Therefore, the aim of this study was to isolate pathogenic bacteria from the external surfaces of the cockroaches and to determine their antimicrobial resistance statuses.

MATERIAL AND METHODS

Study area: Jazan region (Fig. 1) is situated in the subtropical zone, Southwest Saudi Arabia, lies between 16°-12, and 18°-25, latitude north. It is surrounded by the Red Sea (260 km) from the west and by Arabic Republic of Yemen (120km) from the south and east and Asir region from the north, with total area of about 22,000 km2 (Al-Sheikh, 2011) and 1.6 million populations (GASTAT 2017: https://www.stats.gov.sa/en/5655).

Sampling and identification of cockroaches: Cockroaches were randomly trapped from 37 different households of Gizan City. Cockroaches were trapped in food-baited pitfall traps. Traps were placed at night in kitchens, toilets, and bathrooms and collected the next morning and transported to the laboratory of the Saudi Center for Disease Prevention and Control (SCDC) in Jazan for identification and further processing.

Figure 1: Map of Saudi Arabia showing Jazan region.

Cockroaches were identified to the species level using CDC pictorial key: (https://www.cdc.gov/nceh/ehs/docs/pictorial_keys/cockroaches.pdf). and (Harwood and James, 1979).

Bacterial isolation: Trapped cockroaches were frozen at 0 ^{0}C for 10 minutes. Then, each cockroach was placed in a sterile test tube and 5 ml. of sterile 0.9% normal saline was added to each test tube and thoroughly shaken for vigorously washing in the centrifuge for 2 minutes at 3000 rpm. A loop full of each suspension was cultured on MacConkey agar (MAC) and blood agar plate and incubated overnight at 37 ^{0}C.

Identification of bacterial isolates and antimicrobial susceptibility testing (AST): Following the manufacturer's instructions of Vitek 2 Automated Microbiology System, Biomerieux®; two plastic tubes (the first fills with 3 ml of 0.45% NaCl and the second is empty) were prepared.

Then a suitable number of every pure colony from the culture media was taken by sterile loop and placed in the 3 ml saline tube. The suspension was then centrifuged at 15,000g for 3 minute and measured for McFarland turbidity by DensiChek (McFarland range for GNB and GNB is 0.5 - 0.63). The former two steps were repeated to get the needed McFarland turbidity.ID card was placed in the suspension tube and AST card placed in the empty tube, and both tubes were put in the cassette, then the cassette was loaded in a smart carrier to enter needed data. The cassette then was loaded to the Vitek 2 to start the run for bacteria identification and Susceptibility testing. The cards were read by kinetic fluorescence measurement and the results reported within11 to 24 hrs.

RESULTS AND DISCUSSION

One hundred fifty two cockroaches were collected from 37 different households of Gizan City. Out of these, 98 (64.5%) were German cockroaches (*B. germanica*), and 54 (35.5%) were American cockroaches (*P. americana*) (Table 1). In this study, both *Blattella germanica* and *Periplaneta americana* were identified in the households of Gizan City.

Table 1. Bacteria species isolated from *Periplaneta americana* and *Blattella germanica* in Gizan City, Saudi Arabia

Bacteria species	Gram (-ve /+ve)	Cockroaches (N = 152)		Total bacteria species No (%)
		B. germanica No (%)	*P. americana* No (%)	
Klebsiella				
Klebsiella pneumoniae	-ve	15	18	33 (21.7%)
Klebsiella oxytoca	-ve	8	-	8 (5.3%)
Klebsiella pneumoniae spp. ozaenae	-	5	5 (3.3%)	
Klebsiella pneumoniae	-ve			
spp. pneumoniae	-ve	-	3	3 (1.9%)
spp. pneumoniae				
Citrobacter				
Citrobacter gillenii	-ve	5	-	5 (3.3%)
Citrobacter werkmanii	-ve	4	-	4 (2.6%)
Citrobacter braaakii	-ve	2	-	2 (1.3%)
Citrobacter freundii	-ve	-	1	1 (0.66%)
Serratia				
Serratia marcescens	-ve	26	-	26 (17.1%)
Serratia plymuthica	-ve	7	-	7 (4.6%)
Serratia fonticola	-ve	-	8	8 (5.3%)
Enerobacter				
Enerobacter aerogenes	-ve	3	-	3 (1.9%)
Enerobacter cloacae complex	-ve	-	2	2 (1.3%)
Pseudomonas				
Pseudomonas aeruginosa	-ve	4	-	4 (2.6%)
Pseudomonas luteola	-ve	1	-	1 (0.66%)
Aeromonas				
Aeromonas samonicida	-ve	-	1	1 (0.66%)
Aeromonas hydrophila complex	-ve	-	5	5 (3.3%)
Pantoea				
Pantoea agglomerans	-ve	20	-	20 (13.2%)
Pantoea spp.	-ve	-	6	6 (3.95%)
Enterococcus				
Enterococcus durans	+ve	-	4	4 (2.61%)
Kluyvera				
Kluyvera ascorbata	-ve	3	-	3 (1.9%)
Hafnia				
Hafnia alveii	-ve	-	1	1 (0.66%)
Total	-	98 (64.5%)	54 (35.5%)	152 (100%)

Cockroaches are widely distributed in Saudi Arabia. For example, in Jeddah province, four species had been identified; German cockroach (*Blattella germanica*), American cockroach (*Periplaneta americana*), brown-banded cockroach (*Supella longipalpa*), and Oriental cockroach (*Blatta orientalis*) (Noureldin and Farrag, 2010). *Blattella germanica* was the most dominant species in both households and other properties. Cockroaches possess nocturnal as well as omnivorous features; thus considered the ideal vectors of pathogenic microorganisms including protozoa, bacteria, helminthes, fungus, and virus (Tatfeng et al., 2005: Salehzadeh et al., 2007). They are also of public health significance due to their ability to produce potent allergens (Özdemir, 2014). Among others, including house dust mites (HDMs), molds, pets, and rodents, cockroaches are considered one of the common indoor allergens (Al-Ghamdi et al., 2019).

Twenty two species of bacteria belonging to ten genera were identified from all cockroaches (Table 1). However, *Klebsiella pneumoniae* 33 (21.7%), *Serratia marcescens* 26 (17.1%), and *Pantoea agglomerans* 20 (13.1%) were the predominant isolates, followed by *Klebsiella oxytoca* and *Serratia fonticola* 8 (5.3%), and *Serratia plymuthica* 7 (4.6%).Cockroaches are common in many human dwellings, particularly in places where food is handled or stored. They are also found in wards and laboratory rooms of hospitals (Donkor, 2019).

Table 2. Resistance status of bacterial isolates identified from cockroaches collected from households in Gizan City, Saudi Arabia

Bacteria isolates	Antibiotics*																			
	AMC	AMP	CEZ	CEX	CER	CRA	CEL	MER	IMP	AZT	FOS	CLN	ERY	NIT	TE	TRS	CEF	CED	PIP	TIG
Klebsiella pneumoniae	R	R	R	R	S	S	S	S	S	R	R	S	S	S	S	R	R	I	R	I
Klebsiella oxytoca	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S
Klebsiella pneumoniae spp. ozaenae	S	R	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S
Klebsiella pneumoniae spp. pneumoniae	S	R	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S
Citrobacter gillenii	S	S	S	I	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Citrobacter werkmanii	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Citrobacter braaakii	S	I	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Citrobacter freundii	R	S	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
Serratia marcescens	R	R	R	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
Serratia plymuthica	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Serratia fonticola	R	R	R	I	I	S	S	S	S	S	R	S	S	S	R	S	S	S	S	S
Enerobacter aerogenes	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Enerobacter cloacae complex	R	S	S	R	S	S	R	S	S	S	S	S	S	I	R	S	S	S	S	S
Pseudomonas aeruginosa	R	R	R	R	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S
Pseudomonas luteola	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	R	I	S	S	S
Aeromonas samonicida	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Aeromonas hydrophila complex	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
Pantoea agglomerans	S	I	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	I	S
Pantoea spp.	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Enterococcus durans	S	S	S	S	R	R	S	S	S	S	S	I	R	S	S	S	S	S	S	S
Hafina alvei	R	R	S	R	S	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S
Kluyvera ascorbata	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Resistance Percentage	41%	41%	27 %	36 %	9 %	5 %	9 %	5 %	5 %	9 %	27 %	5 %	5 %	9 %	14 %	14 %	5 %	0 %	5 *	0 AMC:

Amoxicillin-clavulanic acid, AMP: Ampicillin, CEZ: Cefazolin, CEX: Cefoxitin, CER: Cefuroxime, CRA: Cefuroxime Axetil, CEL: Cefalotin, MER: Meropenem, IMP: Imipenem, AZT: Azteronam, FOS: Fosfomycin, CLN: Clindamycin, ERY: Erythromycin, NIT: Nitrofurantoin, TE: Tetracycline, TRS: Trimeth/Sulfa, CEF: Cefotaxime, CED: Ceftazidime, PIP: Piperacillin, TIG: Tigecycine. R: Resistant, S: Susceptible, I: Intermediate, NR: Not Reported.

Similar results were obtained in Ethiopia where *Klebsiella pneumoniae* 32 (17.7%), *Escherichia coli* 29 (16%), and *Citrobacter spp.* 27 (15%) were the predominant isolates (Moges et al., 2016). It is also reported that *Klebsiella pneumonia* was the most prevalent nosocomial bacteria in a tertiary hospital in Ghana (Patience et al. 2013). In

Iraq, *Klebsiella* (42.56%), *Pseudomonas* (38.61%), and *Proteus* (35.34%) were found to be the primary bacterial isolates in households and hospitals (Hams et al., 2014). *Blattella germanica* was found to carry 12 species of bacteria isolates (54.5%) (11Gram-negative and 1 Gram-positive), while *Periplaneta americana* harboured 10 spescies (45.5%) (all Gram-negative).Only one Gram-positive bacteria speices (*Enterococcus durans*) was found on the cuticle of *B. germanica*. Interestingly, *Klebsiella pneumonia* was the only bacteria species found on both *B. germanica* and *P. americana*.

Both *Blattella germanica* and *Periplaneta americana* in this study were found to harbor bacterial pathogens associated with food spoilage organisms and foodborne illness through their bodies. With this in mind, cockroaches could be potential vectors and reservoirs for foodborne bacteria and nosocomial infections in Gizan City.Cockroaches are reported to harbor variety of pathogens that potentially cause serious diseases such as typhoid, diarrheal syndromes and gastroenteritis (Graczyk et al., 2005). *Klebsiella pneumonia* is well known as a gram-negative pneumonia that can cause both nosocomial and community-acquired pneumonia. It is responsible for a range of serious infections involving the lungs, abdominal cavity, soft tissues surgical sites, intra-vascular devices, urinary tract and causing bacteraemia (Shon et al., 2013).

Serratia marcescens on other hand, causes variety of infections including meningitis, urinary tract infection, septicaemia, wound infection and respiratory tract infection (Gouin et al., 1993; Cox, 1985; Komer et al., 1994). It has also been reported to infect the left side of the heart causing endocarditis in the community and in hospitals (Cohen et al., 1980). This species has been implicated in ICU [21%] followed by male medical [18.5%] and emergency department [12.3%] in a hospital in Mekkah, Saudi Arabia (Faidah et al, 2015).*Pantoea agglomerans*, formerly known as *Enterobacter agglomerans* may cause a wide variety of nosocomial infections, including meningitis, urinary tract infections, wound and burn infections, pneumonia, infections of intravascular and other prosthetic devices (Donnenberg, 2015).

Worryingly, the present study demonstrated high resistance percentages to Ampicillin and Amoxicillin clavulanate (41%) followed by Cefoxitin (36%) and each of Cefazolin and Fosfomycin (27%). While least resistance rate was observed to Imipenem, Meropenem, Piperacillin, Eryhromycin and others (5%) (Table 2).In Mekkah city of Saudi Arabia, the resistance of *Serratia* strains to the tested antibiotics was high, except for Imipenem and Meropenem. The resistance was higher with Ampicillin (97.5%), Cefoxitin (90%) and Tetracycline (86%) (Faidha et al, 2015).

It has been reported that the resistance of bacterial isolates from cockroaches to Ampicillin in Taiwan is ranged between 13.7% to 100%, Chloramphenicol (14.3% to 71.4%), Tetracycline (14.3% to 73.3%), and Trimethoprim-sulfamethoxazole (14.3% to 57.1%). This was found in two gram-positive and five gram-negative bacteria including *Klebsiella pneumonia* and *Serratia marcescens* (Pai et al., 2004).In Indonesia, resistance of bacterial isolates, including *Klebsiella ozaenae*, to Amoxicillin, Vancomycin, and Chloramphenicol were detected but still sensitive to Ciprofloxacin and Ofloxacin (Astiti et al., 2018).

This study demonstrated that for individual bacterial strains, *Kl. Pneumoniae* had multi-resistance to 9 of the tested antibiotics, *Se. marcescens*, and *Pseudomonas aeruginosa* to 6, and *Hafnia alvei*, *Se. fonticola* to 5 (Table 2). In Libya, multiple resistance to at least 6 different antibiotics was observed among the bacteria isolated from the hospital and household cockroaches including *Klebsiella*, *Enterobacter* and *Serratia* species (Elgedri et al., 2006). Moreover, most bacterial isolates of *Klebsiella* from households and hospital cockroaches in India revealed drug resistance to at least 4 antimicrobial agents (Fotedar et al., 1991).

All isolates in this study showed 100% susceptibility to Ceftazidime and Tigecycine. This would help when tailoring effective reserve therapies against resistant bacterial isolates indicated in the present results. Results of this study also showed that half of the isolates 11 (50. %) were multidrug-resistant strains. Association of nosocomial infections with multidrug resistance organisms including bacteria are regarded as a major cause of morbidity and mortality (Faidah et al., 2015). It is well known that multi drug resistance strain could arise due to expression of genes that code for multidrug efflux pumps or due to accumulation of resistant genes in a single bacterial cell (Nikaido, 2009). Antibiotic resistance is often associated with selection and its subsequent proliferation of multi drug resistance strains or the horizontal transfer of genetic elements such as plasmids that encoding resistance (Chmelnitsky et al., 2013).

There are several mechanisms contribute towards virulence and antimicrobial resistance in Gram-negative bacteria. This include inactivating of the antimicrobial agent by resistance determinants, decreasing antimicrobial drug concentrations within the cell and modifying the antibiotic or its target sites (Nordmann and Poirel, 2008; Fernández et al., 2011; Kumar et al., 2011). In combating antimicrobial resistance, the World Health Organization (WHO) have identified several key shortcomings. These issues include: (i) irrational use and unconfirmed drug quality (ii) infections poor prevention and control (iii) lack of commitment and data, and (iv) languishing research into new antimicrobial agents and tools, including diagnostic tests and antimicrobials (Leung et al., 2011). This study indicates the importance of identifying and detecting of multi-drug-resistant bacterial strains from cockroaches for the first time in Jazan region and Saudi Arabia, which in turn cause therapeutic difficulties or failures. Therefore, therapies for such bacterial infections must be tailored to individual bacterial isolates and should be based on antimicrobial susceptibility testing (Donnenberg, 2015).

CONCLUSION

Cockroaches in Gizan City are potential source of pathogenic bacteria with multidrug resistant strains. Twenty tow species of bacteria belonging to ten genera were identified from cockroaches with *Klebsiella pneumoniae*, *Serratia marcescens* and *Pantoea agglomerans* being the predominant isolates. Half of the isolates were multidrug-resistant strains. High resistance percentages were noted to Ampicillin and Amoxicillin clavulanate, followed by Cefoxitin, Cefazolin and Fosfomycin.This fact implies epidemiological risks and complicating therapeutics and leads to more medical costs in urban environments. Preventive and control measures in households and other facilities focusing on hygiene measures within an integrated vector management approach are highly needed to minimize cockroach related food-borne diseases and other nosocomial infections that may arise in Gizan City.

Conflict of interest:
Authors declared that they have no conflict of interest.

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Correlation Between Lower Pharyngeal Airway and Chin Throat Angle in Class II Div 2 Malocclusion

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ABSTRACT

Normal airway is one of the important factors for the growth and development of craniofacial structures. Narrow lower pharyngeal airway is considered as one of the factors for obstructive sleep apnea but there is no clinical evidence for early detection of narrowed lower pharyngeal airway , hence the aim of the present study was to find any correlation between degree of submental cervical angle and lower pharyngeal airway dimensions in class II div 2 subjects and also to evaluate quantitatively the influence of submental cervical angle on lower pharyngeal airway to aid clinicians in early diagnosis of narrow lower pharyngeal airway. This retrospective cross-sectional study was performed on the pre-treatment lateral cephalometric films of 50 Class II div 2 subjects of aged between 14 and 30 years from multicentres in Tamil Nadu . All cephalograms were traced digitally by using FACAD software. The assessment of Chin Throat Angle (CTA) and Lower Pharyngeal Airway(LPA) was done according to Legan and Burstone analysis and McNamara airway analysis respectively. Independent sample t test and Pearson correlation coefficient were analyzed .Mean and standard deviation of lower pharyngeal airway and chin throat angle was 7.91 and 3.04 and 125.03 and 10.61 respectively. The value of -.340 indicates a highly negative significant correlation between CTA and LPA. Highly negative significant correlation was found between Chin Throat Angle (CTA) and Lower Pharyngeal Airway(LPA). If chin throat angle is increased correspondingly, lower pharyngeal airway is decreased in class II div 2 subjects.

KEY WORDS: CHIN THROAT ANGLE (CTA), LOWER PHARYNGEAL AIRWAY(LPA), SKELETAL CLASS II DIV2 MALOCCLUSION.

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INTRODUCTION

The function of respiration is important in orthodontic diagnosis and treatment planning. Normal airway is one of the important factors for the growth and development of the craniofacial structures (Claudino et al. 2013; Indriksone & Jakobsone 2015; McNamara 1981). On an average normal upper pharyngeal airway space is 15-20 mm while lower pharyngeal airway (LPA) space is 11-14 mm. Skeletal features such as retrognathic maxilla and mandible and vertical maxillary excess in class II patients may lead to narrower anteroposterior dimensions of the airway(Dunn, Green & Cunat 1973; Joseph et al. 1998). The lower oropharyngeal airway dimension is decreased in skeletal class II, division 2 group, (Uslu-Akcam 2017, McNamara 1984; Ravikumar et al. 2019).

Submental-cervical angle is an important factor in perception of facial profile attractiveness (Moreno, Bell & You 1994). Surgical procedures like mandibular and/or chin setback procedures may lead to an increase in submental fullness, an obtuse submental-cervical angle due to reduction in submental length leading to a potential deterioration in submento-cervical aesthetics. Conversely, mandibular advancement and/or advancement osseous genioplasty tend to improve submento-cervical aesthetics(Naini 2011).

The magnitude of the deviation of chin-throat angle ,whether it is due to an underlying dentoskeletal discrepancy, the overlying submental-cervical soft tissues or a combination of the two, is an important factor in deciding requirement of surgery. In an excessive submental-cervical angle, the treatment planning may be straight forward. However, in borderline cases, the decision may be transferred from subjective clinical judgement to objective, evidence-based guidance based on data from studies investigating perceptions of facial attractiveness (Naini et al. 2012). Rainbow scale has been validated as reliable measurement tool for the assessment of the cervicomental angle(Van Dongen et al. 2020). Normal cervicomental angle is 105-120 degree (Ellenbogen & Karlin 1980; Patel 2006).

The use of lateral cephalograms in determining the pharyngeal airway is acceptable as Cephalometric films are highly reliable and reproducible in determining airway dimensions (Malkoc et al. 2005).Assessment of dental and skeletal anomalies as well as soft tissue structures and form can be done with cephalometry. Clinical detection of narrowing of the pharyngeal airway may facilitate early recognition of obstructive sleep apnea. Many studies have assessed the airway by means of cephalometry in subjects obstructive sleep apnea, different malocclusions and also in patients following orthodontic treatments)(Arya et al. 2010; Batool et al. 2010; Hora et al. 2007); Joy et al. 2020). Therefore, the aim of the present study was to find any correlation between degree of submental cervical angle and lower pharyngeal airway dimensions in class II div 2 subjects and also to evaluate quantitatively the influence of submental cervical angle on lower pharyngeal airway to aid clinicians in early diagnosis of narrow lower pharyngeal airway which is considered as one of the factors for obstructive sleep apnea.

MATERIAL AND METHODS

This retrospective cross-sectional study was performed on the pre-treatment lateral cephalograms of 50 individuals who were selected from the databases of multi centres in Tamilnadu. The primary inclusion criteria were subjects of Tamil Nadu origin, only skeletal Class II div 2 malocclusion of either gender (Ceylan & Oktay 1995; Gupta & Subrahmanya 2014; Linder-Aronson & Leighton 1983), ANB angle greater than 4 degree confirmed after cephalometric tracing,(Ali 2018; Ardani, Sanjaya & Sjamsudin 2018; Chang 1987; Ferrazzini 1976; Oktay 1991; Riedel 1957; Santo & Del Santo 2006), between the age group of 14 and 30 years(Gonçalves, Raveli & Pinto 2011; King 1952) and no previous history of orthodontic treatment and any other pathologic pharyngeal or nasal obstructions .

Exclusion criteria were subjects with craniofacial syndromes or any other asymmetry, enlarged adenoids or tonsils, history of any other respiratory disorders,skeletal Class I or III malocclusions and radiographs with poor quality which made it difficult to identify the soft tissue landmarks were excluded.All cephalograms had been taken by using a standardized technique, with Frankfort horizontal plane parallel to the floor, with lips in a relaxed position(Arnett, William Arnett & Gunson 2004; Raghav et al. 2014). All collected cephalograms were traced by using FACAD software for the confirmation of class II div 2 malocclusion. After the confirmation of the obtained data, cephalograms were traced again for evaluating correlation between lower pharyngeal airway and chin-throat angle.Cephalograms were traced and measured using the following landmarks and reference lines Soft tissue menton (me'), the lowest point on the contour of the soft tissue chin found by dropping a perpendicular from horizontal plane through menton.

C, intersection between submental line and cervical line
Submental line(sm) is a tangent to the submental contour passing through soft tissue menton (me')

Cervical line(ce) tangent to the anterior soft tissue contour of the neck above and below the thyroid prominence.

Legan and Burstone analysis was used to measure Submental-cervical (chin-throat angle) (sm-ce) angle, formed by the intersection of Sm line and Ce line(Legan & Burstone 1980).

McNamara airway analysis was used to measure the lower pharyngeal airway. Lower pharyngeal width was measured from the intersection of the posterior border of the tongue and inferior border of mandible to closest point on the posterior pharyngeal wall(McNamara 1981).

10 cephs were retraced by the same observer after a period of 2 weeks to assess intraoperator bias.

Statistical analysis: Independent sample t test was done to determine the mean and standard deviation of chin throat angle and lower pharyngeal airway. Pearson correlation coefficient was done to determine the correlation between chin throat angle and lower pharyngeal airway.

Figure 1: Legan and burstone analysis was used to measure Submento-cervical (chin-throat angle) (sm-ce) angle, formed by the intersection of Sm line, submental plane and Ce line ,cervical plane

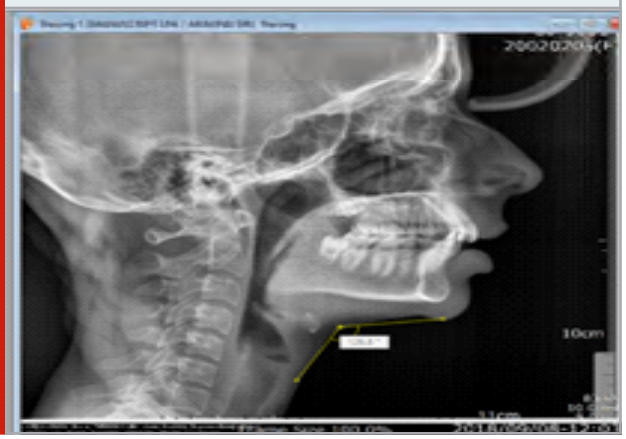
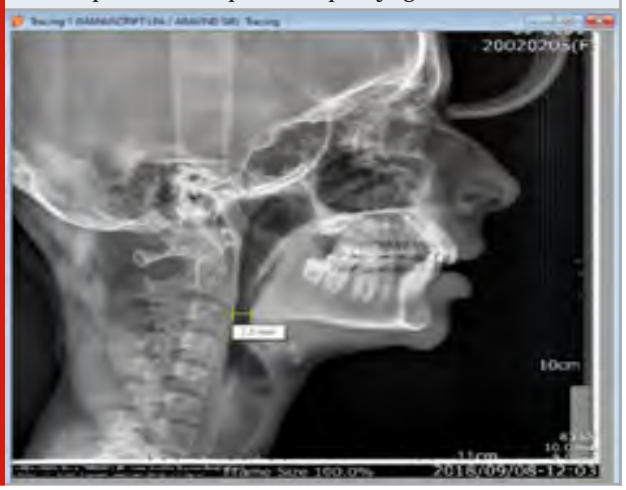


Figure 2: 2: McNamara airway analysis was used to measure the lower pharyngeal airway. Lower pharyngeal width was measured from the intersection of the posterior border of the tongue and inferior border of mandible to closest point on the posterior pharyngeal wall.



RESULTS AND DISCUSSION

Results of 50 patients are reported.Mean and standard deviation of lower pharyngeal airway was 7.91 and 3.04 (Table 1)Mean and standard deviation of chin throat angle was 125.03 and 10.61 (Table 1).Pearson correlation coefficient was done to determine the correlation between chin throat angle and lower pharyngeal airway. The value of -.340 between chin throat angle and lower pharyngeal airway indicates highly negative significant correlation between chin throat angle and lower pharyngeal airway (Table 2).

Orthodontists should have a knowledge of various factors that contribute to craniofacial development , since it can influence the orthodontist's decision on diagnosis and treatment planning (Tourné 1991). Normal anatomical dimensions of the airway are dependent for normal respiration and the function of respiration is in turn important for the cranio- facial growth and development which is very complex and multifactorial. Cephalograms have been used as a reliable diagnostic tool for many years to evaluate facial growth and development and for analysis of dental and skeletal anomalies as well as soft-tissue structures and form.In this cross-sectional study, we have included only skeletal Class II div 2 subjects with no abnormalities to eliminate the confounding effects of sagittal discrepancies. This is the first study that introduced the norms for correlation between chin-throat angle and lower pharyngeal airway in class II division 2 malocclusions by using cephalometric values.

Table 1. Independent sample test to determine the Mean and Standard Deviation of lower pharyngeal airway and chin throat angle.

	N	Mean	Standard Deviation
LPA	50	7.91	3.04
CTA	50	125.03	10.61

Table 2. Pearson correlation coefficient of lower pharyngeal airway and chin throat angle.

		LPA	CTA
LPA	Pearson Correlation	1	-.340*
	Sig. (2-tailed)		.016
	N	50	50
CTA	Pearson Correlation	-.340*	1
	Sig. (2-tailed)	.016	
	N	50	50

*Correlation is significant at the 0.05 level (2-tailed).

It is evident that measurements on a two-dimensional cephalometric radiograph cannot reveal the transverse dimension of the airway. For this reason, three-dimensional imaging such as CT, MRI, CBCT scans have been introduced for orthodontic patients but except for some clinical conditions such as obstructions or any other pathology ,impactions, severe asymmetries, craniofacial abnormalities up to now there is no substantial evidence indicating these advanced diagnostic scans to use as

routine radiograph in general orthodontic patients due to high economic cost and high exposure of radiation and ethical issues, (El & Palomo 2010; Liedke et al. 2012; Mouhanna-Fattal et al. 2019; Sedentext 2011). Malkoc et al (2005) showed that cephalometric films were significantly reliable and reproducible in determining the pharyngeal airway dimensions(Malkoc et al. 2005) and further Parkkinen et al (2011) confirm in their study that the lateral cephalograms is a valid method for measuring dimensions of pharyngeal airway(Pirilä-Parkkinen et al. 2011).

ANB angle is considered the most commonly used cephalometric measurement for evaluation of anteroposterior skeletal discrepancies(Ali, Manjunath & Sheetal n.d.; Ardani, Sanjaya & Sjamsudin 2018; Chang 1987; Ferrazzini 1976; Oktay 1991; Riedel 1957; Santo & Del Santo 2006) and also considered as one of the most reliable and accurate measurements of the anteroposterior jaw relationship (Ishikawa et al. 2000; Oktay 1991). The pharynx continue to grow rapidly until 13 years of age and then there is minimal growth until adulthood(King 1952). The upper pharyngeal airway depth increases with age, whereas the lower pharyngeal airway depth is established in early life (Handelman & Osborne 1976). The upper pharyngeal airway width increases with age (Ceylan & Oktay 1995; García-Martínez et al. 2016; Gonçalves, Raveli & Pinto 2011) but lower pharyngeal airway width does not show significant difference among the age groups(Gonçalves, Raveli & Pinto 2011). Therefore the age group of present study selected between 14 and 30 years to avoid probability of growth changes.

Gender discrimination was found in Class I and III subjects. No sex differences were detected in Class II subjects (Ceylan & Oktay 1995; Gupta & Subrahmanya 2014; Handelman & Osborne 1976; Linder-Aronson & Leighton 1983; Taloumtzi et al. 2020). Therefore the present study does not discriminate against gender. Basically, orthodontists rated the treatment needs based on soft tissue ,facial appearance and function. Submental-cervical angle is an important factor in perception of facial profile attractiveness. One of the possible reasons for poor submental-cervical aesthetics is retrognathic mandible (Moreno, Bell & You 1994). Morphology of the chin-neck region in profile view is a potentially important determinant of perceived attractiveness and is important for clinicians in correcting facial deformities(Naini 2011). The chin-throat angle is critical in defining chin extension: an acute angle indicates anterior projection and a significantly obtuse angle conveys the impression of reduced extension and the latter is characteristic of aging, along with the development of a double chin, particularly associated with weight gain(Gupta & Subrahmanya 2014).One study reported that pretreatment averages for CTA in class I (116° ± 6.87°), Class II (132.13° ± 13.13°) and Class III (112.22° ± 13.11°) subjects(Haddad & Ghafari 2017).

There are five visual criteria in restoring the youthful neck, in which one of the criteria is cervical mental angle between 105 degree and 120 degree (Ellenbogen & Karlin 1980).In Class II , obtuse CTA angle was less esthetic than other malocclusions and gives the clinical impression of a double chin or heavy neck(Haddad & Ghafari 2017; Naini et al. 2016). Abnormalities in the craniofacial region have been recognized as a part of the pathophysiology of OSA . Most common abnormalities are narrow posterior airway , elongation of the soft palate, mandibular deficiency, bimaxillary retrusion,and inferiorly positioned hyoid bone (Cistulli 1996). Among these mandibular deficiency in skeletal class II has been reported as predisposing factor to OSA and leads to decrease in the inferior oropharyngeal airway space(Abu Allhaija & Al-Khateeb 2005; Grauer et al. 2009; Hänggi et al. 2008; Miller et al. 2009).Small mandibular length was also a prime causative factor for OSA(El & Palomo 2011; Trenouth & Timms 1999; Zhang et al. 2019).

Lower pharyngeal airway is significantly decreased in OSA patients(deBerry-Borowiecki, Kukwa & Blanks 1988; Riley et al. 1983).Beneficial results have also been obtained in OSA after correcting the posteriorly placed mandible by mandibular advancement surgery or by functional appliances (Kyung, Park & Pae 2005; V et al. 2019). Oropharyngeal airway dimensions became smaller with the increase in ANB angle may be attributable to the different location of tongue and mandibular position with respect to cranial base in Class II malocclusion compared with other skeletal configurations in accordance with the statement given by Balters' philosophy(El & Palomo 2011; Rai et al. 2015).Subjects with skeletal class II division 1 mainly hyperdivergent growth pattern showed decreased lower pharyngeal airway due to mandibular deficiency compared to skeletal class I ('Evaluation of upper and lower pharyngeal airway in hypo and hyper divergent Class I, II and III malocclusions in a group of Egyptian patients' 2015; Kirjavainen & Kirjavainen 2007)

Some studies found that upper pharyngeal airway width is influenced by craniofacial growth pattern alone but not by malocclusion type and lower pharyngeal airway is not influenced by both growth pattern and type of malocclusion. This contradicts the present study(Ackerman & Klapper 1981; Tarkar et al. 2016; 'Upper and lower pharyngeal airways in subjects with Class I and Class II malocclusions and different growth patterns' 2006).

One study found that smallest dimension of lower pharyngeal airway was recorded in the skeletal class II, division 2, hence, the oropharyngeal airway dimension should be carefully considered for treatment timing(Uslu-Akcam 2017).If there is no finding of upper nasopharyngeal airway pathology related to enlarged adenoids or tonsils , early correction of a skeletal class II, division 2 malocclusion might have eliminated the possibility of disturbed respiratory function during sleep, such as snoring(Murat Özbek et al. 1998). Hence in this study, we evaluated the correlation between chin-throat angle and lower pharyngeal airway only in skeletal class II division 2 subjects. In this study, there is

a significant correlation between chin-throat angle and lower pharyngeal airway.

Thus, clinical evaluation of chin-throat angle helps in early diagnosis of obstructive sleep apnea and leads to early correction in growing patients and early correction of skeletal class II division 2 malocclusion might have eliminated the possibility of having disturbed respiratory function and OSA later especially in those patients who have retrognathic mandible and smaller lower pharyngeal dimensions. The limitations of the present study is small sample size and 2 D digital cephalograms hence ,we recommended to use 3 D cone-beam computed tomography on large sample size for better assessment of airway dimensions and also to check this correlation between lower pharyngeal airway and chin throat angle.

CONCLUSION

Highly negative significant correlation was found between Chin Throat Angle (CTA) and Lower Pharyngeal Airway(LPA). If chin throat angle is increased ,correspondingly lower pharyngeal airway is decreased in class II div 2 subjects. Hence, Norms of our study was as follows,

IF CTA IS ABOVE 130 DEGREE, LPA IS BETWEEN 3-5 MM,

IF CTA IS ABOVE 120 AND BELOW 130 DEGREE, LPA IS BETWEEN 5-8 MM,

IF CTA IS BELOW 120 DEGREE, LPA IS BETWEEN 9-12MM.

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In silico Modelling of Antibacterial Protein Transferrin from *Periplaneta americana* and its Interaction Analysis with Membrane Protein of MRSA

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ABSTRACT

Antibiotics are used for human therapy, farm animals and even in aquaculture. The overuse and misuse of these antibiotics result in pathogenic bacteria resistant to multiple drugs, which can be generated either by accumulating multiple genes on-resistance (R) plasmids or increased expression of multidrug efflux pump genes. The development of resistance in bacteria to conventional antibiotics restricts their use for treatment lead to the need for searching for an alternative to avoid this scenario. An antimicrobial protein of plant and animal origin is one of the most trending solutions for this issue. In our previous study, 7 such proteins were isolated from brain tissue lysate of *Periplaneta americana*. These proteins were further identified and characterized to be transferrin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Mass Spectrometry techniques. The current study is aimed at analysing structure homology modeling and docking of transferring protein with its receptor on cell membrane of MRSA using the GRAMM-X docking server and further analysis using PDBsum. The results indicated significant interactions of Transferrin with its GAPDH receptor on MRSA. The results can be of future use to design an analogous drug based on the mechanism of action and binding site of transferrin on MRSA as well as probable ways of drug delivery for a better results.

KEY WORDS: ANTIBACTERIAL PROTEIN, ANTIBIOTIC RESISTANCE, MASS SPECTROMETRY, PROTEIN DOCKING.

INTRODUCTION

Bacterial infections and diseases are controlled by antibacterial drugs against pathogenic bacterial. The drug resistant bacteria ares one of the important causes for the death of about 50,000 new born annually. Insects are known to play an important role as carriers of these bacteria and well as spreading them amongst humans. The cockroach is found to harbor several gram-positive and gram-negative bacteria both internal and external of their digestive system (Sisai et al., 2006). In all around 114 multidrug-resistant strains were isolated from cockroach (Feleke et al., 2016). Almost all living organisms have innate immunity where they have the capability of developing antibacterial protein. Isolation of these proteins from different plant and animal sources has been trending these days (Zasloff, 2002). The 10 KDa protein isolated from brain tissue lysate of locust was found to be showing its antibacterial action against *S.epidermis*, *S.auerus*, MRSA and neuropathogenic

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E.coli K1. The antibacterial activity of the protein is heat resistant and is effective at a concentration as low as 5 µg. (Simon et al., 2012).

The polymyxins B and E (the latter is also known as colistin) are examples of antimicrobial peptides (AMPs) in clinical use since the 1950s. They have been applied for both topical and systemic treatment of infections (Landman et al., 2008). Their therapeutic use has increased to combat multidrug-resistant pathogens (Falagas and Kasiakou, 2005). However, their intravenous use has been limited by high nephrotoxicity and neurotoxicity (Falagas and Kasiakou, 2006). Identification of antibacterial proteins and their characterization is a very important aspect of proteomics nowadays. Without the identification of protein, its structure and its characteristics, it is difficult to reveal its action within the cell. Several techniques have been developed for the identification of the protein. Of them, Mass spectrometry has evolved as one of the most primary tools in protein identification (Thomson, 1913; Brunett et al,2020).

The use of mass spectrometry for biological applications dates from the 1950s (Beynon, 1965), and its use in peptide identification dates from the 1960s (Biemann et al., 1966). Accuracy, speed, and sample weight range have seen improvements spanning many orders of magnitude in recent decades (Henzel et al., 2003), making mass spectrometry one of the greatest scientific success stories of the twentieth century. For better extraction and identification of protein, mass spectrometry is combined with different other techniques like liquid chromatography (LC), gas chromatography (GC), time of flight (TOF), matrix-assisted laser desorption/ ionization (MALDI), etc. (Jamer, 2009; Bilal et al., 2017; Francis et al., 2020). Nano-capillary liquid chromatography -Electro Spray Ionization -Ion Trap -Time of Flight-Mass spectrometry/Mass Spectrometry (Nanocapillary LC–ESI–IT–TOF–MS/MS) has been successfully applied in clinical microbiology because of its economical and diagnostic benefits. It was used for rapid identification and comparison of S. aureus and its extracellular profile (Jones et al. 2008).

Most of the Antibacterial proteins show cationic property and using this they tend to destruct bacterial cell envelope. Antibacterial proteins target cell membranes through the formation of ion channels or trans-membrane pores and in this way destroy the bacterial cell (Duclohier, 2002; Park and Hahm, 2005). Apart from the membrane destruction, some AMPs, i.e. Pyrrhocoricin, Drosocin, and Apidaecin, may exert antibacterial activity by interactions with intracellular targets thus disrupting intracellular processes (Kragol et al., 2002; Li et al., 2006; Nicolas, 2009). The properties of protein can be better understood using in-silico tools at preliminary level and analyze their interactions with other proteins and those identified in/on drug resistant bacteria.

Interaction of antibacterial protein with the bacterial protein can be studied much better by identifying the structure of protein firstly. NMR or X-Ray crystallography are two standard techniques to identify the structure of the protein, however, it is time-consuming and expensive Gupta et al., 2014). Hence, the 3D structure prediction tool was used for the identification of the structure of nifA protein from *Rhizobium leguminoserum* (Sadam et al., 2018). To produce the tertiary structures of proteins, templates were selected from PDB (Protein Data Bank) (Bernstein et al., 1977; Goodsell et al, 2020) by using the BLASTp algorithm (Altschul et al., 1990; Lobanov et al,2020).

Sequences of proteins that are more similar to the query sequence, were selected as templates. The modeling of the three-dimensional structure of the proteins was performed by three homology modeling programs, Phrye2 (Kelley et al., 2015), SWISS MODEL (Arnold et al., 2006) and Modeller (Sali et al., 1993). Bioinformatics tool gives great ease in predicting the protein-protein interaction using several algorithms which can later help in drug designing (Schmidt, 2013). Several Fast Fourier Transform - based docking programs have been made available as web servers e.g. ClusPro (Comeau et al., 2004), GRAMM-X (Tovchgrechko and Vakser , 2006) and ZDOCK (Chen et al., 2003). Overexpression of human epidermal growth factor receptor 2 (HER2) in the patients denotes poor prognosis leading to a reduced survival rate compared to other subtypes of breast cancer. Using GRAMM-X protein docking server interaction between Her2 receptor and protein from Pseudomonas exotoxin A (PE38) and A subunit of Shiga toxin 2a (Stx2a) was analyzed and gave promising results. (Goleij et al., 2019).

The analyses of docking are primarily image-based and include protein secondary structure, protein-ligand, and protein-DNA interactions, PROCHECK analyses of structural quality, and many others. This can be best achieved by the use of the PDBsum server (Roman et al., 2018). HAWKDOCK server uses Molecular Mechanics/ Generalized Born Surface Area (MM/GBSA), which are more theoretically rigorous than scoring functions, have been widely used to predict binding free energies and identify correct binding conformations for protein-protein systems (Gaogi,2019). During the current study an attempt has been made to predict protein-protein interaction between transferrin and GAPDH as receptor on MRSA which can give us a lead for further research on its antibacterial pathway.

MATERIAL AND METHODS

Protein identification by TOF LC-MS/MS: LC-MS/MS was performed on an MS Q-TOF G65504A system. HPLC- Chip was used for ionization. After in-gel tryptic digestion peptides were eluted into the nano pump at the flow rate of 0.3 µL/min. Peptides were separated using the mobile phase gradient solvent A and Solvent B viz. Water and Acetonitrile respectively. Separation along mobile phase was in ratio of A 80% and B 20% for first 2 min, A 2% and B 98% for next 15 min, A 2% and B 98% for next 20 min, A 97% and B 3% for next 25min , A 97% B3% for next 35 min. The m/z ratio was between

300 – 3200 with an MS scan rate of 5.0 spectra/sec and MS/MS scan rate of 3.0 spectra/sec. LC-MS/MS data were acquired and protein was identified using NCBInr (https://www.ncbi.nlm.nih.gov/protein/) data.

Protein structure modelling: The structure of Protein identified using LC-TOF MS/MS was predicted using homology modeling server SWISS-MODEL (http://swissmodel.expasy.org/). The SWISS-MODEL server is based on homology-based modeling i.e. structure of the protein is predicted on sequence homology of the existing structure from the PDB database. SWISS-MODEL will search for templates initially form the PDB database, it allows selection of best template found and then generates the model with QMEAN4 score and Local quality estimate as evaluation parameter (Marco et al.,2014). The amino acid sequence of transferrin protein of cockroach and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the surface of the cell membrane of MRSA was extracted from the BLASTp server using accession no. 372292427 and KST21754.1 respectively. The FASTA sequence was entered and the server was allowed to form templates using its algorithms. Templates with maximum sequence identity and QMEAN value closest to 1 were selected for modeling the structure. The obtained structure prediction was downloaded in .pdb format and used for further docking.

Protein-Protein docking: GRAMM-X (http://vakser.bioinformatics.ku.edu/resources/gramm/grammx) server was used for online protein docking. GRAMM-X grew out of the original Fast Fourier Transformation (FFT) GRAMM methodology. It represents a new implementation that uses a smoothed Lennard-Jones potential on a fine grid during the global search FFT stage, followed by the refinement optimization in continuous coordinates and rescoring with several knowledge-based potential terms. The best surface match between molecules is determined by the correlation technique using FFT. An important feature of GRAMM is the ability to smooth the protein surface representation to account for possible conformational change upon binding within the rigid body docking approach (Andrey and Ilya, 2006).

Table 1. Identification of protein using TOF LC-MS/MS analysis method.

Band no.	Num Spectra	Num Peps Unique	Score Unique	% Coverage	MW (Da)	pI	Species	Database	Accession no.	Entry name
2	36	21	358.64	47.4	79914.4	5.43	*Periplaneta americana*	NCBInr	372292427	Transferrin
4	17	11	188.27	48.4	35585.1	6.98	*Periplaneta americana*	NCBInr	343965965	Glyceraldehyde-3-phosphate dehydrogenase

The amino acid sequence of Transferrin was entered in ligand slot and an amino acid sequence of glyceraldehyde-3-phosphate dehydrogenase of MRSA cell membrane surface was entered in receptor slot. Visualization of different binding modeling was done using Discovery Studio (figure 2). HAWKDOCK server was used to identify the best binding pose for protein-protein in docking based on binding free energy score. The more the negative value more is the interaction and complex suitable to bind. The best model obtained was analyzed using a free online PDBsum server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html). PDBsum-Generate module, at PDBSum allows the users to load protein (receptor) – protein (ligand) docked and analyze the structural characteristics and interactions are provided in simplified graphics. The analyses are primarily image-based and include protein secondary structure, protein-ligand, and protein-DNA interactions, PROCHECK analyses of structural quality (Roman et al., 2018).

The. pdb file obtained as result by GRAMM-X was uploaded on the PDBsum server. The link to the analytic result was obtained via mail. The functional annotation of the proteins was determined using the Universal Protein Resource (UniProt) database (**http://www.uniprot.org/**).

RESULTS AND DISCUSSION

Previously, around 7 antibacterial protein was isolated from brain tissue lysate of cockroach using Polyacrylamide Gel Electrophoresis (PAGE). These proteins were of molecular weight ranging from 97.4 kDa to 14.3 kDa. Amongst these, two proteins that showed the best antibacterial activity were chosen for its identification. TOF LC-MS/MS analysis revealed amino acid sequence and identified protein to be transferrin and glyceraldehyde-3-phosphate dehydrogenase having molecular weight 79.9 kDa and 35.5 kDa respectively (Table 1).

Protein structure modelling: The amino acid sequence and protein model of Transferrin of cockroach (figure 1a) and GAPDH of cell membrane surface of MRSA (figure 1b) was obtained using NCBInr and are as follows:

Transferrin:

MSKLSLLLLLQLPAALLLAIPTPHVYKVCVPD
GALNDCEQMASETALHMHCVPARDRTACLD
KIQHHDADFVPVDPEDIFLASKITNQSFIVFKE
IRTKEEPDEEFRYEAVAVIHKNQNITSVQGLR
GLKSCHTGVGRNVGYKIPITKLRKMGVLTNL
NDPDMTPRENELHALSQLFSKACLVGKWAP
DPAQNQALKERYPNLCALCEHPEQCDYPDK
YSGYDGALRCLAENGGEVAWTKVYYVKKH
FGLPIGAGEAVPTGEDPDNYAFLCPDGTKKPI
TGRPCIWAARPWQGYITNTDVEDMSELRSQI
SLADNIGETEHAAWLSKVLDLNNKTVAVDN
GGPFSPQQYLDKANYTDVIERDTGDPHRPVR
FCVTSDTELEKCHVLRRAAFSRDIRPAFDCVQ
ESTNQDCMATVRDNGADVITLDGGDVFTAM
REYNLKPIIAEQYGEHGSMYYAVAVVKKSSS
YQSIADLRGAKSCHTGYGRTAGWNVPLYLLL
NQSLISRTSCPYSEAVSTFFSGGSCVPGVPHGP
ELLCSLCAGNLDTGDRTYACSASNNESFFGY
TGAFRCLASGAGDVAFVKHTTVAENTDGNN
TAAWAAGLHSSDFELLCPNGGRAPVEQYSRC
HLAEVPPHMVVTSNDKSDNVLNEIRHAVLAA
GDLYSRRPDLFKLFGDFDGTKDLLFKNSATG
LRAVDTGTPVMQHYTEMLDVIRTCENQTPA
QE

GAPDH at cell membrane of MRSA:

MSTNIAINGMGRIGRMVLRIALQNKNLN
VVAINASYPPETIAHLINYDTTHGKYNLK
VEPIENGLQVGDHKIKLVADRNPENLPW
KELDIDIAIDATGKFNHGDKAIAHIKAGA
KKVLLTGPSKGGHVQMVVKGVNDNQLD
IEAFDIFSNASCTTNCIGPVAKVLNNQFGI
VNGLMTTVHAITNDQKNIDNPHKDLRRA
RSCNESIIPTSTGAAKALKEVLPELEGKLH
GMALRVPTKNVSLVDLVVDLEKEVTAEE
VNQTFENAGLEGIIEVEHQPLVSVDFNTN
PNSAIIDAKSTMVMSGNKVKVIAWYDNE
WGYSNRVVDVAEQIGALLTSKETVSAS

Protein-Protein Interaction: GRAMM-X online server showed multiple interactions of transferrin with three chains of GAPDH. Interactions were observed using PDBsum. It gave a good image (figure 3a) and picturesque prediction of interaction and bonds. Transferrin is denoted by letter E and chains of GAPDH is denoted by letter A, B, C, and D. It showed three types of interaction 1. Salt bridges are indicated by the red line, 2. Hydrogen bonds are indicated by blue line 3. Non bonded contacts are indicated by the orange line (figure 3b). Docking failed to show any disulfide interactions between proteins (generally indicated by the yellow line).

The GRAMM-X .pdb file showing the interaction between Transferrin and GAPDH was observed in Discovery Studio (figure 2). Out of 10 models of protein interaction that were obtained using GRAMM-X, model 6 was considered the best model due to lowest binding energy i.e -39.54 kcal/mol. The detail of same is given in figure 10 and table 3. EMBL-EBI's PDBsum generated detailed reports of interaction. The following figure is a picturesque depiction of the interaction between transferrin which is denoted by Chain E and GAPDH consisting of four Chain A, Chain B, Chain C, and Chain D (figure 3b). Linear structure of protein is depicted in (figure 7).

Figure 1: SWISS MODEL structure view in RasMol a. Transferrin, b. GAPDH of MRSA.

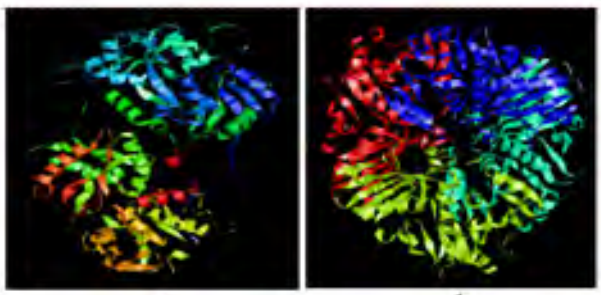


Figure 2: Model 1-10:- 3D View of protein interaction

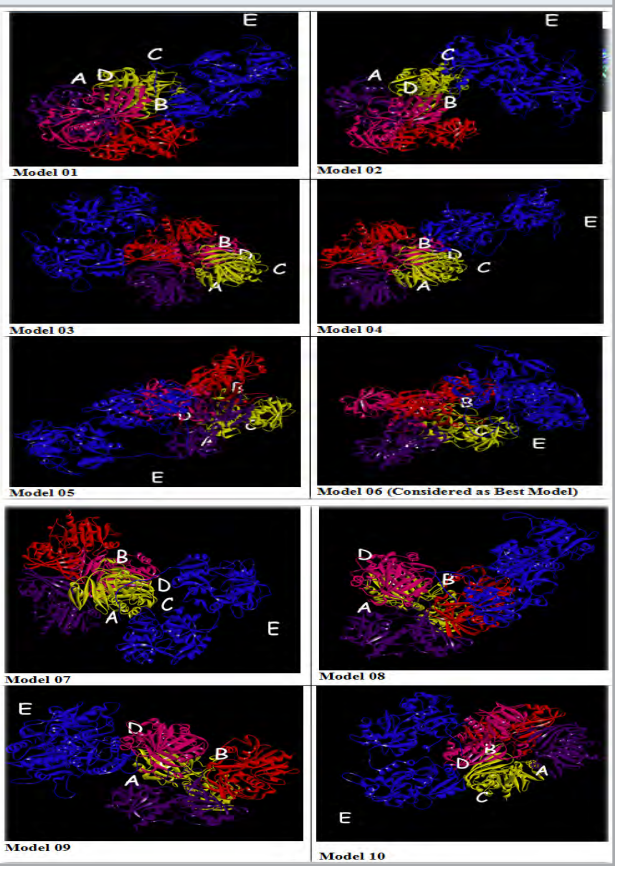


Figure 3: PDBsum report of GRAMM-X docking interaction of transferrin i.e E with chain A, B and C of GAPDH a. Cartoon model b. Picturesque of interaction

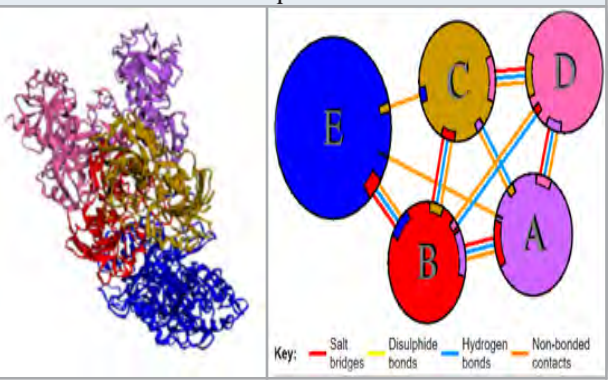


Table 2 : List of types of interaction within the chain of GAPDH and between chains of GAPDH and transferrin.

Interface statistics

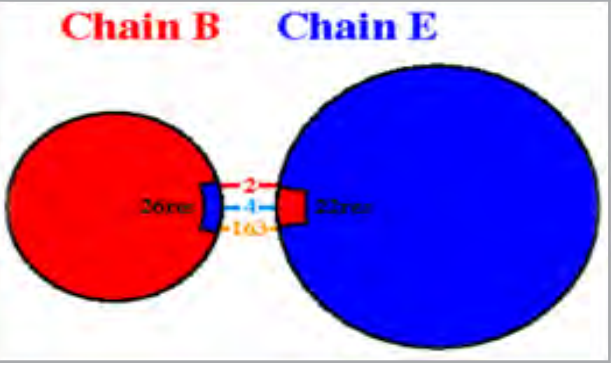
Chains	No. of interface residues	Interface area (Å²)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A+B	42 : 43	2030:2012	8	-	[illegible]	216
& B+C	39 : 39	1966:1912	6	-	[illegible]	206
A+C	9 : 11	470:470	-	-	[illegible]	31
& B+C	8 : 9	413:414	-	-	[illegible]	30
A+D	14 : 16	1026:1027	3	-	1	86
& B+C	13 : 13	888:919	2	-	[illegible]	81
A+E	1 : 1	48:58	-	-	[illegible]	2
B+E	26 : 22	1384:1580	2	-	[illegible]	163
C+E	11 : 11	638:626	-	-	[illegible]	114

Note. Indented interfaces in the table are equivalent to the last prior non-indented interface. Equivalent chains are listed below.

Equivalent chains: A = B = C = D = E

Interaction between Chain B of GAPDH with E: Chain B and Chain E share four hydrogen bonds, one between Asn82 and Thr513, Gln129 and Thr22, Glu143 and Ser311 and Asn136 and His24 each. There are 2 salt bridge, one between Lys111 and Glu509 and Glu143 and Arg307 each. Somewhere around 163 non-bonded contacts are observed. For these interactions, 26 amino acids of chain B and 22 amino acid residues of protein E are involved (figure 4 and figure 6). Figure 5 depicts cartoon model of interaction between Chain B of GAPDH and Chain E.

Figure 4: Picturesque Interaction between Chain E i.e transferrin and Chain B of GAPDH



Interaction between Chain C of GAPDH with E: Chain C and Chain E around 114 non-bonded contacts. For these interactions, 11 amino acids of chain C and 11 amino acid residues of protein E are involved (figure 2)

Figure 5: Cartoon Image if interaction of Chain B GAPDH and Transferrin using PDBsum sever

Figure 6: Detailed interactions of specific amino acids of Chain B of GAPDH and transferrin i.e Chain E

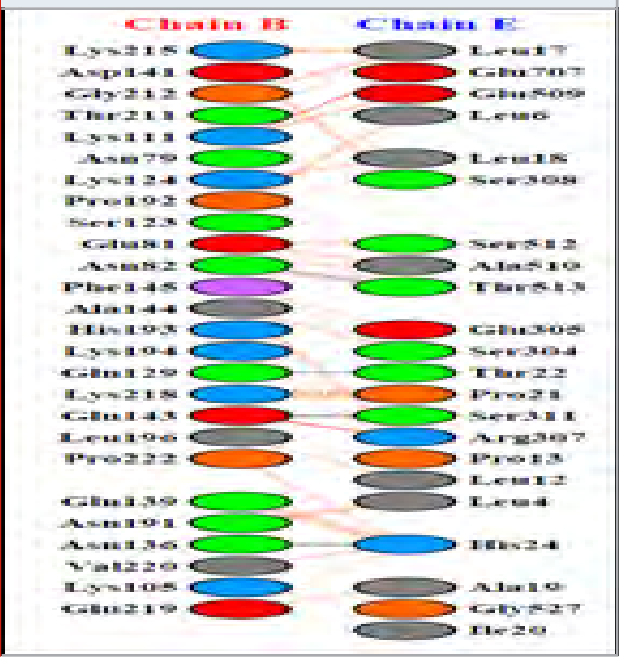


Figure 7: Secondary structure of Protein chain A, B, C, D and E

Figure 8. Ramachandran plot of detailed PROCHECK analyses

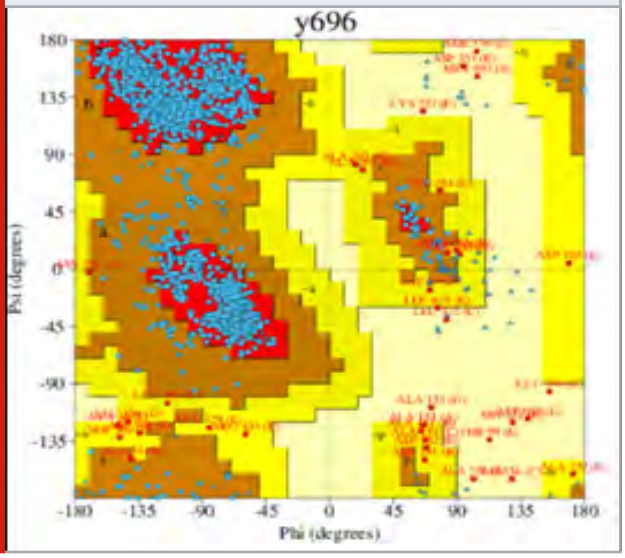


Figure 9: Detailed analysis of PROCHECK


PROCHECK statistics

1. Ramachandran Plot statistics

2. G-Factors

Parameter | Score | Average Score

Dihedral angles:
Covalent-force angles:
Overall average:

G factors provide a measure of how unusual, or out-of-the-ordinary, a property is.

Values below -0.5 - unusual
Values below -1.0 - highly unusual

Important note: The main-chain bond-lengths and bond-angles are compared with the Engh & Huber (1991) ideal values derived from small-molecule data. Therefore, structures refined using different restraints may show apparently large deviations from normality.

Interaction between Chain A of GAPDH with E: Chain A and Chain E share around 2 non-bonded contacts. For these interactions, 1 amino acids of chain A and 1 amino acid residues of protein E are involved (figure 2).

(Above interaction may not be associated in one model but are given as combined from 10 different model)

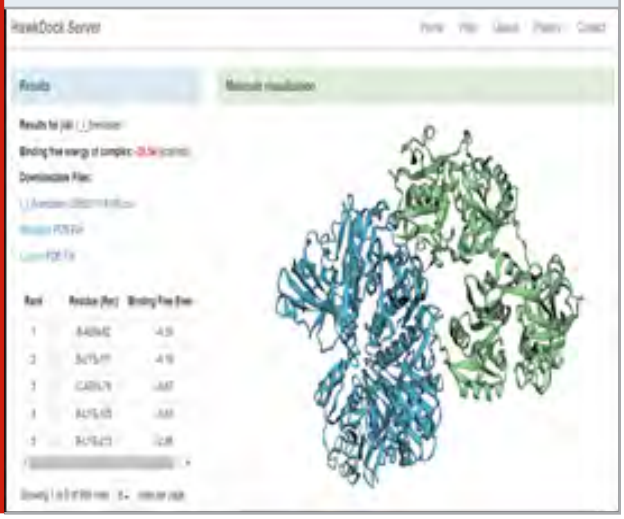
The phenomenon of antimicrobial resistance has been known even before the introduction of penicillin as an antibiotic (Abraham and Chain, 1940) and over the last century, it has been observed that resistance arises independently from the chemical group of the drug. The issue has recently raised global awareness due to the increase of resistant organisms, the affected geographic locations and the breadth of resistance in single organisms (Levy and Marshall 2004; Chukwu et al.,2020).

Over the past twenty years, several studies have investigated the potential of cationic AMPs as novel anti-infective agents (Hancock and Lehrer, 1998; Marr et al., 2006). These naturally occurring compounds have been a model for the extensive design of new classes of antibacterial compounds (McGrath et al., 2013). Multiple such antibacterial proteins ranging from 64 kDa to 10 kDa have been isolated from cockroach and found to be effective against multiple drug-resistant and pathogenic bacteria, (Simon et al., 2012 Siddharth and Rao, 2015).

Table 3. Calculating Binding Free energy for each complex generated from protein-protein docking

Protein-Protein Docked complex from GRAMM-X Server	Binding Free energy Obtained from HAWKDOCK server (kcal/mol)
Complex 01 (r_l_1.pdb)	+09.20
Complex 02 (r_l_2.pdb)	-04.28
Complex 03 (r_l_3.pdb)	+00.59
Complex 04 (r_l_4.pdb)	-01.65
Complex 05 (r_l_5.pdb)	-04.36
Complex 06 (r_l_6.pdb)	-39.54
Complex 07 (r_l_7.pdb)	-06.91
Complex 08 (r_l_8.pdb)	+10.63
Complex 09 (r_l_9.pdb)	+08.76
Complex 10 (r_l_10.pdb)	+15.62

Figure 10: Best Model Identified on Bases of Binding Free Energy Complex Using Hawkdock server



Insects produce these products and secrete in their hemolymph as their first line of defense mechanism

(Seraj et al., 2003). Muga silkworm (*Antheraea assamensis*) larvae, when injected with *Candida albicans*; AMPs were isolated from the hemolymph and evaluated for antimicrobial activity against fungal and bacterial pathogens. Isolated peptides were confirmed by SDS-PAGE and TLC, and its molecular mass was determined as 9.052 kDa by MALDI-TOF mass spectrometry. From the mass fingerprinting analysis of this peptide after trypsin digestion, a peptide fragment with the molecular mass of 2622.7 Da was obtained. De novo sequencing of this peptide fragment following MS/MS analysis resembled with gloverin peptide of A. mylitta, comprising few aminoalkanoic acid residues as "KSGGGGWGS" with a complete score of 46.9. The peptide inhibited biofilm formation of the Gram-negative bacteria pathogens (Ellison et al., 1988). Iron-binding proteins like transferrin and lactoferrin express their antibacterial property by damaging the outer membrane of gram-negative and alter bacterial outer membrane permeability from an entry of iron (Waterhouse, 2018).

Net charge and amphipathic structure are the two interrelated features that confer antibacterial activity to AMPs. Often such peptides cause lysis of the bacterial cell and it is believed that cell lysis originates from the interaction between the bacterial anionic membranes and the positively charged peptides, (Skarnes and Watson, 1957; Raguse et al., 2002). Such interactions can best be predicted by using Bioinformatics tools. The X-ray structure of homodimer in chain A and B were used for autolysin while pneumolysin and pneumococcal surface protein A (pspA) was homology modeled with SWISS-MODEL where the peptides were based on NMR solution structure data of indolicidin peptide derivative (Le et al., 2015).Antimicrobial peptide interaction with the spike protein of the Coronavirus was checked using In silico tools Piper & GRAMM-X (Sabeena et al., 2019; Siddharth et al.,2020).

The mechanism of interaction between Death associated kinase 1 (DAPK1) and a subunit of antidepressant GluN2B-CT1290-1310 was predicted by GRAMM-X online server (Gao et al., 2018). GRAMM-X is the easy and reliable tool for prediction of protein-protein interaction (Pourjafar-Dehkordi et al., 2020). The stereochemical stability of the predicted models can be further verified using various protein quality based parameters such as percentage residues lying in favored and allowed regions, the number of glycine and proline residues and orientation of dihedral angles including phi (ϕ) and psi (ψ) and backbone conformation using PROCHECK module of the PDBSum server (Laskowski et al., 2005). Validation of docked complexes generated from GAMM-X suggested Model 6 had the best mode of binding with the free energy of -39.54 Kcal (Sarkar et al., 2020; Siddharth et al., 2020).

Majority of predicted residue were observed to be non-bonded interaction two for Chain A-E, 114 for Chain C-E. And residues from Chain B (ASN82, GLN129, GLY143, ASN136) shared four hydrogen bonding with Chain E (THR513, THR22, SER311, HIS24), also LYS111, GLU143(chain B) with GLU707, ARG307(chain E) formed two salt bridges that together with other interaction may act important from the perspective of stability of the complex and produce the required effect.

CONCLUSION

Transferrin protein that is produced in the brain tissue of cockroach showed an excellent antimicrobial capability against multiple bacteria both gram-negative and gram-positive. Glyceraldehyde-3-phosphate dehydrogenase found on the cell membrane of MRSA acts as a receptor for transferrin. This can be confirmed by using In silico tools like GRAMM-X which is a docking app helping us with providing information about protein interaction. Interaction can be understood in detail using EMBL-EBI's PDBsum server.

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Escalating Rhizospheric Chromium Pollution Grades as Plant Foes or Friends?-Evaluation by Enzyme Assay, Physiological Growth and Photosynthetic Phytochemicals of Mash [*Vigna mungo* (L.) Hepper] genotypes.

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ABSTRACT

The presence of heavy metals in soils may be toxic or beneficial to the environment. The plants may require some of these elements considered as essentials (like Fe, Cu, Zn, Cr or Mo) in trace quantities, but at higher concentrations they may be poisonous. An experiment was conducted on four mash bean (*Vigna mungo* (L.) Hepper) genotypes to evaluate the toxic or beneficial effects of chromium (III) applied in rhizosperic environment of plant. Photosynthetic phytochemicals in the form of Chlorophyll a, Chlorphyll b, total caretenoids, Plant nitrate reductase activity and physiological growth as leaf area index (LAI) were recorded against 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 mg kg^{-1} chromium (III) concentrations. Seeds of four genotypes sterilized with 10% (V/V) hydrogen peroxide were sown in earthen pots filled with homogenized loamy soil. Chromium was added in soil as $CrCl_3$ solutions after twenty days of germination. Data were collected on expiry of twenty five days after chromium addition. Increasing amount of chromium (Cr) appeared to be responsible for gradual reduction in Nitrate Reductase Activity (NRA), photosynthetic pigments and physiological growth of leaf in term of Leaf Area Index (LAI). The lowest significantly effective) dose was 20 mg kg^{-1} in this regard. While the most effective proven dose was 60 mg kg^{-1} for each attribute. The observations were excluded from the ongoing trend when 10mg kg^{-1} chromium (Cr) reflected an increase in studied characteristics at the most being 13.59% for Nitrate Reductase Activity (NRA) at this level. Of the genotypes, MASH 80 was the most sensitive while MASH 88 was the least sensitive to chromium stress

KEY WORDS: CHROMIUM; MASH; NITRATE REDUCTASE; CHLOROPHYLL ; CAROTENOID; LEAF AREA INDEX.

ARTICLE INFORMATION

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INTRODUCTION

Chromium (Cr) is an element naturally occurring in rocky soils and volcanic dust. It has been classified as a carcinogen agent according to the International Agency for Research on Cancer. Therefore, this metal needs an accurate understanding and thorough investigation in soil–plant systems. Due to its high solubility, Cr (VI) is regarded as a hazardous ion, which contaminates groundwater and is transferred to food chain through soil plant interaction system. Chromium also has negative impacts on the growth of plants by impairing their essential metabolic processes (Sharma et al. 2020). Many industries like dyeing, electroplating, leather, tanning and steel discharge effluents of chromium causing significant rise in environmental Cr contents (Joutey et al. 2015). Chromium high solubility contaminates groundwater and enters into food chain through soil plant interconnection (Joutey et al. 2015;Kumar et al. 2019;Kumar et al. 2019). The Agency for Toxic Substances and Disease Registry (CERCLA, 2019). Chromium is ranked 17th among the most hazardous metals (Agency for Toxic Substances and Disease Registry, 2019).

Its high redox potential enables chromium to change oxidation state easily (Shahid et al., 2017; Prado et al. 2016). It s most common oxidation states are hexavalent and trivalent (Ashraf et al., 2017). These oxidation forms differ in respect of their bioavailability, toxicity and translocation in plants (Shahid et al. 2017). Of these Cr (III) is the most stable form of Chromium, whereas Cr (VI) is the highest noxious one for plants. When enter into the cell, these oxidation states attack proteins, lipids and DNA (Tchounwou et al. 2012;Stambulska et al. 2018).

In plants excess Chromium concentration disrupts many biochemical and physiological phenomenon (UdDin et al. 2015;Kamran et al. 2017). Toxicity of metal rely upon its complex interactions with signal transduction, genetics and macromolecules (Santos et al. 2012; Eleftheriou et al. 2015;Kumari et al. 2016). Chromium toxicity affects growth of plant by many ways including changes in structure of cell membrane, chloroplast, pigments, disturbing water balnce, mineral nutrition, enzymes and assimilation process (Reale et al. 2016; Ali et al. 2015;Farooq et al. 2016;Cervantes and Campos-Garcia, 2007;Anjum et al. 2017). The major toxic effects of chromium are due to its role in production of reactive oxygen species (ROS), which alters the redox balance in plants (Anjum et al. 2017).

Taking all into consideration, we review the literature that addresses Cr uptake, translocation and sub-cellular distribution in plants. We also discuss different effects of Cr on plant pigments, photosynthetic parameters, enzymatic and non-enzymatic antioxidative system and various endogenous levels of plant hormones. Roots of some plant secrete organic molecules as acids which act as ligand and can modify solubility of metals in the soil (Khanna et al. 2019;Kaur et al. 2017;Kaur et al. 2018; Kohli et al. 2018). Plant uptake Cr through active and passive mechanism (Shanker et al. 2005; Cervantes2001;Appenroth et al. 2008;De Oliveira et al. 2013). Due to it has structural structural similarity with sulphate and phosphate, uptake of chromium by root cells is through sulphate or phosphate (De Oliveira et al. 2013;De Oliveira et al. 2016). After entry into plant cells, the Cr (VI) is converted to Cr (III) which by binding to the cell walls can block the further transport of Cr within plant (Kabata-Pendias and Szteke, 2015).

Mash bean (*Vigna mungo* (L.) Hepper), belonging to family Papillionaceae, is among the most important pulse crops of the world. In Pakistan, it is amongst the least researched pulse crops inspite of its high nutritive and economic value. Chemical analysis of mash bean seed indicates that it contains oil, fats, protein, carbohydrates and a fair amount of vitamin A and B (James, 1981). It fix free atmospheric N_2 for its consumption and enriches with N for next crop (Sen, 1996). Considering the importance of mash bean and considering the ever increasing toxicity of chromium in environment, the present experiment was devised to find out the extent of chromium concentration which could be toxic to plant.

MATERIAL AND METHODS

To find out whether chromium metal is toxic or beneficial element for plant, an experiment was devised to evaluate the Photosynthetic pigments, Nitrate Reductase (EC 1.6.6.1) Activity (NRA) and leaf area index of four mash bean (*Vigna mungo* (L.) Hepper) genotypes under various chromium applied concentrations.After the initial survey, effluents hazards free sandy loam soil was selected. Soil was air-dried ground, passed through 2mm sieve and mixed well. Seeds of four mash genotypes i.e., MASH 80, MASH 88, MASH 97 and MASH ES^{-1} were used in the experiment. The genotypes have their origin in Ayub Agricultural Research Institute (AARI), Faisalabad (Pakistan) and National Agricultural Research Centre (NARC) Islamabad (Pakistan). These were obtained from Pulse Section, Ayub Agricultural Research Institute (AARI), Faisalabad (Pakistan). For imposing metal pollution in soil, chloride of chromium of Sigma Aldrich, Japan was used.

Experiment was designed with complete randomization of treatments and genotypes to avoid unequal exposure of environmental factors. Each treatment was repeated four times by pots and plants. Pots of 30 cm diameter were filled with 10 kg sandy loam soils and lined with polyethylene bags ensuring seepage prevention. These were arranged in completely randomized design. Seeds sterilized with 0.1% (V/V) $HgCl_2$, similar in size and weight, were germinated and thinning was performed after germination to maintain one seedling in each pot in order to avoid the imbalanced uptake of nutrients by plants. Weeds were uprooted from time to time by hand weeding and hoeing in order to avoid weed crop competition. Insects and pests were control by foliar spray of Thiodon insecticides of Hoechst (Pvt) Ltd, Pakistan. Plants were irrigated with normal irrigation water.

Quantified amounts of chromium chloride were added in soil accordingly to raise the chromium levels of 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 mg kg^{-1} soil. Metals salts were applied in soil as a water solution of $CrCl_3$, method similar to that used by (Stoeva and Bineva, 2003) after twenty days of sowing while the pots without the addition of metals salts acted as control.Photosynthetic phytochemicals in the form of pigments contents were measured by using the formula of Arnon, 1949) after twenty five days of metal imposition. The leaves were extracted with 80% acetone. By using spectrophotometer (Hitachi Model-U 2001 Japan), the absorbances were measured at 645nm and 663nm for Chl a and b contents respectively and at 480nm for carotenoids. Carotenoids contents were calculated after (Goodwin, 1965) and chlorophylls were calculated according to the (Lichtenthaler, 1987) formulae

Table 1. Chlorophyll a contents (mg g^{-1} leaf F. wt) of 45 days old mash (*Vigna mungo* (L.) Hepper) grown in chromium (III) supplemented soil (0, 10, 20, 30, 40 50 and 60mg/kg soil) (Values represent means ± SE). Values in parentheses represent % age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions (upper case letters).

Chromium (mg kg^{-1} soil)	MASH 80	MASH 88	MASH 97	MASH ES-1	TREATMENTSMEANS (LSD=0.044 ;n=16)
		(LSD=0.088 ;n=4)			
Control	1.204±0.002 (A)	1.055±0.054 (BC)	0.979±0.031 (CD)	0.995±0.085 (BC)	1.058a±0.103
10	1.192±0.115 (0.996) (A)	1.236±0.129 (-17.156) (A)	1.070±0.053 (-9.295) (B)	0.903±0.068 (9.246) (DE)	1.100a±0.159 (-3.969)
20	0.827±0.057 (31.312) (EF)	0.992±0.089 (5.971) (BC)	0.782±0.120 (20.127) (F)	0.508±0.100 (48.944) (GH)	0.777b±0.198 (26.559)
30	0.436±0.061 (63.787) (H)	0.586±0.027 (44.450) (G)	0.449±0.090 (54.136) (H)	0.342±0.035 (65.628) (I)	0.453c±0.104 (57.183)
40	0.241±0.016 (79.983) (JK)	0.331±0.010 (68.625) (I)	0.296±0.091 (69.765) (IJ)	0.167±0.030 (83.216) (KL)	0.259d±0.077 (75.519)
50	0.113±0.028 (90.614) (LM)	0.161±0.02 (84.739) (KLM)	0.153±0.040 (84.371) (LM)	0.087±0.016 (91.250) (LM)	0.129e±0.040 (87.807)
60	0.078±0.013 (93.521) (M)	0.157±0.032 (85.118) (KLM)	0.084±0.004 (91.419) (M)	0.075±0.009 (92.462) (M)	0.098e±0.038 (90.737)
GENOTYPES MEANS →	0.585b±0.462	0.645a±0.427 (-10.256)	0.545c±0.382 (6.837)	0.439d±0.362 (24.957)	0.553±0.412
		(LSD=0.033 ; n=28)			

Carotenoids (mg g^{-1} leaf fresh weight) = (Acar/EM) × 1000. Chl a (mg g^{-1} leaf fresh weight) = (12.7(OD663) - 2.69 (OD645)) × V/1000 ×W.Chl b (mg g^{-1} leaf fresh weight) = (22.9(OD645) - 4.68(OD663)) × V/1000 ×W. Where Acar = OD480+0.114(OD663)-0.638(OD645); EM (100%) =2500; OD =Optical density; V = Volume of sample; W = Weight of sample.

Nitrate reductase activity was determined on the expiry of twenty five days after metal imposition using the method of (Sym, 1984).

The leaf area index (LAI) was calculated by using the following formula given by Puttaswamy et al., (1976).

LAI = L × W × N × K Where, L= length of the leaf (cm);W= maximum width of the leaf in cm;N= number of leaves per plant;K= constant (0.65 for legume crops).

The data collected were analyzed for analysis of variance using COSTAT computer package (CoHort Software, Berkeley, CA). Duncan's New Multiple Range test at 5% level of probability (Duncan, 1955) was used to compare means. Significant F values were tested for mean differences by LSD tests at 0.05% significance level, by using MSTAT-C Computer Statistical Programme (MSTAT Development Team, 1989)

RESULTS AND DISCUSSION

According to Duncan's Multiple Range Test (Table: 1), increasing intensity of metal stress by its escalating levels appeared to be responsible for gradual reduction in chlorophyll a contents. Index of variability in chlorophyll

a revealed that chromium (III) application above the limit of 10mg kg-1 caused statistically marked reduction in the pigment concentration. Maximum effect in all the genotypes was predominantly observed by 60mg kg^{-1}. A deviation from the ongoing role of metal was noted when 10mg kg^{-1} metal was supplemented to soil medium of MASH 88 and MASH 97 plants which exhibited an increase in chlorophyll a contents by 17.156% and 9.295% respectively from control. Among the genotypes, MASH 88 revealed maximum (0.645) and MASH ES-1 revealed minimum (0.439) values. MASH 97 differed from MASH 80 by a value of 6.837%.

Table 2. Chlorophyll b contents (mg g^{-1} leaf F. wt) of 45 days old mash (*Vigna mungo* (L.) Hepper) grown in chromium (III) supplemented soil (0, 10, 20, 30, 40 50 and 60mg/kg soil) (Values represent means ± SE). Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions (upper case letters).

Chromium (mg kg^{-1} soil)	MASH 80	MASH 88 (LSD=0.089 ;n=4)	MASH 97	MASH ES-1	TREATMENTSMEANS (LSD=0.049 ;n=16)
Control	0.639±0.043 (F)	0.935±0.135 (BC)	0.915±0.126 (C)	0.860±0.170 (CD)	0.837a±0.166
10	0.624±0.101 (2.347) (F)	1.039±0.061 (-11.122) (A)	1.008±0.050 (-10.163) (AB)	0.830±0.063 (3.488) (D)	0.875a±0.182 (-4.540)
20	0.402±0.0289 (37.089) (H)	0.896±0.082 (3.660) (CD)	0.733±0.114 (19.890) (E)	0.461±0.093 (46.395) (GH)	0.623b±0.221 (25.567)
30	0.205±0.031 (67.918) (JK)	0.520±0.024 (44.385) (G)	0.416±0.086 (54.535) (H)	0.305±0.032 (64.534) (I)	0.361c±0.129 (56.869)
40	0.107±0.007 (83.255) (LM)	0.290±0.009 (68.983) (IJ)	0.269±0.087 (70.601) (IJ)	0.142±0.028 (83.488) (KL)	0.202d±0.091 (75.866)
50	0.042±0.014 (93.427) (MN)	0.134±0.022 (85.668) (KL)	0.133±0.039 (85.464) (KL)	0.063±0.023 (91.970) (LMN)	0.093e±0.048 (88.888)
60	0.012±0.013 (98.122) (N)	0.060±0.029 (35.187) (LMN)	0.032±0.014 (96.502) (MN)	0.027±0.009 (96.860) (MN)	0.033f±0.024 (96.057)
GENOTYPES MEANS →	0.290d±0.254	0.553a±0.387 (-90.689)	0.501b±0.372 (-72.758)	0.384 c±0.335 (-32.413)	0.432±0.359
(LSD=0.037 ; n=28)					

Chromium (III) stress imposition induced a reduction in chlorophyll b contents and established an inverse correlation between the two (Table: 2). A remarkable and statistically non significant effect of chromium (III) in decreasing pigment concentration was when supplied above 10mg kg^{-1} concentration. Maximum effect in all the genotypes was by 60mg kg-1 but in MASH 88 was by 50mg kg^{-1}. Metal level of 10mg kg^{-1} in MASH 88 and MASH 97 provided an opposite index of its action and increased the chlorophyll b contents by 11.122% and 10.163% from untreated control plants. Among the genotypes, MASH 88 revealed maximum (0.553) and MASH 80 revealed minimum (0.590) values. Differences of 72.758% and 90.689% were statistically obvious for MASH 97 and MASH 88 respectively from MASH 80. Higher concentrations of chromium (III) induced reduction in carotenoids contents corresponded to its levels of application (Table: 7).

Carotenoids were affected significantly by chromium (III) concentrations of not less than 20mg kg^{-1}. Maximum effect in all the genotypes was by 60mg kg^{-1}. Though irregularly, chromium (III) at lower level of its concentration, exhibited enhancement effects as 10mg kg^{-1} revealed an increase of 16.760 % and 10.606% for plants of MASH 88 and MASH 97 respectively. Similarly 20mg kg^{-1} chromium (III) increased the carotenoids contents of MASH 88 by 21.092%. Differences of 69.587% and 78.350% were statistically obvious for MASH 97 and MASH 88 respectively from MASH 80.Genotypes, MASH 88 revealed maximum (0.346) and MASH 80 revealed minimum (0.194) values for total carotenoids contents.

From the data for mean values, it could be inferred that the chromium (III) supply impaired the activity of nitrate reductase for its reduction potential. The decrease in Nitrate Reductase Activity (NRA) established an inverse correlation with metal concentration (Table: 4). Nitrate Reductase Activity (NRA) value, when measured under the influence of more than 10mg kg^{-1} chromium (III), was found to be statistically lower than that of untreated control. Maximum effect (42.382%) was conceived by metal toxicity of 60mg kg^{-1}. A similar pattern of metal stress was extendable to all the genotypes. However, the application of 10mg kg^{-1} chromium (III) to the plants of MASH 97 and MASH ES-1 stimulated the Nitrate Reductase Activity (NRA) by 1.433 % and 13.593% respectively. Different sensitivity range for chromium (III) was found in genotypes. Among the genotypes, MASH 88 revealed maximum (0.732) and MASH 80 revealed minimum (0.626) values.

Table 3. Total Carotenoids contents (mg g^{-1} leaf F. wt) of 45 days old mash (*Vigna mungo* (L.) Hepper) grown in chromium (III) supplemented soil (0, 10, 20, 30, 40 50 and 60mg/kg soil) (Values represent means ± SE). Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions (upper case letters).

Chromium (mg kg^{-1} soil)	MASH 80	MASH 88 (LSD=0.077 ;n=4)	MASH 97 (LSD=0.077 ;n=4)	MASH ES-1	TREATMENTSMEANS (LSD=0.041 ;n=16)
Control	0.432±0.57 (FG)	0.531±0.119 (DE)	0.594±0.017 (CD)	0.773±0.164 (A)	0.582a±0.159
10	0.403±0.102 (6.712) (G)	0.672±0.044 (-16.760) (B)	0.657±0.067 (-10.606) (BC)	0.545±0.039 (29.495) (DE)	0.569a±0.126 (2.233)
20	0.197±0.033 (54.398) (I)	0.643±0.076 (-21.092) (BC)	0.480±0.059 (19.191) (EF)	0.275±0.056 (64.424) (H)	0.399 b±0.188 (31.443)
30	0.130±0.023 (69.907) (IJ)	0.285±0.052 (46.327) (H)	0.298±0.062 (49.831) (H)	0.177±0.024 (77.102) (I)	0.222 c±0.083 (61.855)
40	0.059±0.008 (86.342) (JK)	0.186±0.007 (64.971) (I)	0.180±0.068 (69.696) (I)	0.072±0.018 (90.685) (JK)	0.125d±0.069 (78.522)
50	0.069±0.056 (84.027) (JK)	0.079±0.014 (85.122) (JK)	0.081±0.017 (86.363) (JK)	0.036±0.010 (95.342) (K)	0.066 e±0.033 (88.659)
60	0.068±0.064 (84.259) (JK)	0.027±0.017 (94.915) (K)	0.016±0.005 (97.306) (K)	0.019±0.004 (97.542) (K)	0.032 e±0.037 (94.501)
GENOTYPES MEANS →	0.194 c±0.159	0.346 a±0.258 (-78.350)	0.329 a±0.242 (-69.587)	0.271 b±0.277 (-39.690)	0.285±0.242
(LSD=0.031 ; n=28)					

From the data for mean values, it could be inferred that the chromium (III) supply reduced the leaf area index (Table: 8). The decrease in leaf area index (LAI) established an inverse correlation with metal concentration. Leaf area index (LAI) value, when measured under the influence of more than 10mg kg^{-1} chromium (III), was found to be statistically lower than that of untreated control. Maximum effect (42.036%) was conceived by metal toxicity of 60mg kg^{-1}. A similar pattern of metal stress was extendable to all the genotypes. However, the application of 10mg kg^{-1} chromium (III) stimulated the leaf area index (LAI) in all genotypes Different sensitivity range for chromium (III) was found in genotypes. Among the genotypes, MASH ES-1 revealed maximum (340.992) leaf area index (LAI) and MASH 88 revealed minimum (297.288) values.

Our results revealed that chlorophyll concentration was decreased by chromium application in a concentration dependent manner (Table and 2). A significant reduction of chlorophyll in chromium treated plant is reported by many researchers (Rai et al. 2014; Rajendran et al. 2019;Sinha et al. 2004;Balal et al. 2017;Islam et al. 2016;Zou et al. 2009; Amin et al. 2013;Tang et al. 2012;Amin et al. 2014). Chlorophyll reduction might be either due to inhibiting biosynthesis of chlorophyll

(Lushchak, 2010; Sharma et al. 2019; Chandra and Kulshreshtha, 2004) or destruction of chlorophyll molecule in Cr treated plants (Valko et al. 2006). Reduction in chlorophyll contents may be due to the increased activity chlorophyllase enzyme and nutrients deficiency because of higher concentration of metal translocation toward shoots (Khan et al. 2016;Shakoor et al. 2014). Plants exposed to Cr stress showed depleted chlorophyll contents that might be due to the disrupted chlorophyll biosynthesis (Chandra and Kulshreshtha, 2004). Chromium reduces chlorophyll contents by inhibiting activity of δ-aminolevulinic acid dehydratase (ALAD) enzyme which is involved in chlorophyll synthesis (Hayat et al. 2012).

Table 4. Nitrate Reductase (EC 1.6.6.1) Activity (NRA) of 45 days old mash (*Vigna mungo* (L.) Hepper) grown in chromium (III) supplemented soil (0, 10, 20, 30, 40 50 and 60mg/kg soil) (Values represent means ± SE). Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions (upper case letters).

Chromium (mg kg^{-1} soil)	MASH 80	MASH 88 (LSD=0.0444 ;n=4)	MASH 97	MASH ES-1	TREATMENTSMEANS (LSD=0.026 ;n=16)
Control	0.743±0.023 (E)	0.963±0.039 (A)	0.837±0.030 (D)	0.951±0.037 (AB)	0.873a±0.097
10	0.844±0.029 (-13.593) (D)	0.902±0.026 (6.334) (C)	0.849±0.036 (-1.433) (D)	0.910±0.025 (4.3112) (BC)	0.876a±0.040 (-0.343)
20	0.609±0.012 (18.034) (JK)	0.837±0.030 (13.084) (D)	0.763±0.010 (8.841) (E)	0.721±0.052 (24.185) (EF)	0.733b±0.089 (16.036)
30	0.583±0.013 (21.534) (KL)	0.658±0.043 (31.671) (GHI)	0.726±0.007 (13.261) (EF)	0.616±0.014 (35.226) (IJK)	0.646c±0.059 (26.002)
40	0.551±0.014 (25.841) (LM)	0.670±0.017 (30.425) (GH)	0.692±0.024 (17.323) (FG)	0.579±0.009 (42.481) (KL)	0.623c±0.063 (28.636)
50	0.522±0.002 (29.744) (MIN)	0.634±0.018 (34.164) (HIJ)	0.640±0.011 (23.536) (HIJ)	0.547±0.022 (43.112) (LM)	0.586d±0.055 (32.875)
60	0.533±0.027 (28.263) (MN)	0.462±0.060 (52.024) (O)	0.490±0.032 (42.457) (NO)	0.528±0.135 (44.479) (MIN)	0.503e±0.0753 (42.382)
GENOTYPES MEANS →	0.626c±0.115 (9.668)	0.732a±0.168 (-5.627)	0.714a±0.118 (-3.030)	0.693b±0.171	0.691±0.149
(LSD=0.020 ; n=28)					

Metal stress reduced carotenoids contents (Table 3). Reduction in carotenoids contents might be attributed to activation of osmotic stress leading the biosynthesis of abscisic acid (ABA) by carotenoid cleavage catalyzed by a 9-cis epoxycarotenoid dioxygenase (NCED). Another possible reason for reduction in carotenoids contents might be biosynthesis of anthocyanins. The anthocyanins are synthesized during stress and interfere with carotenoids (Burger and Edwards, 1996). The experimental results revealed, as a general trend, reduction in nitrate reductase activity by metal stress (Table 4). Inhibition of NRA by metal might be caused either by reduction of biosynthesis enzyme or by suppression of activity of existing enzyme. Depolarization of NR thiol or SH groups by metal also reduces enzyme activity (Jones and Mhuimhneachain 1995). It has been suggested that NR activity depends upon active photosynthesis or products of photosynthesis as it requires photosynthetically generated reductant (NADH) and energy (Raghuram and Sopory 1995).

Reduced NRA may be attributed to reduced N contents availability to plant either due to shortage in soil or consumption by plant (Campbell 1999). Stress mediated decreased cytokinin levels might cause a reduction in nitrate reductase activity (Bueno et al 1994). Through Phosphorus limitation (Gniazdowska and Rychter 2000). Another reason for NRA might be due to reduced chlorophyll contents or reduced rate of photosynthesis (Rai et al 1992; Li et al. 2012; Zhang et al 2018). The experimental results revealed a gradual reduction in leaf area index with increasing concentration of metal (Table 5). Leaf area reduction can be due to growth inhibition

in metal treated plants (Ouariti and Ghorbal, 1997). Leaf growth reduction might be the result of low water potential due to very negative solute potential in the soil solution (Hayward and Spurr, 1944).

Table 5. Leaf Area Index (LAI) of 45 days old mash (*Vigna mungo* (L.) Hepper) grown in chromium (III) supplemented soil (0, 10, 20, 30, 40 50 and 60mg/kg soil) (Values represent means ± SE). Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions (upper case letters).

Chromium (mg kg^{-1} soil)	MASH 80	MASH 88	MASH 97	MASH ES-1	TREATMENTSMEANS (LSD=17.393 ;n=16)
		(LSD=34.79 ;n=4)			
Control	401.957±12.967 (ABC)	362.462±13.717 (CDE)	447.702±6.819 (AB)	429.642±6.003 (ABC)	410.441b±34.487
10	381.375±10.975 (5.369) (BCD)	421.377±14.754 (-16.254) (ABC)	449.850±23.829 (-0.479) (AB)	463.490±6.982 (-7.878) (A)	429.023a±35.276 (-4.527)
20	301.787±34.864 (25.030) (EFGH)	307.422±13.670 (15.185) (CDE)	309.630±19.615 (30.840) (EFG)	418.030±13.275 (2.702) (ABC)	334.217c±53.842 (18.571)
30	290.52±21.424 (24.920) (FGHI)	363.897±33.022 (-0.384) (CDE)	287.690±27.565 (35.740) (FGHI)	313.842±28.829 (26.952) (DEF)	288.987d±31.012 (29.591)
40	267.355±28.422 (7.723) (FGHI)	249.967±28.425 (31.036) (FGHI)	263.34±11.829 (41.101) (FGHI)	253.887±24.070 (40.907) (FGHI)	258.637e±22.787 (36.985)
50	245.197±14.083 (33.480) (FGHI)	242.267±19.941 (33.160) (JHI)	251.845±19.081 (43.747) (FGHI)	260.230±28.350 (39.430) (FGHI)	249.885ef±20.091 (39.117)
60	243.377±77.637 (38.999) (GHI)	233.627±9.060 (35.544) (HI)	226.802±16.358 (49.340) (I)	247.827±12.767 (42.317) (FGHI)	237.908f±37.148 (42.036)
GENOTYPES MEANS →	304.510c±67.758	297.288c±69.293 (2.371)	319.551b±88.408 (-4.939)	340.992a±89.694 (-11.980)	315.585±80.156
	(LSD=13.148 ; n=28)				

Reduced cytokinin contents by metal might be responsible for growth reduction by inhibition of cell division and cell elongation.The results of the experiment indicated that plants could tolerate chromium up to 10 mg/kg soil as no toxicity was recorded at this concentration. These effects of chromium may simply be due to a dose dependent response of the seedlings where the low dose stimulates the growth while high dose suppresses the growth (Shah et al. 2008). The absence of effect at low concentration could be attributed to the fact that low dose of metal accumulates in roots than in the shoot and the effect is restricted to the root but not in the shoot (Selvam and Wong, 2008).

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Optimization of Exo-Polygalacturonase Production Activity by Various Factors by Soil Isolate *Bacillus megaterium*

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ABSTRACT

Polygalacturonase represent an important member of pectinase group of enzymes with immense industrial applications that hydrolyzes external and internal α (1–4) glycosidic bonds of pectin to decrease the viscosity of fruits juices and vegetable smashes. Several bacterial strains were isolated from soils of decomposed pectin waste from mango processing industries screened for polygalacturonase production. The strain which produced maximum polygalacturonase was identified as *Bacillus sp*., based on the morphological, biochemical and physiological tests performed which was further confirmed at species level as *Bacillus megaterium* (mpb2) by the molecular characterization with the assistance of IMTECH, Chandigarh, India. Under partially different optimized conditions like temperature, pH, substrate concentration, metal ions, carbon sources, nitrogen sources, surfactants, effect of time and stability of exo-polygalacturonase enzyme activities were studied. Results indicated that the maximum polygalacturonase production by *B. megaterium* were at pH (0.96 U/ml) range of 6.0 to 8.0 and temperature (0.97 U/ml) of 55°C and substrate concentration of 1% citrus pectin (0.73 U/ml). Lactose (1.5 U/ml) as carbon source, Peptone (0.66 U/ml) as nitrogen source, Triton X-100 (0.37 U/ml) as surfactant, $CaCl_2$ (0.25 U/ml) as metal ion, effect of time for 15 minutes at 55°C (0.73 U/ml) and the enzyme stability for one hour at 65oC was (0.73 U/ml) were observed as optimum for the polygalacturonase production by *B. megaterium* respectively. The main objective is to study the various optimum factors on production of exo-polygalacturonase by *Bacillus megaterium* isolated from soil samples collected from pectin industry waste of Vinsari and Varsha mango fruit processing industries around Tirupati, Chittoor district of Andhra Pradesh.

KEY WORDS: EXO-POLYGALACTURONASE, BACILLUS MEGATERIUM, METAL ION, OPTIMIZATION, SURFACTANTS,.

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INTRODUCTION

The microbial enzyme production had greatly influenced by environmental and nutritional factors. Therefore, the selection of microbial source (wild type, recombinant, mutagenized) along with variations in various parameters; pH, metal ions, temperature affects the polygalacturonase production. Surfactants such as tween-20, Tween - 80 increase the enzyme production due to favourable effect on cell membrane permeability which leads to secretion of the enzyme. Pectinase synthesis is inhibited by SDS PAGE because of the denaturation of enzyme. The degrading ability of the enzyme is enhanced by agitation (Ahlawat et al., 2009). The purified polygalacturonase characterization is an important that used for research since it exhibited on being able to distinguish between the enzymatic complex components of the substrate degradation mechanisms, optimum conditions for enzymatic activity and the regulation of enzyme by inhibiters (Sathyanarayana and Panda 2003).

Of the many microorganisms *Bacillus spp*. are known to produce variety of extracellular enzymes and they have a wide range of industrial applications (Annamalai et al., 2011; Amin et al., 2017). The advantage of using micro-organisms for the production of enzymes is that these are not influenced by climatic and seasonal factors, and can be subjected to genetic and environmental manipulations to increase the yield (Zeni et al., 2020)

The microbial pectinase production varies according to the composition of growth medium and the cultivation conditions i.e., pH, temp, aeration, agitation and incubation time (Thakur et al., 2010). Carbon sources also effect the production of pectinase. As observed PGA, lactose, pectin increased pectinase production (Kashyap et al., 2003). In *Aspergillus fumigatus* sucrose yielded maximum pectinase production (Phutela et al., 2005). Among the nitrogen sources for pectinase production the maximum yield of pectinase was shown by yeast extract pectinase production is inhibited by glycine, urea, ammonium nitrate while wheat bran, peptone, ammonium chloride, yeast extract enhance pectinase production (Colla et al., 2017). The optimum pH for growth and pectinase production for most of the bacteria is 7-10 (Ahlawat et al., 2009; Tepe and Dursun 2014). Some bacterial strains; *Streptomyces* QG-11-3 and *Aspergillus aculeatus* produced PGase active at pH 3.0 (Torres et al., 2006).

The activity of pectinases depends on thermal stability. Pectinase production by *Bacillus subtilis* the optimum temperature was found to be 37°C (Ahlawat et al., 2009). PGase from fungi have optimum activity at 50°C while from yeast the temperature varies from 40°C to 60°C (Torres et al., 2006). The activity of Endo PGase is reduced due to Cu^{2+} and Hg^{2+}. Metal ions Hg^{2+}, Zn^{2+}, Mg^{2+} inhibit enzyme production due to inhibition by thiol group blocking agents as there is possible involvement of the thiol group in the enzyme s active site (Ahlawat et al., 2009; Suneetha and Khan 2011). Mn^{2+} increases the PGase activity; however Li^{2+}, Fe^{2+}, Rb^{2+} have no effect on the activity.

The time of fermentation i.e. incubation period of organisms had a profound effect on microbial product formation (Bennamoun et al., 2016; Murad and Foda 1992; Murad and Salem 2001). Reda et al. (2008) found that the level of polygalacturonase increased gradually with increasing the incubation period up to a maximum of 96 h by *Bacillus firmus*-1-10104 under solid state fermentation conditions. The effects of various organic and inorganic nitrogen sources on the pectinase production were extensively studied. The observations of Hours suggested that lower levels of $(NH_4)2So_4$ (0.16%), or K_2HPO_4 (0.1%) added to the growth medium as inorganic nitrogen sources did not influence pectinase yield (Hours et al., 1988). Reda found that the maximum value of polygalacturonase productivity by *Bacillus firmus*-1-10104 reached up to 350 U mL^{-1} in the presence of peptone as a nitrogen source in the growth medium. In addition to this organic nitrogen sources showed higher endo, exo pectinases activities than inorganic nitrogen sources (Vivek et al., 2010).

Optimum growth affecting the growth of organism and its metabolism can be achieved by an adequate supply of carbon as energy source. Reda et al. (2008) reported that *Solanum tuberosum* (ST) peels was the best carbon source for polygalacturonase production by *Bacillus firmus*-1-10104 under solid state condition. *B. megaterium* enzymes and products have been used in industrial applications for several years and are also effective in immobilized systems, making industrial processes even more efficient. An extensive review of B. megaterium emphasizing its commercial applications has been published (Vary, 1992). In the present study, an efficient exo-polygalacturonase producing strain was isolated from potent sites of pectin waste and identified as *Bacillus megaterium* followed by optimizing favourable conditions for growth and exo-polygalacturonase production.

MATERIAL AND METHODS

Cultures were selectively isolated from untreated non-rhizospheric potent soil mainly from pectin producing industrial wastes initially by serial dilution and plating technique using pectin enrichment method. Sample was inoculated into Nutrient Agar medium and incubated for 24 to 48 hours at 37 °C and isolates were subjected for their pectinolytic property by puncturing the medium on Citrus pectin agar (CPA) medium which is a quantitative test for pectin degrading bacteria. Potential isolates were achieved at mesophilic temperatures ranging from 30 to 37 °C. All morphological colonies were purified by repeated streaking.The medium was the same used for isolation of cultures, supplemented with 2% agar agar. Pure cultures were inoculated by puncture in the medium and incubated for 48h at 30°C. After the colonies reached around 3 mm, iodine –potassium iodide solution (1.0 gm Iodine, 5g of KI and 330 ml H_2O) was added to detect clearance zones (Zeni et al., 2020).

The potent pectinolytic isolates were also checked for cellulolytic, proteolytic and amylolytic activities by using carboxymethylcellulose (CMC) agar medium, casein agar medium and starch agar medium through getting clearing zones. Strains presenting large clearing zones were used for enzyme production on liquid medium same used for screening. Cultures were grown in 150 mL Erlenmeyers flasks with 100 ml of medium in rotary shaker of 150 rpm at 30 °C. After 48 hours the biomass was separated by centrifugation at 10000xg for 20 minutes and the supernatant was used to evaluate polygalacturonase (PG) activity. The supernatant was used for further enzymatic activity studies (Soares et al., 2001).

Exo polygalacturonase activity was determined by measuring the release of reducing groups using the dinitro salicyclic acid reagent (DNS) assay by Miller (1959). The reaction mixture containing 0.8 ml of 1% citrus pectin with 67% metoxilation in 0.2 M citrate phosphate buffer, pH 6.0 buffers and 0.2 ml of culture supernatant, was incubated at 37 °C for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid released per minute. Citrus Pectin medium (100 ml in 500 ml Erlenmeyer flasks) was inoculated with 1 ml overnight culture of *Bacillus megaterium* and incubated at 37 °C with vigorous aeration in a shaker at 150 rpm for 5 days. Cells were separated by centrifugation at 8000xg for 20 minutes at 4 °C. The cell free culture (supernatant) was used as crude enzyme source. All steps of purification were carried out at 4 °C. The enzyme in the crude preparation was precipitated by the addition of Ammonium sulphate (30-90% saturation) and kept overnight at 4 °C and centrifuged in a refrigerated centrifuge (Remi C24) at 10000g for 30 min. The precipitate was removed and supernatant was again subjected with further addition of ammonium sulphate in view to remove other proteinaceous material.

The resultant precipitate bearing high pectinase activity was dissolved in the small quantity of 0.01M Tris-HCl buffer (pH 6.0) and dialyzed (in dialysis bag, Sigma 10-12 kDa) overnight against the same buffer with constant stirring and was subjected to further purification. During the course of dialysis, the buffer was frequently changed with the fresh lot until no traces of ammonium were found upon testing with Nessler's reagent (Afifi et al., 2002). Production medium was supplemented with different nitrogen sources at an equimolar of nitrogen that present in diammonium sulphate in pectin medium. The applied nitrogen sources were ammonium molybdate, ammonium chloride, ammonium oxalate, ammonium nitrate, diammonium hydrogen phosphate, potassium nitrate, gelatin, peptone, casein and urea. Different carbon sources were introduced into the pectin medium at an equimolecular amount located at 1% (W/V) glucose. The carbon sources were represented by glucose, sucrose, starch, lactose, maltose, galactose, fructose, cellulose and pectin.

The optimum pH was determined with tri sodium citrate 1% (w/v) as the substrate dissolved in different buffers (Tris-Hcl 3.0, Citrate phosphate, pH 5.0, sodium phosphate 7.0, glycine-NaOH pH 9). The pH was adjusted at different pH values 2, 4, 6, 8, 10, 12 by using sodium citrate buffer. The effect of pH was determined by pre-incubating enzyme and substrate at different pH values (2-12) for 24 hours at room temperature. The effect of temperature on enzyme activity was determined by performing standard assay procedure within a temperature range from 0 to 75 °C for 24 hours.

The effect of different metal ions on pectinase activity was determined by the addition of the corresponding ion at a final concentration of 0.1 mM and the reaction mixture was assayed under standard conditions. The enzyme was carried out in the presence of CaCl2, $MgSO_4$, $CuSO_4$ and inhibitor like EDTA. The effect of surfactants on exo-PG was determined by using the corresponding ions at a concentration of 0.1 mM and the reaction mixture was assayed under standard conditions. The enzyme was carried out in the presence of surfactants like Tween 80, Tween 20 and Triton X-100.Different concentrations of citrus pectin (0.5%, 1%, 1.5% and 2%) were applied to determine the effect of substrate concentration on exo-PG activity. Exo-PG enzyme activity was estimated for different time intervals (5-30 minutes) at 55 °C. Enzyme stability was measured at different temperatures (35 to 85 °C) for one hour.

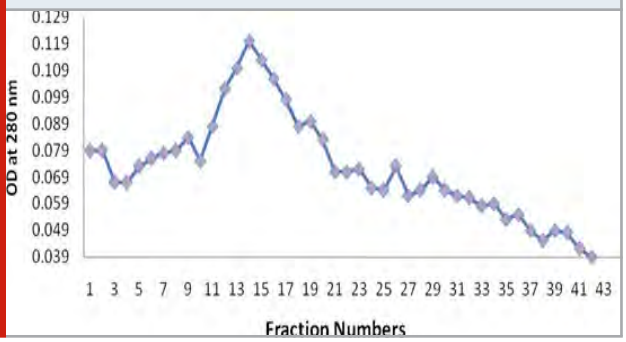
RESULTS AND DISCUSSION

Based on the above experiments it is clearly evidenced that *B. megaterium* was showing maximum activity. Exo- PG (EC 3.2.1.67) is an essential component of pectinase complex and is the key enzyme used by the bacteria to decompose pectin. Exo-PG randomly hydrolyzed internal glyosidic linkages, which result in rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. In view of its importance in saccharification process of pectic substrates and also poor production in some bacterial strains, its search in other organisms attracts a great deal of attention. Therefore, production and purification of exo-PG by *B. megaterium* isolated in this study was emphasized. The cell free culture filtrate obtained by orbital shaker in 100 mM Tris-HCl was used as crude enzyme source. In the culture filtrate a total activity of 0.98 U and total protein content of 2.35 mg with specific activity of 0.41 U/mg, with recovery 100%.

The supernatant was fractionated by precipitation with ammonium sulphate at 30-90% saturation. The precipitate was suspended in 0.1 M Tris-HCl buffer (pH 6.0) and dialyzed against same buffer with constant stirring. After dialysis protein content was determined. The purified fraction from ammonium sulphate has a total activity of 0.6 U, with total protein content of 0.68 mg with specific activity of 0.882 U/mg with 67.4% recovery with purification fold of 2.578. Further the fraction from ammonium sulphate precipitation was subjected to ion exchange chromatography by means of DEAE cellulose CL21 column. Fraction was obtained showing a total activity of 0.80 U and total protein with 0.82 mg. The specific activity calculated was 0.97 U/mg with recovery

of 81% and purification fold of 2.3 (Figure 1).

Figure 1: Fractions from Sephadex G100 column



The fraction from DEAE cellulose CL 21 chromatography was further subjected to gel filtration using Sephadex G100 column. This fraction had total activity of 0.63 U with a recorded total protein content of 0.174 mg with specific activity of 3.6 U/mg. The enzyme was recovered with a purification fold of 8.8 and 64% recovered. The fraction from Sephadex G100 column was subjected to SDS- PAGE to determine purity of enzyme (Table 1).

The influence of different physiological factors like pH, temperature, metal ions, carbon sources, nitrogen sources, surfactants, substrate concentration, time and temperature stability was observed on exo-PG activity. Influence of pH on exo-PG activity by bacterial isolates was examined. Individual enzyme components of exo-PG had shown maximum activity at pH 6 to 8 in both isolates. As the isolate was found active at pH 7, it can be used in degradation of organic matter, detergents and sewage treatment (Table 2). High activities of exo-PG (0.96 U/ml) produced at pH 6.0 to 8.0. Above and below the pH range of 6-8, a decrease in the growth was observed.

Table 1. Purification of exo-Polygalacturonase

Purification Step	Total Protein (mg)	Activity (U)	Specific Activity (U/mg)	Yield (100%)	Purification Fold
Crude Enzyme	2.35	0.98	0.41	100	1
Ammonium Sulphate precipitation	0.68	0.6	0.882	67.4	2.578
Dialysis	0.82	0.8	0.97	81	2.3
Sephadex G – 100	0.174	0.63	3.6	64	8.8

Table 2. Effect of pH on exo-PG activity of *B. megaterium*

	pH	N	Mean	Std. Deviation	F-value	P-value
	2	3	0.3333 a	0.01528		
	4	3	0.4200 b	0.01000		
	6	3	0.9633 c	0.01528		
B. megaterium	8	3	0.9500 c	0.02000	971.242**	0.000
	10	3	0.5333 d	0.01528		
	12	3	0.4433 b	0.01528		
	Total	18	0.6072	0.26155		

** Significant at 1% level; Note: The same letter indicates insignificant difference in any pair of media pH according to Duncan's Multiple Range Test (DMRT).

Results were noted in Table 2. From the F-value one can understand that there is significant difference among the impact of pH on the exo-PG activity in both organisms. Maximum activity was observed at pH 6 and 8 in both isolates. Further DMRT suggest that the same level of growth was observed at pH 6 and 8 of *B. megaterium* and then followed by pH 4 and 12. Temperature highly influences the metabolic reactions through enzymatic activities, thereby affecting the growth of the organism. Effect of temperatures on enzyme production indicated that maximum exo-PG enzyme was shown at 55oC. The growth, extracellular protein, total soluble sugar content and exo-PG enzymes were assayed at 72 hr of incubation (Table 3). This culture filtrate also yielded higher activity in terms of exo-PG with 0.97 U/ml in *B. megaterium*. Exo-PG activity was showing significant difference at 1% level among the temperatures. Since p - value 0.000< 0.01 for the corresponding F- value (376.167**). At 55 °C *B. megaterium* show maximum activity 0.97 u/ml, respectively. This temperature is most useful for the

degradation purposes of organic matter when compare to fungal pectinase.

various temperatures.Effect of different metal ions and inhibitors on exo-PG production in *B. megaterium* was studied. Metal ions tested had shown stimulatory as well as inhibitory effect on microorganisms. We found that $CaCl_2$ showed increased exo-PG activity when compare to other metal ions. This culture filtrate also yielded higher activity in terms of exo-PG with 0.25 U/ml. Inhibitors like EDTA, NaN3 at 1mM concentration inhibited the activity i.e., (0.07U/ml) **. One way ANOVA results revealed that metal ions and inhibitors play an important role on exo-PG activity (Table 4). $CaCl_2$ has shown stimulatory effect on exo-PG activity. Inhibitors like EDTA and NaN_3 were also greatly influencing the exo PG activity.

Table 3. Effect of Temperature on exo pg activity by *B. megaterium*

	Temperature	N	Mean	Std. Deviation	F-Value	P-Value
	25	3	0.3467 a	0.02517		
	35	3	0.4167 b	0.01528		
	45	3	0.4933 c	0.01528		
B. megaterium	55	3	0.9700 c	0.02000	376.167**	0.000
	65	3	0.4400 d	0.02646		
	75	3	0.3700 b	0.02000		
	Total	18	0.5061	0.21966		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the

Table 4. Effect of metal ions on exo-PG activity by *B. megaterium*

	Metal ion	N	Mean	Std. Deviation	F-value	P-value
	$CuSO_4$	3	0.1933 a	0.01528		
	$MnSO_4$	3	0.2233 b	0.02082		
B. megaterium	$CaCl_2$	3	0.2500 b	0.01000	85.690**	0.000
	EDTA	3	0.0700 c	0.02000		
	NaN3	3	0.0567 c	0.01528		
	Total	15	0.1587	0.08400		

** Significant at 1% level

Exo-PG production and secretion of extracellular protein on citrus pectin medium with different carbon sources at 1% level was compared for the organism. Results of this comparative study are represented in Table 5. Of all the carbon sources tested in this study, Lactose and Citrus Pectin elicited the production of highest titres of exo-PG activity with 1.5 U/ml in *B. megaterium*. Lactose was the best carbon source among all the carbon sources.

Nitrogen is one of the important elements required for growth of microorganisms. Provision of utilizable form of nitrogen source to organisms is the basic requirement to be fulfilled for optimal growth. In order to find out the best utilizable form of nitrogen source for growth, extracellular protein and pectinase production by bacteria on citrus pectin medium supplemented with different nitrogen source such as ammonium sulphate, ammonium molybdate, ammonium chloride, ammonium oxalate, ammonium nitrate, K_2HPO_4, KNO_3, gelatin, peptone, casein, and urea were determined. The results obtained from this experiment were presented in table and graph. Peptone followed by casein and K_2HPO_4 were the best nitrogen sources for *B. megaterium*. The highest activity of exo-PG produced was 0.66 U/ml (Table 6). The growth of the both isolates showing a significant difference at 1% level among the nitrogen sources. Since, p value $0.000<0.01$ for the corresponding F–value is 294.221. The results suggested that the difference between the same alphabets was insignificant.

Influence of surfactants such as Triton-X-100, Tween-80, and Tween-20 on exo-PG production by *B. megaterium* was determined in the same manner. Exo-PG production on the medium amended with surfactants was showed in Table 13 and Figure 14. The medium with Triton X-100 had yielded more extracellular protein and highest

pectinolytic activity of 0.37 U/ml (Table 7). Since p –value 0.000 <0.01 for the corresponding F–Value 141.23. Triton X-100 showed maximum exo-PG activity.

Substrate concentration also plays an important role on the exo pg activity. CP medium was amended with 0.5%, 1%, 1.5%, 2%, 2.5% etc and checked for the better activity. 1% citrus pectin shows maximum activity on exo-PG compare to other concentrations. One way ANOVA reveals that there is significant substrate concentration on exo-PG activity. One percent citrus pectin shows maximum activity on exo-PG activity 0.73 U/ml. Since p-value 0.000<0.01 for the corresponding F-value is 138.29**.

Table 5. Effect of different carbon sources on exo-PG production

	Carbon sources	N	Mean	Std. Deviation	F-value	P-value
	Lactose	3	1.5367 a	0.01528		
	Sucrose	3	0.3600 b	0.01000		
	Starch	3	0.9467 c	0.01528	1576.858**	0.000
	Glucose	3	0.4367 d	0.02082		
B. megaterium	Maltose	3	0.5633 e	0.01528		
	Galactose	3	0.7400 f	0.02000		
	Fructose	3	0.5600 e	0.01000		
	Cellulose	3	0.4333 d	0.01528		
	Pectin	3	0.9667 c	0.02082		
	Total	27	0.7270	0.35996		

** Significant at 1% level; **Note:** The same letter indicates insignificant difference among the various carbon sources.

Table 6. Effect of nitrogen sources on exo-PG activity by *B. megaterium*

	Nitrogen Sources	N	Mean	Std. Deviation	F-Value	P-Value
	Ammonium	3	0.2333 a	0.02082		
	Sulphate					
	Ammonium	3	0.1767 b	0.01528		
	molybdate					
B. megaterium	Ammonium	3	0.2567 c	0.01528	294.221*	0.000
	chloride					
	Ammonium	3	0.2333 a	0.01528		
	oxalate					
	Ammonium	3	0.3133 d	0.01528		
	nitrate					
	K2HPO4	3	0.5633 e	0.01528		
	KNO3	3	0.4867 f	0.01528		
	Gelatin	3	0.4367 g	0.02082		
	Peptone	3	0.6667 h	0.01528		
	Casein	3	0.5733 e	.01528		
	Urea	3	0.2767 c	.02082		
	Total	33	0.3833	.16330		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the various nitrogen sources.

Stability of exo-PG was studied for one hour at 65^{0}C temperature. One way ANOVA was carried out whether stability of the exo-PG was significant. At 65 °C temperature the enzyme stability was for one hour was 0.73 U/ml. Based on DMRT analysis the influence of different temperatures for one hour was significant. The alphabets showed insignificant among the temperatures for one hour.

The activity of each enzyme component is greatly influenced by factors like pH, temperature, metal ions / inhibitors, surfactants, substrate concentrations, time,

temperature stability and nutrients which can be best monitored for the maximum activity of the enzyme complex thus the degradation. Pectinolytic enzymes or pectinases are a heterogeneous group of enzymes that hydrolyze the pectic substances present in plants. They include polygalacturonase, pectin lyase, and pectin methyl esterase that hydrolyze the glycosidic bonds of pectic substances. Endo polygalacturonase (EC 3.2.1.15) and exo polygalacturonase (EC 3.2.1.67) are the enzymes of particular interest to industry because they act on pectin, hydrolyzing its internal and external glycosidic bonds, producing shorter pectin molecular structures, decreasing the viscosity, increasing the yield of juices, and determining the crystalline structure of the final product. Some of the bacterial species producing pectinases are *Agrobacterium tumefaciens, Bacteroides thetaiotamicron, Ralstonia solanacearum,* and *Bacillus sp* (Bennamoun et al., 2016; Murad and Foda 1992). After identification of bacterial cultures, exo polygalacturonase production was done in citrus pectin agar (CPA) medium. Pectinolytic activity of bacterial cultures was determined according to dinitro salicillic acid by Miller (1959) and Colla et al (2017).

Table 7. Effect of surfactants on exo-PG activity by *B. megaterium*

	Surfactants	N	Mean	Std. Deviation	F-value	p-value
	Tween80	3	0.1900 a	0.01000		
B. megaterium	Tween20	3	0.2433 b	0.01528	141.235**	0.000
	Triton X-100	3	0.3733 c	0.01528		
	Total	9	0.2689	0.08253		

** Significant at 1% level

Table 8. Effect of substrate concentration on exo-PG activity by *B. megaterium*

	Substrate Conc.	N	Mean	Std. Deviation	F-value	p-value
	0.50%	3	0.4733 a	0.01528		
	1%	3	0.7333 b	0.01528		
B. megaterium	1.50%	3	0.6267 c	0.01528	138.293**	0.000
	2%	3	0.5267 d	0.01528		
	2.50%	3	0.4733 a	0.02082		
	Total	15	0.5667	0.10486		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the various concentrations of substrate.

Individual enzyme components of exo PG secreted into the culture medium of *B. megaterium* was estimated in accordance with methods listed by (Amin et al., 2017). The PGase activity was assayed by estimating the amount of reducing sugar released under assay conditions. The reducing sugar produced in the reaction mixture was determined by dinitro salicylic acid (DNS) method. One unit of exo polygalactoronic acid activity was defined as the amount of enzyme releasing 1 µ mole of galactuornic acid per minute. The extracellular exo polygalacturonase enzyme of *B. megaterium* sps, was purified 8.8 fold with an yield of 31.4%. Its specific activity was 3.6 U/mg. The Physiological conditions play a vital role in enzyme production (Zeni et al 2020).

These conditions include environmental factors such as temperature, pH, substrate concentration, metal ions, carbon sources, nitrogen sources, surfactants and stability of exo-polygalacturonase which have shown significant effect on exo-PG activity of bacterial isolates (Tepe and Dursun 2014). The purified enzyme exhibited optimum polygalacturonase activity at initial pH of the production medium was adjusted to 6-8 (Table 2) and there was a drastic decrease (80%) in enzyme activity at above or below pH range of 6-8. A pH range of 6–8 has been reported for maximum PGase production of *B. megaterium*. High activities of exo-pg (0.96 U/ml) production were observed at pH 6.0 to 8.0.

Most favourable production temperature for PGase production was found to be 55 °C (Table 3). *B. megaterium* produced maximum PGase activity when incubated at 55 °C for 72 hours. Further effect of different metal ions and inhibitors on exoPG production was studied. Metal

ions tested had shown stimulatory as well as inhibitory effect on microorganisms. Stimulatory effect was shown by $CaCl_2$ which increased exo-pg activity when compare to other metal ions. Inhibitors like EDTA, NaN3 at 1mM concentration inhibited the activity in both the organisms (Table 4). This culture filtrate also yielded higher activity in terms of exo pg with 0.21 U/ml and 0.25 U/ml respectively. *B. megaterium* pectinolytic potential was further assessed by determining the effects of nutrients on exo-pg production. These nutrients include Carbon sources, Nitrogen sources, surfactants, substrate concentration etc. of all the carbon sources tested in this study, Lactose and Citrus Pectin elicited the production of highest titres of exo-PG activity with 1.5 U/ml of B. Megaterium (Table 5).Nitrogen is one of the important elements required for growth of microorganisms. Peptone followed by casein and K_2HPO_4 were the best nitrogen sources for *B. megaterium*. The highest activity of exo polygalacturonase with peptone was 0.66 U/ml by *Bacillus megaterium* (Table 6). Influence of surfactants such as Triton-X-100, Tween-80, and Tween-20 on exo polygalacturonase production by *B. megaterium* was also determined in the same manner. The medium with Triton x-100 had yielded more extracellular protein and highest pectinolytic activity of 0.25U/ml and 0.37 U/ml respectively (Table 7).

Substrate concentration also plays an important role on the exo pg activity. CP medium was amended with 0.5%, 1%, 1.5%, 2%, 2.5% etc and checked for the better activity. 1% citrus pectin shows maximum activity on exo-PG compare to other concentrations (Table 8). Stability of exo PG was also studied for one hour. At 65oC temperature the enzyme stability was for one hour was 0.73 U/ml. *B. megaterium* shows best activity at 65oC for one hour.The maximum activity of polygalacturonase from *B. megaterium* was obtained using 0.5 ml of enzyme after 15 minutes' incubation at 50 °C and pH of 5.0 (Table 9). The activity of each enzyme component is greatly influenced by factors like pH, temperature, metal ions / inhibitors, surfactants, substrate concentrations, time, temperature stability and nutrients which can be best monitored for the maximum activity of the polygalacturonase production.

Table 9. Temperature Stability of exo-PG by *B. megaterium*

	Temp. Stability	N	Mean	Std. Deviation	F-Value	P-Value
	35	3	0.4300 a	0.02000		
	45	3	0.5100 b	0.02646		
	55	3	0.6700 c	0.02000		
B. megaterium	65	3	0.7367 d	0.01528	284.015**	0.000
	75	3	0.3067 e	0.01528		
	85	3	0.2833 f	0.01528		
	Total	18	0.4894	0.17575		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the various temperature stabilities.

CONCLUSION

Enzyme production is one of the broad areas of biotechnology which accounts for about 1.5 billion of the world market. The enzymes from microbial origin were found to be more advantageous than others. Pectinase accounts for 10% of global industrial enzymes produced and their market is increasing day by day. Pectinolytic enzymes are produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. In the present study production of exo polygalacturonase using mango fruit waste was carried out and the production conditions optimized. Several microorganisms are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental conditions.

Serial diluted samples of fruit waste were screened for exo-PG produced by bacteria. Out of the 16 bacteria grown on screening medium (citrus pectin agar medium) only 6 strains showed clear zones. Among the six isolates, 1 strain showed maximum clearing zones and found to be pectinase producers. These strains were preliminarily identified. Then it was identified as *Bacillus megaterium* by IMTECH, Chandigarh. Enzyme activity was measured and protein estimation was done for the exo PG activity. Optimum conditions for the exo PG production was studied pH 6-8, temp-55 °C, metal ions-$CaCl_2$, carbon sources-lactose and pectin, nitrogen sources peptone and casein surfactants like Triton X-100, substrate concentration 1%, effect of time 15 to 20 minutes, stability of the enzyme 65 °C for 1 hour.

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Isolation and Characterization of *Bacillus tequilensis* from Gut Content of *Perionyx excavatus* and Evaluation of its Starch Hydrolyzing Property

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ABSTRACT

Starch degrading enzyme amylase has manifold importance with enormous industrial application. Although it can be obtained from various sources, bacteria are the most imperative sources for amylase production. The objective of the present research work is to isolate and characterize bacteria from the gut of earthworm (*Perionyx excavatus*) those having the ability to degrade starch. In addition, the amylase production by the bacterial isolate EGB3 is determined quantitatively. The bacterial isolate was gram positive, rod shaped, and spore former. EGB3 was found to be non pathogenic and could ferment a wide range of sugars like lactose, xylose, ducitol, sorbitol, galactose, arabinose, inositol, salicin, trehalose, dextrose, fructose, maltose, mannose, sucrose, and cellobiose. The isolate EGB3 produced large halo-zone on starch agar medium and starch degrading index was 2.43. Enzyme production by the isolate EGB3 was 29.45U/mL after 24 h growth in 1% starch broth at 37^0 C. The bacterial isolate also able to hydrolyze protein (gelatin) and lipid. From the morphological, biochemical and 16S rRNA gene sequence the isolate EGB3 was identified as *Bacillus tequilensis*.

KEY WORDS: STARCH HYDROLYSIS, AMYLASE, AMYLOLYTIC BACTERIA, BACILLUS TEQUILENSIS, CHARACTERIZATION, EARTHWORM.

INTRODUCTION

Microorganisms especially bacteria have huge importance in production of industrial enzymes (Sadhu and Maiti, 2013; Bharathi et al., 2019). Amylase is one such enzyme which has manifold importance with enormous application in food, paper, fermentation and textile industries (Mishra and Behera, 2008). Amylases constitute a group of industrial enzymes occupying roughly 25% of the marketable enzymes (Sidhu et al., 1997). Although it can be obtained from various sources, bacteria are the most imperative sources for amylase production (Kathiresan and Manivannan, 2006). But not only for the industrial use, the enzyme amylase could be a potential tool in organic waste management. Along with escalating industrialization, urbanization and economic growth, production of solid waste around the Globe is also increasing. Recently, it is assumed to increase the world's solid waste production up to three billion tons by the year of 2025 (Charles et al., 2009).

According to Pappu et al. (2007) in India, 960 million tons solid wastes are formed each year. Under this circumstance, waste management has become a massive problem and one of the most challenging issues now. Organic wastes from agro-based industries and households chiefly constitute with huge quantities of

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starch (Jesse et al., 2002). This massive waste can be degraded by the application of amylolytic bacteria. There are diverse reports on starch hydrolyzing microorganisms from various sources (Nimisha et al., 2019).

Earthworms are voracious feeder of organic compounds and their gut content hold a diverse community of microorganisms which are able to degrade macromolecule like starch (Parthi et al., 2019). Indeed, the microorganisms residing in earthworm's gut are responsible for degradation of organic matter and thus they help in production of vermicompost. Sereval species of *Bacillus* like *Bacillus subtilis, B. Stearothermophilus, B. myocodes , Bacillus amyloliquefaciens, B. polymyxa, B. licheniformis, B. gavealeus, B.mesentericus, , B.vulgates, B. aterrimus* were reported as amylase producer. Especially, *Bacillus subtilis*, *B.licheniformis* and Bacillus *amyloliquefaciens* have been reported to produce adequate quantity of alpha amylase (Singh et al., 2011). During present study, starch hydrolyzing *Bacillus tequilensis* (EGB3) isolated from earthworm gut has been characterized by morphological, biochemical and molecular methods and identified through polyphasic methods. The starch hydrolyzing ability of the bacterial isolate was determined both qualitatively as well as quantitatively.

MATERIAL AND METHODS

Live earthworms (*Perionyx excavatus*) used in vermicompost preparation, were collected in sterile plastic container from Kulti vermicomposting farm (23°12′ N, 88°30′ E) of Purba Bardhaman district of West Bengal, India. After collection, earthworms were brought to Parasitology and Microbiology Research Laboratory of the Department of Zoology, the University of Burdwan for further microbial and biochemical analysis. Live earthworms were surface sterilized with 50% alcohol and quickly transferred to a dissection tray. The gut was cut open and the gut content was collected with a sterile loop into an Eppendorf tube. The semi solid gut content was diluted with sterile distilled water and pour plated on Nutrient Agar (NA) medium (Himedia, India) [Composition: peptone 5.0, Yeast extract 1.5, HM peptone 1.5, NaCl 5.0, agar 15 g l^{-1}]. The plates were incubated at 30±1^{0}C for 24-48h. At the end of incubation, bacterial colonies were picked up from plates and streaked repeatedly on NA plate and maintained in pure culture at 4^{0}C for further study.

The morphological and biochemical characterization of bacterial isolate were done following standard methods (Holt, 1984; Smibert et al. 1995; Logan et al., 2009). The Gram staining was performed using Gram staining kit (Himedia, India). SIM agar was used to perform the motility test of the bacterial isolate by stabbing into the slant (Czaban et al., 2007). The biochemical characteristics such as Methyl-Red (MR), catalase, Voges-Proskauer (VP), citrate utilization, nitrate reduction, indole production, urease, oxidase, and carbohydrate fermentation tests were performed. For the determination of pathogenic nature the isolate blood hemolysis and DNase test were conducted (Benson et al., 2012).

To prepare a scanning electron micrograph a thin smear was made on cover slip, fixed using 2.5% glutaraldehyde and gradually dehydrated with graded alcohol. Finally the specimen was gold coated and observed under scanning electron microscope (ZEISS). Antibiotic sensitivity test was performed with standard antibiotics discs (Himedia, India) following Brown (2004) and sugar fermentation test was carried out with sugar fermentation discs like inositol, maltose, sucrose, adonitol, fructose, mannose, cellobiose, raffinose, xylose, dextrose, galactose, lactose, trehalose, rhamnose, ducitol, melibiose, salicin, sorbitol, mannitol, and arabinose (Himedia, India). The sensitivity of the isolate against antibiotics was tested using nalidixic acid, kanamycin, tetracycline, ofloxacin, ampicillin, amoxicillin, neomycin, gentamycin, ciprofloxacin, azithromycin, erythromycin, bacitracin, chloramphenicol, penicillin, rifampicin, and doxycycline. The zone diameter of inhibition (ZDI) values were interpreted following CLSI (2011). Multiple Antibiotic Resistance (MAR) index were calculated following Krumperman (1983).

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate showed resistance}}{\text{Number of total antibiotics exposed to the isolate}}$$

For molecular characterization, Genomic DNA of bacterial isolate was extracted using DNeasy Ultra Clean Microbial Kit of Qiagen. Fragment of ~1.5 kb rDNA was amplified by Polymerase Chain Reaction (First cycle at 94^{0}C, thirty five cycles at 58^{0}C and finally at 72^{0}C) using 27F forward (5′AGAGTTTGATCATGGCTCAG 3′) and 1492 reverse (5′GGT TAC CTT GTT ACG ACTT3′) primer. Then the PCR product was purified with MinElutePCR purification kit, Qiagen. Purified PCR product was sequenced using universal bacterial primer in DNA sequencer. Sequenced information was aligned by ClustalW (http://www.ebi.ac.uk/clustalw) and analyzed using MEGA 4.0.2 software (Thompson et al., 1994). Evolutionary distances of mostly related bacteria were calculated following the method of Jukes and Cantor 1969 and phylogenetic tree of the bacterial isolate was constructed following Tamura et al., (2007).

For screening of amylolytic bacteria starch hydrolysis test was performed using iodine and starch agar (Himedia, India) [Meat Extract 3.0; Peptic digest of animal tissue 5.0; Starch, soluble 2.0; Agar 15.0 g l^{-1}; pH 7.2±0.1]. Colonies of the bacterial isolate were transferred on to starch agar plates and incubated at 37±1^{o}C for 48 h. Then the plate with bacterial colony was flooded with Gram's iodine. If a isolate was amylolytic then it would produce a clear zone around the colony. This clear zone is formed as enzyme amylase starts hydrolyzing the starch molecule. Selection was made as per colonies with and without clear and transparent zone as starch hydrolyzing and starch non-hydrolyzing isolate, respectively.

The Hydrolyzing Capacity of the bacterial isolate was determined by Starch Degrading Index (SDI) and it was indexed as the diameter of the colony plus the clear

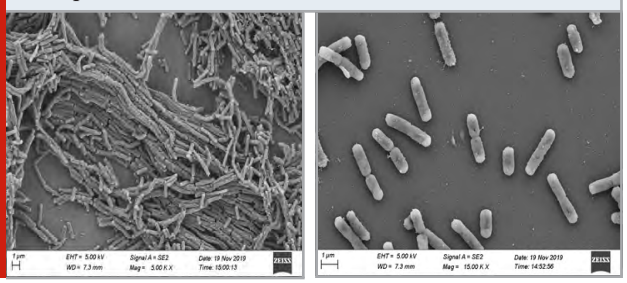
zone around it divided by the diameter of the colony (Nusrat and Rahman, 2007). The largest ratio represented the highest activity. The amylase assay was carried out using 3-5-dinitrosalicylic acid reagent (DNS Reagent) (Bernfeld, 1955).

The bacterial isolate was inoculated in 20ml Minimal Starch Broth and incubated in the shaker incubator for 24 h at 30±1°C at 200rpm. 1ml of this broth was then inoculated in 100ml of minimal broth containing 1% starch and incubated in shaker incubator at 200rpm for 48 h at 30 °C. There after the culture was centrifuged at 2500 rpm. The supernatant was used as a source of crude enzyme extract. 1 ml of bacterial crude enzyme was taken in a test tube containing 2 ml of 1% starch broth. Then the reaction mixture was incubated at 50°C for 60 minutes. To arrest the enzyme action, 1ml of DNS reagent was added. The test tubes were kept in boiling condition upon water bath for 5 minutes. 1ml of 40% sodium potassium tartarate was then added into the test tubes and allowed to cool. The optical density of the tubes was measured at 540 nm. Enzyme activity was expressed in units (Mishra and Behera, 2008).

RESULTS AND DISCUSSION

The colony of the bacterial isolate EGB3 appeared on NA plate was rounded in shape with a diameter of 3-3.5 mm, moderately elevated with smooth margin, opaque and offwhite in colour. EGB3 was a Gram positive, spore former, rod shaped bacterium with a length of 784.6-802.5 nm and breadth of 3.378-3.423 µm (Figure 1). The motility test of the isolate indicated its motile character. EGB3 was found to be non pathogenic against blood hemolysis test and DNase test. The bacterial isolate was able to produce enzyme catalase, oxidase and utilized citrate as carbon source, but it was unable to produce urease. The isolate was positive for both MR and VP test. EGB3 was able to degrade arginine, tryptophan and positive for ONPG test. The isolate EGB3 could hydrolyze gelatin, lipid as well as starch (Table 1).

Figure 1: Scanning microscopy of *Bacillus tequilensis* (EGB3) showing single cell and chain formation by multiple bacteria.



The bacterial isolate (EGB3) showed the ability to ferment inositil, galactose, xylose, salicin, trehalose, rhamnose, arabinose, dextrose, dulcitol, sorbiol, fructose, maltose, mannose, sucrose, lactose and cellobiose (Table 2). Bacterial isolate EGB3 showed sensitivity to the recommended doses of antibiotics like tetracycline (30 µg/ disc), ofloxacin (5 µg/ disc), doxycycline (30 µg/ disc), nalidixic acid (30 µg/ disc), erythromycin (15 µg/disc), neomycin (30 µg/disc), amoxicillin (10 µg/ disc), bacitracin (10/disc µg), levofloxacin (5 µg/ disc), streptomycin (10 µg/ disc), azithromycin (30 µg/ disc), gentamycin (50 µg/ disc), vancomycin (30 µg/ disc), chloramphenicol (30 µg/ disc), kanamycin (30 µg/ disc) and norfloxacin (10 µg/disc) but resistance to rifampicin (5 µg/ disc), ampicillin (10 µg/ disc) and penicillin (10/ disc µg) (Figure 2) . MAR index of EGB3 was 0.15.

Figure 2: Antibiotic sensitivity and ZDI values of the isolate EGB3 against standard antibiotics

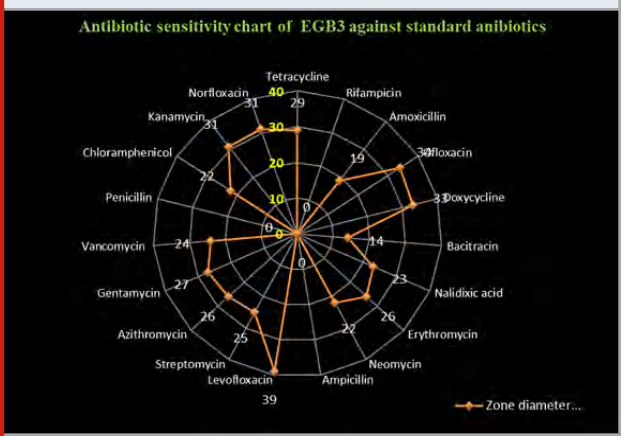


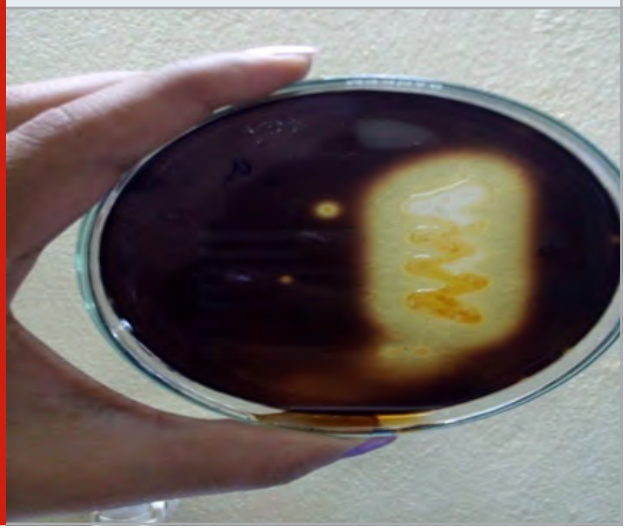
Table 1. Morphological and biochemical characteristics of isolated strain Bacillus tequilensis EGB3.

Test	Response
Test	Response
Gram's staining	Gram positive
Shape	Rod
Length of bacterium	784.6-802.5 nm
Breadth of bacterium	3.378-3.423 µm
Motility	Positive
MR	Positive
VP	Positive
Catalase	Positive
Citrate	Positive
Urease	Negative
Oxidase	Positive
H_2S Production	Negative
Blood hemolysis	Negative
DNase test	Negative
Indole	Positive
Nitrate Reduction to N_2	Positive
Starch hydrolysis	Positive
Protein (gelatin) hydrolysis	Positive

The bacterial isolate (EGB3) was able to hydrolyze starch as it produced large clear zone around the colony (Figure 3). The SDI calculated from the clear zone and colony ratio was found to be 2.43 during the current

study. Enzyme production by the isolate EGB3 was 29.45U/mL after 24 h cultivation in 1% starch broth at 37^{0}C.

Figure 3: The amylase production by *Bacillus tequilensis* EGB3.



Neighbour joining tree has been constructed by partial 16S rRNA sequence of the isolate using *Pseudomonas hussainii* as an out-group (Figure 4). From Neighbour joining tree it was found that the bacterium EGB3 clustered with both *Bacillus subtilis* and *Bacillus tequilensis* [*Bacillus tequilensis* (MK618597; HQ238557; KC172005; HQ238430; KT265077) *Bacillus subtilis* (HQ683932; KJ769215; FJ174586; KF05307; MK367800]. Gatson et al. (2006) previously reported that there was 99% similarity in 16S rRNA gene sequences of *B. tequilensis* and *B. subtilis*. From the16S rDNA gene sequencing it was found that EGB3 might be a strain of *B. tequilensis* or *B. subtilis*. But the biochemical properties like enzymatic and fermentation properties of EGB3 shows higher affinities with *B. tequilensis*. For example, EGB3 and B. tequilensis both could ferment galactose, rhamnose, lactose and dulcitol but *B. subtilis* could not ferment these sugars. In addition, unlike to *B. subtilis*, EGB3 was able to decompose arginine and tryptophan. *B. tequilensis* was also able to degrade arginine, tryptophan and ONPG (Gatson et al., 2006).

Moreover, other biochemical properties like MR, VP, indole and catalase production, citrate and urea utilization, starch and gelatine hydrolysis strongly suggested the similarity between isolate EGB3 and previously reported *B. tequilensis* (Li et al., 2018; Gatson et al., 2006) . The percentage of AT and GC content of 16S rRNA gene sequence of EGB3 were 44% and 56% respectively. From the morphological, biochemical and molecular analysis the isolate EGB3 was identified as *B. tequilensis*. Vermicompost harbours a rich variety of microbial populations such as bacteria, fungi and actinobacteria (Edwards, 1998; Samanta and Das, 2016). Role of earthworms and microbes in decomposition and humification of organic substances has been studied by various researchers (Edwaeds and Bohlen. 1996; Cai et al., 2002; Manivannan et al., 2004; Munnoli and Bhosle, 2014). In the present study, it was found that the gut of Perionyx excavatus contained bacteria that could produce amylase, a starch hydrolysing enzyme. Enzyme α-amylase belongs to a group of endo-amylases which catalysed the hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds (Souza et al., 2010).

Figure 4: Phylogenetic neighbor joining tree constructed based on partial 16S rRNA gene sequence of EGB3 (MT078694) strain along with the other 16S rRNA gene sequnces retrieved from NCBI and RDP.

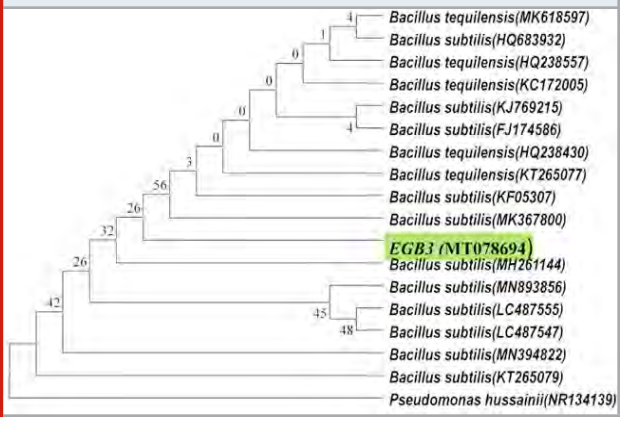


Table 2. Carbohydrate fermentation property of *Bacillus tequilensis* EGB3.

Carbohydrate	Response
Inositol	Positive
Salicin	Positive
Trehalose	Positive
Galactose	Positive
Xylose	Positive
Dextrose	Positive
Adonitol	Negative
Lactose	Positive
Arabinose	Positive
Melibiose	Negative
Fructose	Positive
Maltose	Positive
Mannose	Positive
Sucrose	Positive
Rhamnose	Positive
Cellobiose	Positive
Mannitol	Negative
Ducitol	Positive
Raffinose	Negative
Sorbitol	Positive

Although it could be obtained from various sources, bacteria were the most imperative sources for amylase production (Gangadharan et al., 2006). The microbial amylase has a wide range of industrial applications (Mishra and Behera, 2008). Amylases of microbial

origins are more stable than amylase of animal or plant origin (Tanyildiz iand Elibol, 2005). Moreover, the foremost advantage of using microorganisms for amylase production is the cost-effective mass production capacity and as microbes are uncomplicated to manipulate, enzymes of desired characteristics are obtained easily (Souza et al., 2010). Industrially produced first enzyme was an amylase from fungi in 1894, which was used in making of a pharmaceutical aid (Crueger and Crueger, 1989; Pandey et al., 2000). Biodin and Effront were the pioneers for the production of α-amylase on commercial scale from *B. subtilis* and *B. mesentericus*. (Hoogerheide, 1954).

In this present study the amylolytic bacterial isolate was identified and this result is similar to the findings by many workers (Parthasarathi et al., 2007; Samanta and Das, 2016). Several species of *Bacillus* like *Bacillus subtilis, B. stearothermophilus, B. myocodes , Bacillus amyloliquefaciens, B. polymyxa, B. licheniformis, B. gavealeus, B.mesentericus, , B.vulgates, B. aterrimus* were reported as amylase producer. Especially, *Bacillus subtilis, B.licheniformis* and *Bacillus* amyloliquefaciens have manifold importance in commercial applications as they produce adequate quantity of alpha amylase (Singh et al., 2011). *B. tequilensis* was foremost reported by Gatson et al in 2006 from two thousand year-old Mexican shaft-tomb near the city of Tequila. Kaur and Azmi (2013) found *B. tequilensis* from earthworm's intestinal content which have high collagenase activity. *B. tequilensis* was previously reported as starch hydrolyzer (Prasanth et al., 2017; Li et al 2018).

Mishra and Behera (2008) reported amylolytic *Bacillus spp*. from kitchen waste. The kitchen wastes, predominantly consists of starchy materials and they advocated that bacteria isolated from such places possessed better potential to produce amylase under adverse situation. Amylase production efficiency by the bacteria of same genus even of the same species differs significantly. A branch of factors regulates the production of amylase. Temperature, pH, growth kinetics of isolates, incubation time, size of inoculums, Carbon sources, etc. play important roles in production of bacterial amylase, (Alariya et al., 2013). In an experiment on starch hydrolysis by amylase was exhibited by Dida (2018), who observed that the SDI of rhizospheric *Bacillus spp*. ranged between 1.23 and 2.15. The starch hydrolyzing ability from present research work in relation to SDI was comparable with *Bacillus spp*. (Chatterjee et al., 2019).

According to Pappu et al., (2007) in India, 960 million tons solid waste is produced each year. Under this circumstance, waste management has become a massive problem and one of the most important issues now. Present study showed that the gut bacteria of *Perionyx excavatus* may be a potential tool in removal of agricultural waste through its starch hydrolysing activity. This study is at preliminary level and it requires some more research for better understanding of diversity and potential application of the earthworm gut bacteria in sustainable agricultural practice, organic waste management and other land uses.

CONCLUSION

From the present study it is found that the gut content of earthworm contains amylolytic bacteria like *Bacillus tequilensis* which have a great potentiality to degrade starch. The earthworm *Perionyx excavatus*, or the bacteria itself may be utilized in organic waste management and the amylolytic property of the isolate can be explored commercially in various aspects.

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

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BiOCl Nano Pellets Preparation and their White/Solar Light Mediated Photocatalytic Activities Evaluation on Carbamate Pesticide Oxamyl and Synthetic Dye Azure B

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ABSTRACT

A quick, reliable, simple and one-pot sonochemical method has been discussed for the synthesis of BiOCl nanomaterial by using Bismuth Nitrate as a precursor along with HCl, Ammonia and L – lysine in water medium at 298.15 K under the designed and developed open glass double-walled beaker reactor. The nanomaterial was further characterized by using PSA, DLS, FTIR, BET, FT-RAMAN, Photoluminescence spectroscopy, HRXRD and HRFESEM. As a result, highly pure and well crystalline BiOCl nano pallets were obtained. HR-XRD results revealed their sizes between 40-50 nm followed by PSA, DLS and HRFESEM analysis. The prepared nanomaterial was tested for its photocatalytic activities under the designed and developed reactor on a carbamate pesticide Oxamyl and a synthetic dye Azure B under the white light lamp and solar light. Prepared BiOCl nanomaterial showed the impressive photocatalytic activity against pesticide under solar light only and able to degrade selected dye under both lights. Present results concludes a potential future utilization of BiOCl nanomaterial as great environmental remediation technology for various hazardous as well as persistent compounds removal.

KEY WORDS: BiOCI NANOMATERIAL, SYNTHESIS, CHARACTERIZATION, OXAMYL PESTICIDE, AZURE B DYE AND PHOTOCATALYSIS.

INTRODUCTION

The Bismuth oxychloride has been extensively studied as an impressive material and a promising technology in the field of oxide-based semiconductors and heterogeneous catalytic degradation of various organic contaminants as well as environmental remediation purposes (Chen et al., 2010; Guerrero et al., 2014; Gao et al., 2018; Yang et al., 2018). BiOCl class of nanomaterials are very efficient photocatalytic semiconductor catalysts (Zhao et al., 2014) because of specific layered structures (Zhang et al., 2006), various 1 & 2-dimensional arrangements (Yang et al., 2019) and nanonetwork assemblies (Guo et al., 2018) viz. nanoflakes (Li et al., 2011), nanoflowers (Cheng et al., 2012), nanofibers (Zhang et al., 2016), nanowires (Wu et al., 2016), nanobelts (Wang et al., 2017), nanosheets (Shi et al., 2018) and several other self-similar micro (Mendez-Alvarado et al., 2020) - mesosphere structures (Guo et al., 2012; Xie et al., 2015; Ji et al.,

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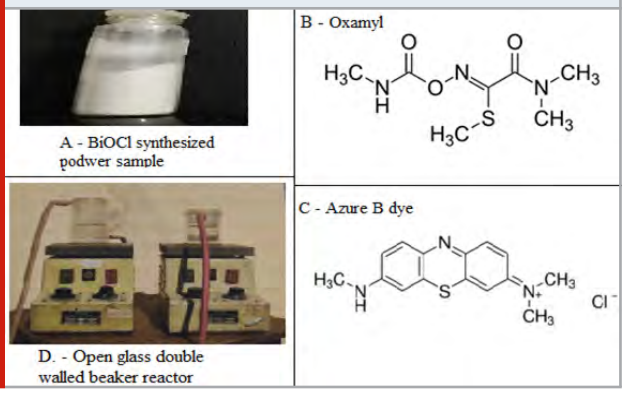
2020) by a variety of reported synthesis methods including sonochemical (Geng et al., 2005), template-assisted routes (Wu et al., 2010), hydrolysis (Li et al., 2011), microwaves (He et al., 2015), hydrothermal synthesis (Hu et al., 2016) and biological/plant assisted (Garg et al., 2018; Yadav et al., 2019).

Over recent decades, due to simple synthesis, low cost, low toxicity, stability and ability to absorb UV as well as sunlight made BiOCl nanomaterials a preferential choice over other oxide based semiconductor materials like polyoxometalates (Mylonas et al., 1996), non-metallic doped TiO_2 (Asahi et al., 2001), complex oxides (Luan et al., 2009), WO_3 (Ashok kumar and Maruthamuthu, 1989; Li et al., 2012), as well as several other solar light-absorbing materials common nanomaterials like TiO_2 (Shaham-Waldmann and Paz, 2016; Lee et al., 2020). Thus the present study was an attempt to understand the synthesis, characterization and photocatalytic activity of BiOCl nanomaterial. The prepared BiOCl nanomaterial was further evaluated for its photocatalytic activity against a Carbamate pesticide Oxamyl (Fig.1B) and a synthetic dye Azure B (Fig. 1C) under visible and solar light.

Figure 1: A. – Prepared BiOCl nanomaterial power in a vial. B- The chemical structure of Oxymal pesticide. C – The chemical structure of Azure B dye. D – The open glass double walled beaker reactor.



MATERIAL AND METHODS

Synthesis of BiOCl: All of the reagents were analytical grade and commercially available. They were used without further purification. Milli-Q water (Millipore SAS 67/20 Mosheim) of 10^{-7}S cm-1 was used for solution preparation. Typically, 10 mmol of Bi $(NO_3)3•5H_2O$ (4.85g) and 0.4 g of L-lysine was dissolved in concentrated HCl (dropwise). Then the mixture was quickly diluted to 100 mL by water for the immediate formation of precipitates. The solution was adjusted to pH = 9 by adding 5 wt. % ammonia solution, the BiOCl product was collected and washed with water and alcohol, (Chen et al 2013).

Characterization methods: Milli-Q water was used for solution preparation as well as setting black in PSA and DLS instruments. A pinch of (0.005 g) power was taken and 30 ml of water was added and sonicated for 30 minutes at 298.15 K. After that centrifugation performed at 7000 rpm for 15 minutes. The supernatant was used as a sample for particle size distribution analysis (Malvern Zetasizer Nano S90) and Dynamic light scattering analysis (MicrotracZetatrac, U2771). X-ray diffraction patterns (XRD) of the nanomaterial was recorded by D8 advance diffractometer (BRUKER). The structural information of nanomaterial was measured by a Fourier transform spectrophotometer (FT-IR, Bruker). The surface morphology of the sample was observed by using High-resolution field emission scanning electron microscope (Zeiss, model name SIGMA VP). Material scattering analysis carried out by FT RAMAN (BRUKER RFS 27 Model). Photoluminescence spectroscopy (Horiba Scientific) was used for the further electron structure elucidation of nanomaterial and specific surface area was calculated by BET analyser. The degradation studies were carried out in the designed and developed reactor by using UV-Vis spectrophotometer.

Measurement of Photocatalytic activity: Pesticide and dye solutions were freshly prepared by dissolving in Milli-Q water. An open glass double-walled beaker reactor was designed and developed to carry out a controlled degradation experiment (Fig. 1D). A space for continue water pump was present between the double-walled beaker to maintain the temperature during the whole experiment. A magnetic shaker was also added beneath the bottom of the double-walled beaker to insure uniform stirring on medium (Fig. 1D). Before light experiments, dark (adsorption) experiments were carried out to know the adsorption limit of pesticide or dye on the catalyst. For solar experiments, pesticide/dye solution of 100 mL was taken with the known amount of the catalyst. The solution was illuminated under bright visible/solar light. At specific time intervals, an aliquot (5 ml) of the mixture withdrawn and centrifuged for 2 minutes to remove the BiOCl particles. A PC based double beam spectrophotometer (Systronics – 2202) was used for measuring absorbance at different time intervals. The intensity of light was measured by a Lux meter (Lutron, LX-101). The pH and conductivity of the solution was constantly been monitored using a pH meter and conductivity meter. The efficiency of photocatalytic process was calculated as:

$$\%\,Efficiency = \frac{C_0 - C}{C_0}\ x\ 100$$

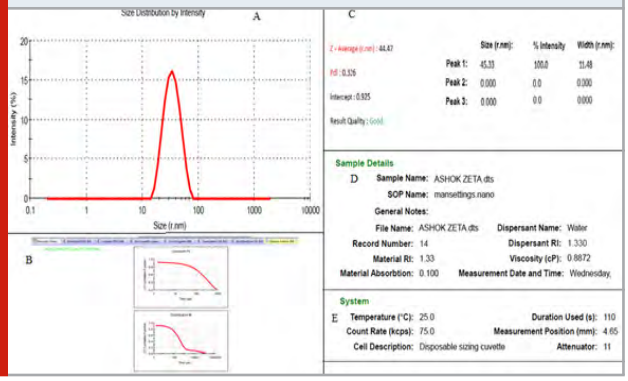
Where C_0 was the initial absorbance and C was the absorbance at different time intervals of photocatalytic process, (Sarwan et al 2014).

RESULTS AND DISCUSSION

A one-pot sonochemical synthesis was performed by using Bismuth nitrate as Bi source. The dropwise HCl used as Cl – source in the medium. The basicity was maintained by addition of the 5 wt. % ammonia solution to restrict the medium pH at 9. The L lysine was used as surfactant or capping agent and water was used as the solvent. After the 10 minutes reaction, the centrifuged sample was dried in the lyophilizer and the white powder

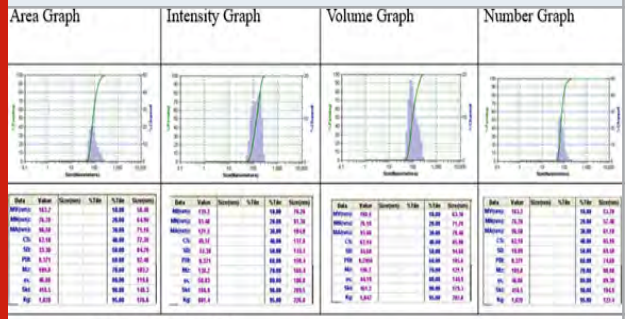
used as a sample for further analysis and kept in the glass vial (Fig. 1A). The pinch of this powder sample was further used for PSA and DLS analysis to understand the size distribution. The PSA evaluation showed the average size of the nanomaterial between 44 nm – 46 nm in the intensity graph along with the Poly Disperse Index (PDI) was 0.326 detected (Fig.2).

Figure 2: The Particle size analyser results of BiOCl nanomaterial. A. - Showing the size distribution pattern according to the intensity. B. – the particle size analysis curves. C – The calculated Size, PDI, intercept and result quality report. D – The sample details and E – system details.



The Dynamic Light Scattering evaluation revealed the whole hydrodynamic volume measurement of water sample between 70 – 135 nm in different graphs of the same sample viz. area, intensity, volume and number graphs (Fig.3). The surface charge calculated was found to be negative and the zeta potential was detected – 18.22 mV (Fig. 3) showed strong stability of synthesized nanomaterial along with PDI of 0.371.

Figure 3: The DLS analysis of BiOCl nanomaterial. The Area, Intensity, volume and number graphs of same material. The Diameters and physicochemical analytic data of sample were present in tables associated and the 0.371 PDI was detected.



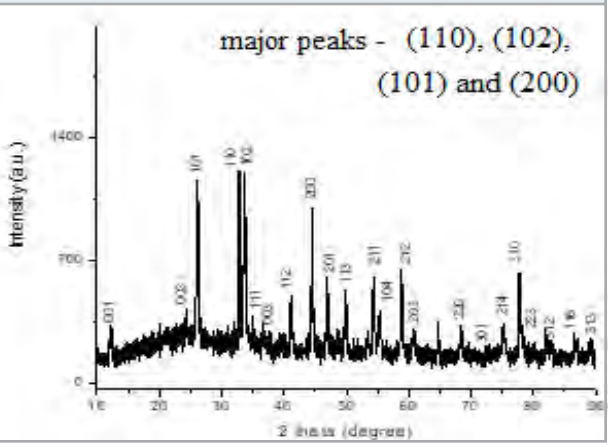
PDI was observed = 0.371

Parameters	Diameter	Volume %	Width
Area	84.20	100%	66.60
Intensity	133.3	100%	108.70
Volume	94.60	100%	89.20
Number	69.50	100%	38.20

Zeta potential	- 18.22 mV
Conductivity	15 uS/cm
Viscosity	0.8620 cp
Mobility	-1.44 u/s/V/cm
Temperature	299.3K
Dielectric cont.	79

The XRD patterns of the BiOCl sample showed a well and high crystalline preparation of nanomaterial along with the reference of standard tetragonal structure (JCPDS 06-0249) and no other diffraction intense peaks were detected. The planes of material found to be in good arrangement regarding the unit cell of the tetragonal structure. The four important diffraction peaks viz. (110), (102), (101) and (200) were found to be prominent, intense and sharper while other peaks were relatively weak (Fig. 4).

Figure 4: The HR-XRD pattern of prepared BiOCl material. Where th 110, 102, 101 and 200 peaks where prominent.



From the x-ray patterns, the broadening of the diffraction peaks of the nanomaterial was obvious. Which was characteristic of nanosized by applying Debye- Scherrer formula.

$$D = \frac{0.9\lambda}{\beta cos\theta}$$

Where D was the mean particle size, λ was the wavelength of incident X-ray (1.5406 ˚A), θ was the degree of the diffraction peak, and β was the full width at half maximum (FWHM) of the XRD peak appearing at the diffraction angle θ. The broadening of the absorption spectrum could be due to the quantum confinement of the nanoparticles. The mean calculated crystallite size was observed between 40 - 50 nm (Tab. 1).

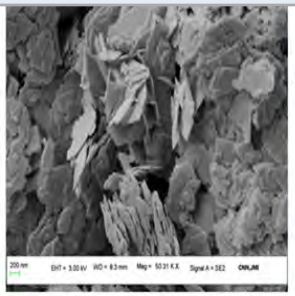
Table1. Summary of XRD analysis for BiOCl nanomaterial.

S.No.	2 θ	plane	d spacing	Size (nm)
1	26.103	101	0.173948	48
2	32.800	110	0.217488	45
3	33.560	102	0.222126	46
4	44.387	210	0.290926	49

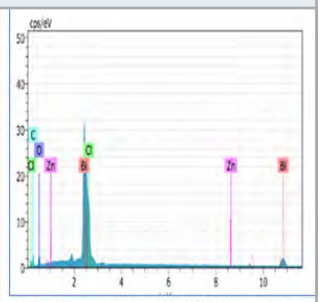
The topological imaging of BiOCl nanomaterial was carried out with FE-SEM analysis. The surface electron

imaging showed many pallets like morphology of overall BiOCl material with a very smooth surface (Fig. 5A). The particle size obtained for PSA, DLS, XRD and FESEM analysis were found to be in good agreement with each other. These results have been found to be consistent with the literature (Baladi et al 2010). The elemental composition was also tested for BiOCl nanomaterial with the help of EDS analysis that revealed the clear detection of Bi, Cl and O in the sample (Fig. 5B & Tab.2).

Figure 5: The surface morphological evaluation and elemental composition analysis of BiOCl. (A) HR-FESEM image of BiOCl and (B) EDS pattern of BiOCl.



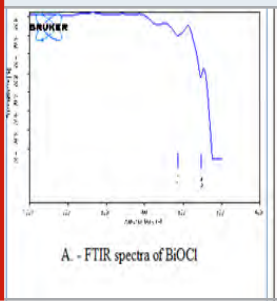
A . - FE SEM image of BiOCl material



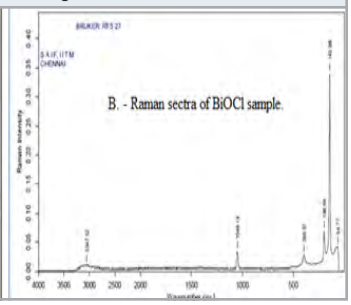
B . - EDS pattern graph of BiOCl material

FT-IR spectra functional group evaluation of BiOCl nanomaterial showed strong absorption bands at low-frequency zone (between, 400–600 cm^{-1}). These peaks were attributed to the Bi–O vibration of chemical bonds in the sample (Fig. 6A) and showed a good agreement with EDS results. (Chang et al 2014).

Figure 6 Spectroscopic analysis of BiOCl. A - FT-IR spectra of the BiOCl and B. – Raman specta of BiOCl.



A. - FTIR spectra of BiOCl



B. - Raman sectra of BiOCl sample.

FT – Raman spectrum analysis was also carried out to assess the vibrational/ structural properties of the crystal. As XRD result indicated that the tetragonal structure of BiOCl belongs to the P_4/nmm (D^7_{4h}) space group with

Table 2. The EDS data of BiOCl nanomaterial.

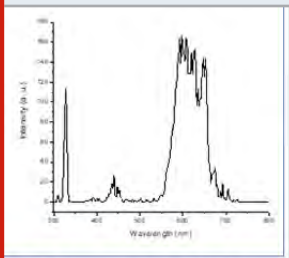
El	AN	Series	unn. [wt.%]	C norm. [wt.%]	C Atom. C [wt.%]	Error (1 SIgma)	[wt.%]
Bi	83	L-series	93.39	78.76	0.08	3.10	
Cl	17	K-series	11.26	9.50	18.40	0.41	
O	8	K-series	9.24	7.79	33.48	1.70	
C	6	K-series	4.59	3.87	22.13	0.91	
Zn	30	K-series	0.10	0.08	0.09	0.04	
Total:			118.58	100.00	100.00		

two molecular formulae per unit cell. For this kind of structure, the correlation method indicated that the optical modes were as follows: $\Gamma = 2A_{1g} + 2A_{2u} + B_{1g} + 3E_g + 2E_u$, where the g modes were Raman-active only and u modes were infrared-active only. Raman spectra of the pallets like nano BiOCl material showed four bands at 64.77 cm^{-1}, 143.98 cm^{-1}, 198.69 cm^{-1}, 395.97 cm^{-1} related to various type of vibrations and an agreement between XRD and Raman data of the sample (Fig. 6B). Same raman shift has been also observed by other authors (Davies 1973, Geng et al 2005 and Cao et al 2009).

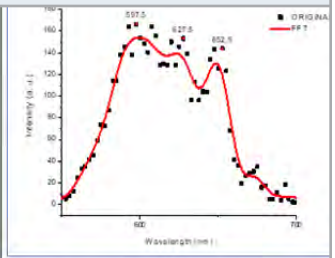
The material further characterized for its photoluminescence spectra (Fig. 7A) and the results were elaborated along with original data and FFT data in visible light zone (Fig. 7B). The thick red curve was the fitted result of the originally scattered dots. The main emission peaks were observed in the visible light area at 597.5 nm and 627.5 nm indicated the orange emission and one more peak at 652.52 nm was also observed which was related to red emission (Fig. 7A-B). The main peak in Visible light region found to be ascribed to the band gap recombination of electron–hole pairs. That indicated the efficient photocatalytic activity in visible/solar light. (Duo et al 2015).

As photocatalytic properties also depend on the surface area of the catalyst so a Brunauer–Emmett–Teller (BET) analysis was carried out to determine the total surface area of the prepared catalysts (Tab. 3). In that analysis 21.591 m^2/g surface area and 7.672 cc/g total pore

Figure 7: Photoluminescence spectroscopy spectra of BiOCl material (A) and the original data and FFT data in visible region (B).



A. - Photoluminescence spectroscopy spectra of BiOCl material.



B. - The original data and FFT data in visible region

volume were observed. That showed a strong correlation along with good agreements with photocatalytic activity and degradation curve data of BiOCl nanomaterial with selected compounds.(Zhang et al 2015).

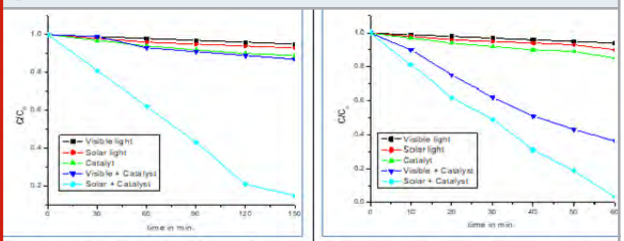
Table 3. The BET analysis results for BiOCl nanomaterial and summary of Average Pore Size, BJH adsorption, DFT method, BET

Average Pore Size summary	
Average pore Radius	7.10672 Å
BJH adsorption summary	
Surface Area	3.096 m²/g
Pore Volume	0.004 cc/g
Pore Radius Dv(r)	16.709 Å
DFT method summary	
Surface Area	11.011 m²/g
Pore Volume	0.012 cc/g
BET summary	
Surface Area	21.591 m²/g
Total Pore Volume	7.672 cc/g

Photocatalytic studies and mechanism of degradation assessment: The photocatalytic activities of nano BiOCl were evaluated with the degradation of Oxamyl pesticide (Fig. 1B) and Azure B dye (Fig.1C) in the presence of solar and visible light. For both pesticide and dye degradation experiments photolysis, adsorption and photocatalysis studies were carried out in the form of five experiments. The activities were calculated as C/C_0 versus time where C was the absorbance at a particular time and C_0 was the initial absorbance respectively. In Oxamyl degradation studies no significant results obtained in the photolysis and absorption phenomena. The degradation of Oxymyl pesticide was found to be very low under visible light as well as solar light in the absence of BiOCl nanomaterial. It was also observed that the degradation of pesticide was unable to initiate in the dark. The BiOCl mediated pesticide degradation initiated when light source applied. It was observed that visible light-mediated degradation was not showed the favourable response but solar light-mediated degradation showed impressive results. It may be possible because the solar light contained UV as well as visible light zone photons and UV light initiated the response and other electromagnetic energy photons favoured further reaction by inducing it in the combination (Fig. 8A).

Same experiment was performed for Azure B dye and approximately the same degradation patterns obtained in the photolysis and absorption phenomena. A major difference was that dye was also degraded to some extent in the presence of visible light. It was also observed that the dye was completely degraded in the presence of solar light because both UV and visible lights were present in solar light. Thus it can be assumed that UV light was responsible to initiate the photocatalysis reaction and solar light further propagated the degradation process. As a result, the photocatalytic degradation of Azure B dye using solar light was the favourable method to mineralise the dye solution and combination of UV and visible light was responsible to induce the process (Fig. 8B), (Pare et al 2017).

Figure 8: The photcatalytic degradation of Oxamyl pesticide results (A) and Azure B dye degradation results (B). The Experimental condition for pesticide degradation studies: [Oxamyl] = 10^{-4} mol dm^{-3}, BiOCl NPs= 80mg/100ml, pH= 6.2. Experimental condition for dye degradation studies [Azure B] = 4.0 × 10^{-5} mol L^{-1}, BiOCl= 30 mg/100 mL pH = 8.0.



A. - Photcatalytic degradation of Oxamyl pesticide.

B. - Photocatalytic degraation of Azure B dve.

CONCLUSION

A very simple, quick, facile and environmentally benign process for BiOCl nano pallets preparation at room temperature was discussed and highlighted in this work. Précised synthesis and various characterization results indicated the formation of remarkable BiOCl nanomaterial that was having sheet-like/ pallets structures at an eye-catching tiny size range. The generated nanomaterial found to be active as a potential photocatalyst capable to degrade carbamate pesticide Oxamyl as well as Azure B dye under the designed and developed open glass double-walled beaker reactor.

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Applications of Zinc Oxide and Hydroxyapatite Nanoparticles in Orthodontics: a Perspective

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ABSTRACT

Nanotechnology is widely applied to orthodontic practices like Brackets, orthodontic wires/ligatures and orthodontic retainers. Fixed orthodontic appliance is inclined to plaque biofilm deposition and furthermore improve the opportunity of complete demineralization (additionally called white spot injuries, WSLs) the underlying indication of caries. These injuries are portrayed by their obscurity and a decline in fluorescence brilliance when contrasted with sound polish surfaces. Despite the fact that mechanical treatment is utilized to expel the dental plaque in the oral disorders, it brings about minimising symptoms and better patient compliance. A portion of the nanoparticles like Zinc oxide, Silver, Gold,Copper oxide, Hydroxyapatite, Titanium oxide possess significant antimicrobial properties and forestalls the microbial attachment or lacquer remineralization, reducing contact in orthodontic therapy. Recent advances contributed to widespread reaching utilization of clinical nanosystems in different areas of dentistry like prevention, prognosis, care, tissue regeneration, and restoration. The advancement of oral medication nanosystems for prophylaxis is huge for ensuring excellent oral care. Nanomaterials in oral cosmetic agents are utilized in toothpaste and other mouthwash to improve oral health. These procedures spread nanoparticles and nanoparticle-based materials, particularly spaces of utilization identified with biofilm the board in cariolog, periodontology and orthodontics. Likewise, nanoparticles have been coordinated in differing restorative produces for the consideration of veneer remineralization and dental hypersensitivity .The aim of the article is to survey the updated literature with Zinc oxide and Hydroxyapatite nanoparticles and its antimicrobial viability ,Sheer bond strength and Nanoparticle based dental filler.

KEY WORDS: ORTHODONTIC THERAPY, FIXED ORTHODONTIC APPLIANCE, ZINCOXIDE, HYDROXY APATITE, NANOPARTICLES.

ARTICLE INFORMATION

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INTRODUCTION

Nanoparticles are currently used in dental practice for several applications in the field of orthodontics nano particles is applied as nano-coatings in arch wires and brackets to reduce friction , Fabrication of hollow wires , orthodontic brackets, as antimicrobial agent to prevent white spot lesion. Direct bonding with resin adhesives has become the most popular method for orthodontic bonding. One of the most important considerations for a good orthodontic adhesive is to have adequate bond strength which is able to withstand both occlusal and orthodontic forces. An orthodontic bonding agent must possess high bond strength balanced with the ability of the material to leave little or no residue on enamel upon removal. The bonding material must be inexpensive and accessible. At present, most orthodontic cements available in the market already have these features, however, finding a bonding agent that may also prevent white spot lesions (WSLs) is a challenge. An obstacle on the crown level during fixed orthodontic procedures (FOP) are white spot, enamel demineralization and tooth decay, (Bishara et al 2007, Uysal, 2010, Panchali, 2016, Florence 2020).

Nanoparticles are incorporated into orthodontic adhesives/cement and can be coated on the surfaces of orthodontic appliances to prevent dental plaque or enamel demineralization during the treatment. Various literature studies showed that the addition of antimicrobial NPs to orthodontic adhesive agents and resin-modified glass ionomers cements (RMGICs) might prevent plaque accumulation and bacterial adhesion, (Gunes 2008) This review provides a useful insight into the antimicrobial effects and orthodontic adhesives incorporated with zinc oxide and hydroxyapatite nanoparticles.

Antimicrobial Activity Based On Literature Review: Dental disorders have affected over 3.47 billion individuals in the world and is one of the three most common reasons for worldwide infections. Among the oral disorders, the most common were dental caries and periodontitis. Together, they contribute to the most widely recognized irresistible human ailment in the world. Regardless of whether caries and periodontitis are multifactorial ailments, the primary etiological factor is the occurrence of pathogenic microorganisms. These microbes are composed inside an extracellular framework to shape a bacterial biofilm, (Frencken et al .2017).

In the biofilm, the microorganisms are amassed to shape a hindrance that opposes anti-toxins furthermore, advances incessant fundamental diseases. Additionally, icroorganisms are multiple times progressively impervious to hostile to microbial treatment. Additionally, in biofilms, microbes can escape the resistant framework by delivering super antigens. To battle these bacterial contaminations, metal, metal oxide, and different NPs give off an impression of being promising choices due to their particular physio-synthetic properties, (Seil and Webster,2012 ;Valm, 2019) Sodager et al (2013) investigated the antibacterial properties of ordinary orthodontic composite containing silver/hydroxyapatite nanoparticles. In his investigation antibacterial properties of the composite groups were tested against *Streptococcus mutans, Lactobacillus acidophilus*, and *Streptococcus sanguinis*. Plate dissemination strategy was followed to discover the zone of hindrance. Antibiofilm activity showed that silver/hydroxyapatite nanoparticles indicated zone of inhibition against all the microorganisms tested. NiTi orthodontic wires were covered with ZnO nanoparticles utilizing the chemical deposition activity. Coating characteristics just as the physical, mechanical and antibacterial properties of the wires were researched. The covered wires introduced up to 21% decrease in the frictional powers and antibacterial action against Streptococcus mutans, ZnO nano coating fundamentally improved the surface nature of NiTi wires (Kachoei et al 2016).

A recent study by Scribante et al (2020) demonstrated the utilization of remineralizing solution which initiated decrease of demineralized areas .Bond quality characteristics were essentially diminished for the two sections and connections subsequent to remineralizing treatment. His investigation additionally indicated higher grip esteems brackets in both conditions tested. Remineralized enamel showed significantly higher micro hardness values than demineralized enamel and lower values than intact enamel, Ohtsu et al (2017) in their examination called attention to the antibacterial viability of ZnO/HAP These discoveries proposed that the coatings utilized in the investigation assumes critical job for the surface adjustment of Ti inserts, with a capacity to consolidate the avoidance of irresistible sicknesses with osteogenic action. In a study involving pure and ZnO (0% – 43%) in various forms doped with nano Hydroxyapatite powders .Sol gel method is used to synthesise the nanoparticles. The characterisation of this nanoparticles were performed using XRD, FTIR assay (Deepa.,2013)

In vitro Antimicrobial assay was carried out against gram negative bacteria in which pure and doped nHAp samples were observed irrespective of the ZnO content.Another study was performed focussing on the antimicrobial efficacy of silver, titanium dioxide and zinc oxide nanoparticles against S*treptococcus mutans* (Reddy et al .,2018) Viable bacterial count is determined in the study which showed a significant difference in the colony forming units among all three concentrations of silver (Ag), titanium dioxide (TiO2) and zinc (ZnO) nanoparticles. The study further proved that the Silver, Zinc oxide and Titanium dioxide which showed significant antimicrobial effects were found to be concentration dependent.

Some other works investigated by Grenho et al (2015) about the development of three dimensional and inter connected porous granules of nanostructured hydroxyapatite. *In vitro* and In vivo experimental models were performed in this study. In the In vitro model ,when the granules exposed to *staphylococcus aureus* and *staphylococcus epidermidis* showed antibacterial

activity cytocompability assay towards osteoblast cell line showed inflammatory response. *In vivo* models also showed antibacterial effects .Altogether these nano HA-ZnO porous granules possess significant antibacterial activity and employed in orthopaedic and dental applications.

Nanoparticle Based Dental Fillers: HA-NPs have been incorporated into oral care products, for example, dentifrices and mouthwash to decrease or erase dental affectability by hindering open dentinal tubules on the outside of the dentin also, associated with the mash, or to advance the remineralization of lacquer by supplanting calcium and phosphate particles in the regions from which minerals dissolved, restoring its integrity and shine, (Jena et al.,2017 ;Vano.,2014) Hydroxyapatite nanoparticle is one of the commonly metal nanoparticle for the teeth filling but the literature regarding its efficacy for filling in infectious mouth is still not clear .study conducted by Konar et al (2019) using Graphene oxide ,hydroxyapatite ,zirconia nanoparticles against *Enterobacter ludwigii* and *Escherichia coli*. field emission scanning electron microscopy (FESEM), fluorescence microscopy and zeta potential techniques. The findings revealed that Zirconia nanoparticles are not efficient dental filler, whereas graphene oxide nanoparticle is the best filler followed by hydroxyapatite nanoparticles, that can reduce the bacterial load significantly.

Sheer Bond Strength: Influence of silver (Ag), zinc oxide (ZnO), and titanium dioxide (TiO2) nanoparticles on shear bond strength (SBS) was investigated by Reddy et al .In the study he observed that there was a significant difference between control, Ag, ZnO, and TiO2 with SBS at 5% level of significance. The Scanning electron microscopic examination confirmed homogenous distribution of nanoparticles in the adhesive in all three groups. Further to mention in the study, it was concluded that the incorporation of various nanoparticles in to adhesive materials in minimal amounts may decrease SBS and may lead to the failure of bracket or adhesive (Reddy et al.,2016). The addition of silver, zinc oxide or titanium dioxide nanoparticles into orthodontic bonding agent at a concentration (1%) did not show any effect on bond strength. These nanoparticles have shear bond strength values above the minimum for clinical routine use.

The findings in the study suggest significant differences is observed in ARI, considerably more adhesive remains on the enamel surface following bracket removal in Ag, ZnO, and TiO2 groups compared to the control group. In an experimental study 80 extracted human pre molars were used and divided in to 4 groups, the study was conducted to evaluate the SBS of resin-modified glass ionomer cements. (RMGICs) modified by nano-zinc oxide (NZnO) and nano-hydroxyapatite (NHA) in comparison with composite resins. Findings of the study showed that the amount of SBS was similar among all groups and addition of NZnO and NHA particles had no negative effect on SBS of RMGIC (Sari et al.,2015). Similar study was based on the addition of silver and Hydroxyapatite nanoparticles on the shear bond strength (SBS) of an orthodontic adhesive.

SEM and EDAX analysis showed significant changes between the study groups and the control .Incorporation of silver/HA nanoparticles containing 5% and 1% silver maintains and increases the SBS of orthodontic adhesives, whereas increasing the amount of particles to 10% has an undesirable effect when compared to the control group (Baratali et al.,2015). Saffarpour et al (2016) suggested that the incorporation of Zincoxide nanoparticles in to the dental adhesives increased the antimicrobial effects without affecting the bond strength properties. Adhesive system with hydroxyapatite nanoparticles as load, characterizing it and evaluate the effectiveness of its bond to dental structure. Evaluating with microshear test and characterization technique. It was found that the nanoparticles showed statistically significant difference between the groups. It has been observed that Combined effect of Zinc oxide and CuO has also been studied and it has been observed that CuO and ZnO/CuO nanoparticles coated brackets have better antimicrobial effect on S.mutans than brackets coated with Zinc oxide or CuO alone (Sarah 2018; Dumont et al 2013).

Clinical Significance: Various research has been published in the application of Nanotechnology in the form of metallic nanoparticles like silver, gold, titanium ,nickel, Copper oxide, zinc oxide, hydroxyapatite coated in the orthodontic adhesives as bonding agent to evaluate the sheer bond strength, nano fillers, enamel remineralising agents, fabrication of brackets. This nanoparticle application in the field of orthodontics is in a growing stage ,although several studies were done in orthodontics incorporating the nanotechnology but they are mostly in the in vitro phase and in future several in vivo research to be carried out in order to investigate the antimicrobial ,anti-inflammatory ,cytotoxicity efficacy to use in the clinical treatment for the patients .

CONCLUSION

Nanotechnology has carried enormous changes to the field of Orthodontics. As of now, these improvements are found for an enormous scope in different field identified with oral prophylaxis. Right now, oral care products like toothpastes and mouthwash which contain NPs with anti-microbial, mitigating, and remineralizing properties. In view of promising outcomes and changed, diversified properties, nanomaterials contain various possibilities, and their uses brief numerous points of view that make it workable for them to be compelling. In any case, the advantages of NPs include similar reasons that make them Safe–Properties like small particle size , surface properties, quantum state, movement, conglomeration, change, and the formation of free radicals. In this manner, Incorporation of NPs is as of now one of the most contemplated component of dentistry, because of the practically boundless fields of utilization, furthermore, subsequently administrative and wellbeing concerns must be considered and addressed, particularly

concerning the utilization of hydroxy /Zinc oxide NPs in oral care products.

Conflict of Interest: Nil

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Biorestoration of Textile Effluent Polluted Soil Through Vermistabilization

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ABSTRACT

The present study is focused on biorestoration of the soil polluted by textile effluent through a process of vermistabilization. The polluted soil (PS) amended with cow dung (CD) is vermistabilized by Eudrilus eugeniae and its efficiency is subsequently evaluated with *Phaseolus radiatus*. A series of admixtures of PS and CD on dry weight basis were studied under laboratory condition for 8 consecutive weeks and the results were monitored on a weekly basis. During the study, maximum viability of worms was observed for CD90 PS10 during the 4th week, and there was peak hatchling production during the 5th week when amended soil was composed of CD50 PS50. However maximum worms weight and cocoon production were recorded for an admixture of CD60 S40 during 4th and 5th weeks respectively. The biorestoration resulted in marked changes in physico-chemical parameters of the final vermicompost (CD50 PS50) with significant increase in TKN (77 %), TP (202 %), TK (48 %), TCa (57 %), Mg (27 %) and reduction in EC (46 %), C:N ratio (75 %), TNa (30 %) and S (50 %). Further, heavy metals such as Cu and Zn have also registered an increase in their concentration while Fe, Mn and Cr exhibit a downward trend. Germination studies and plant growth measurements have also been carried out during the investigation and it has been observed that the amended mixture with CD50 PS50 is the optimal medium for biorestoration through vermistabilization for the soils affected by textile effluent.

KEY WORDS: VERMISTABILIZATION, TEXTILE EFFLUENT, EUDRILUS EUGENIAE, BIORESTORATION, *PHASEOLUS RADIATUS*.

ARTICLE INFORMATION

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INTRODUCTION

India is the second largest producer and exporter of textile and garments globally Corporate Catalyst (2015). Textile industries utilize about 200 L of water to produce 1 kg of fabric. Water is mainly used for application of chemicals onto the fibers and rinsing of the final products (Ghaly et al., 2014). The wastewater produced during this process contains large amount of dyes, salts, chemicals and heavy metals. Textile effluents are mostly discharged after minimal or no pretreatment into the neighboring water channels, streams and estuaries. The presence of chemicals in large quantities in the effluent not only affects water bodies but also collapses the soil properties adjacent to the water bodies and impinges on plant growth. Soil is a vibrant living matrix that is an indispensable part of the terrestrial ecosystem. The textile effluent polluted soil having an excess of any element or compound through direct or secondary exposure causes toxic response to biota or humans, resulting in unacceptable environmental risks (Baskaran et al.2009 Kabir et al. 2019, Garczynska et al 2020).

The Textile belt of Tamil Nadu (Tirupur, Coimbatore, Erode and Salem), India, had been discharging their effluents into wastelands which were once cultivable until strict rules and regulations were passed to confine their effluents (Sadasivam et al. 2015). For the past two decades, the dyeing units located in the textile belt of Tamil Nadu have polluted non-perennial tributaries of Cauvery by discharging the toxic effluents. According to Tamil Nadu Pollution Control Board (TNPCB) 8.8 crore litres of effluents, after primary processing in effluent treatment plants are being let out into the Noyyal River every day. TNPCB specifies that the total dissolved solids (TDS) in the water discharged into the river should not be more than 2,100 parts per million (ppm). But the TDS level in the water in Orathupalayam dam area is above 9,000 ppm; in summer when evaporation shoot up, the level of TDS is still higher (Jayanth et al. 2011).

Environmentally acceptable alternatives to unsustainable treatment methods for textile effluent polluted soils have been sought. Regulators are now starting to recognize the influence of contaminant bioavailability and mobility on environmental risk, and consequently, there is an increasing adoption of a risk-based approach when assessing soil quality (Selvam et al. 2018 Garczynska et al 2020). Organic materials are a popular choice for this as they are derived from biological matter and often require little pre-treatment before they may be directly applied to soils (Selvam et al. 2019). Additionally, such amendment practices may also be a convenient route to the disposal of organic residues surplus to requirement (Beesley et al. 2011). The amendments of textile effluent polluted soil for remediation has been a long standing procedure aimed at reducing the risk of pollutant transfer to proximal waters or receptor organisms (Esmaeili 2020).

Hence, this study focuses on the biorestoration of textile effluent polluted soil of the Perundurai region, Erode, Tamil Nadu, India, through vermistabilization process. Cow dung was the organic material used in this study. Eudrilus eugeniae, commonly called African night crawler, is native of tropical West Africa, is an epigeic earthworm commonly used for vermistabilization as growth, fecundity, maturation and biomass are significantly greater when compared with other species. Hence, *Eudrilus eugeniae* was the choice for this study for vermi stabilization.

MATERIAL AND METHODS

Collection of textile effluent polluted soil (PS), cow dung (CD) and *Eudrilus eugeniae:* The PS was collected from the Common Effluent Treatment Plant (CETP) of State Industries Promotion Corporation of Tamil Nadu (SIPCOT) industrial estate area, Perundurai, Erode district, Tamil Nadu, India. Fresh cow dung was collected from the residential areas in and around Karumandapam, Tiruchirappalli, Tamil Nadu, India. The initial physico-chemical characteristics of PS and CD are given in Table 1. *Eudrilus eugeniae* were obtained from Periyar Research Organization for Bio-Technique and Eco-system (PROBE), Periyar Maniyammai University, Vallam, Thanjavur, Tamil Nadu, India. The experiments were conducted in a specially designed perforated plastic basket (diameter 20 cm and depth 15 cm) named as vermireactors and numbered as per PS and CD concentrations in each mixture.

Table 1. Initial physico-chemical characteristics of CD and PS

Composition	Cow dung (CD)	Polluted soil (PS)
pH	8.13±0.05	7.86±0.01
EC	1.63±0.05	0.62±0.01
TOC	56.30±0.45	4.26±0.01
TKN	12.33±0.05	7.64±0.04
TP	2.30±0.10	1.64±0.45
TK	9.33±0.05	9.40±0.17
C:N ratio	0.74±0.03	0.06±0.17
Cu	0.69±0.00	0.14±0.00
Zn	1.56±0.01	1.88±0.06
Fe	9.63±0.01	15.00±0.01
Cr	0.78±0.03	0.15±0.21

PS and CD were completely air-dried and sieved (2.0 mm mesh) before mixing. PS was mixed with CD in different ratios VO 100% CD ,90:10, 80:20, 70:30,60:40, The study included a control vermireactor having CD only as feed mixture for the earthworms. The ratio of PS and CD in different ratio These mixtures were turned manually every day for 15 days in order to eliminate volatile gases potentially toxic to earthworms. After 15 days, 3 adult individuals of E. eugeniae weighing between 1 g and 1.2 g were introduced into each vermireactor. The experimental period was for 8 consecutive weeks. Care was taken to rear the earthworms with more than

60 % of moisture in the immediate surroundings using water sprinkler. Moisture content was measured based on the previous studies of (Sadasivam et al. 2015). All the vermireactors were kept under ambient conditions (room temperature 25±2 °C; appropriate temperature for E. eugeniae).

The experiments were repeated thrice for each feed mixture. Homogenized samples (free from earthworms, cocoons and hatchlings) of the feed material were drawn on 0, 7, 14, 21, 28, 35, 42, 49 and 56th days from each vermireactors. The initial and final vermicompost samples were air-dried in the shade at room temperature, ground in a stainless steel blender and stored in plastic vials for further chemical analysis. Physico-chemical characteristics and metal composition: The pH was measured using a digital pH meter (Systronics) by dissolving double distilled water suspension of each vermicompost in the ratio of 1:10 (w/v). Electrical conductivity (EC) was determined in double distilled water suspension of each mixture in the ratio of 1:10 (w/w) using a HM digital meter-COM-100. Total Organic Carbon (TOC) was measured on igniting the samples in a Muffle furnace at 550°C for 1 h following the method of (Nelson and Sommers (1996). Total Kjeldhal nitrogen (TKN) was determined by Bremner and Mulvaney (1982) procedure. Total phosphorous (TP) was analyzed using the colorimetric method of molybdenum in sulphuric acid. Total potassium (TK) was determined by flame photometer [Elico, CL 22D, Hyderabad, India]. Total sodium (TNa) was measured using a Systronics flame photometer-128 after digesting the samples in diacid mixtures ($HClO4$: HNO_3 in 4:1 ratio).

Table 2. Initial and final physico-chemical parameters (mean±SE) different concentration of textile effluent polluted soil with cow dung

Parameters		VC	V1	V2	V3	V4
pH	Initial	8.13±0.05	7.77±0.11	7.73±0.12	7.76±0.12	7.63±0.47
	Final	7.86±0.00	7.43±0.02	7.27±0.02	7.58±0.05	7.59±0.05
	% change	-5.33	-4.37	-5.92	-2.37	-0.52
EC (Ds/m)	Initial	1.63±0.05	1.40±0.02	1.30±0.00	1.35±0.51	1.29±0.00
	Final	0.88±0.00	0.56±0.07	0.87±0.00	0.92±0.00	0.80±0.05
	% change	-46.01	-60	-21.18	-20.10	-42.66
TOC (k/kg)	Initial	81.30±0.04	63.33±0.57	64.46±0.55	66.06±0.94	61.30±0.10
	Final	25.63±0.36	25.50±0.55	26.63±0.45	27.20±0.17	28.46±0.11
	% change	-68.17	-57.73	-58.60	-58.82	-53.67
TKN (k/kg)	Initial	12.33±0.05	10.13±0.57	11.43±0.57	11.23±0.57	10.83±0.63
	Final	19.66±0.09	15.64±0.45	16.33±0.39	16.57±0.39	17.46±0.11
	% change	59.44	54.33	42.86	47.55	61.21
C:N ratio	Initial	6.32±0.29	6.25±0.37	5.72±0.40	5.96±0.11	5.34±0.21
	Final	1.36±0.05	1.70±0.03	1.63±0.03	1.62±0.04	1.63±0.14
	% change	-78.48	-72.8	-71.50	-72.81	-69.47
TP (g/kg)	Initial	2.30±0.10	1.90±0.17	1.76±0.57	1.63±0.45	1.60±0.10
	Final	4.57±0.32	3.56±0.11	3.70±0.34	3.56±0.11	4.64±0.45
	% change	98.69	87.36	53.40	110.22	89.29
TK (g/kg)	Initial	9.33±0.05	13.53±0.20	13.56±0.30	13.40±0.26	13.03±0.11
	Final	15.64±0.36	18.66±0.06	17.64±0.45	18.26±0.11	18.73±0.57
	% change	67.63	37.91	52.95	36.26	43.74
Cu (mg/kg)	Initial	0.69±0.00	0.35±0.06	0.30±0.06	0.23±0.32	0.25±0.12
	Final	0.72±0.01	0.79±0.00	0.91±0.01	0.96±0.01	0.82±0.01
	% change	4.35	55.69	67.03	317.12	227.99
Zn (mg/kg)	Initial	1.56±0.01	1.57±0.12	1.71±0.04	1.57±0.06	2.01±0.07
	Final	2.36±0.00	1.77±0.19	1.85±0.11	1.80±0.19	2.08±0.08
	% change	51.28	13.47	8.18	14.67	3.48
Fe (mg/kg)	Initial	9.63±0.01	16.44±0.02	14.36±0.18	15.33±0.00	15.20±0.00
	Final	9.12±0.00	12.68±0.11	12.89±0.00	11.69±0.00	12.58±0.01
	% change	-5.29	-22.87	-10.23	-23.74	-17.23
Cr (mg/kg)	Initial	0.12±0.01	0.39±0.00	0.48±0.01	0.53±0.04	0.47±0.01
	Final	0.05±0.00	0.04±0.01	0.05±0.00	0.02±0.00	0.06±0.11
	% change	-58.33	-89.74	-89.58	-96.22	-87.23

Significance level was determined by Tukey test $p \leq 0.05$ Weight in (g/kg) and (mg/kg)

Table 2. Initial and final physico-chemical parameters (mean±SE) different concentration of textile effluent polluted soil with cow dung

Parameters		V5	V6	V7	V8	V9	V10
pH	Initial	7.62±0.49	7.66±0.10	7.62±0.42	7.52±0.82	8.02±0.01	8.89±0.01
	Final	7.59±0.05	7.58±0.55	7.53±0.05	7.46±0.05	7.94±0.41	7.89±0.01
	% change	-0.39	-1.04	-1.18	-0.79	0.99	-12
EC (Ds/m)	Initial	1.10±0.07	1.17±0.05	1.10±0.05	1.10±0.01	0.59±0.00	0.62±0.05
	Final	0.92±0.00	0.57±0.05	0.87±0.00	0.78±0.00	0.49±0.00	0.32±0.05
	% change	-38.66	-52.99	-20.90	-29.09	-16.94	-48.38
TOC (k/kg)	Initial	61.10±0.56	28.96±0.56	21.43±0.15	19.16±0.20	6.41±0.30	5.26±0.12
	Final	25.64±0.45	22.23±0.05	12.33±0.57	10.64±0.45	5.63±0.05	4.26±0.11
	% change	-59.67	-23.18	-42.46	-44.62	-12.16	-19.01
TKN (k/kg)	Initial	9.73±0.57	10.80±0.60	9.43±0.57	10.76±0.57	8.64±0.45	7.64±0.45
	Final	17.23±0.05	16.83±0.05	16.16±0.05	15.54±0.05	9.24±0.45	8.64±0.45
	% change	77.08	55.83	71.36	44.42	6.94	13.08
C:N ratio	Initial	4.27±0.46	2.43±0.21	2.27±0.14	1.78±0.08	1.10±0.04	1.00±0.02
	Final	1.49±0.04	1.52±0.34	1.27±0.18	1.47±0.16	1.90±0.00	1.89±0.01
	% change	-76.23	37.44	-44.05	-17.41	-72.77	-88.99
TP (g/kg)	Initial	2.53±0.15	1.76±0.49	1.40±0.16	1.40±0.17	1.31±0.15	1.20±0.14
	Final	4.73±0.05	3.73±0.11	2.16±0.45	2.26±0.11	1.90±0.15	1.25±0.14
	% change	86.95	111.93	54.28	61.42	45.30	4.16
TK (g/kg)	Initial	12.43±0.11	12.56±0.64	11.13±0.11	11.10±0.10	11.11±0.90	10.10±0.90
	Final	18.43±0.57	19.26±0.11	19.26±0.11	18.64±0.45	13.17±0.90	11.85±0.64
	% change	48.23	53.34	73.04	67.92	18.54	17.32
Cu (mg/kg)	Initial	0.29±0.30	0.54±0.26	0.42±0.12	0.35±0.60	0.24±0.60	0.14±0.00
	Final	0.84±0.00	0.92±0.00	0.76±0.01	0.62±0.04	0.54±0.04	0.16±0.64
	% change	189.65	70.37	80.95	77.14	125.00	14.28
Zn (mg/kg)	Initial	1.84±0.13	1.50±0.17	1.79±0.71	1.85±0.84	1.90±0.84	1.88±0.06
	Final	2.25±0.95	2.36±0.11	2.39±0.49	2.45±0.00	2.32±0.00	1.99±0.00
	% change	22.28	57.33	33.51	32.42	22.10	5.85
Fe (mg/kg)	Initial	16.18±0.05	18.24±0.10	14.58±0.04	15.58±0.01	15.01±0.01	15.00±0.01
	Final	11.56±0.11	11.36±0.00	12.65±0.00	13.45±0.00	13.45±0.00	14.11±0.10
	% change	-28.55	-37.71	-13.23	-13.67	-10.39	-5.99
Cr (mg/kg)	Initial	0.42±0.00	0.43±0.04	0.52±0.00	0.61±0.02	0.13±0.00	0.16±0.00
	Final	0.04±0.01	0.05±0.01	0.03±0.01	0.02±0.00	0.05±0.05	0.12±0.21
	% change	-90.47	-88.37	-94.23	-96.77	-61.53	-25.00

Heavy metals were measured by using Agilent AA 240 model atomic absorption spectrophotometer after digesting the samples in diacid mixtures.

Germination studies and plant growth measurements: Germination studies and plant growth measurements were done in Phaseolus radiatus (Green gram) to assess the manure quality of the biorestored soil. 1–5 % of the final vermicompost from the best vermireactor was mixed with normal soil to check germination and plant growth of *P. radiatus* for 4 consecutive days. Direct seed germination test (Warman 2010) of Phaseolus radiatus was conducted in triplicate by mixing final vermicompost of the best vermireactor with normal soil. Based on the previous studies carried out by David et al. (2014), the percentage amendment of vermicompost of the best vermireactor with normal soil chosen for this study ranged from 1 to 5 %. Amendments of the vermicompost of the best vermireactor with the normal soil were named as AST1 to AST5. Three controls were chosen (i) positive control comprising 100 % vermicompost of the best vermireactors (ASC1), (ii) negative control comprising 100 % polluted soil (ASC2) and (iii) overall control comprising 100 % normal soil (ASC3).

Each amended soil was seeded with 16 seeds of *Phaseolus radiatus*. The cell packs were moistened with potable water (as required) and kept under 12-14 h of light and 25±2°C in the area where vermicomposting had been carried out. Germination count of the seeds was noted after 48 hours of seeding. The plants were harvested after 4 days of sowing and repeatedly washed with tap water. Furthermore, these were rinsed with 10 mM CaCl2 solution and washed with deionised water. Wet biomass of root, shoot and leaves was obtained after taking off excess water with tissue paper and using a Shimadzu

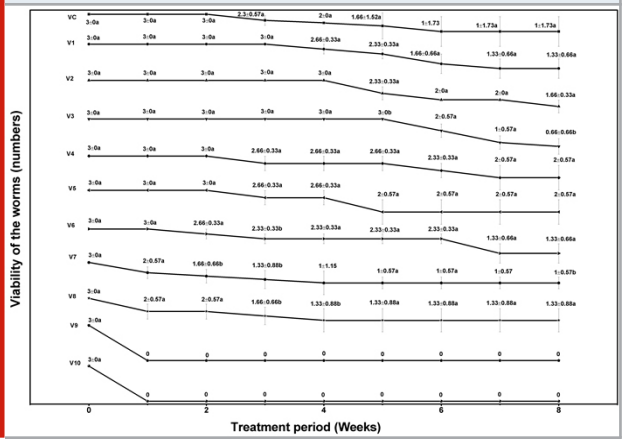
weighing balance. Dry biomass of root, shoot and leaves was calculated by drying at 72° C in an oven for 72 h Wong et al. (1996).

Statistical analysis: All the samples were analyzed in triplicate (n = 3), and the results were averaged. Statistical analyses were performed by one-way analysis of variance (ANOVA) to evaluate the significant difference between vermireactors for growth and fecundity, physico-chemical and metal composition studies. Tukey's post t-test was done to identify the homogenous types of vermireactors. The number of seedlings and plants was counted and statistically analyzed using ANOVA (SPSS 16.0).

RESULTS AND DISCUSSION

The mean viability of worms was found to be highest in V4 and lowest in V7 of all the vermireactors, and mean viability was greatest at 2nd week of the experimental period Fig. 1. Viability of *E. eugeniae* was zero in V9 & V10 from 1st to 8th week of the experimental period. The net mortality rate was found to be higher in V7 & V8 and consequently in Vc. Significant changes were observed in V7 at 2nd week, V6, V7 & V8 at 3rd week, V8 at 4th week, V3 at 5th week and V7 & V8 at 8th week when compared with all the other vermireactors in their respective week. The median viability of E. eugeniae was observed in V1

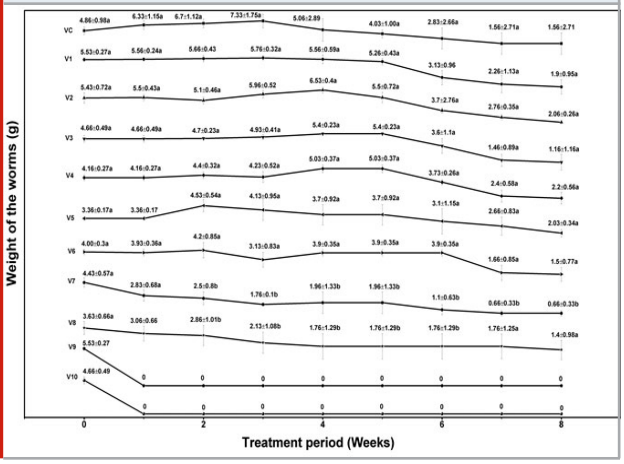
Figure 1: All values are mean (n=3) S.D. Mean viability of the earthworms was compared among all the vermireactors having different concentration of CD+PS, where (a) highly significant and (b) less significant when compared with the control at (One Way ANOVA F ratio =0.988,TukeyTest



and at 4th week of the experimental period. The viability of the worms was found to be decreasing from 4th week, and thereby, a gradual decrease in viability of worms was monitored till the end of the experiment.

Mortality rate was directly proportional to increase in the concentration of polluted soil in the vermireactors. Increased concentration of PS in CD attributed mortality to degradation processes that result in changes in environmental characteristics (Garg and Kaushik. 2005). Addition of at-least 50 % CD in PS was essential for the survival of *E. eugeniae*. Finally, these results suggested that feed mixtures in V8, V9 & V10 cannot be used as substrate for vermicomposting by *E. eugeniae*, but must be supplemented with more CD. Similar observations had been reported by (Elvira et al 1998) for paper mill sludge vermicomposting by *E. andrei* and for textile mill sludge vermicomposting by E. foetida (Kaushik and Garg 2004). The mean worm weight of earthworm was found to be maximum in V2 and lowest in V10, and the highest mean worm weight was observed at 2nd week

Figure 2: All values are insignificantly different (One Way ANOVA: =0.502, Tukey Test) where (a) highly significant and (b) significant when compared with the control

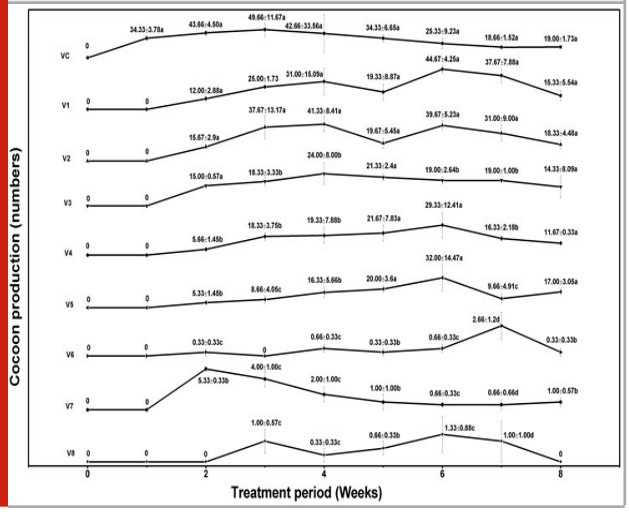


of the experimental period Fig. 2. Significant changes were observed when V7 & V8 (except 0th week of V7 and 0th, 1st, 7th and 8th week of V8) were compared with rest of all the vermireactors from 1st week to 8th week of the experimental period.

Increasing concentration of PS in vermireactors promoted a decrease in the weight of E. eugeniae, but did not show a regular pattern. The median worm weight was observed in V4 when compared with all the other vermireactors and at 4th week when compared with rest of all the weeks throughout the experimental period. Initial increase in worm weight (till 3rd week in all the vermireactors except V2 in which worm weight increased till 4th week) was followed by stabilization. Later weight loss was observed in all the vermireactors from 4th week except in V2. The loss in worm weight can be attributed to the exhaustion of food (Renuka and Garg, 2007). When E. eugeniae received the food below a maintenance level, it lost weight at a rate which depended upon the quantity and nature of its ingestible substrates. This was in consonance with the study carried out by Renuka et al. 2007 in E. foetida. (Garczynska et al 2020)

Cocoon production: The net cocoon production peaked in Vc and decreased in V8 and V6 in comparison with all the vermireactors; mean cocoon production increased at 6th week and dropped at 8th week of the experimental period Fig. 3. From 0th to 8th week, no cocoon production was observed in V9 & V10. However, least cocoon production was observed in V8 from 3rd to 7th week. Significant alterations in cocoon production were observed when V6 was compared with V4, V5 and V7 at 2nd week;

Figure 3: All values are mean (n=3) S.D. Mean cocoon production was compared among all vermireactors having different concentration of CD + PS where (a) highly significant, (b) significant, (c) less significant, and (d) least significant when compared with the control at different letters in some column are significantly different (One Way ANOVA: F ratio 4.01, tukey test). V9 and V10 were not plotted in the graph since no cocoon production was observed throughout the experimental period.

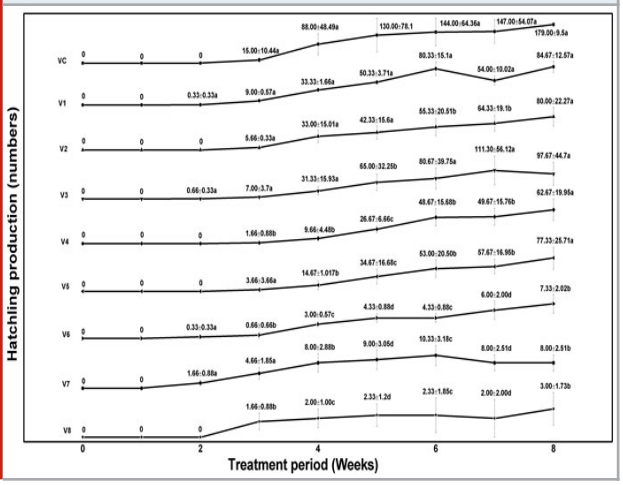


V8 with V5, V7 ,V3 & V4 at 3rd week; V6, V7 V8 with V3, V4 &V5 at 4th week; V6, V7 &V8 with rest of the vermireactors at 5th week; V6, V7 &V8 with V3 at 6th week; V6, V7 & V8 with V5, V3& V4 at 7th week and finally when V6 & V7 were compared with the remaining vermireactors at 8th week.

The differences among rates of cocoon production in different vermireactors could be related to the biochemical quality of the feed mixtures, which is one of the important factors in determining the onset of cocoon production (Edwards et al.1998). The present study reported the maximum cocoon production in V4 and at 5th week during the entire treatment period. This is in consonance with the studies conducted by (Xie et al. 2012) which stated that addition of cow dung with sludge of animal wastewater treatment plant improved the mutual interaction between earthworm and microbes; the maximum proliferation of *E. fetida* was noted in sludge mixed with 40 % CD and proved to be a suitable growth medium for the fecundity of *E. fetida*.

Number of hatchling: The greatest mean number of hatchlings was produced in Vc and diminished in V8. This observation was same as in the case of cocoon production. Dissimilarity in mean number of hatchlings was noted when maximum number of hatchlings peaked at 7th week for all the vermireactors Fig. 4. No significant changes were observed at 2nd week of the treatment period. However, significant variations were examined when V4, V6 &V8 were compared with all the other vermireactors at 3rd week; V6 &V8 with V4, V5 & V7 at 4th week; V6, V7 &V8 compared with V4 &V5 at 5th week; V6, V7 & V8 with V3, V4 & V5 at 6th and 7 th week and V6, V7 & V8 with rest of all the vermireactors at 8th week. The median cocoon production was observed in V4 and at 5th week among the entire treatment period. These data suggested that greater percentage of PS in feed mixtures significantly affected the development of hatchlings per cocoon. The feed, which provide earthworms with sufficient

Figure 4: All values are mean (n=3) S.D. Mean hatchling production was compared among all vermireactors having different concentration of CD+PS where (a) highly significant, (b) significant, (c) less significant, and (d) least significant when compared with the control at different letters in some column are significantly different (One Way ANOVA) : P < 0.05, tukey test).



amount of easily metabolizable organic matter and non-assimilated carbohydrates, favors the growth and reproduction of the earthworms (Garg and Kaushik. 2005). The maximum number of hatchlings in V4 was supported by the studies conducted by (Bhat et al. 2015) where maximum number of hatchlings was observed in 60-100% amendment of cow dung with sugar beet mud using *E. fetida*.

Physico-chemical properties: The physico-chemical characteristics of initial and final feed mixtures of textile effluent are shown in Table 2.Significant difference in pH was not observed in all the vermireactors of the initial feed mixtures. The pH of the initial feed mixtures ranged from 7.52 to 8.89, near neutral to alkaline. But the pH of the final vermicompost was near neutral in all the vermireactors except V9 & V10. The optimum pH was observed in V3 & V6 in the final vermicompost. It has been reported (Ndegwa and Thompson, 2000) that the pH shift is dynamic and substrate dependent. The shift in pH during vermicomposting process could be attributed to the production of metabolic compounds of aerobic digestion of organic stuffs such as CO2, ammonia, NO3- and organic acids (Lopez et al., 2002).

Significant decrease in EC was observed in the final vermicompost except in V9 & V10 when compared with the initial feed mixtures of all the vermireactors. The

net EC loss was 42 % in the final vermicompost when compared with the initial feed mixtures. The optimum EC was observed in V4 & V5 of the final vermicompost. Decrease in EC of the final vermicompost may be due to stabilization of mixtures (Singh et al. 2010). They also observed a decline in EC in the vermicompost from the biosludge of a beverage industry.

Total Organic Carbon (TOC): TOC of the final vermicompost was significantly reduced when :compared with the initial feed mixtures. The optimum TOC was observed in V5 of the final vermicompost. There was a net loss of 52 % TOC in the vermireactors at the end of vermicomposting period. This finding was supported by (Kaviraj and Sharma, 2003) who reported 45% loss of carbon during vermicomposting of municipality and industrial wastes. (Suthar, 2006) reported that earthworms promoted microclimatic conditions in the vermireactors that increased the loss of TOC from substrate through microbial respiration (Abbas Esmaeili et al 2020)

Total Kjeldhal Nitrogen (TKN): A significant increase in TKN was recorded in all the vermireactors at the end of the study period. The net TKN of the final vermicompost increased upto 50 % and the nitrogen enhancement rate was optimum in V3. Body secretions of earthworm (excreta, mucus) add nitrogen in substrate if earthworms were inoculated in organic wastes for longer periods. Earthworms also alter the microclimatic conditions of vermireactors which consequently promote microbial populations responsible for nitrogen enrichment (Suthar et al. 2012). Hence in this study, the combination of the organic waste (CD) and polluted soil acted as good energy stuff for nitrogen fixing bacteria enhanced by E. eugeniae.

Carbon Nitrogen ratio (C: N ratio):C:N ratio was found to decrease significantly (75 % net loss) in all the vermireactors of the final vermicompost when compared with initial feed mixtures. The optimal C:N ratio was observed in V5 & V6 of the final vermicompost. Decline in C:N ratio in this study was due to higher loss of carbon through microbial respiration in the form of CO2 along with an increase in nitrogen and stabilization of waste by the action of E. eugeniae. Similar results were observed by (Bhat et al. 2014). Earlier studies have suggested that a C:N ratio below 20 is an indicative of acceptable maturity, while a ratio of 15 or lower is being preferred for agronomic use of composts. The vermicomposts obtained in this study showed C:N ratio within the preferable limits as described by (Morais and Queda, 2003).

Total Phosphorous (TP): Trivial changes were observed in phosphorous content in all the vermireactors of initial feed mixtures. The net P content in vermicomposted material was 118 % higher than in the initial feed mixtures. The optimal P content was observed in V3 of the final vermicompost. Significant changes in phosphorous content were observed in all the vermireactors of the final vermicompost except in V9 & V10. The concentration of phosphorous in vermicomposted material may reflect the amount of organic forms of phosphorous in PS, but its mineralization rate is directly affected by the nature of the amendment material and activities of P-mineralizing microflora in decomposting wastes. (Satchell and Martin 1984) found an increase of 25 % in total P of paper-waste sludge after vermicomposting. In the present study there was 118 % increase in P content.

Table 3. Effect of different concentration of bio-restored soil from V5 with normal soil on root, shoot, and leaves length and their respective biomass of Phaseolus radiatus

Treatment	No. of grown plants after 4th day	Root length (cm)	Wet biomass of root (g/plant)	Dry biomass of root (g/plant)	Shoot length (cm)	Wet biomass of shoot (g/plant)	Dry biomass of shoot (g/plant)	Length of leaves (cm)	Wet biomass of leaves (g/plant)	Dry biomass of leaves (g/plant)
ASC3	11.66±0.57	9.55±0.32	0.020	0.0050	10.39±0.23	0.200	0.021	1.86±0.23	0.027	0.0079
AST1	11.66±0.57	10.23±0.94	0.267	0.0061	12.36±1.34	0.247	0.022	2.03±0.15	0.054	0.0088
AST2	12.66±0.57	10.90±1.52	0.267	0.0056	12.50±1.89	0.220	0.021	1.93±0.11	0.054	0.0088
AST3	12.66±1.15	9.91±0.43	0.267	0.0058	9.86±0.74	0.240	0.023	2.56±0.20	0.054	0.0088
AST4	13.20±2.64	9.86±0.74	0.040	0.0062	11.95±0.80	0.250	0.021	2.46±0.32	0.054	0.0091
AST5	11.33±0.57	9.86±0.74	0.053	0.0059	11.95±0.80	0.240	0.021	1.86±0.20	0.054	0.0088

All values are mean (n = 3) ±S.D.
ASC3 : Amended Soil Control 3 (Overall control: 100% garden soil) AST: Amended Soil Treated
AST1 : 198 : 2 g (Garden soil : g)
AST2 : 196 : 4 g
AST3 :194 : 6 g
AST4 : 192 : 8 g
AST5 : 190 : 10 g

This increase might be due to the release of P in available forms which were partly performed from earthworm gut phosphatases, and further release of P might be attributed to the P-solubilizing microorganisms present in worm casts as suggested by (Suthar et al. 2012). The results clearly suggest that P enrichment is directly related to the quality of material used in the vermireactors.

Total Potassium (TK): High potassium content was observed in the initial feed mixtures, but did not show a regular pattern and started diminishing after V7. Subsequently, significant increase of mean TK content (43%) was observed in the final vermicompost when compared with initial feed mixtures and started diminishing after V8. The optimal K content was observed in V5 & V8. Most of the earlier works on vermicomposting (Bhargava 2009). (Hait and Tare 2011) had reported higher K levels at the end of vermicomposting. The increase in K content indicates that when organic waste passes through the gut of earthworm, some fraction of organic materials is converted into more available types of nutrients (i.e. exchangeable forms) due to the action of endogenic and/ or exogenic enzymes Suthar (2010).

Heavy metal composition: Copper and Zinc (Cu & Zn):Transition metals Cu and Zn increased significantly ($P<0.05$) over initial feed mixtures in all the vermireactors except V10. Table 2. The mean increase in Cu and Zn content was 148% and 23%, respectively, in the final vermicompost. The optimal Cu and Zn content was observed in V4 and V5, respectively. In the final vermicompost, a little increase in Cu and Zn was noticed, and their contents increased with the increasing proportion of PS in the vermireactors. Increase in Cu and Zn metal contents may be due to decline in the weight and volume of the feed mixtures as suggested by Deolalikar et al. 2005. An increase in the content of transition metals in vermicompost from industrial sludge was also observed by Singh et al. 2010.

Iron, and Chromium (Fe, and Cr): Significant decrease of Fe and Cr content was observed within and between initial feed mixtures and final vermicompost of all the vermireactors. Table 2. The net decrease in Fe, and Cr content in the final vermicompost was 20 %, 20 % and 86 %, respectively. The optimal Fe content was observed in V4 and optimal . Heavy metal content of Fe, and Cr in this study was found to be decreased in the final vermicompost when compared with initial feed mixtures. Similar observations were made in the studies conducted by Garg and Kaushik, (2005) on vermistabilization of textile mill sludge and by Bhat et al. (2015) on vermistabilization of sugar beet by E. foetida. The decrease in the heavy metal content of Fe, and Cr in the present study suggested that the vermicompost can be used in the fields without any ill effects on soil.

Germination studies and plant growth measurements Based on the optimal characteristics of almost all the physico-chemical parameters, metal composition, growth and fecundity studies, the final vermicompost of V5¬ was chosen for amendment with normal soil in germination and plant growth measurements. Seed germination and plant growth were observed in all the amended soils for except the 100% polluted soil (ASC2). Charring of seeds was observed in ASC2. 100 % of the final vermicompost of V5¬ (ASC2) showed germination of the seeds, but plant growth was absent Table 3. Significant changes were observed when ASC1 and ASC2 were compared with ASC3 and the rest of the treated amended soil (AST1–AST5). As the concentration of biorestored soil in the amended soil increased, plant growth of *P. radiatus* also increased except in the case of AST5.

Vegetative growth parameters of *P. radiatus* (root, shoot and leaf length) at all tested concentrations of amended soil were similar to normal soil. The maximum root and shoot length were observed in AST3 and maximum leaf length in AST4. Maximum wet biomass of root, shoot and leaves was observed in AST1-AST3, AST4 and all the amended soils except ASC3, respectively. Maximum dry biomass of root, shoot and leaves was observed in AST4, AST1 and all amended soils except ASC3, respectively. The maximum protein content for root was observed in AST4, for shoot in AST5 and for leaves in AST3 Table 3. Similar seed germination and vegetative growth were observed in Phaseolus mungo when 10% distillery sludge was amended with garden soil by Chandra et al. 2008.

CONCLUSION

Restoration of textile effluent polluted soil by environmentally acceptable means has become a great challenge and a major concern of the present decade. This study had demonstrated vermistabilization by E. eugeniae as an appropriate technology for management of textile effluent polluted soil. Growth and fecundity studies of E. eugeniae revealed that a high degree of PS stabilization was achieved in (V4 and V5) (CD40 PS60 and CD50 PC50) at 4th and/or 5th week of the study period. The optimal CD+PS composition for almost all the favorable Physico-chemical parameters and metal concentrations ranged from (V3 to V6) (CD70 PS30 to CD40 PS60) when compared with Vc (CD100 PS0) (V9 & V10) (CD90 PC10) and (PC 100), while (V1 & V2) (CD90, PS10 CD20 and PC80) on the other hand Vc ,(CD100), (V9 and V10) (CD10 PC90 and CD0 PS100) were not found to be suitable for the growth of E. eugeniae. Germination studies and plant growth measurements of Phaseolus radiatus showed that 1-5% amendment of the vermicompost of V5 would serve as a soil conditioner or manure in the agricultural fields. The study revealed the fact that PS could be restored and transformed into good quality manure by vermicomposting when mixed with at least 30-50 % CD.

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

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Characterization of Potential Plant Growth Promoting Rhizobacteria Excerpted from Wheat, *Triticum aestivum* Rhizosphere of Saurashtra Region

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ABSTRACT

In last several decades the properties of soil are damaged due to modern agricultural practices. Synthetic fertilizers damaged the natural microbial flora of soil which was maintaining the fertility of the soil. In present study we were characterization the plant growth promoting rhizobacteria isolated from the rhizospheric area of wheat (*Triticum aestivum L.*) of Saurastra region of Gujarat, India. All the isolates were screened for plant growth promoting trait to utilize them for the sustainable agriculture. Total thirty-four organisms were purified from the three different ecological region (Dhandhusar, Gir-gadhada, Gingani) of Saurashtra region. Among the 34 bacterial isolates twenty-eight were able to produce indole-3- acetic acid in tryptophan supplemented medium; twenty were able to solubilize inorganic phosphate and zinc in vitro. PE-1was found to produce high amount of IAA i.e. 46.01 µg/ml, JG-13 solubilizes maximum inorganic phosphate (635 µgml–1) followed by GG-12 (603 µgml–1), JZ-8 gives 25mm zone on ZnO2 medium around colony. Present study indicates the potentiality of PGPR that can be utilize as a biofertilizer for better enhancement of productivity and health of wheat crop.

KEY WORDS: RHIZOBACTERIA, WHEAT, IAA, PHOSPHATE.

INTRODUCTION

In Saurashtra region, wheat is another major crop of winter season. Many biotic and abiotic factors affected on the production rate of wheat. The state agriculture department has estimated wheat production in Gujarat in the financial year 2016 to be at about 4.80 million tonnes that is 5 percent of total production in India. The overall production of wheat is set back by some biotic factors (plant pathogens) and abiotic factor (environmental factor). Therefore, there is an urgent need to find alternative strategies that can ensure competitive crop yields, provide environmental safety, and protection while maintain long term ecological balance in agro-ecosystem. Use of microbial inoculants or plant growth promoting rhizobateria (PGPR) for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world, (Alori and Babalola 2018, Santos et al 2019).

Plant growth promoting rhizobacteria are free living soil bacteria that aggregate around rood surface (rhizosphere) and enhance the growth and yield of crops when directly applied on seeds (Kumar et al., 2014). The bacteria that

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provide some beneficial effect to the plants are of two types, those that form a symbiotic relationship with the plant and those that are free living in the soil, but are often found on or near to the roots and inside the roots (Kloepper et al., 1988, Van Peer and Schippers, 1989). Beneficial free-living bacteria are called plant growth promoting rhizobacteria or PGPR (Kloepper et al., 1989). PGPR can affect plant growth in two ways, indirectly or directly. There are many mechanisms by which PGPR exhibit plant growth promotion. PGPR can fix nitrogen and supply it to plants; they synthesize siderophores that can sequester iron from soil and provide it to plant cells; produce phytohormones and solubilize inorganic phosphorus and low molecular mass compounds (Davison, 1988; Lambert and Joos 1989; Glick et al., 1994a, 1994b), ammonification, etc. In our recent study was planned to isolate rhizosperic bacterial of wheat from different climatic location and examine their role in plant growth promotion. These bacteria were screened for their in-vitro potentiality by biochemical test. In our further study we are going to examine their role along with some other microbial population.

Figure 1: IAA production by PGPR

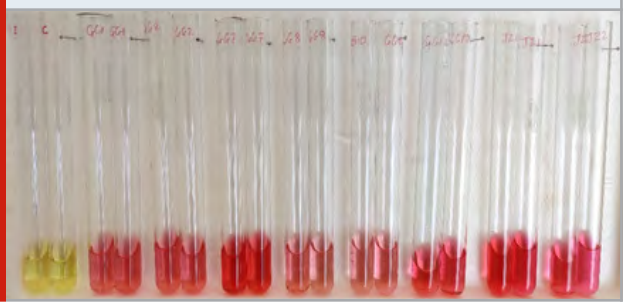


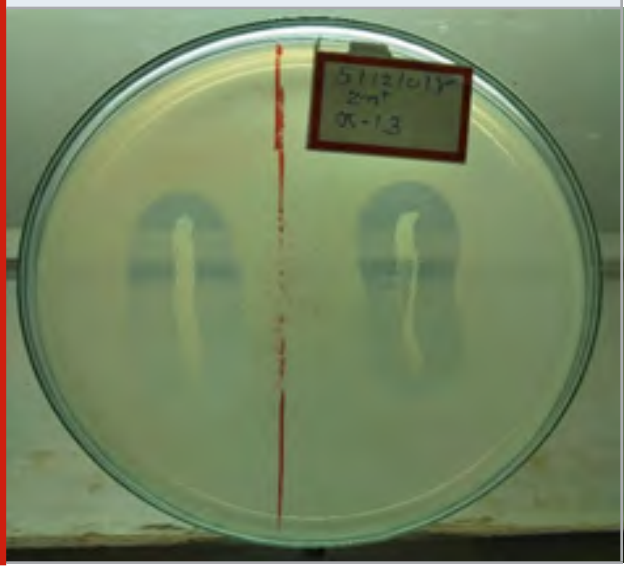
Table 1: Geographical location of rhizosperic isolates

Sampling site	Geographical location	Isolates ID
Dhandhusar, Dist. Junagadh, Gujarat, India	21.552687, 70.357483	GG-1, GG-2, GG-3, GG-4, GG-5 GG-6, GG-7, GG-8, GG-9, GG-10 GG-11, GG-12
Gir-gadhada, Dist. Junagadh, Gujarat, India	20.922836, 70.929627	JZ-1, JZ-2, JZ-3, JZ-4, JZ-5, JZ-6 JZ-7, JZ-8
Gingani, Dist. Junagadh, Gujarat, India	21.882330, 70.072294	JG-1, JG-2, JG-3, JG-4, JG-5, JG-6 JG-7, JG-8, JG-9, JG-10, JG-11, JG-12, JG-13, JG-14

MATERIALS AND METHODS

Selection of sampling site and isolation of bacteria: Wheat (Triticum aestivum L.) variety LOK-1 were collected from different region of saurashtra region according to physiological and chemical properties of soil for the isolation of rhizospheric bacteria (Table1). Samples were collected from the different geographical location on of Saurashtra region i.e Dhandhusar (21.552687, 70.357483), Gir-gadhada (20.922836, 70.929627), Gingani (21.882330, 70.072294).

Figure 2: Zinc Solubilization by PGPR



Isolation of PGPR from wheat rhizospheric soil: Rhizospheric bacteria were isolated from 1g soil tightly adhering to the root by serial dilution plating on nutrient agar plates and King's medium B (King et al., 1954) as described (Somasegaranand Hoben, 1994). The plates were incubated at 30 ± 2°C for 24hrs than up to 48hrs. Individual colonies were picked and streaked on nutrient agar plate for further purification. A total of one hundred and fifty-four Rhizobacterial isolates were obtained. The isolates were incubated at 30 ± 2°C during culturing and maintained as glycerol stocks (35% glycerol+ KB broth) at -20°C.

Morphological and biochemical analyses of wheat PGPR: Colony morphology, size, colour, shape, gum production, and growth pattern were recorded after 24hrs of growth on King'B agar plate at 30 ± 2°C as described by Somasegaran and Hoben (1994) with some modification. Cell size and motility was observed by light microscopy. Acid/alkali production was tested on nutrient broth containing phenol red as pH indicator. Gram's reaction was carried out as described by Vincent and Humphrey (1970).

Bioassay for plant growth promotion activity: All isolates collected from wheat rhizosphere were screened for detecting and quantifying the production of indole acetic acid (IAA) like substances. All rhizobacterial isolates were grown in 100 ml Erlenmeyer flasks containing 25 ml of Tryptone yeast extract (TY) broth and incubated on a rotary shaker in the dark at 30 ± 2°C for 4 days (Sarwar and Kremer (1995). Than 25 ml of culture broth

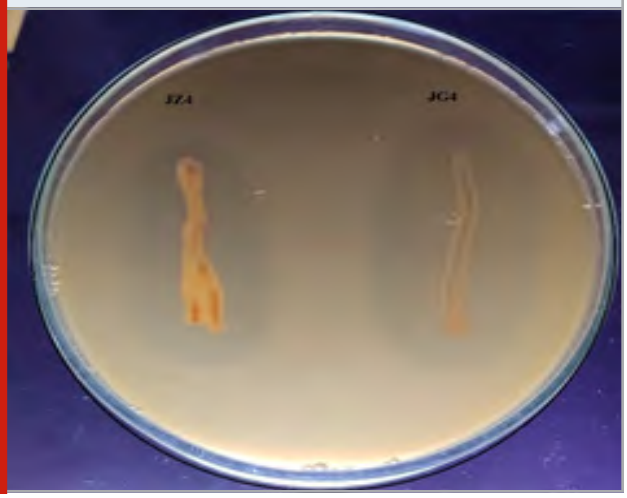
were centrifuged at 2000 rpm for 20 mins to remove cell debris. 1ml of clear supernatant was transfer in another tube to determine concentration of IAA and add 1 ml of Salkowsky reagent (1 ml of 0.5 M FeCl3 in 50 ml of 3.5% HClO4) to developed colour. The reaction mixture was incubated in the dark for 1h. Pink colour developed which conformed which IAA production. Amount of IAA was quantified by taking supernatant in 1 ml of the reagent, the reaction mixture was incubated in the dark for 60 min for colour development.

Table 2. Colony morphology of wheat rhizosperic isolates

Sr. No.	Isolates ID	Gram's Nature	Cell Shape	Motility
1	GG-1	Gram-Negative	Cocci	Motile
2	GG-2	Gram-Positive	Long Rod	Non-motile
3	GG-3	Gram-Positive	Long Rod	Non-motile
4	GG-4	Gram-Positive	Rod	Motile
5	GG-5	Gram positive	Rod	Motile
6	GG-6	Gram positive	Rod	Motile
7	GG-7	Gram positive	Short rod	Motile
8	GG-8	Gram positive	Rod	Motile
9	GG-9	Gram-Positive	Rod	Motile
10	GG-10	Gram-Positive	Rod	Non-motile
11	GG-11	Gram-Positive	Rod	Non-motile
12	GG-12	Gram-Negative	Short Rod	Motile
13	JZ-1	Gram positive	Rod	Non motile
14	JZ-2	Gram positive	Rod	Motile
15	JZ-3	Gram positive	Long rod	Non motile
16	JZ-4	Gram positive	Rod	Motile
17	JZ-5	Gram positive	Rod	Motile
18	JZ-6	Gram positive	Rod	Motile
19	JZ-7	Gram positive	Short Rod	Non motile
20	JZ-8	Gram negative	Rod	Motile
21	JG-1	Gram-Negative	Short Rod	Motile
22	JG-2	Gram-Negative	Short Rod	Non-motile
23	JG-3	Gram-Positive	Long Rod	Motile
24	JG-4	Gram-Negative	Short Rod	Motile
25	JG-5	Gram-Negative	Short Rod	Non-motile
26	JG-6	Gram-Negative	Cocci	Motile
27	JG-7	Gram-Negative	Short Rod	Motile
28	JG-8	Gram-Negative	Short Rod	Motile
29	JG-9	Gram-Negative	Long Rod	Motile
30	JG-10	Gram-Negative	Cocci	Motile
31	JG-11	Gram-Positive	Filamentous	Non-motile
32	JG-12	Gram-Positive	Cocci	Motile
33	JG-13	Gram-Negative	Cocci	Motile
34	JG-14	Gram-Negative	Cocci	Motile

Absorbance was taken in visible spectrophotometer at 535 nm. The amount of IAA produced was measured from the standard curve. Solubilisation of insoluble phosphate by the rhizobacterial isolates was tested in Pikovskaya's medium (Pikovskaya, 1948), containing tri-calcium phosphate. Spot inoculation was done with the rhizobacterial isolates and plates were incubated at 32°C for 4-7 days. Observation was taken for clearing or solubilization zone around the colonies. The solubilization of tri-calcium phosphate was quantified in Pikovskaya's broth (Pikovskaya, 1948), following the protocol described by Dey et al. (2004). The result of solubilisation in measured after 6 days of incubation.

Figure 3: Phosphate Solubilization



RESULTS AND DISCUSSION

Isolation and morphological analysis of wheat rhizospheric bacteria: Bacteria were isolated form the rhizosphere of wheat (Triticum aestivum L.) during winter season from the three different location of Saurashtra region (Table1). Total 65 organisms were isolated after observing colony morphology. Finally, after observing colony morphology different 34 colonies were selected for further study. The bacteria showed mostly are non-pigmented and others give pigments like bluish, yellow, and greyish with variable sizes, margins and elevation on KB's agar plates. Shape of cells were short rods, long rods and cocci observed under light microscope. Twenty organisms gives gram's positive reaction and sixteen gram's negative reaction with KOH solution and grams staining.In microscopic observation using several techniques like gram staining, negative staining and hanging drop method of different isolates are illustrated in table 2. Total 24 isolates were motile, six were cocci shaped and others were short rods or long rods. Bacterial with different colony morphology were selected and maintained on nutrient agar slants and 60% glycerol at -40°C.

Colony morphology and cell morphology of wheat rhizospheric bacteria: All organisms show different colony morphology viz. brown, green, yellowish pigmentation, round, oval shape, small to large size colony and viscous, moist, dry like consistency were observed in different isolates of wheat rhizosphere. Regarding cell shape and gram-staining, 20 isolates were gram-positive and 14 were gram-negative. Long rod, short rod and cocci shaped cell morphology were observed under microscopic observation (Table 2).

Carbohydrate utilization efficiency of Wheat rhizospheric bacteria: Carbohydrate utilization like Glucose, Sucrose, Lactose and Mannitol were tested on all selected PGPR. It was observed that out of 34 isolates 22 having capacity to utilize glucose. In carbohydrate utilization test glucose, lactose, sucrose, mannitol sugars were supplemented in media and noticed glucose was utilized by the 22 isolates, Lactose was utilized by the 23 isolates and Sucrose was utilized by the 25 isolates and Mannitol was utilized by the 22 isolates. Not all glucose utilizer was able to produced acid through carbohydrate metabolism.

IAA production of wheat rhizospheric bacteria: The ability of PGPR to produce IAA found in different species had reported earlier (Mansour et al., 1994; Zahir et al., 2000). The various pathways, enzymes complexes and genes involved for the biosynthesis of IAA (Patten and Glick, 1996). The production of IAA by bacteria isolated from rhizosphere of different crops, i.e., peanut, wheat, and rice had already been reported in number of studies (Dey et al., 2004; A. Khalid et al. 2004, Prakash Nathan et al. 2011).

Out of thirty-six isolates twenty-eight were able to synthesis the IAA from the tryptophan amino acids. IAA produced by all isolates ranged in between 3.15 µg/ml to 46.01µg/ml. Among all isolates, JG-7 was found to produce high amount of IAA i.e. 46.01µg/ml. some of the isolates were not able to produced IAA were GG-3, GG-4, GG-5, GG-6, GG-9, GG-11, JZ-4, JZ-5, JG-10. 74% of total population were able to produce plant growth hormone, indole acetic acid. In vitro plant growth promotion traits of the rhizobacteria are described in Table 2.

Table 3. Plant growth promotional traits of rhizosperic isolates

Sr. No.	Phosphate Solubilisation			Zinc Solubilisation	IAA production
	Isolates ID	Zone of clearance (mm)	Concentration (µg/ml)	Zone of clearance (mm)	Concentration (µg/ml)
1	GG-1	11	268	6	8.02
2	GG-2	17	483	15	7.60
3	GG-3	10	244	5	0.00
4	GG-4	14	362	10	0.00
5	GG-5	0	0	0	0.00
6	GG-6	3	42	10	0.00
7	GG-7	20	552	16	28.50
8	GG-8	0	0	0	7.60
9	GG-9	7	122	0	0.00
10	GG-10	6	141	3	5.43
11	GG-11	0	0	2	0.00
12	GG-12	26	603	29	12.44
13	JZ-1	0	0	0	34.88
14	JZ-2	6	89	20	12.71
15	JZ-3	0	0	10	11.76
16	JZ-4	0	0	0	0.00
17	JZ-5	7	136	0	0.00
18	JZ-6	0	0	0	17.97
19	JZ-7	10	236	10	13.45
20	JZ-8	19	489	25	12.50
21	JG-1	10	263	6	40.74
22	JG-2	0	0	0	17.66
23	JG-3	0	0	0	15.22
24	JG-4	14	342	7	36.66
25	JG-5	0	0	0	3.49
26	JG-6	0	0	13	11.17
27	JG-7	20	485	9	46.13
28	JG-8	11	242	0	29.31
29	JG-9	0	0	3	3.11
30	JG-10	0	0	0	0
31	JG-11	5	103	0	8.33
32	JG-12	0	0	0	19.57
33	JG-13	32	635	0	26.45
34	JG-14	15	326	0	5.98

Phosphate and Zinc solubilisation by wheat rhizospheric bacteria: Quantitative estimation of P-solubilizing activity was done in Pikovskaya's medium (Sherathia et al. 2016). Phosphorus is the second most important nutrient, next to nitrogen (N) required for growth of plants. A greater portion of phosphorus in soil is in the form of insoluble phosphates and cannot be used directly by the plants (Pradhan et al. 2006). In the above study, isolates were found to give clear zone on Pikovskaya agar containing insoluble mineral phosphate such as tri-calcium phosphate (Table 3). More or less this isolates were able to utilize zinc also. Several researchers proved that the phosphate solubilizers are more around the rhizosphere in comparison to bulk soil.

Twenty bacteria were able to give form clear zone on Pikovskaya agar plates after 7 days of incubation. All twenty isolates were quantified for phosphate solubilisation in tri-calcium phosphate liquid media. From the all isolates JG-13 solubilize maximum inorganic phosphate JG-13 (635 µgml–1) followed by GG-2 (603 µgml–1). Another were also able to solubilized good amount of inorganic phosphate. Zinc solubilisation ability of the bacterial strains was evaluated by determining the zone of solubilisation of zinc (ZnO_2). About 31% of total isolates were show more than 10mm zone around colony and 40% were not solubilize zinc. GG-12 shown maximum zinc solubilisation (29mm) Medium, JZ-8 gives 25mm zone on ZnO_2 medium. All 34 isolates solubilize zinc on ZnO_2 supplemented medium (Table 3)

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Isolation, Screening and Identification of Lactic Acid Bacteria from Human Milk

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ABSTRACT

The present study shows the successful isolation and identification of lactic acid bacteria from mother's milk. A total of three lactic acid bacteria designated as M2P1, S1 and M2L2 were isolated on MRS medium. Morphological analysis revealed that M2P1 produces small, creamy white, entire, convex and opaque colonies, S1 produces small, off white, entire, raised and opaque colonies, while M2L2 produces medium, creamy white, round shaped, raised and opaque colonies. Microscopic observation reveled that all the isolates are Gram-positive. To confirm the molecular identity of isolates, 16S rRNA sequencing was performed. The isolates M2P1, S1 and M2L2 were identified as *Lactobacillus fermentum*, *Lactobacillus oris* and *Lactobacillus fermentum*, respectively. Further characterization of all three isolates to check their potential as probiotics is under progress

KEY WORDS: ISOLATION; LACTIC ACID BACTERIA; MOTHER'S MILK; SCREENING.

INTRODUCTION

Milk is a prime source of nutrition for young mammals. It is considered as a complete food with all the nutrients. Colostrum is early-lactation milk which carries the mother's antibodies to the baby and reduces the risk of many diseases in the baby. Fermentation of milk is the first technique developed by humans for food preservation and it has played many important roles in nutrition. Lactic acid bacteria (LAB) are non-taxonomic group of Gram-positive, non-spore forming, fastidious facultative anaerobes having low mol% G+C content (Zhu. et al., 2009; Taguchi et al., 2008; Mercenier et al., 2000). The genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* are considered as food associated LAB (Makarova et al., 2006). They require certain specific nutrients such as carbohydrates, amino acids, peptides, vitamins etc for their growth, (Dipak, & Sheela, 2015, Huidrom & Sharma, 2018, Adams & Gutiérrez, 2019 and Talashi & Sharma, 2019).

LAB have ability to survive when they passed through the gastrointestinal tract and under acidic conditions. Apart from that they are known as 'generally regarded as safe (GRAS)' lactic acid producers (Bilkova et al, 2008). Above properties make them suitable candidate for their use in various fermentation, food and pharmaceutical processes. Especially, in food industries, LAB is used to enhance the flavor and texture of the food in addition to preserve the fermented food. LAB includes various Lactobacillus species which are naturally indigenous

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microflora of the fermented milk products. In recent time interest of using various *Lactobacillus* species as probiotics (according to FAO report probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host) is increased because they are resistant to antibiotics. In fact, probiotics have been used for as long as people started the use of fermented foods (Pangallo et al., 2008, Shin et al., 2019).

Probiotic bacteria show positive affect in the individual by enhancing the properties of the indigenous microflora and maintaining microintestinal balance. It also competes with disease causing bacteria for nutrients absorption and villi attachment sites. Use of probiotics have reduced symptoms of lactose intolerance, improved immune function, cholesterol lowering function, antimutagenic activity and treatment of diarrhea (Shu et al. 2017, Mahmoudi et al., 2018 and Lockyer and Stanner, 2019).

However, in 20th century, Elie Metchnikoff proposed that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract. He suggested that Lactobacillus species are important for human health and longevity, and thus, he promoted the use of yogurt and other fermented foods as healthy diet (Metchnikoff, 2004). LAB of human origin is more suitable to be used as a source of probiotics because they are adapted to the normal conditions prevailing in the gastrointestinal tract and therefore they are more competitive than others. In addition to that some *Lactobacillus* species have shown inhibitory action against common human pathogens. *Lactobacillus* species are also a producer of bacteriocin which has therapeutic importance as well as acting as a good bio-preservative of food products (Mobarez et al., 2008). Looking towards these many benefits there is urgent need to develop a potential LAB culture having ability to act as a source of probiotics.By considering the above values in mind, the present study was designed for the isolation and screening of potential lactic acid bacteria from mother's milk sample. After the isolation, a representative lactic acid bacterial culture was identified through 16S rRNA sequencing.

MATERIAL AND METHODS

Mother's milk is a rich source of health beneficial microflora. So, in the present research mother milk was selected to isolate various strains of Lactic acid bacteria. Before isolation the milk sample was stored at low refrigerated temperature i.e. -4°C to retain the normal milk flora and avoid contamination. Primary screening of lactic acid bacteria from mother's milk was performed by the enrichment of milk flora in de Man Rogosa Sharpe broth. For the enrichment of milk flora 1 mL of the milk sample was added into 9 mL of de Man Rogosa Sharpe (MRS) broth, and it was incubated at 37°C for 48h. For the secondary screening of lactic acid bacteria the enriched MRS broth was serially diluted, and plated on different MRS agar plates followed by the incubation of all plates at 37°C until visible colonies of bacteria were observed. Selected bacterial colonies which grew on MRS plates were streaked on fresh MRS agar plates to obtain pure cultures. The pure cultures were maintained in 15% skimmed milk at -4°C. To retain the culture in active state, they were regularly transferred to the freshly prepared MRS medium and then stored as mentioned above. The purity of isolated bacterial cultures was primarily done through morphological and microscopic observations.

The bacterial cultures were identified through 16S rRNA sequencing according to the protocol shown by (Sambrook, 2001; Ikram et al., 2016) with slight modifications. In brief, 1.0 mL of 12h grown bacterial culture in MRS broth was centrifuged at 10,000 x g for 2 min and then, the cell pellet was washed with the sterile deionized water. Later, the cell pellet was suspended in 0.2 ml of 10 mM Tris-HCl, PH 8.0, 10 mM EDTA, pH 8.0, 20 mM glucose, 50 mM Sodium chloride mixture containing lysozyme and incubated at 37°C for 30 min. Following to that 25 µL of proteinase K (20 mg/ml) and 100 µL of 10 % SDS were added to it and the mixture was incubated for 1 h at 60°C. After that 300 µl of 3M sodium acetate (pH5.2) was added to the mixture and kept on ice for 5 min to allow the precipitation of nucleic acid.

The cell debris was removed by centrifugation at 10,000×g for 15 min at 4°C, and then the supernatant was collected in separate sterile micro-centrifuge tube. In the supernatant an equal volumes of phenol-chloroform-isoamylalcohol (25:24:1 v/v) was added with gentle shaking. Later on the preparation was centrifuged at 15000×g for 10 min to separate out two phases. DNA containing upper aqueous layer was carefully transferred in sterile micro-centrifuge tube. In next step, DNA was precipitated by adding two volumes of ice-cold ethanol with gentle shaking, and then it was incubated at 4°C for 1 h. The mixture was again centrifuged at 15000×g at 4°C for 10 min. The pellet was then rinsed with 70% (v/v) ice-cold ethanol and dried it in air. Then the dried mass was dissolved in 50µL of deionized water. Following to that 10µL of 10 mg/mL RNase was added into the preparation and it was incubated at 37°C for 15 min. Finally, the sample was kept at 4°C for PCR reaction and at -15°C for long term usage.

16S rRNA gene sequencing using PCR: Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The 16s rRNA sequence was blast using NCBI blast similarity search tool. Thephylogeny analysis of query sequence with the closely related sequence of blast resultswas performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes

alignmentnoise) (Talavera and Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs usingsimulated data, while being one order of magnitude faster. PhyML was shown to be atleast as accurate as other existing phylogeny programs using simulated data, while beingone order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper, 2008; Reis et al., 2016).

Table 1. Colony characteristics of lactic acid bacteria

Colony character	M2P1	S1	M2L2
Size	Small	Small	Medium
Shape	Round	Round	Round
Margin	Entire	Entire	Entire
Texture	Smooth	Smooth	Smooth
Elevation	Convex	Raised	Raised
Consistency	Moist	Moist	Moist
Pigmentation	Creamy white	Off white	Creamy white
Opacity	Opaque	Opaque	Opaque

RESULTS AND DISCUSSION

After extensive screening a total of three different lactic acid bacteria were isolated on MRS agar plates. They were designated as M2P1, S1 and M2L2. On MRS agar plate isolate M2P1 produced small, creamy white, entire, convex and opaque colonies, S1 produced small, off white, entire, raised and opaque colonies, while M2L2 produced medium, creamy white, round shaped, raised and opaque colonies. Detailed colony characteristics of all three isolates are shown in Table 1. During their studies Baradaran et al. (2012) successfully selected three lactic acid bacteria designated as K1, K3 and K5 out of total 12 different isolates which were isolated from the leaves of Malaysian herb Kesum. In their study all three isolates were observed to produce colonies with round, convex and creamy white appearances which were similar to the isolates of our study. In another study, Chowdhury et al., (2012) isolated four different lactic acid bacteria from buffalo yogurt.

Figure 1: Phylogenetic analysis of isolated lactic acid bacteria.
Figure 1 (A): Based on phylogenetic analysis an isolate M2P1 was identified as Lactobacillus fermentum.
Figure 1 (B): Based on phylogenetic analysis an isolate S1 was identified as Lactobacillus oris.
Figure 1 (C): Based on phylogenetic analysis an isolate M2L2 was identified as *Lactobacillus fermentum*.

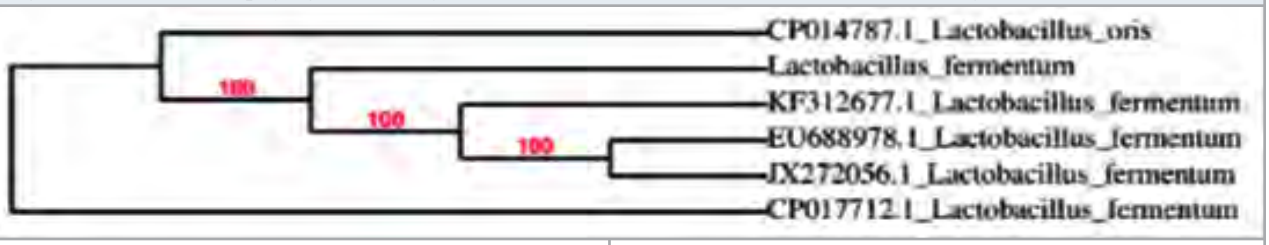

Fig. 1 (A)

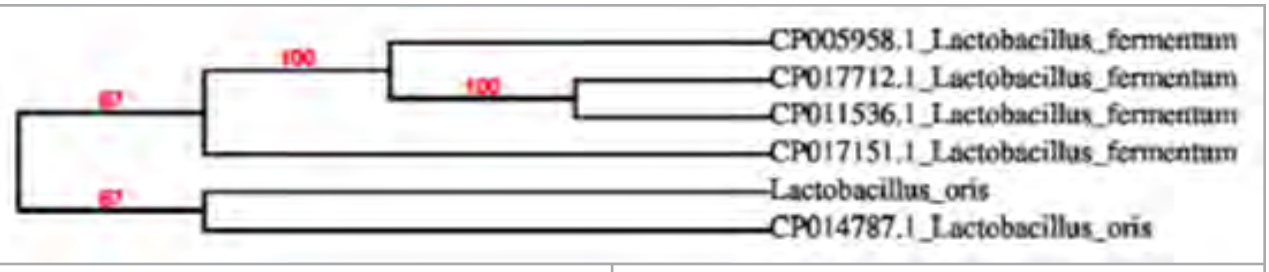

Fig. 1 (B)

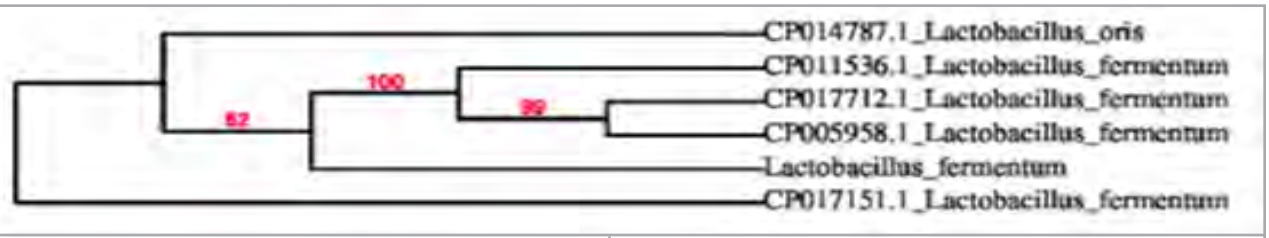

Fig. 1 (C)

Microscopic observation of lactic acid bacteria: All three isolates namely, M2P1, S1 and M2L2 were studied for Gram's reaction followed by microscopic observation. During microscopic observation M2P1, S1 and M2L2 observed as Gram-positive, rod shaped non-spore forming bacteria which indicated that they have characteristics of Lactic acid bacteria. Baradaran et al. (2012) shown that lactic acid bacteria designated as K1, K3 and K5 isolated from the leaves of Kesum were Gram-positive and catalase negative upon microscopic and biochemical characterization, respectively.

Identification of Lactic acid bacteria: Ben Amor et al. (2007) suggested that physicochemical and biochemical analysis based identification of lactic acid bacteria is often unreliable because many different bacterial species exhibit similar morphological and nutritional requirements. So, in our study, after microscopic observation all three isolates were identified through 16S rRNA gene sequencing. The 16s rDNA sequences were aligned through BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Tilhaun et al., 2018).

Later, sequences of all three isolates were compared with the published sequences of 16S rDNA gene of different lactic acid bacteria strains available at NCBI database. Based on 16S rRNA gene sequencing results bacteria designated as M2P1, S1 and M2L2 were identified as *Lactobacillus fermentum, Lactobacillus oris* and *Lactobacillus fermentum*, respectively. Results of phylogenetic analysis of all three isolates are shown in Fig. 1. On the basis of 16S rRNA sequencing results lactic acid bacteria K1, K3 and K5 were identified as *Lactococcus lactis, Pediococcus pentosaceus* and *Lactobacillus curvatus,* respectively (Baradaran et al., 2012).

Several parameters have to be kept in mind while using the probiotics. These include type of prebiotics, concentrations, presence of other components, probiotic microorganism to be used, incubation time and fate of metabolism of prebiotics (Sharma et al., 2017; Singla and Chakkaravarthi, 2017).

CONCLUSION

The present research work highlights successful isolation and identification of lactic acid bacteria from mother's milk. In the present study a total of three different bacteria namely, M2P1, S1 and M2L2 were isolated on MRS agar medium. All isolates were identified as Gram-positive bacteria through microscopic observations. Finally, on the basis of 16S rRNA sequencing results isolates M2P1, S1 and M2L2 were identified as *Lactobacillus fermentum, Lactobacillus oris* and *Lactobacillus fermentum*, respectively. Further characterization to check the potential of isolated lactic acid bacteria as probiotics is under progress.

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Computational Characterization of Human Vascular Endothelial Growth Factor Proteins

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ABSTRACT

We present an in silico learning method to discriminate the pathologically important vascular endothelial growth factor (VEGF) protein through proteomic tools. Primary structure analysis showed most of the VEGF human proteins are rich in hydrophilic residues. The average molecular weight of VEGF human proteins calculated as 76244 Dalton. Grand Average hydropathy (GRAVY) index of all the VEGF human proteins are ranging from -0.2 to 0.1 except the protein O14495 which has comparatively high GRAVY value. Antigenic sites for all the proteins are recognized as C, Y, L, V, P, and K residues-EMBOSS antigenic program. The computed pI value indicates that most of the proteins are basic (pI>7) in nature. SOPM and SOPMA program shows that all the VEGF human proteins are different in secondary structural content. The presence of disulfide bridges are identified by CYS_REC tool, also visualized through 3D structure. The SOSUI server classifies the proteins P15692, P49765 and O43915 as soluble proteins and other proteins as transmembrane proteins. The projected technique provides more accurate information about 3D structure, geometry, cystines involved in the disulfide bond. It would provide biological insights about protein hubs and their roles in interaction networks.

KEY WORDS: VEGF PROTEINS, PROTEOMIC TOOLS, HOMOLOGY MODELING, TRANSMEMBRANE PROTEINS, DISULFIDE BRIDGES.

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INTRODUCTION

Over the past few decade the occurrence of chronic diseases was increased due to change in life style, exposed to carcinogens that results in endothelial damage and failure to repair these injuries are the main cause of vascular injuries. Endothelial damage led to development of pulmonary disease, cancer and cardiovascular diseases. In all such chronic diseases, angiogenesis growth factor plays a predominant role. It is a physiologic process it involves formation of new blood vessels from pre-existing microvasculature (Goldmann, 1907, Okada, 2014; Yang, et al; 2017; Hulse, 2017).

The importance of this process widely known to be essential for growth of developing organs, wound healing, ovulation and pregnancy (Alitalo & Carmeliet, 2002). Ocular conditions related to angiogenesis also the leading cause of irreversible vision loss (Penna et al., 2008). Vascular endothelial growth factor (VEGF) - a dimeric glycoprotein and platelet derived growth factor (PDGF) have critical role in tumor-associated angiogenesis (Rivera & Bergers, 2015), cardiovascular diseases (Anthony Ware & Michael Simons, 1997) and principal causes of blindness (Penna et al., 2008). Where, the increase in vascular permeability to plasma proteins, induction of endothelial cell division and migration are widely reported in tumor angiogenesis (Dvorak, 2002; Hicklin & Ellis, 2005). About five glycoproteins are considered as the family of VEGF (VEGF-A, VEGF-B, VEGF-C, VEGF-D/F14F) and placental growth factor (PlGF) (Lee, Ellis & Daniel, 2008; Dvorak, 2002; Hicklin & Ellis, 2005).

VEGF-A isoform known to play a dominant role in solid tumors. The activation of VEGF ligands happens through binding to type III receptor tyrosine kinases, designated VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4) (Waltenberger et al., 1994; Hiratsuka et al., 1998). The functional diversity of each tyrosine kinases depends on binding to specific receptors. Where, the expression of VEGFR1 is on vasculature and the exact role in vascular endothelium remain to be elucidated. Both VEGFR1 and VEGFR2 selectively expressed on vascular endothelial cells. In case of VEGFR3 has fundamental character in lymphatic system and embryogenesis (Laakkonen et al., 2007). In addition to these receptor tyrosine kinases (RTKs), VEGF interacts with a family of co-receptors, the neuropilins. The neuropilin receptor (NRP-1) expressed on vascular endothelium and neurons.

Table 1. Human VEGF protein sequences retrieved from Swiss-Prot Knowledgebase

Accession Number	Entry name	Protein Names	Gene names	Length
O14495	LPP3_HUMAN	Vascular endothelial growth factor and type I collagen-inducible protein (VCIP)	PPAP2B (LPP3)	311
O14786	NRP1_HUMAN	Vascular endothelial cell growth factor 165 receptor	NRP1 (NRP) (VEGF165R)	923
O60462	NRP2_HUMAN	Vascular endothelial cell growth factor 165 receptor 2	NRP2 (VEGF165R2)	931
P58294	PROK1_HUMAN	Endocrine-gland-derived vascular endothelial growth factor (EG-VEGF)	PROK1 (UNQ600/PRO1186)	105
P15692	VEGFA_HUMAN	Vascular endothelial growth factor A (VEGF-A) (Vascular permeability factor) (VPF)	VEGFA (VEGF)	232
P49765	VEGFB_HUMAN	Vascular endothelial growth factor B (VEGF-B) (VEGF-related factor) (VRF)	VEGFB (VRF)	207
P49767	VEGFC_HUMAN	Vascular endothelial growth factor C (VEGF-C) (Vascular endothelial growth factor-related protein) (VRP)	VEGFC	419
O43915	VEGFD_HUMAN	Vascular endothelial growth factor D (VEGF-D)	FIGF (VEGFD)	354
P17948	VGFR1_HUMAN	Vascular endothelial growth factor receptor 1 (VEGFR-1) (Vascular permeability factor receptor)	FLT1 (FLT) (FRT)	1338
P35968	VGFR2_HUMAN	Vascular endothelial growth factor receptor 2 (VEGFR-2)	KDR (FLK1)	1356
P35916	VGFR3_HUMAN	Vascular endothelial growth factor receptor 3 (VEGFR-3)	FLT4	1298

VEGF-B (also called VEGF-related factor/VRF) is expressed more abundant in the heart and in the skeletal muscle cells (Olofsson et al., 1998). VEGF-C, it produced as a single propeptide, the N-terminal and C-terminal ends are proteolytically processed to generate a protein with high affinity for VEGFR-2 and VEGFR-3. VEGF-C induces mitogenesis and migration of EC (Kukk et al., 1996). In this regard, identifying specific regions is fundamental for scientific disciplines that require detailed characterization of proteins to explain essential biological systems. The biochemical and physicochemical characterization of VEGF proteins have not been done so far. By characterizing the VEGF proteins, we can impact our understanding of the relationship between protein flexibility and function. From this investigation, we report for the computational analysis and characterization of 11 Human VEGF proteins using proteomic tools and online prediction servers.

Proteomic tools and methods: Protein sequence retrieval and selection: Vascular Endothelial Growth Factor (VEGF) human proteins were retrieved from the UniProtKB/ Swiss-Prot release 57.0 (http://www.expasy.org/sprot) protein sequence database. The Swiss-Prot database was scanned for the keyword "vascular endothelial growth factor, Homo sapiens (Human) [9606]" through the search interface available in the Swiss-Prot database. The search yielded 65 proteins; all these protein sequences were downloaded in FASTA format (Lipman & Pearson, 1985). These 65 protein sequences were matched with each other using the online server "Blast 2 sequences" (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and finally 11 dissimilar protein sequences were selected for analysis. The details of vascular endothelial growth factor human proteins selected for analysis are tabulated in Table 1.

Table 2. Amino acid composition (in %) of VEGF proteins

Amino	Accession Numbers										
Acids	O14495	O14786	O60462	P58294	P15692	P49765	P49767	O43915	P17948	P35968	P35916
Ala	7	4	5	5	3	14	7	4	5	5	7
Cys	4	2	3	10	8	4	9	8	2	2	3
Asp	4	6	6	4	3	4	4	4	5	5	5
Glu	3	7	7	4	7	3	7	8	6	8	7
Phe	6	4	4	4	3	0	4	4	4	3	3
Gly	6	9	8	9	6	6	5	3	5	6	6
His	3	3	2	4	5	3	2	4	2	2	3
Ile	8	7	6	4	3	2	2	4	6	6	4
Lys	5	6	4	4	9	3	6	7	8	6	4
Leu	10	7	8	10	7	8	7	7	9	9	11
Met	3	2	2	4	3	2	2	2	2	2	2
Asn	5	5	4	3	3	0	5	3	4	4	3
Pro	5	6	6	6	6	13	6	7	5	5	5
Gln	3	3	4	1	6	6	5	4	3	4	4
Arg	6	4	5	10	8	6	6	7	5	5	6
Ser	8	8	9	6	6	8	8	10	10	8	8
Thr	5	6	6	6	3	6	6	6	7	7	5
Val	5	6	5	6	5	8	4	5	6	8	7
Trp	0	2	2	1	2	1	1	1	1	1	2
Tyr	5	4	4	1	3	1	3	1	4	4	3

Proteomic tools and servers: The amino acid composition of human vascular endothelial growth factor proteins were computed using the tool BioEdit 5.0.9 (Hall, 1999). Percentages of hydrophobic and hydrophilic residues were computed using the primary structural data. The physico-chemical parameters such as theoretical isoelectric point (pI), molecular weight, extinction coefficient (Gill & Von Hippel, 1989), half-life (Bachmair et al., 1986; Gonda et al., 1989; Tobias et al., 1991; Ciechanover & Schwartz, 1989; Varshavsky, 1997), instability index (Guruprasad et al., 1990), aliphatic index (Ikai, 1980), antigenic site and grand average of hydropathy (GRAVY) (Kyte & Doolittle, 1982) values were computed using the Expasy's ProtParam prediction server (Gasteiger et al., 2005).

The correlation between the number of acidic and basic residues is calculated on this server. The SOPM and SOPMA tools were used for the secondary structure prediction (Geourjon, Deleage, 1994 & Geourjon, Deleage, 1995). Secondary Structural Content Prediction (http://coot.embl.de/SSCP/) server is used for the computation of percentages of α-helical, β-strand (Eisenhaber et al., 1996). The SOSUI server (Takatsugu Hirokawa et al., 1998) allowed the identification of transmembrane regions in VEGF human proteins. The tool BioEdit was used to compute the Kyte and Dolittle mean hydrophobicity profile of the transmembrane regions (Hall, 1999). Multiple sequence alignment of transmembrane regions computed using the MSA tool

to generate the sequence logo of transmembrane regions (Lipman et al., 1989; Schneider & Stephens 1990). The ScanProsite tool was used to identify the profiles with a high probability of occurrence in the PROSITE database (Edouard de Castro et al., 2006; Falquet et al., 2002).

Prediction of disulfide bridges-SS bound cysteines: A disulfide bridge in VEGF proteins is predicted by two different methods. The first method defines the presence of disulfide bonds (SS) and total number of cysteines using the protein sequences (FASTA format) submitted in the CYS_REC tool. In a second method SS bonds are identified through visualization of three-dimensional (3D) structure of proteins; the 3D structure of five proteins was predicted by homology modeling using the Esypred server and visualized in the RasMol tool.

Structure analysis and validation: For comparative modeling, the five protein sequences are selected based on disulfide bridges predicted in CYS_REC tool. The similar 3D structures were predicted in Protein Data Bank (www.rscb.org) through BLASTP analysis with the expectation value of 0.01 for the O60462, P15692, P49765 & P58294 proteins. For, the other protein (O14495) a similar 3D structure was predicted with expectation value of 10. The modeled 3D structures were validated using servers Rampage (Ramachandran plot), ProQ (Protein Quality server), and ProSA (Protein Structure Analysis) (Lovell et al., 2002; Cristobal et al., 2001; Wiederstein & Sippl, 2007).

RESULTS AND DISCUSSION

Primary structure analysis suggest that most of the studied human VEGF proteins are hydrophilic in nature (Table 2 and Table 3) except O14495 (VEGF type I collagen-inducible) and P58294 (Endocrine-gland-derived VEGF) protein. The amino acids asparagines, lysine, aspartic acid, glutamine, histidine, arginine and glutamic acid are responsible for hydrophilic property. The average molecular weight of VEGF human proteins calculated is 76244 Dalton. Hydrophilic molecules are polar charged residues and capable of hydrogen bonding, dissolve more readily in water than oil. As reported earlier, the beneficial effect of hydrophilic drugs (Statin & Provastatin) in decreasing hemodynamically compromising rejection, attenuation of rise in lipid profiles; also in favor of a reduction in allograft coronary artery disease (Mandeep R. Mehra et al., 2004) [34]. Followed by, the antigenic sites (Asite) for all the VEGF proteins are identified through EMBOSS antigenic program (table 2). It attempts to understand the role of protein structure and thermodynamics of protein interactions during pathological conditions through binding at specific epitopes.

Table 3. Hydrophobic and hydrophilic residues content

Accession Number	Percentage of hydrophobic residues	Percentage of hydrophilic residues	Net hydrophilic residues content
O14495	54.7	45.3	Low
O14786	47.5	52.5	High
O60462	46.2	53.8	High
P58294	53.3	46.7	Low
P15692	43.5	56.5	High
P49765	46.4	53.6	High
P49767	45.3	54.7	High
O43915	40.4	59.6	High
P17948	45.2	54.8	High
P35968	46.8	53.2	High
P35916	48.5	51.5	High

Table 4. Physiochemical parameters computed using BioEdit, EMBOSS, and Expasy's ProtParam tool

Accession No.	Length	M.Wt.	pI	-R	+R	EC	HL (hours)	II	AI	GRAVY	A Site
O14495	311	35116	9.31	21	35	29965	30	39.49	94.41	0.104	268C
O14786	923	103134	5.58	117	94	156995	30	35.56	72.54	-0.441	880Y
O60462	931	104858	5.04	126	87	175110	30	47.57	72.59	-0.47	889Y
P58294	105	11714	9.01	8	15	7615	30	40.64	77.05	-0.038	14V
P15692	232	27042	9.21	24	40	39055	30	52.3	57.54	-0.783	15L
P49765	207	21601	8.46	16	19	14480	30	59.27	75.51	-0.232	55P
P49767	419	46883	7.77	46	49	47755	30	58.1	57.09	-0.496	159V
O43915	354	40444	8.16	43	48	36825	30	56.81	61.67	-0.556	274K
P17948	1338	150768	8.66	151	169	164500	30	46.14	82.19	-0.35	12C
P35968	1356	151526	5.6	172	145	178020	30	45.34	87.21	-0.273	415L
P35916	1298	145598	5.89	163	139	214235	30	49.19	85.79	-0.282	159L

M.Wt. - Molecular weight; pI - Isoelectric point; R - residues; EC - Extinction coefficient at 280nm; II - Instability Index; AI - Aliphatic Index; GRAVY - Grand Average Hydropathy; A Site - Antigenic Site.

Table 5. Computed theoretical isoelectric point (pI) and number of acidic and basic amino acids

Accession Number	pI	No. of Basic Amino acids	No. of Acidic Amino acids	Property
O14495	9.31	35	21	Basic
O14786	5.58	94	117	Acidic
O60462	5.04	87	126	Acidic
P58294	9.01	15	8	Basic
P15692	9.21	40	24	Basic
P49765	8.46	19	16	Basic
P49767	7.77	49	46	Basic
O43915	8.16	48	43	Basic
P17948	8.66	169	151	Basic
P35968	5.6	145	172	Acidic
P35916	5.89	139	163	Acidic

Expasy's ProtParam computes the extinction coefficient (EC) for a range of (276, 278, 280 and 282nm) wavelength. The EC value at 280nm is favoured because proteins absorb strongly while, the other substances commonly in protein solutions do not. Extinction Coefficient (EC) of VEGF human proteins at 280nm is ranging from 7615 to 214235 M-1 cm-1 with respect to the concentration of Cys, Trp and Tyr (Table 4). Expasy's ProtParam classifies most of the VEGF human proteins as unstable on the basis of Instability index (II>40) and the two proteins (O14495, O14786) as stable (II<40) proteins in the room temperature. The aliphatic index (AI) that is defined as the relative volume of a protein occupied by aliphatic side chains (Ala, Val, Ile and Leu) is regarded as a positive factor for increase of thermal stability of globular proteins is low (57-94) for all of the VEGF human proteins and it infers that the VEGF proteins may become unstable at high temperature.

Table 6. Percentage of residues forming alpha, beta, and coil structures

Accession Number	Alpha Helix	Extended Strand	Beta Turn	Random Coil	Class
O14495	46.6	12.5	3.2	37.6	Mixed
O14786	16.1	24.9	7.4	51.6	Beta
O60462	14.5	24.0	7.3	54.2	Mixed
P58294	9.5	17.1	5.7	67.6	Beta
P15692	29.3	15.1	3.9	51.7	Alpha
P49765	28.0	10.1	5.8	56.0	Alpha
P49767	33.9	7.2	4.3	54.7	Alpha
O43915	30.5	9.0	3.7	56.8	Mixed
P17948	21.5	23.1	4.9	50.5	Mixed
P35968	22.6	23.8	6.1	47.6	Mixed
P35916	22.4	23.0	5.9	48.6	Mixed

Table 7. Transmembrane regions identified using SOSUI server

Accession Number	Transmembrane region	Type	Length
O14495	VLLICLDLFC LFMAGLPFLIIET	Primary	23
	NDAVLCAVGIV IAILAIITGEFY	Primary	23
	IQNPYVAALYKQ VGCFLFGCAIS	Secondary	23
O14786	ERGLPLLCAVLA LVLAPAGAFRN	Primary	23
	ILITIIAMSALGVL LGAVCGVVL	Primary	23
O60462	ITIIAMSSLGV LLGATCAGLLLY	Primary	23
	MDMFPLTW VFLALYFSRHQ	Secondary	19
P58294	GATRVSIMLL LVTVSDCAVITGA	Primary	23
	RDVQCGAGTC CAISLWLRGLRMC	Secondary	23
P15692	Soluble		
P49765	Soluble		
P49767	MHLLGFFSVA CSLLAAALLPGP	Primary	23
O43915	Soluble		
P17948	SYWDTGVLL CALLSCLLLTGSSS	Primary	23
	ELITLTCTC VAATLFWLLLTLFI	Primary	23
P35968	IIILVGTAVI AMFFWLLLVIILR	Primary	23
P35916	IVILVGTGVIA VFFWVLLLLIFC	Primary	23
	GAALCLRLWLCL GLLDGLVSGYS	Secondary	23

Grand Average hydropathy (GRAVY) index of all the VEGF human proteins are ranging from -0.2 to 0.1 and this indicates that all these proteins may interact equally and easily with water except the protein O14495 which has comparatively high GRAVY value. Isoelectric point (pI) is the pH at which the surface of protein is covered with charge but net charge of the protein is zero. At pI proteins are stable and compact. The computed pI value indicates that most of the proteins are basic (pI>7) in nature (Table 5). The number of basic and acidic amino acids in each VEGF human proteins correlates well with the pI computed. The computed isoelectric point (pI) will be useful for developing buffer systems for purification by Isoelectric focusing method. The computed protein concentration and extinction coefficients help in the quantitative study of protein-protein and protein-ligand interactions in solution.

3.3. Secondary structure analysis: The secondary structure was predicted for all the proteins using SOPM and SOPMA tools; it shows that all the VEGF human proteins have different secondary structural content. The computed percentage of residues forming α-helices, β-strands and coils are shown in Table 6.

Table 8. Organization of profiles identified in the VEGF protein sequences

Accession	Identified profiles	Domains
O14495	No hit	
O14786		CUB domain Coagulation factors 5/8 type C domain
O60462		MAM domain CUB domain Coagulation factors 5/8 type C domain MAM domain
P58294	No hit	
P15692		Platelet-derived growth factor
P49765		Platelet-derived growth factor
P49767		Platelet-derived growth factor
O43915		Platelet-derived growth factor
P17948		Ig-like domain Protein kinase domain
P35968		Ig-like domain Protein kinase domain
P35916		Ig-like domain Protein kinase domain

The SOSUI server classifies the proteins P15692, P49765 and O43915 as soluble proteins and other proteins as transmembrane proteins. The various primary and secondary transmembrane regions identified by SOSUI server were shown in Table 7. The identified transmembrane regions were found to have more hydrophobic residues and it is well documented by the Kyte and Doolittle mean hydrophobicity profile (Fig.1) in which all the peaks are above the zero line. The sequence logo of transmembrane regions (generated from the multiple sequence alignment of transmembrane regions) is shown in Figure 2. The height of each letter in the sequence logo is proportional to the frequency of the amino acid at that position. The presence of more leucine amino acid in the transmembrane region is identified from sequence logo. Generally, the amino acid

Table 9. Presence of cysteine residues and disulphide bond patterns predicted by CYS_REC tool and visualized by RasMol in 3D protein structures.

Accession Number	PDB template	No. of Cysteines	Disulfide bridges CYS REC	RasMol
O14495	2AKC	11	Cys68-Cys162, Cys171-Cys181	Cys38-Cys43, Cys132-Cys181 Cys268-Cys269
O14786	Too large	22	Too large	---
O60462	2QQJ	24	Cys28-Cys277, Cys55-Cys927, Cys149-Cys646, Cys230-Cys883, Cys626-Cys892, Cys655-Cys902,	Cys277-Cys427, Cys434-Cys592
P58294	1IMT	11	Cys26-Cys38, Cys32-Cys96, Cys37-Cys78, Cys50-Cys86, Cys60-Cys80	Cys26-Cys38, Cys32-Cys50, Cys37-Cys78, Cys60-Cys86, Cys96-Cys80
P15692	3V2A	18	Cys52-Cys94, Cys77-Cys172, Cys83-Cys128, Cys86-Cys171, Cys87-Cys130, Cys184-Cys202, Cys187-Cys204, Cys206-Cys225, Cys213-Cys227	Cys52-Cys94 Cys83-Cys128 Cys87-Cys130
P49765	2VWE	8	Cys72-Cys81, Cys78-Cys122, Cys82-Cys124	Cys47-Cys89 Cys82-Cys124 Cys78-Cys122
P49767	Too large	38	Too large	---
O43915	Too large	30	Too large	---
P17948	Too large	33	Too large	---
P35968	Too large	33	Too large	---
P35916	Too large	35	Too large	---

Table 10. Validation of modeled VEGF proteins- Ramachandran Plot, ProQ and ProSA.

Accession Number	Rampage Residues in Favored region (%)	ProQ		ProSA Z Score	Protein quality
		LGscore	Max Sub		
O14495	88	0.96	0.059	-1.35	Fairly good model
O60462	95.9	4.740	0.292	-6.91	Extremely good model
P58294	96.1	0.968	0.119	-5.08	Fairly good model
P15692	97.8	1.210	0.133	-4.44	
P49765	96.8	2.083	0.239	-3	Very good model

Figure 1: Kyte and Doolittle mean hydrophobicity profile of all the transmembrane regions.

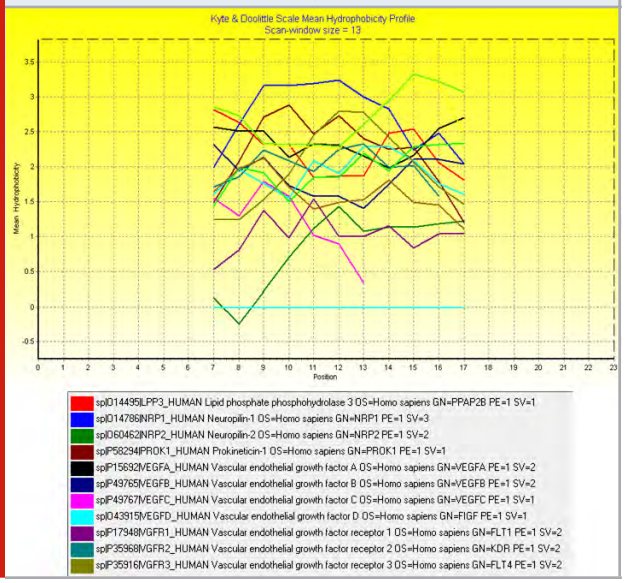


Figure 3: Homology modeling-3D structure of VEGF proteins (Ribbon) and Cysteines (SS) bonds (ball and stick) viewed by RasMol tool. A) O60462; B) P58294; C) P15692; D) P49765

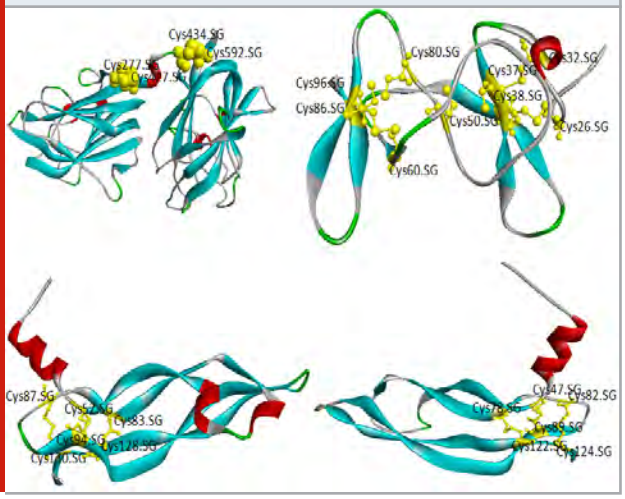


Figure 2: Sequence logo representation of the (generated using the multiple sequence alignment) transmembrane regions.

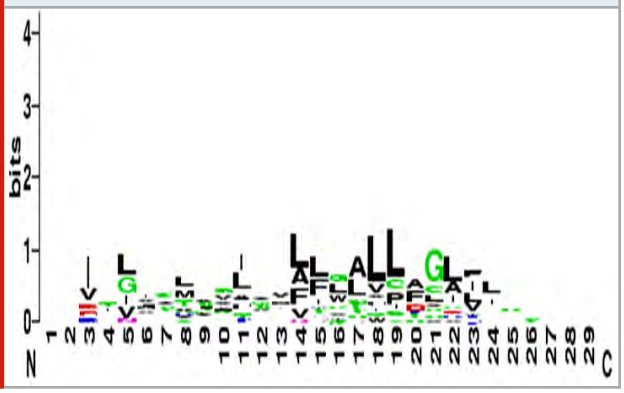
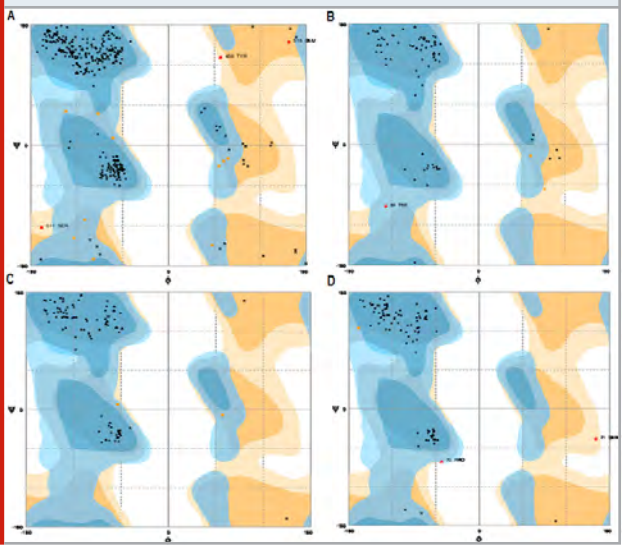


Figure 4: Validation of 3D structure of VEGF proteins- Ramachandran plot. A) O60462; B) P58294; C) P15692; D) P49765.

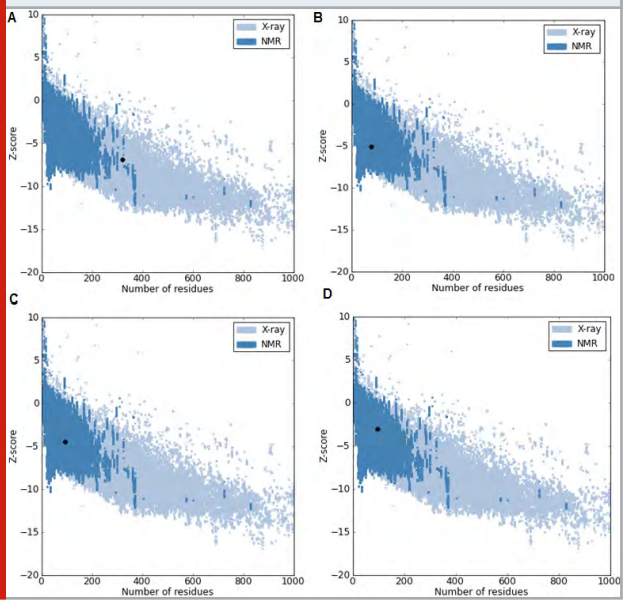


Leucine has the capacity to stimulate protein synthesis in muscles (Etzel, 2004).

The Scanprosite server identified different profiles in VEGF proteins except the protein O14495 (Vascular

endothelial growth factor and type I collagen-inducible protein [VCIP]), and P58294 (Endocrine-gland-derived VEGF protein). The organization of all the identified profiles is given in table 8.

Figure 5: Validation of 3D structure of VEGF proteins-ProQ. A) O60462; B) P58294; C) P15692; D) P49765.



The location of disulfide bridges in all the proteins are screened using CYS_REC tool. VEGF proteins showed cysteine residues (~1-35) and the positions of most probable SS bond patterns are predicted. Disulfide bridges identified only in few proteins, the selection of pairs are skipped due to too large in SS-bound cysteines as shown in table 9. In another method, the cysteines and the SS bond positions are identified in 3D structure of proteins. Where, few proteins did not have probable SS bond patterns as visualized in 3D structure. There are few unpaired cysteines identified (Fig.3A,B,C & D).. The 3D structures of proteins are validated through Ramachandran plot, ProQ and ProSA, the score were within the acceptable limits (Table 10; Fig. 4 & 5) (Lovell et al., 2002; Cristobal et al., 2001; Wiederstein & Sippl, 2007). The profile of 3D VEGF protein structures match its own sequences with Z scores, it expresses backbone identical to that of the template. This means not only the positions of alpha carbons, but also phi and psi angles with secondary structure are identical to the template.

Prediction of disulfide bonds in these proteins able to show the stability and structure of proteins. It's a covalent bond between sulfur atoms that binds two peptide chains or different parts of individual peptide chain oxidized to create a stable R-S-S-R bond and is a structural determinant in many of the protein molecules. These are essential to antibodies in creating the cell-surface receptors for target cells, as well as being part of the surface receptors of cells.

CONCLUSION

Sequence-based approaches have proven to be very useful for functional prediction, entire map of protein complexes, architecture of network and their cell signaling factors for known active sites or binding regions. This information would be integrated in various experimental conditions so that overall signaling networks are characterized which gives unique characteristics on targeted protein-drug interactions. Hence, the data and concepts discussed here offer a sense of direction for harnessing the proteomic tools towards protein characterization. Such knowledge may then be channeled to the development of improved targets for biomedicine in the near future. The present work paves the way towards meaningful new areas in which technologies may be further exploited, especially using proteomic tools in order to advance innovate therapies and diagnostics.

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Cladistic Analysis and Comparative Account of Different Invasive Weeds and their Dominance Using Various Bioinformatics Tools

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ABSTRACT

Cassia uniflora Mill. non Spreng. and *Synedrella nodiflora* (L.) Gaertn. are invasive alien weeds on Deccan plateau. Both the weeds are spreading on agricultural and fallow lands at an alarming rate replacing many native and exotic species. Along with the detail studies of their morpho-physiological, metabolomic and chemical attributes, an attempt was made to explore their ecological survival and dominance, which is responsible for development of huge monothickets. Their invasion success probably is due to containment of similar ecological behaviour and evolutionary relatedness to potential plant invaders. In the present study we have extensively employed different tools of bioinformatics such as BLAST, FASTA, servers like SWISSMODEL, CLUSTAL OMEGA and software like MEGA-X, and carried out phylo-genomics and evolutionary cladistic analyses for protein structures of important enzymes such as Rubisco and Maturase –K. The selected weeds were compared using molecular data from gene sequences with each other and other co-dominant native and exotic species. The results revealed that all the species under focus shared larger part (80%) of MSAs (Multiple Sequence Alignments). The data indicated that *Cassia uniflora* and *Synedrella nodiflora* exhibited parallel resistance to the environmental stresses, similar evolutionary patterns and highlighted their dominance amongst different species of Cassia and respective genera of Asteraceae. Based on further phylogenetic studies it can be proposed that *C. auriculata* and Lactuca indica would be the future successful invaders on Deccan plateau. The present investigation based on the results of MSA, deep view analyses and phylogenomics of weeds may predict the changes in weed flora of Deccan pleateau due to environmental changes.

KEY WORDS: INVASIVE WEEDS, MATURASE-K, MEGA-X, PHYLOGENY, RUBISCO.

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INTRODUCTION

The alien species highly out-compete the native species or escape from adverse environmental conditions and dominate the community (MacDougall and Turkington 2005).Their diversity is controlled by population, ecosystem dynamics, disturbances, nutrient supply and climatic factors. The biotic restrictions also force them to skip from their previous habitat and start surviving in new habitats, helping in the process of invasion (Mack et al. 2000). Many a times these phyto-invasives become very aggressive due to production of some defensive chemicals (Carpenter and Cappuccino 2005). These invasions pose many ecological, economic and social problems. Because of this the studies on plant invasions and its mechanism and consequences of them on global biodiversity and ecosystem functioning are of urgent need. This is because slowly and gradually these invasives become aggressive and encroach cultivable lands and pose a great problem (Chauhan et al. 2017).

Cassia uniflora Mill. non Spreng. (family- Caesalpinaceae) is annual, erect herb with yellow flowers and clustered pods. It originated in tropical South America and now distributed worldwide. This invasive weed grows luxuriantly at many places (Almeida, 2003). *Synedrella nodiflora* (L.) Gaertn. (family- Asteraceae) originated in tropical America, is an annual, erect, dichotomously branched herb distributed all over India (Almeida, 2003). Their dominance is attributed to wide adaptability to diverse habitat, different morpho-physiological characters and defensive allelo-chemicals (Ghayal et al.2007a,b; 2009; 2013).

Limited research has been done on the allelopathic effect or phyto-toxicity of *Cassia uniflora* to other plants. There is a general temper of agreement now-a-days that invasive plants displace the local biodiversity through their harmful effects including allelopathy (Cronk and Fuller, 1995). Allelopathic effects may due to the presence of allelochemicals in *Cassia* and *Synedrella*, like different types of phenolic compounds, alkaloids, triterpenoides, essential oils and flavonoids, biocides, juvenile hormones, growth hormones. They may be interacting with various physiological processes (Chatterjee et. al. 2012). From current literature review, it revealed that little research has been done on distribution, evolutionary studies and impact of *Cassia uniflora* and *Synedrella nodiflora* on co-occuring species by using various tools of bioinformatics. The work done till now on the metabolic compounds and various allelochemicals has been restricted only to the wet lab methods and very little is known about the gene level expression of all such compounds. Some advanced researches show the gene expression analysis on different weeds that indicate several compounds responsible for the invasion of weeds into new environments (Chen, 2013).

For bioinformatics study, several soft-wares and tools were used to analyse the data present on both the weeds. Due to lack of research on the weed plants, it was difficult to retrieve the molecular data. Hence the common enzymes in Cassia uniflora Mill. non Spreng, and *Synedrella nodiflora* (L) Gaertn, were selected for further analysis. The enzymes or proteins that were studied in both the invasive weeds are – Maturase K [EC 2.7.10.2] and Ribulose-1,5-bisphosphate carboxylase / oxygenase(Rubisco/ rbcL)[EC 4.1.1.39]. Maturase K is a plant plastidial gene. The protein it encodes is an intron Maturase, a protein that splices introns. Mat-K is proposed as the only chloroplast-encoded group II intron Maturase, thus implicating Mat-K in chloroplast posttranscriptional processing. For a protein-coding gene, mat-K has an unusual evolutionary significance, including relatively high substitution rates at both the nucleotide and amino acids levels, (Barthet et. al.2015).

Table 1. Phylogenetic trees of invasive species *Cassia* and *Synedrella*

No.	Type of Phylogenetic tree	Enzyme	MSA %
1.	Tree and herb species of *Cassia/ Senna*	Maturase-K	90%
2.	Tree and herb species of *Cassia/ Senna*	Rubisco	Negligible - the authenticity of this clad was very low and hence was not considered for comparison
3.	Weed species of *Cassia/ Senna*	Maturase-K	95%
4.	Weed species of *Cassia/ Senna*	Rubisco	90%
5.	*Synedrella nodiflora* and other weed species	Maturase-K	60%
6.	*Synedrella nodiflora* and other weed species	Rubisco	90%
7.	*Cassia/ Senna, Synedrella* and other related genera and species	Maturase-K	80%
8.	*Cassia/ Senna, Synedrella* and other related genera and species	Rubisco	85-90%

The other enzyme that has been studied in both plants was ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) is the major enzyme assimilating CO2 into the biosphere. At the same time Rubisco is an extremely inefficient catalyst and its carboxylase activity is compromised by an opposing oxygenase activity involving atmospheric O2. These enzymes were considered for checking the probable similarity in the protein sequences of these two weeds. The nucleotide research on these enzymes is done intensively since both the enzymes play crucial role in plant metabolism. The focus of the study was to acquire the common factor based on genomic data in both the weeds that is responsible for their dominance and to understand and interpret their ecological and evolutionary significance.

The present work was carried out for correlating different genes or proteins in Cassia uniflora Mill. non Spreng and Synedrella nodiflora (L) Gaertn. responsible for their invasiveness by using different bioinformatics tools. Scanty information is available on the molecular level work, gene identification and sequencing of the invasive weeds as compared to the crop plants. Therefore present attempt was made to fulfil this gap in above mentioned weeds. It has also helped to get the idea about the ecological corridor developed by current dominant invasive weeds. Not only that but it has also helped to predict the future changes in weed flora and their ecological status.

MATERIAL AND METHODS

A) By performing Multiple Sequence Alignment (MSA): The Multiple Sequence Alignment of the 2 or more sequences was done to check whether the sequences align exactly similar to each other. Here, sequence homology is applied to assess if the sequences are sharing evolutionary origins. B) By using 'Deep-view software': This software was used for direct analysis of the similar or distant proteins. The proteins were modelled from the server 'SWISSMODEL' in PDB format to get the 3D structure. And then these proteins were analysed in the software. C) Preparation of phylogenetic tree: It was carried out to check the homology between the selected protein sequences. Cladistics analysis was performed by using software MEGA-X with the help of maximum likelihood method. Website used - www.ncbi.nlm.nih.gov

RESULTS AND DISCUSSION

For tracking down the upshots on invasive species *Cassia uniflora/ Senna uniflora* and *Synedrella nodiflora* the efforts were carried out in the following manner –A) By performing Multiple Sequence Alignment (MSA) – For Maturase-K and Rubisco for *Cassia uniflora/ Senna uniflora* and *Synedrella nodiflora*. B) By using 'Deep-view software'- For protein structure of Maturase-K and Rubisco of *Cassia uniflora/ Senna uniflora* and

Table 2: Showing details of two weeds used for MSA of Maturase-K enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	ARR68700.1	280	Included	All these species
Synedrella nodiflora	AAR02837.1	508	Included	show about 70% sequence similarity

Figure 2: Showing Multiple Sequence Alignment for Rubisco

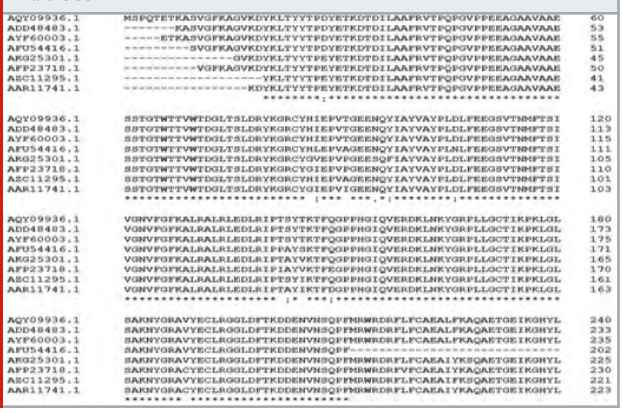


Figure 1: Showing Multiple Sequence Alignment for Maturase-K enzyme

Synedrella nodiflora. C) Preparation of phylogenetic tree / Cladistic analyses

A) Results of Multiple Sequence Alignment (MSA): Multiple sequence alignment showed that the sequence of enzyme 'Maturase-K' is exactly similar in plants *Cassia uniflora* and *Synedrella nodiflora*. This result revealed the sequences with almost homologous regions showing '*'symbol as exactly matching sequences (Fig.1, Table 2). Similar MSA was carried out on Rubisco for the same two plants *Cassia uniflora* and *Synedrella nodiflora* and equivalent similarity was observed. The figure and table for this are not included to avoid repetition of the sets.

When similar MSA (Multiple sequence alignment/s) was carried out for Rubisco enzyme, *Cassia auriculata* had to beexcepted because it has different base pairs than all other plants which was affecting the comparison among the selected plants. The comparison of remaining plants showed almost similar sequences in all the species related to the weed species under consideration i.e. *Cassia uniflora* and *Synedrella* (Fig. 2, Table 3). Similar comparison is done for Maturase-K enzyme and it shows similar results except the inclusion of *Cassia auriculata*. Hence it can be predicted that these invasive species could have evolved in similar way for the enzymes like 'Maturase-K' and Rubisco.

Results of using 'Deep-View' software: When the models of protein structures for both the enzymes 'Maturase-K' and 'Rubisco' were run in Deep view software, models exhibited similar sequences of both the enzymes that implied the similar structures and hence all the models tracked in the software were able to get merged. Results of deep view analysis (Fig. 3 & 4) showed that the enzymes studied had identical structures and the highlighted portion in both the images showed shared structures of the same protein but in two different plants

Figure 3: Circled portion indicates common sequence coding similar structure of enzyme MATURSE-K in *Cassia uniflora* and *Synedrella nodiflora*

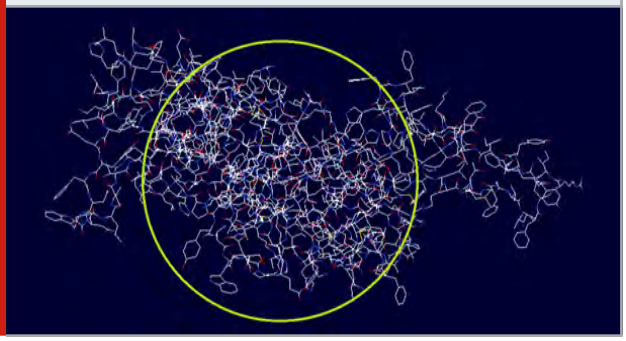


Figure 4: Circled portion indicates common sequence coding similar structure of enzyme RUBISCO in *Cassia uniflora* and *Synedrella nodiflora*

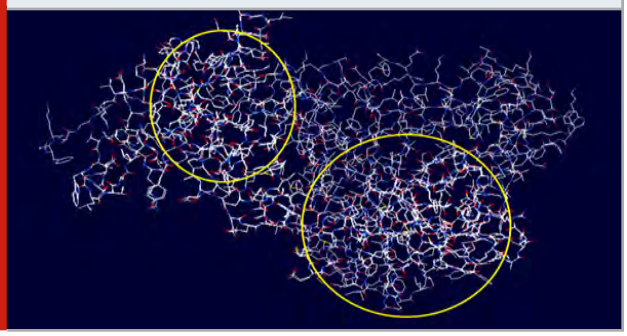


Table 3: Showing details of nine plants used for MSA of Rubisco enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	AQY09936.1	249	Included	
Cassia obtusifolia	ADD48483.1	234	Included	
Cassia tora	AYF60003.1	238	Included	All these species
Cassia auriculata	AII33731.1	290	Excluded	together show
Cassia occidentalis	AZC11295.1	416	Included	about 80 %
Cassia sophera	AFU54416.1	202	Included	sequence
Synedrella nodiflora	ARR11741.1	468	Included	similarity
Tridax procumbens	AFP23718.1	461	Included	
Pulicaria dysenterica	AKG25301.1	439	Included	

Cassia / Senna uniflora and *Synedrella*. This probable further confirms *simultaneous* evolution of these invasive species and their imperative enzymes.

Preparation of phylogenetic trees / Cladistic analyses: The construction of phylogenetic trees was carried out with the interest of searching the evolutionary associations of Cassia/ *Senna uniflora* with the other native, weedy and less dominant species of *Cassia*. In case of *Synedrella* searching was attempted with the same approach but due to very much insubstantial outcomes, the cladograms were developed based on few weedy, local and fairly prevailing genera and species from the same family Asteraceae, as this family is well known to contain globally distributed, highly dominant invasive weed genera other than *Synedrella*.

Figure 5: Comparison between different species of *Cassia* for maturase-K enzyme

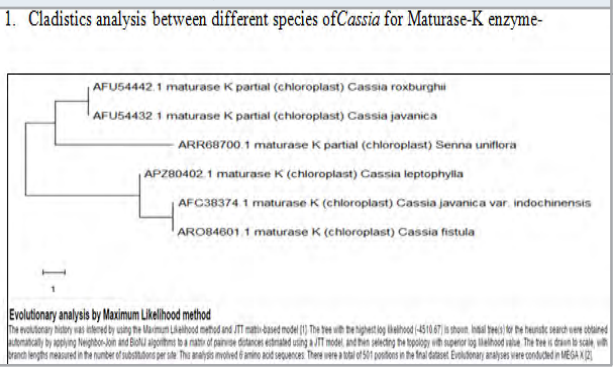
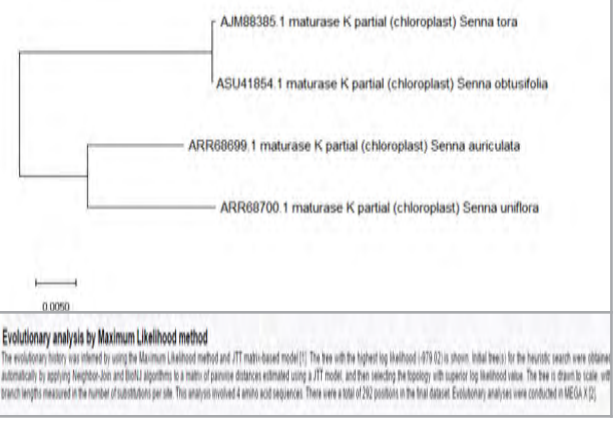


Figure 6: Showing Phylogenetic tree of four weed species for Maturase-K enzyme



The Cladistic analysis (Fig. 5, Table 4) performed on different herb and tree species of *Cassia* revealed that *Cassia uniflora* had a distant phylogeny and did not share any recent ancestry with any of the other Cassia species for Maturase-K enzyme.As against that when the phylogenetic comparison of different weed species of *Cassia*/ Senna was carried out for these two enzymes, it was observed that *Cassia uniflora* and *Cassia auriculata* might have progressed in the most parallel way and from very recent common ancestor in due course of evolution (Figures 6, 7; Tables 5,6). It can also be confirmed by the habitat of both the species of *Cassia*. Both Cassia uniflora and *Cassia* auriculata grow intensively in semi-arid conditions.

The similar phylogeny here, in both the enzymes, Maturase-K and Rubisco, suggests that these two plants must have acquired similar properties that help them survive under the unfavourable conditions and this could be the key to their dominance over other native plants. The results showed that the evolution of *Cassia uniflora* and *Cassia auriculata* is relatively similar to each other over other species of *Cassia*. *Cassia uniflora* is the most dominant species of the genus Cassia presently and *Cassia auriculata* might evolve further as dominant species in subsequent situations of environment, as *Cassia uniflora* is today.

The other species of *Cassia* such as *Cassia* occidentalis and *Cassia* tora have shown distant phylogeny. Both *Cassia uniflora* and *Cassia auriculata* are distantly evolved from other species of *Cassia* and hence show significantly different habitat and possibly also the chemical properties than other members of genus Cassia. The comparison of *Synedrella* in cladogram with the other genera of asteraceae for Maturase-K showed that, number of base pairs varies greatly among genera and hence the MSA percentage has fallen (60%) but the matching base pairs show completely identical sequences for all the plants. *Synedrella nodiflora* indicated distant phylogeny from Tridax procumbens and Pulicaria dysenterica but is relatively nearer to Lactuca indica (Fig. 8, Table 7). These three genera of Asteraceae show their frequent but less dominant occurrence than Synedrella.For the same members of asteraceae, the building up of phylogenetic tree for Rubisco was performed excluding *Lactuca indica* as its protein sequence was unavailable. Here, it was observed that *Tridax* procumbens and Pulicaria dysenterica had the closest phylogeny showing *Synedrella nodiflora* distantly placed (Fig. 9, Table 8).

The comparison of both *Cassia uniflora* and *Synedrella nodiflora* for Maturase-K showed very distant origin. *Cassia uniflora* showed quite distant phylogeny from other *Cassia* species (Fig. 10, Table 9) whereas; *Synedrella nodiflora* shares similar phylogeny with Lactuca indica. In contrast the comparison for Rubisco showed common ancestry for *Cassia uniflora* and *Synedrella nodiflora* as they are placed very close to each other (Fig. 11, Table 10). This shows that both the plants might have developed different evolutionary patterns than all the other plants under consideration which resulted in distant phylogeny of both. In this clad, *Lactuca indica* was not considered for the comparison as its protein sequence for Rubisco was unavailable (Fig. 11, Table 10). The importance of genomic tactics for understanding the weedy and invasive behaviours of plants, their evolution and resistant response to environmental fluctuations is better realised now as a part of weed biology (Stewart et al. 2009).

It further opens a new research route for perception of reckless growth and evolution of phyto-invasives and their functioning under harsh stress conditions. It also shares knowledge about weed management, herbicide resistance mechanism of allelopathy and evolution of invasiveness of such plant species (Thomas and Klaper, 2004). Such phylogenetic studies using various methods help to better understand causes of invasion success, ecosystem disturbance and alterations in biodiversity (Forest et al., 2007; Proches et al., 2008; Winter et al., 2009; Dawson et al., 2009). Some studies have also claimed that phylogenetic and functional attributes of alien species readdress different aspects of ecosystem functioning and variations produced at the level of organisms (Chen, 2013; Ricotta et al. 2009, 2010; Cadotte et al. 2009).

According to some researchers unusual characters and ancestral relations with natives probably are promoting the aliens to become invasive very swiftly in non-native ranges of global vegetation (Clements and Ditommaso, 2011).Bezeng et al. (2013) have claimed that phylo-genomic studies of invasive species with reference to their native co-survivors are the most important drivers of ecosystem change, which can alter the vegetational set up of a particular area. Similar studies on Maturase-K and Rubisco of island flora have been carried out by them to understand the causes of invasion on Robben island, South Africa. The results recorded in the figures 1 and 2 and Tables 2 and 3 showed that multiple sequence alignments are shared for Maturase-K and Rubisco of *Cassia uniflora* and *Synedrella*. Deep view analysis (Figures 3 and 4) also revealed major portions identical for both the enzyme proteins for C. uniflora and Synedrella. This further indicates that these two enzymes might be the drivers in the invasion success of *C. uniflora* and *Synedrella*(Bezeng et al., 2013), since these enzymes have prime importance in the plant metabolism.

When evaluation of different species of *Cassia* for Maturase-K was carried out (Fig. 5, Table 4), it exhibited minor likelihood of *C. uniflora* from others. Further, when

Table 4: Showing details of different species of *Cassia* for Maturase-K enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	ARR68700.1	280	Included	
Cassia roxburghii	AFU54442.1	278	Included	All these species show about 90 % sequence similarity
Cassia javanica	AFU54432.1	278	Included	
Cassia leptophylla	APZ80402.1	499	Included	
Cassia javanica var. indochinensis	AFC38374.1	501	Included	
Cassia fistula	ARO84601.1	501	Included	

Table 5: Showing details of four weed species of Cassia used for MSA of Maturase-K enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	ARR68700.1	280	Included	All these species show about 95 % sequence similarity
Cassia obtusifolia	ASU41854.1	276	Included	
Cassia tora	AJM88385.1	248	Included	
Cassia auriculata	ARR68699.1	261	Included	

we examined the cladistics patterns of different species of Cassia for both the enzymes (Figures 6, 7 and Tables 5, 6), it was observed that *C. auriculata* is lineally related to *C. uniflora*, indicating similar or parallel functional traits which are essential for invasion. Martyniuk et al. (2009) have phyletically compared members of Amaranthaceae in the same way for pollen structures based on these two enzymes.The evaluation of different asteraceae members along with the invasive alien species *Synedrella nodiflora* suggested its racial link with Lactuca indica for Maturase-K (Fig.8, Table 7). When *Synedrella nodiflora* was (Fig. 9, Table 8) compared with same herb species from asteraceae for Rubisco revealed distant phylogeny. The consideration for phyletic relatedness when was performed for Maturase-K (Fig. 10, Table 9), showed totally separate placement of *C. uniflora* but indicating probable common ancestry. This clad suggested co-evolution of *Synedrella nodiflora* and Lactuca indica. The same set of plants was used to prepare clad for Rubisco revealed (Fig. 11, Table 10) the diversification from the common inherited line. The studies on evolutionary population genomics in the Asteraceae family have been carried out by Stevens (2007), Barker et al. (2008), Broz et al. (2007) and Mandel et al. (2017).

Figure 7: Showing phylogenetic tree of weedspecies for Rubisco enzyme

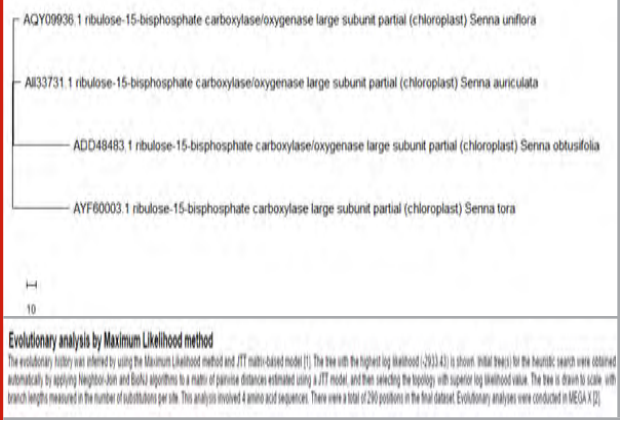


Figure 8: Showing Phylogenetic tree of herb species of members of Asteraceae for Maturase-K enzyme

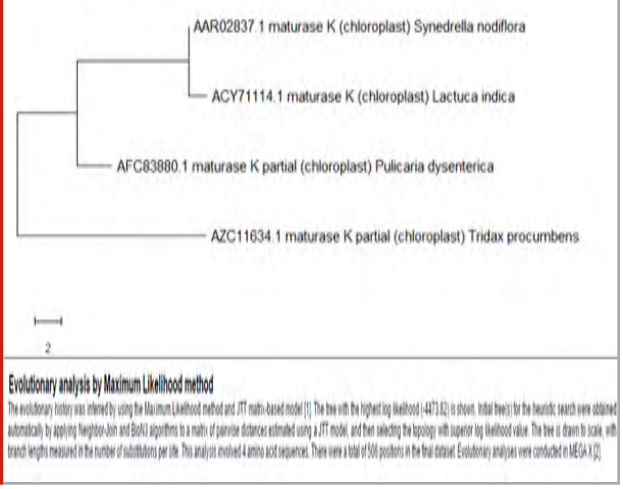


Figure 9: Showing Phylogenetic tree of herb species of members of Asteraceae for Rubisco enzyme

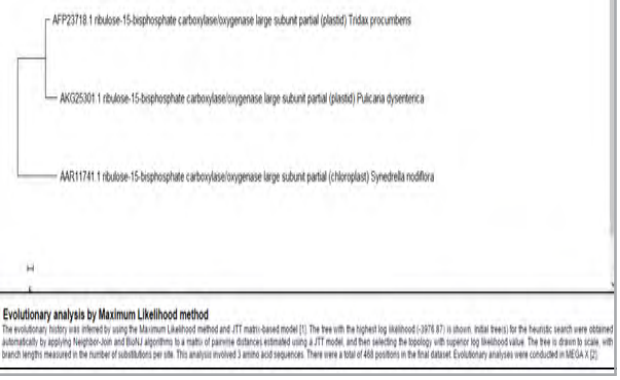


Figure 10: Showing Phylogenetic tree of all 9 species for Maturase-K enzyme

6. Cladistics analysis for weed related species of both *Cassia uniflora* and *Synedrella nodiflora* for Maturase-K enzyme –

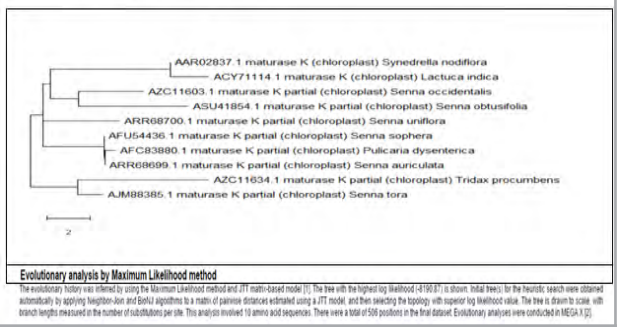


Figure 11: Showing Phylogenetic tree of all 9 species for Rubisco enzyme

7. Cladistics analysis for weed related species of both *Cassia uniflora* and *Synedrella nodiflora* for Rubisco enzyme –

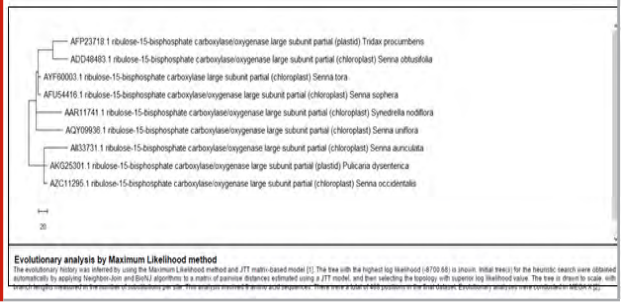


Table 6: Showing details of weedspecies for Rubisco enzyme

Name of the Plant	Accession Number (Amino acids)	Number of Base Pairs	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	AQY09936.1	249	Included	All these species show about 90 % sequence similarity
Cassia obtusifolia	ADD48483.1	234	Included	
Cassia tora	AYF60003.1	238	Included	
Cassia auriculata	AII33731.1	290	Excluded	

Table 7: Showing details of asteraceae members herb species for Maturase-K enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Synedrella nodiflora	AAR02837.1	506	Included	All these species show about 60 % sequence similarity
Tridax procumbens	AZC11634.1	254	Included	
Lactuca indica	ACY71114.1	506	Included	
Pulicaria dysenterica	AFC83880.1	324	Included	

Table 8: Showing details of herb species of members of Asteraceae for Rubisco enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Synedrella nodiflora	AAR11741.1	468	Included	All these species show about 90 % sequence similarity
Tridax procumbens	AFP23718.1	461	Included	
Lactuca indica	-	-	Excluded	
Pulicaria dysenterica	AKG25301.1	439	Included	

Thus the present study has facilitated to establish the correlations of the invasive species *Cassia uniflora* and *Synedrella nodiflora* with the other innate and aggressive species either of the same genus or family. The phylogenomic studies using bio-computing tools enabled to understand the protein nature of Maturase-K and Rubisco of these two weeds mainly and also of other species. There could be generated various clades giving insights into the evolutionary connections of these aliens developing monothickets with each other along with the other plants. Further it will help to know the changing and dominating weed flora in the same area.

Table 9. Showing details of weedspecies for Maturase-K enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	ARR68700.1	280	Included	All these species together show about 80 % sequence similarity
Cassia obtusifolia	ASU41854.1	276	Included	
Cassia tora	AJM88385.1	248	Included	
Cassia auriculata	ARR68699.1	261	Included	
Cassia occidentalis	AZC11603.1	269	Included	
Cassia sophera	AFU54436.1	278	Included	
Synedrella nodiflora	AAR02837.1	506	Included	
Tridax procumbens	AZC11634.1	254	Included	
Lactuca indica	ACY71114.1	506	Included	
Pulicaria dysenterica	AFC83880.1	324	Included	

Table 10. Showing details of weed species for Rubisco enzyme

Name of the Plant	Accession Number	Number of Base Pairs (Amino acids)	Included or Excluded	MSA percentage of Sequence Similarity
Cassia uniflora	AQY09936.1	249	Included	All these species together show about 90 % sequence similarity
Cassia obtusifolia	ADD48483.1	234	Included	
Cassia tora	AYF60003.1	238	Included	
Cassia auriculata	AII33731.1	290	Included	
Cassia occidentalis	AZC11295.1	416	Included	
Cassia sophera	AFU54416.1	202	Included	
Synedrella nodiflora	AAR11741.1	468	Included	
Tridax procumbens	AFP23718.1	461	Included	
Lactuca indica	-	-	Excluded	
Pulicaria dysenterica	AKG25301.1	439	Included	

CONCLUSION

Overall this research work points out to the protein based phylogenetic similarities and distinctiveness of alien taxa with respect to the other genera as significant details deciding their invasion success (Ordonez, 2014). This enhances to the idea of phylogenetic and metabolic patterns of successfully invaded species. Further these studies have focussed light on the future invasive followers of them on Deccan plateau through different probability patterns.

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On the Allelic Variation and Heterozygosity of Bikaneri, Jaisalmeri and Kachchhi Camels using Microsatellite Markers

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ABSTRACT

The genetic diversity of most livestock species is reducing and it is not possible to preserve all livestock breeds. The blood samples for isolation of genomic DNA were collected from NRCC, Bikaner and Field. The genomic DNA was isolated by phenol-chloroform extraction method with minor modification. The Qualitative and Quantitative estimation of genomic DNA was determined by spectrophotometer and agarose gel electrophoresis. The polymerase chain reaction using eleven microsatellite primer pairs was done for amplification of microsatellite loci. PCR products were electrophoresed in 1% agarose gel in 1X TBE buffer system. Eight out of eleven microsatellite primer pairs were optimized. Primers VOLP-03, VOLP-10, YWLL-38, YWLL-44, YWLL-58, VOLP-32, LCA-37 and YWLL-46 amplified the specific bands. Number of alleles ranged from two to five was observed in Bikaneri, Jaisalmeri and Kachchhi camels at five microsatellite loci. The most polymorphic primers were VOLP-10 and VOLP-03 in which a total of 5 Bikaneri camels ranged from 0.28 to 0.741. The expected heterozygosity in at the locus YWLL-44 followed by YWLL-38 in Bikaneri camel. Rest of the three microsatellite loci viz. VOLP-03, VOLP-10 and YWLl-58 revealed more than 58% expected heterozygosity. This indicates that the above five primers can very well be utilized for further genetic studies, which may include characterization, conservation and production enhancement.

KEY WORDS: BIKANERI CAMEL, ELECTROPHORESIS, JAISALMERI CAMEL, KACHCHHI CAMEL MICROSATELLITE MARKER, PCR, PRIMERS

INTRODUCTION

The world population of camel is 35.0 million. Somalia has highest camel population ie 7.15 million followed by Sudan 4.79 million. Ethiopia 1.16 million, India has 0.38 million and ranks 12th in World. (FAO, 2012) Rajasthan state has 3.26 lakhs and ranks 1st in country. (Livestock sensor, SOI, 2012).

Camel has the ability to sustain water scarcity condition and has excellent adaptive mechanism. Camels are being used by the poor farmers in villages and cite to earn their livelihood. Camel is beast of burden and provider of milk, meat, and hides. The camel has shown to be better adapted to extreme conditions in most aspects than other domestic ruminants (Al-Baka, 2016). Camel is used for short distance transportation and for agricultural operations. Camels are able to sustain 20 to 22 per cent of body weight loss during severe scarcity conditions where as other livestock species

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(cattle, buffaloes) cannot sustain beyond 10 to 12 per cent loss in body weight. For survival in desert environment, camels have physiological, anatomical and behavioral adaptation mechanisms. Water conservation ability, the unique features of blood, thermoregulation, and efficient digestion and metabolism are among the physiological adaptations, (Gebreyohanes and Assen, 2017 Liang et al 2020).

Bikaneri breed is developed by selective interbreeding of Sindhi, Baluchi. Afghan and Indigenous camels. Breed characterization at the phenotypic and molecular genetic level has become essential to know the present status of different species and their breeds in different ago climatic zones. Due to extensive cross breeding, Indigenous breeds with unique genetic characteristics are declining numerically and are at risk. Genetic characterization can play a major role in preventing the erosion and Genetic Diversity, which is a part of earth's natural heritage. The loss of a unique breed is an irreplaceable reduction in natural profusion of life forms.

MATERIAL AND METHODS

Collection of Blood and DNA isolation: Venous blood was collected from unrelated Bikaneri, Jaisalmeri and Kachchhi camels, maintained at the National Research Center Camel, Bikaner and from breeding tract of respective breed. EDTA was used as an anticoagulant. Blood samples were transported on ice and kept at 4° c until use. The genomic DNA was isolated by using Phenol- chloroform method. The DNA samples were stored at 20 °C for further use by adding sufficient amount of TE buffer. (Cao et al., 2009)

Electrophoresis PCR method: The master mix for optimization of PCR was prepared as (25µl reaction volume) Genomic DNA (template) 50ng, Primer 5pmol, dNTP 0.25mM each, Taq DNA polymerase 1.5U, Taq polymerase buffer 10X 2.5µl. Subsequent to optimization of microsatellite loci the protocol used is as (12.5µl reaction volume), Genomic DNA (template) 50ng, Primer 5pmol, Taq DNA polymerase 0.75U, Taq polymerase buffer 10X 1.25µl. In a 200ml PCR tube 24µl of PCR mix was taken and subsequently 50ng of genomic DNA was added. PCR amplification was carried out in a thermal cycler at 95 ° c for 5 minutes. Followed by 30cycles each 94 ° c for 45 seconds, 58 ° c for 1 minute, and 72 ° c for 1 minute with final extension at 72 ° c for 15 minutes.

The PCR amplified products were resolved by denaturing sequencing gel electrophoresis using Sequi-Gen GT electrophoresis apparatus. The properly cleaned and wiped with ethanol assembly taken and arranged properly. The gel was prepared by using constituents as Acrylamide 11.4gm, Bis-acrylamide 0.6gm, 10X TBE 10ml, urea 84gm, Distilled water to make 200ml. This mixture was filtered by 3mm Whatmann filter paper to remove undissolved particles. Freshly prepared 10% ammonium per sulfate solution 500µl was dissolved and 40µl TEMED was added. The gel was sucked up into a barrel of 120ml syringe without any air bubble. The gel then pushed into the assembly with taking care of any trapping of air bubbles. The gel was polymerized for 60 minutes at room temperature.

Then the lower and upper buffer tanks of the electrophoresis apparatus were filled with 1X TBE. The gel was then pre run at constant temperature of 72 ° c at 150volt current for 45 minutes. Then the samples 1.5 to 3µl PCR product was mixed with an equal amount of 2X formamide dye. The samples were heated to 95 ° c for 5 minutes for denaturation of DNA and immediately transferred on ice. The product was loaded slowly in the wells. DNA ladder (50bp) was loaded along the side of sample as size marker. Then gel was allowed to run at constant voltage (75W) for 1 to 2 hours depending upon the allele size. On completion of electrophoresis, power was turned off. The gel assembly was dismantled. Then the gel was stained with silver nitrate. The gel was fixed in fixer solution for 20 minutes, then impregnated with staining solution for 30 minutes. The tray was kept in dark; the tray was regularly agitated. Then the gel was incubated in chilled developer solution. After appearance of bands, the reaction was stopped with 10% acetic acid solution. Then the gel was washed with distilled water for 2 minutes. The Whatmann filter paper (3mm) sheet was laid on the gel and gently pressed with a tissue paper flat so that the gel stuck to the paper. The gel was then dried under vacuumed at 80 ° c for 45minutes to 1 hour.

Kim et al. (2002) evaluated the genetic diversity of northeast Asian cattle based on microsatellite data. Thirteen microsatellite loci were analyzed for a total of 200 individuals including Korean, Chinese, Japanese Black and European Holstein cattle. Observed and expected heterozygosity, two estimators (F st and G st) of gene differentiation and Nei's DA distance were evaluated. The lowest genetic diversity was exhibited in Japanese Black cattle (HE=0.471), Korean cattle reveled relatively high degree of genetic diversity (HE=0.728).

The scoring was done manually by comparing the band size with the standard 50bp ladder. Wu Wei et al. (2001) reported the genetic structure of five Chinese and foreign cattle breeds using microsatellite DNA markers. Genetic structure including gene frequency, heterozygosity, PIC, numbers of effective alleles and genetic distance were studied in five cattle types (Nan yang, Yanbian, Han woo, Simmental and Piemontese) using four microsatellite markers. Cluster analysis was conducted based on microsatellite polymorphism,(Drabik et al., 2016).

General PCR Program: PCR involves a process of heating and cooling called thermal cycling which is carried out by machine. There are three main stages as follow,

1. Denaturing: when the double stranded template DNA is heated to separate it into two single strands.
2. Annealing: When the temperature is lowered to enable the DNA primers to attach to the template DNA.

3. Extending: When the temperature is raised and the new strand of DNA is made by the Taq polymerase enzyme.

These three stages are repeated 20 to 40 times, doubling the number of DNA copies each time.

RESULTS AND DISCUSSION

PCR is a method for amplifying specific DNA sequences in in vitro conditions. PCR makes possible the genetic analysis of tiny samples of DNA by a relatively simple process. The micro satellite primers were used to amplify the specific bands by making little variation in the annealing temperature and cycling conditions in PCR. The number of alleles at different marker loci and their frequencies are simple indicators of the genetic variability. At YWLL 38 microsatellite locus, three numbers of alleles with allele size ranging from 180 bp186bp were observed in DNA samples of Bikaneri, Jaisalmeri and Kachchhi breeds (Table 1, 2 and 3). The allele size range reported by Lang et al. (1996) is in close agreement with the present findings. However, Jailin et al. (2000) reported four numbers of alleles in the size range of 185bp-190bp in dromedary.

At YWLL 46 microsatellite locus a single band of 99bp. At VOLP-32 microsatellite locus, a single band of 260bp. And at LCA-37 microsatellite locus a single band of 178bp. was observed Bikaneri, Jaisalmeri and Kachchhi breeds (Table 1). Similar band size at these microsatellite loci has been reported by Lang et al. (1996) and Pendo et al. (1998). However, these loci have been polymorphic in new world camelids but they were observed to be monomorphic in Indian dromedary. This indicates that the genetic variation is relatively less in Indian dromedary population. Variation in the number of alleles and their frequencies, reported in this study and by many other works, within breeds over different markers indicated existence of genetic variation within and among species. The number of alleles and allele frequency could be of tremendous use in calculating the genetic distance between different strains or breeds of livestock species.

Heterozygosity is an appropriate measure of genetic variability within a population. At microsatellite locus VOLP-03, expected heterozygosity and polymorphic information content were observed to be 0.741 and 0.675, respectively in thirty samples of Bikaneri breed (Table 4). However, in Jaisalmeri breed expected heterozygosity and polymorphic information content were observed to be 0.573 and 0.522 respectively, In Kachchhi breed, expected heterozygosity and polymorphic information contents were observed to be 0.515 and 0.460, respectively. Similar, expected heterozygosity and PIC in alpacas has been reported by Oberque et al. (1998) and in dromedary by Jianlin et al. (2000).

At VOLP- 10 microsatellite locus, expected heterozygosity and PIC were observed to be 0.695 and 0.642, respectively in thirty samples of Bikaneri breed (Table 4). However, the expected heterozygosity and PIC in Jaisalmeri breed were observed to be 0.686 and 0.621 and in Kachchhi 0.694 and 0.560 respectively (Table 5 and 6). The expected heterozygosity (0.569) and PIC value (0.521) reported in alpaca by Oberque et al. (2000) reported relative higher expected heterozygosity (0.75) in dromedary camels due to the existence of higher genetic variation as replaced by a greater number of allies at this locus in the population studied. At YWLL 44 microsatellite locus, expected heterozygosity and PIC were observed to be 0.280 and 0.239, respectively in thirty samples of Bikaneri breed (Table 4). However, in Jaisalmeri and Kachchhi breed they were observed to be 0.218 and 0.914 and 0.320 and 0.270 respectively (Table 5 and 6). However, higher PIC (0.845) in alpacas and lamas and expected heterozygosity (0.66) in dromedary camel have been reported by Lang et al. (1996) and Jianlin et al. (2000).

Table 1: Allele number, allele size and allele frequency at five microsatellite loci in Bikaneri camel.

Locus	No. of Samples(n)	Allele No.	Allele size and Allele frequency				
			A	B	C	D	E
VOLP- 03	30	5	168bp 0.08	166bp 0.33	148bp 0.05	147bp 0.28	145bp 0.25
VOLP-10	30	5	265bp 0.1	263bp 0.05	261bp 0.4	252bp 0.35	250bp 0.1
YWLL-44	30	2	107bp 0.027	104bp 0.69	-	-	-
YWLL-58	30	3	177bp 0.316	175bp 0.5	173bp 0.183	-	-
YWLL-38	30	3	186bp 0.5	182bp 0.45	180bp 0.05	-	-
YWLL-46	30	1	99bp	-	-	-	-
VOLP-32	30	1	260bp	-	-	-	-
LCA-37	30	1	178bp	-	-	-	-

Table 2: Allele number, allele size and allele frequency at five microsatellite loci in Jaisalmeri camel.

Locus	No. of Samples (n)	Allele No.	Allele size and Allele frequency				
			A	B	C	D	E
VOLP-03	15	3	168bp 0.270	166bp 0.170	148bp 0.570	--	--
VOLP-10	13	4	--	263bp 0.038	261bp 0.346	252bp 0.346	250bp 0.270
YWLL-44	12	2	107bp 0.125	104bp 0.875	--	--	--
YWLL-58	13	3	177bp 0.153	175bp 0.500	173bp 0.346	--	--
YWLL-38	18	3	186bp 0.416	182bp 0.50	180bp 0.08	--	--

Table 3: Allele number, allele size and allele frequency at five microsatellite loci in Kachchhi camel.

Locus	No. of Samples (n)	Allele No.	Allele size and Allele frequency				
			A	B	C	D	E
VOLP-03	10	3	168bp 0.2	166bp 0.150	148bp 0.650	--	--
VOLP-10	7	4	265bp 0.071	--	261bp 0.357	252bp 0.357	250bp 0.214
YWLL-44	10	2	107bp 0.2	104bp 0.8	--	--	--
YWLL-58	7	3	177bp 0.214	175bp 0.5	173bp 0.285	--	--
YWLL-38	18	3	186bp 0.450	182bp 0.527	180bp 0.028	--	--

Table 4: Heterozygosity and polymorphic information content at five microsatellite loci in Bikaneri camel.

Locus	Observed Heterozygosity	Nei's Expected Heterozygosity	PIC Value
VOLP-03	0.43	0.741	0.675
VOLP-10	0.80	0.695	0.642
YWLL-44	0.33	0.280	0.239
YWLL-58	0.10	0.616	0.540
YWLL-38	0.63	0.545	0.440

Table 5: Heterozygosity and polymorphic information content at five microsatellite loci in Jaisalmeri camel.

Locus	Observed Heterozygosity	Nei's Expected Heterozygosity	PIC Value
VOLP-03	0.466	0.573	0.522
VOLP-10	0.460	0.686	0.621
YWLL-44	0.250	0.218	0.194
YWLL-58	1.00	0.606	0.528
YWLL-38	0.660	0.570	0.475

The higher PCI and expected heterozygosity were expected due to the same reasons as explained above. At YWLL 58 microsatellite locus, expected heterozygosity and PIC were observed to be 0.616 and 0.540, respectively in thirty samples of Bikaneri breed (Table 4). However, in Jaisalmeri and Kachchhi breed they were observed to be 0.606 and 0.620 and 0.50 respectively (Table 5 and 6). The present results are in close agreement with the findings (PCI = 0.06) of Lang et al. (1996) in Alpacas and llamas. At YWLL 38 microsatellite locus, expected heterozygosity and polymorphic information content were observed to 0.45 and 0.44 respectively in Bikaneri breed (Table 4).

Table 6: Heterozygosity and polymorphic information content at five microsatellite loci in Kachchhi camel.

Locus	Observed Heterozygosity	Nei's Expected Heterozygosity	PIC Value
VOLP-03	0.70	0.515	0.46
VOLP-10	0.85	0.694	0.56
YWLL-44	0.40	0.32	0.27
YWLL-58	1.0	0.62	0.50
YWLL-38	0.611	0.522	0.42

Figure 1: Photograph showing alleles at microsatellite locus (VOLP-03) in Jaisalmeri, Bikaneri and Kachchhi camel.

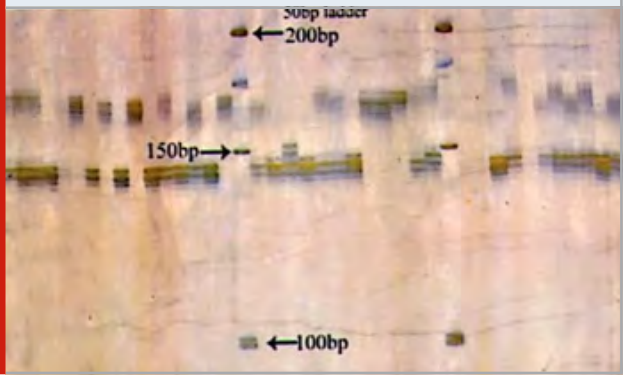


Figure 2: Photograph showing alleles at microsatellite locus (VOLP-10) in Jaisalmeri, Bikaneri and Kachchhi camel.

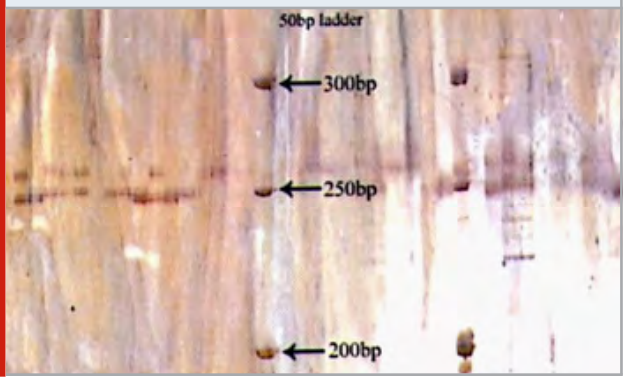


Figure 3: Photograph showing alleles at microsatellite locus (YWLL-44) in Jaisalmeri, Bikaneri and Kachchhi camel.

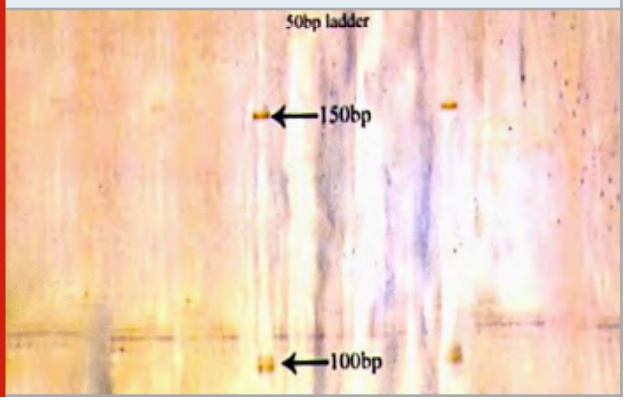


Figure 4: Photograph showing alleles at microsatellite locus (YWLL-58) in Jaisalmeri, Bikaneri and Kachchhi camel.

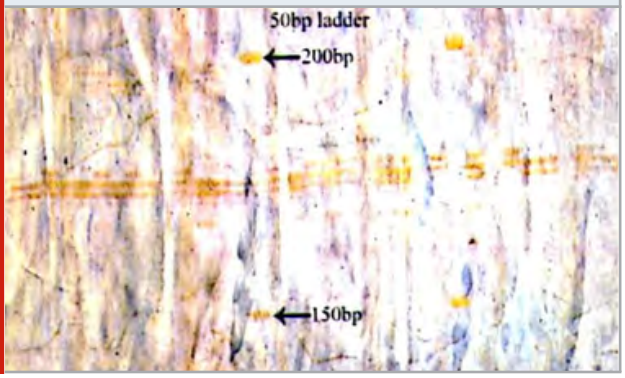


Figure 5: Photograph showing alleles at microsatellite locus (YWLL-38) in Jaisalmeri and Kachchhi camel.

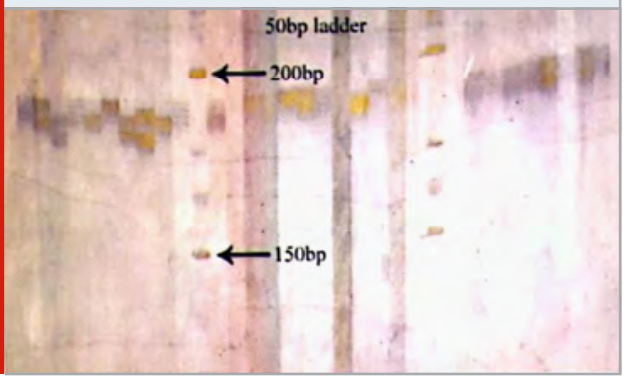


Figure 6: Photograph showing alleles at microsatellite locus (YWLL-38) in Jaisalmeri and Bikaneri camel.

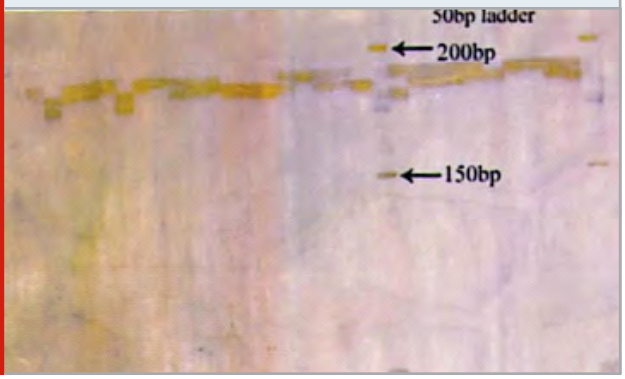


Figure 7: Photograph showing alleles at microsatellite locus (YWLL-38) in Bikaneri camel

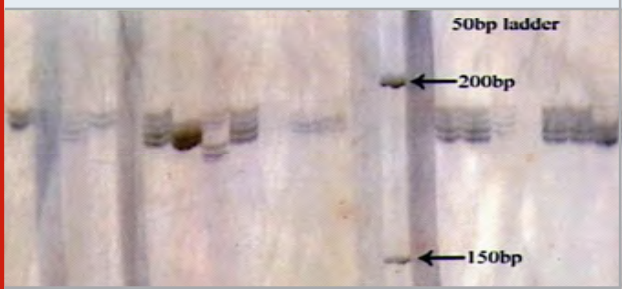


Figure 8: Photograph showing alleles at microsatellite locus (VOLP-32) in Bikaneri camel

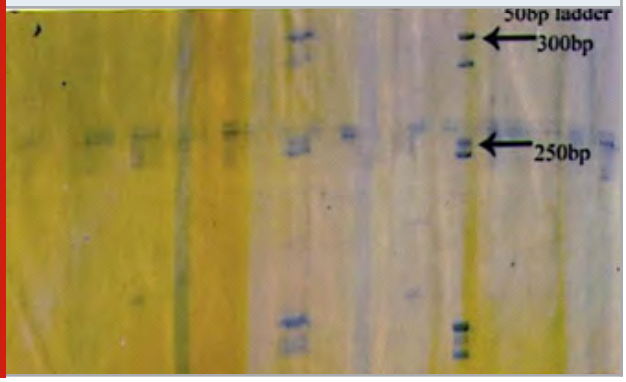


Figure 9: Photograph showing alleles at microsatellite locus (YWLL-46) in Bikaneri camel.

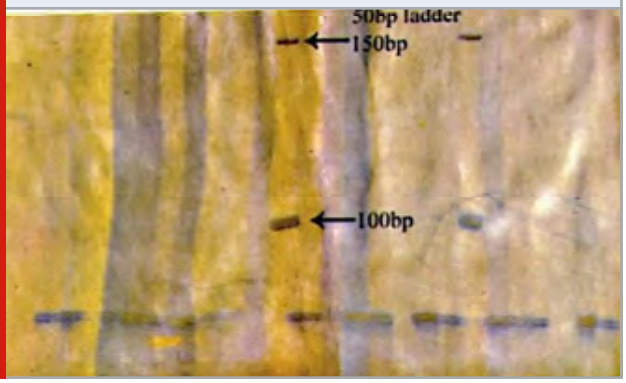
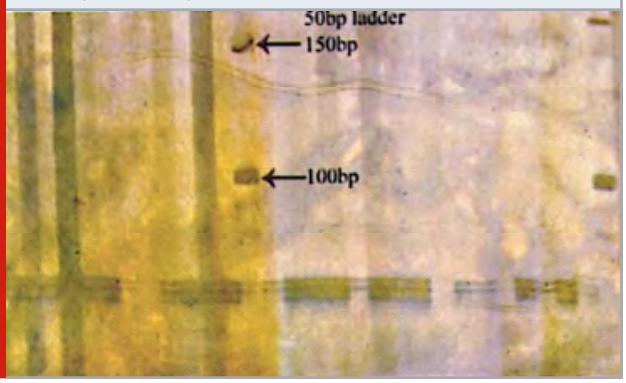


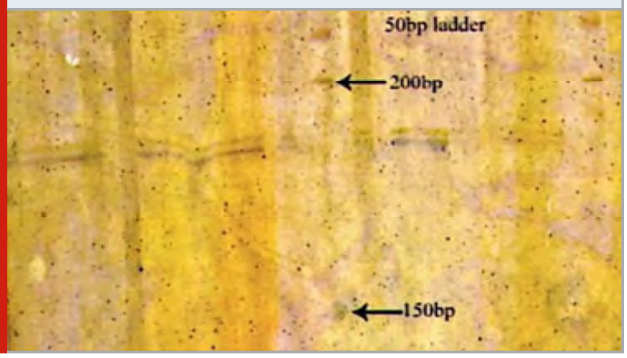
Figure 10: Photograph showing alleles at microsatellite locus (YWLL-46) in Jaisalmeri and Kachchhi camel.



However, in Jaisalmeri and Kachchhi breed, the expected heterozygosity and PIC were observed to be 0.57 and 0.475 and 0.522 and 0.420 respectively (Table 5 and 6). The present investigation in Bikaneri camel, the expected heterozygosity was lowest (0.28) at the locus YWLL -44 followed by YWLL -38 with the expected heterozygosity of 0.54 Rest of the three microsatellite loci viz. VOLP -03, VOLP-10 and YWLL -38 with the expected heterozygosity of 0.57. Rest of the three microsatellite loci viz. VOLP-03, VOLP-10 and YWLL-58 expected heterozygosity was more than 57%. In case of Kachchhi camel, the expected heterozygosity was lowest (0.32) at the locus YWLL-44 followed by VOLP -03 with expected heterozygosity of 0.515. Rest of the three microsatellite loci viz. VOLP-10, YWLL-58 and YWLL-38 expected heterozygosity was more than 52%. This indicates that the above five primers can very well be utilized for further genetic studies, which may include characterization, conservation and production enhancement.

The information derived from microsatellite marker study for individual breed may help the conservation scientists in deciding the purity of the breed and to take necessary steps for in vitro and in vivo conservation of the breed.

Figure 11: Photograph showing alleles at microsatellite locus (LCA-37) in Jaisalmeri, Bikaneri and Kachchhi camel.



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Cogent Manifestation of Nutri–Mix Powder to Heavy Working Mothers to Scuffle with Undernourishment

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ABSTRACT

Undernourishment among heavy working mothers is very common in India. An undernourished mother faces so many health problems which generally includes anemia, infectious diseases, lower immune response, decreased wound healing, mensural issues, increased rate of mortality and morbidity decreased convalescence and hence to reduce the possibilities of undernutrition. The present study was undertaken to formulate and develop cereal and millet based Nutri-mix powder to heavy working women to affray with menstrual losses and undernourishment. Among with the supplementation the study focusses on effective demonstration of multiple sample menus. The nutritional status of the heavy working subjects which includes anthropometry, biochemical, clinical dietary, REAP, frequency consumption of common foods, of the selected subjects were assessed and observed. The combination of cereals and millets were collected randomly. The questionnaire which comprised of 30 questions was mounted to assess the nutritional knowledge attitude and practice (KAP) of the selected subjects. Nutrition education was given by using cogent substantiation and charts. Before and after evaluation of KAP of the subjects were done by using questionnaire to assess the progress of the subjects. The improvement of the selected subjects was measured (98.3%). Scores obtained from pretest was increased (53.3 %) significantly after the nutrition education of the subjects.

KEY WORDS: FORMULATION, DEVELOPMENT, DEMONSTRATION, NUTRITION EDUCATION, IMPACT ASSESSMENT.

INTRODUCTION

Woman is an integral part of the society, traditionally; Indian women had been home makers. In late decades, with the spread of education and better awareness, along with increasing cost of living, women have shifted from home to career. India has provided a stage for growth and development for women. Inadequate and improper utilization of health facilities and wide spread anemia among all the reproductive age women, leading to high maternal mortality. Poor health has repercussions not only for women but also their families. Women with poor health and nutrition are more likely to give birth to low weight infants. Finally, a women's health affects the household economic wellbeing, and as a women with poor health will be less productive in the labor force. While malnutrition is prevalent among all segments of the population, poor nutrition among women begins infancy and continues throughout their lifetime. Because of prevailing culture and traditional practices in India, the health and nutritional status of women becoming worse effected. Earlier, attempts have been made to assess the diet and nutrition profile of women in rural and tribal areas of certain States in India, (Fan et al., , 2017, Abishek and Gayatri 2018, Caroline et al., 2020).

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Nutritional Status of heavy Working Women's the major challenge today before women working is to overcome the resource limitation that deliver them to low levels of productivity and wellbeing (Majied and Sadiqa 2015). For heavy working mother energy has given about 2850 kcal/kg body weight for their needs. While women's role in the food chain is essential to produce most important resource, food which paradoxically does not guarantee women even minimum levels of nutrition, (Rao et al., 2019).

Malnutrition adversely affects women's participation in the economic system and their productivity. To break this fierce paradox it is important to focus simultaneously on women's nutrition-related roles and their nutritional status (Mathur, et al., 2015). Nutri–mix is a type of instant food which can be used as supplementation for providing considerable amount of vitamins and minerals in a daily basis, (Srivastava, et al., 2019). Supplementation of fatty acids (omega3) reduces the premature birth, (Seymour et al., 2020 Daniel and Bremer (2020).

The present research focusses on analyzing the nutritional status of the heavy working mother; to formulate and develop nutri-mix powder; to substantiate the developed sample menus along with the product; and to evaluate the progression on the nutritional knowledge of the selected heavy working mothers. The present findings are based on the data available with National Nutrition Monitoring Bureau (NNMB) collected during 1998-99 and 2005-06 on tribal and rural population respectively.

MATERIAL AND METHODS

Seventy-five women with menstrual complication and malnutrition were selected for investigation. The subjects were around the age range of 25 to 60 years. Nutritional assessment method includes Anthropometric measurement, biochemical profile, clinical assessment, dietary profile, the frequency of food consumption, Rapid Eating Assessment of the Patients (REAP). To study the status on under nutrition and menstrual complication questionnaires (50) related to the food eating habits, health issues and nutritional assessment of Knowledge Attitude and Practical questions were collected and requested to respond and graded with one mark for each questions and documented; to compensate the menstrual losses and undernourishment cereals and millets were collected randomly with different proportion and it was processed as nutri – mix powder to enhance the energy value with the help of India Food Composition Table by NIN, (Longvah et al., 2017).

Different kinds of menu were framed by combining high nutrient compact foods. The investigator suggested the nutri-mix powder and the sample menus to balance the nutritional status. The selected cereals and millets were given in Figure I. The formulated nutri mix powder was substantiated for their preparation method and selection of choice based ingredients to prepare on their own in home level. The investigator also prepared one day menu which comprised with the combination of high nutrient dens food and it was demonstrated to the subjects. The importance of high nutrient compact foods and the RDA was educated. The role of food in balancing the under nutrition and menstrual complication has been explained to the subjects to integrate effective self-management and also to improve the nutritional knowledge and self-confidence. The nutrition education was given in the form of demonstration of one day sample menu by oral and charts.

RESULTS AND DISCUSSION

Table 1. Proportion of Nutri-Mix Powder

Ingredients	Grams
Green Gram	100
Thinai	50
Black Gram	50
Horse Gram	50
Rice	100
Roasted Gram Dal	50
Wheat	100
Ragi Flour	200
Cardamon	5
Dry Ginger Powder	1 Piece

Table 2. Comparative Nutritive Value of Sample Menu

S.No	Nutrients	Nutritive Value Of Sample Menu/ Day	RDA* (For Heavy Working Women)/ Day
1	Energy (k.calories)	2950.57	2850
2	Protein (g)	64.07	55
3	Carbohydrate (g)	454.13	427
4	Fat (g)	19.58	30
5	Fibre (g)	59.71	38
6	Calcium (g)	1005.11	600
7	Iron (g)	28	21

RDA* (Srilakshmi 2014)

The above table describes the ingredient proportions used for the preparations of Nutri-Mix Powder. Providing nutri-mix as supplementation using local food ingredients which have minimum processing is one of the best way to reduce undernourishment (Agashe, and Ghugre 2019).

Table III reveals the status of body mass index which expresses 35% of the subjects were underweight where they needs nutritional support for their improvement of health lack of healthy dietary habits were determined

as major reasons of anemia, (Stiller et al., 2020). Poor nutritional intake during the period of pregnancy and lactation results to vulnerable in infections (Raiten et al., 2020).

Table 3. BMI Distribution of the Subjects

S.No	BMI	Status	Nos	Percentage %
1	<18.5	Under weight	26	35
2	18.5-24.9	Normal	43	57
3	24.9-29.9	Overweight	4	5
4	30 and above	Obese	2	3
		TOTAL	75	100

Table 4. Shows the considerable increase in the knowledge of the selected subjects which evident that nutritional education with effective demonstration and suggestions might increases the quality of life of women. Progressed intervention programmes and nutrition education might results in good Improvement in the nutritional status of the subjects.(Bredbenner et al., 2017)

S.no	Tests	Scores	Percentage %	T value	P value
1	Pre-test	16	53.3		
2	Post-test	29.5	98.3	1.6657	9.87-36
3	Gain in scores	13.5	45		

CONCLUSION

Heavy working women are facing so much of physical stress and health issues like menstrual losses and undernourishment which is highly prevalent in India. Conducting programmes in relation with awareness and education can helps the women to improve the nutritional status and balance the regular diet. The utilization of local and antioxidants foods supports the healthy life of women.

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Biorestoration of Textile Industry Sludge Through Vermistabilization and Subsequent Genotoxic Assessment in *Allium cepa*

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ABSTRACT

Biorestoration of the textile industry sludge (TS) was carried out through a process of vermistabilization involving earthworms, Eudrilus eugeniae. TS were mixed with cow dung (CD) at different concentrations, which were subjected to vermicomposting studies for a period of 12 weeks. During the study, maximum viability of worms and cocoons production were observed for CD70 and TS30 during the sixth week of incubation. The maximum worm weights were observed on CD90 and TS10 at 4th week, while hatchling production was observed maximum on CD90 and TS10 during twelfth week. The physico-chemical parameters such as pH, EC, TDS, OC were found to be significantly decreased in the final product of vermicompost while parameters such as Ca,N,P,K,Na was found to be significantly increased in the final product of vermicompost. Further, Fe, Cr, Cu, and Zn also decreased in the final product. *Allium cepa* root chromosomal aberration assay was used to assess the genotoxicity of pre-vermicompost and post-vermicompost samples of TS to understand the effect of vermistabilization on the reduction of toxicity. Genotoxicity analysis of vermistablilized TS revealed 68% decline in the chromosomal aberration frequencies.

KEY WORDS: TEXTILE SLUDGE, VERMISTABILIZATION, EUDRILUS EUGENIAE ,GENOTOXICITY, CHROMOSOMAL ABERRATION, ALLIUM CEPA.

INTRODUCTION

There are numerous textile industries present in the world which produces high volumes of textile effluent. During the treatment process of these effluents, a large volume of textile sludge (TS) is produced which is a potential source of contamination of agricultural land, surface, and ground water systems causing public health hazards. The space requirement, stringent national waste discarding system and public consciousness have made land filling highly expensive and impractical (Ndegwa and Thompson 2001). Vermicomposting is seen as an alternative technology for the bioconversion of this textile sludge into organic manure. During vermicomposting, earthworms degrade organic matter present in the waste and convert it into high-grade fertilizer called vermicompost. This is a simple and low cost technique used for the breakdown of complex chemical into nontoxic forms. Application of vermicomposting in sludge management is named vermistabilization (Garg et al., 2005; Datta et al., 2018).

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Application of earthworms and biodegrading microorganisms in vermicomposting offers an efficient technique for the recycling of various nutrients. Application of vermistablilized sludge into land can increase water holding capacity, decrease soil bulk density, increase soil aeration, root penetration and stimulate microbial activity Munawar (2016) and Ramu et al. (2019). The aim of the present study is to biorestore textile industry sludge amended with cow dung through vermistabilization involving *E. eugeniae*. Further, the efficiency of vermistabilization in the reduction of toxicity is assessed in Allium cepa. Toxicity evaluation through A. cepa root chromosomal aberration is the most suitable Genotoxicity study recommended by the International program on Plant Bioassay (IPPB) for testing environmental pollutants (Chandrakant et a., 2016) and especially many researchers have used it to evaluate genotoxicity of industrial effluents (Bhat et al., 2014; Shivika et al., 2018).

MATERIALS AND METHODS

Collection of textile Sludge (TS), cow dung (CD) and Eudrilus eugeniae: Textile Sludge (TS) was collected from the Common Effluent Treatment Plant (CETP) of State Industries Promotion Corporation of Tamil Nadu (SIPCOT), Perundurai, Erode District, Tamil Nadu, India. Cow dung (CD) was collected from a single source in a local cattle farm , Karumandapam, Trichy. The initial physico-chemical characteristics of PS and CD are given in (Table 1). *Eudrilus eugeniae* were collected from Periyar Research Organization for Bio-Technique and Eco-system (PROBE), Periyar Maniyammai University, Vallam, Thanjavur, Tamil Nadu, India.

Experimental design: The experiments were conducted in a specially designed perforated plastic basket (diameter 21.5cm and depth 11.5cm). TS and CD were completely air-dried and sieved (2.0mm mesh) before mixing. The experiments were repeated thrice for each feed mixture. TS were mixed with CD in different ratios and 100 percent CD was used as control. TS was mixed with CD in different ratios V0 0:100, V190:10, V2 80:20, V370:30, V4 60:40, V550:50, V640:60, V730:70, V820:80, V910:90, V100:100. These mixtures were turned manually every day for 30 days in order to eliminate volatile gases which are potentially toxic to earthworms. After 30 days, *E. eugeniae* (20 gram) were introduced into each vermireactors. Moisture content was maintained at 70% by periodic sprinkling of water. All the vermireactors were kept under ambient conditions (room temperature 25±2°C which is the appropriate temperature for *E. eugeniae*). At the end of experiment total earthworm number, cocoon and hatchling was counted. The 0th day refers to the time of initial mixing of textile sludge and cow dung that is before pre-composting. The samples were air-dried in the shade at room temperature and stored in plastic bags for further chemical analysis.

Physico-chemical analysis: pH , TDS and EC was measured by using digital meter (Eutech Instrument, PCST test 35 series) by dissolving double distilled water suspension of each sample in the ratio 1:10 (w/v). Organic Carbon (OC) was measured on igniting the samples in a Muffle furnace at 550°C for 1 hour by the method of (Nelson and Sommers 1996). Nitrogen (N) was determined by Bremner and Mulvaney (1982) procedure. Phosphorous (P) was analyzed using the colorimetric method of molybdenum in sulphuric acid. Potassium (K), Sodium (Na) and Calcium (Ca) was Sodium (Na) was measured using a Systronics Flame Photometer-128 after digesting the samples in diacid mixtures (HClO4:HNO3 in 4:1 ratio). Heavy metals were measured by using Agilent AA 240 model Atomic Absorption Spectrophotometer (AAS).

Preparation of extract for genotoxicity analysis: Pre-vermicomposted and post-vermicomposted samples were prepared by mixing 50g of samples in 500 ml of distilled water, after which the sample were continuously stirred for 24 h. Then all sample was filtered through Whatman Filter paper 1 A (pore size 11µm) and was tested for genotoxicity effect. 100% of raw textile sludge was used as positive control and sterile distilled water served as negative control according to French Standardized Method (Ferrari et al., 1999; Sharma et al., 2012).

Experimental procedure: The *Allium cepa* (commonly called as small onion) was purchased at local market and sorted for equal size of onion bulb for all treatment along with control. The surface scales of the onion bulbs were gently removed from the bottom of the root. The rings of the root primordial were left intact Genotoxicity test was described by Rank and Nielsen (1994). The experiments were performed in triplicates and four bulbs were used per treatment. The onion bulbs were inoculated for 24-48 h in distilled water until the development of roots of 0.5-1cm lenght. Then the onion bulbs were exposed to the various concentrations of pre and post vermicomposted extract (10%, 20%, 30%, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, and 100%) as well as positive and negative control. It was exposed of 3 and 6 h in arrange to evaluate occurrence of chromosomal aberration and abnormalities before and after treatment. Later the bulb is washed with distilled and the roots are gently removed and were fixed the Farmer's fluid (glacial acetic acid and ethanol at 1:3 ratio) for 24 h and stored at 4°C. After fixation the root tips were hydrolyzed in 1N HCl for 2 min and then squashed in 4% of aceto carmine after intermittent heating for for 2-4 min. (Sharma et al. 2012).

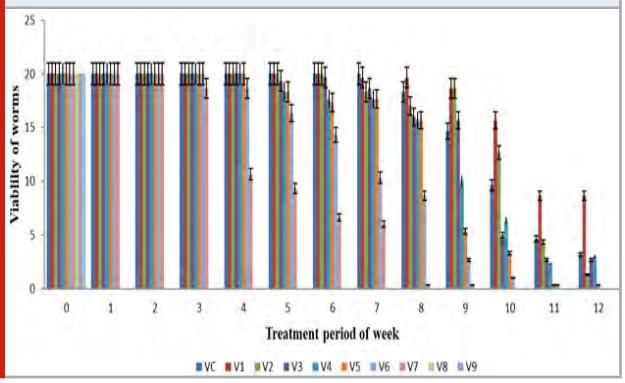
Later the root caps are removed from the well-stained root tips and were immersed in a drop of 45% acetic acid on a clean glass slide. Further squashed under a cover slip with matchstick, sealed (with nail polish) and were examined under light microscope. The presence of chromosome aberrations was observed in a minimum of 500 cells for each treatment groups. The genotoxicity analysis was performed from the pre to post vermicomposted samples. The Mitotic Index (IM) was calculated by observing a minimum of 500 cells per slides for each treatment. The root growth inhibition experiment (root length) was formed after a 12h exposure test on pre-vermicomposted and post-vermicomposted extract (Rank 2003). After the treatment onion bulb was washed with tap water and

then the root length was measured. The mitotic index was calculated by using the following formula:

$$Mitotic\ Index\ (MI) = \frac{Number\ of\ dividing\ cell}{Total\ number\ of\ Cells} \times 100$$

Statistical analysis: One–way ANOVA was used to calculate the differences among various feed mixtures. Tukey's post–hoc analysis was used to compare the means (n=3) between initial and final compost values of different physico chemical parameters. For MI, and root length, means ± SE was calculated and the stage of significant was determined by Tukey's post–hoc test. For chromosomal aberration the linear relationship between different concentration was obtained by regression and correlation study. The experimental data is accessible as (means ± SE) triplicate experiment. The statistical analysis was done with help of SPSS version 16.0 among Origin pro 8 software programs.

Figure 1: Viability of earthworm (numbers) in various concentration of textile sludge mixture with cowdung :All values are mean (n=3) ± S.D Viability earthworm in different vermireactors during the experiment of 12 weeks are different feed mixture of textile sludge and cow dung

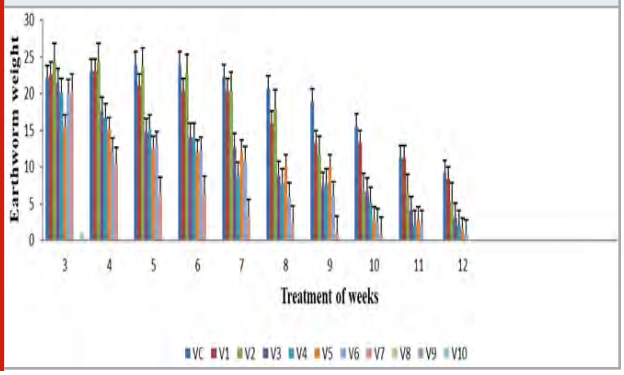


RESULTS AND DISCUSSION

The highest viability of worms (in numbers) were observed in V0 (20±0.0), V1 (20±0.0), V2 (20±0.0) and V3 (20±0.0) in the 6th week and the lowest viability of worms were observed on V5 (3.33±0.33) and V6 (8.66±0.33) in the 10 week but V7 (0.33±0.33) almost declined in the 6th week. The mean viability of worms observed on various concentration of feed mixture was statistically significant (p≤0.05). Viability of *E. eugeniae* was zero in V8, V9 and V10 from 1st day to 12th week of the experiment period. The net mortality rate were noted on V6 and V7 on 10th week and then in V4 and V5 on 12th week but no mortality was observed in V0 till end of experiment. Significant change was noted on V1 and V2 on 8th week followed by V3 on 4th week and subsequently in V4, V5,V6 on 5th week when compared with all other vermireactors in their respective week (Figure 1). The viability of worms gradually decreased in V2 and V3 from 8th week to 12th week while V4, V5 and V6 from 4th week except V7 till end of the experiment. The mortality rate was directly proportional to the concentration of textile sludge in the vermireactors. Similar observations had been reported by Elvira et al. (1998), Garge and Priya (2003) for *E. andrei* and on *E. foetida* by Kaushik and Garg (2004) in textile mill sludge vermistabilization.

The highest earthworm weight was observed in V1 (22.2 ±0.00), V2 (24±0.39) & V3 (21.56±0.76) during 4th week and lowest worm weight was observed in V4 (7.8±0.03), V5 (10.03±0.03), V6 (5.93±0.03) & V7 (2.43±0.23) during 8th week of the experiment period (Figure 2) .The worm's weight at concentrations of feed mixture was statistically different (p≤0.05). Significant changes were observed in V1 & V2 on 8th week; in V3, V4, V5, V6 & V7 for the 6th week compared with all vermireactors from the 1st week up to 12th week of the experimental period. The earthworm weight showed insignificant decreased from V0 to V3 from 6th week and V4 to V7 from 2nd week of the experimental period. The loss in worm weight can be recognized to the exhaustion of food Renuka and Garg (2007). When *E. eugeniae* conventional the food under a maintenance level, it lost weight at a rate which depended upon the quantity and nature of its ingestible substrates by Yadav and Garg (2009) .This was in compatibility with the study carried out by Neuhauser et al. 1980; Renuka et al (2007) in *E. foetida*.

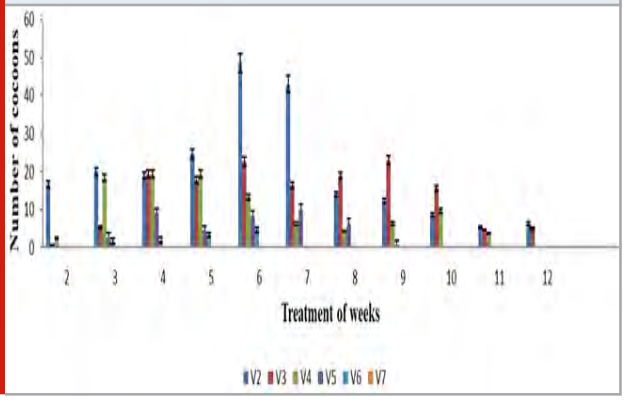
Figure 2: Earthworm weight (g) in various concentration of textile sludge mixture with cow dung. All values are mean (n=3) ± S.D Earthworm weight in different vermireactors during the experiment of 12 weeks are different feed mixture of textile sludge and cow dung



The cocoon production started from the 2nd week up to 12th week of the experiment. The highest number of cocoon was noted in V1 (48±1.11) and V2 (43±1.00) on 8th week and V0 (36.33±1.45) on 6th week during the experimental periods. The lowest cocoons production was observed in V4 (3.00±0.33) on 10th week and V5 (1.33±0.33) in 6th week as well as V6 (4.66±0.33), while no cocoons production was observed in V7, V8, V9 and V10 (Figure 3). The cocoon production in various concentrations of feed mixture was also statistically significant (p≤0.05). The differences among rates of cocoon production in different vermireactors could be related to the biochemical quality of the feed mixtures, which was one of the important factors in determining the onset of cocoon production Edwards et al (1998).

Results reveal the maximum cocoon production in V2 at 7th week during the entire treatment period. The results recommend that if higher proportion of TS added, it could not be suitable for cocoon production by *E.eugeniae*. Similar results were also observed by Sartaj et al (2014) on Press Mud (PM) where it is found that the higher percentage of PM in the feed mixtures significantly affected cocoon production and delayed the sexually maturity and reproduction of *E.fetida*.

Figure 3: Number of cocoon production in various concentration of textile sludge mixture with cow dung :All values are mean (n=3) ± S.D Cocoon production in different vermireactors during the experimental period of 12 weeks are feed mixture of textile sludge and cow dung V8, V9 & V10 were not plotted in the graph since no cocoon production was observed throughout the experiment period.



The hatchling started from the 4th week of experiment in V1, V2, V3, V4, V5 and V0. The highest number of hatchling were observed in V1 (126.05±0.06), V2 (118.33±0.20) and V3 (37.33±6.65) on 12th week followed by V0 with 156.33±2.62 while the lowest number of hatchling was observed in V5 (6.33±0.33) on 8th week. It was observed that hatchling had gradually increased in V1, V2, V3 and V4 except V5 (Figure 4). The hatchling production in various concentrations of feed mixture was also statistically significant ($p \leq 0.05$). The hatchling production was not observed in V6, V7, V8, V9 and V10 which may be due to high concentration of TS. Thus present study shows that higher proportion of TS could not suitable for hatchling production by *E. eugeniae*. The maximum number of hatchlings in V4 was supported by the studies conducted by Bhat et al (2015) where maximum number of hatchlings was observed in 60 - 100% amendment of cow dung with sugar beet mud in *E. fetida*.

In the present study, a decrease of pH was observed in the final product with maximum decrease in V2 (6.57%) and minimum in V6 (1.67%). The pH showed significant difference ($p \leq 0.05$) in different concentration of feed mixtures (Table 2 and 2 a). The lower level of pH in the final vermicompost is attributed to the production of CO2 and organic acids by microbial activity through the process of bioconversion of various substrates in the feed given to earthworms (Gupta and Garg 2007). The pH change towards neutral is attributed to mineralization of the nitrogen and phosphorus into nitrites/ nitrates and orthosphates and bioconversion of the organic materials into intermediate species of organic acids (Yadav and Garg 2009).

Figure 4: Hatchling production in different various concentration of textile sludge mixture with cow dung

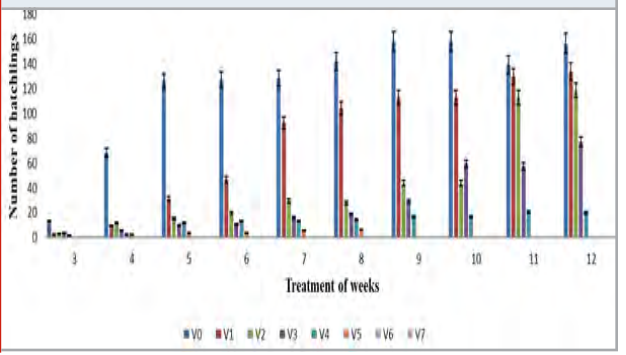


Table 1. Initial Physico- chemical parameters of raw textile sludge and cow dung (Mean ±SE)

S. No.	Physico-chemical parameters	Textile sludge (TS)	Cow dung (CD)
1	pH	8.78±0.10	7.69±0.24
2	EC(Ms/cm)	12.32±0.04	12.54±0.40
3	TDS(mg/L)	21.83±2.09	1.84±0.05
4	Na(g/kg)	3.43±0.22	3.60±0.09
5	K(g/kg)	0.15±0.02	6.88±0.11
6	Ca(g/kg)	274.47±4.00	18.48±0.54
7	OC(g/kg)	5.93±0.15	38.15±0.74
8	P(g/kg)	1.48±0.24	1.68±0.62
9	N(g/kg)	9.47±0.64	16.12±0.22
10	C:N ratio	0.85±0.18	2.55±0.38
11	Zn(mg/g)	2.41±0.07	1.1±0.10
12	Cu(mg/g)	1.8±0.11	0.88±0.08
13	Cr(mg/g)	1.02±0.06	0.15±0.01
14	Fe(mg/g)	8.07±1.19	1.03±0.16

The electrical conductivity (EC) was found to be significantly decreased in vermicompost as compared to initial feed mixture. The maximum decrease in EC was observed in V1, V2, V3, V4, V5, and V6 while minimum decrease was observed in V7, V8, V9 and V10. Decrease in EC in vermicompost is due to stabilization of mixtures. Singh et al. (2010) also observed a decline in EC in the vermicompost from the bio sludge of a beverage industry. Bhat et al (2015) have reported that the acceptance limit of EC for plant growth as 4.0 m Scm−1 and our results are in accordance to it. The total dissolved solids (TDS) were found to be significantly decreased in vermicompost when compared to initial the initial feed mixture. Minimum decrease was observed in V8 (0.33%) and maximum decrease of TDS was observed in V4 (52.15 %). The calcium significantly increased in

vermicompost as compared to initial feed mixture and the maximum increase was observed in V1 (182.35%) and minimum increase in V4 (10.27%). Previous study have found calcium metabolism in earthworm is mostly associated with gut secreted enzymes (Suthar 2010).

A significant increase ($p \leq 0.05$) in K in final product was observed as compared to the initial feed mixture in all different concentration. Maximum increase in potassium was observed in V4 (132.3%) and lowest was in V9 (7.09%). Most of previous report on vermicompost have reported a higher K at the end of experiment (Khwairakpam and Bhargava 2009; Hait and Tare 2011). The acid production by microorganism is the major role for solubilizing of insoluble potassium. It is evident that the inoculation of earthworms in waste feedstock enhances the waste mineralization process. Basically organic waste, during the respiration period pass through the earthworm gut and some fraction of minerals is changed in to the additional available nutrients (i.e exchangeable forms) due to the work endogenic and/or exogenic enzyme (Suthar et a., 2012).

Table 2. Initial and final physico-chemical parameters (mean ±SE) different proportion of textile sludge mixed with cow dung*

Parameters		VC	V1	V2	V3	V4
pH	Initial	7.49±0.06	7.72±0.13	7.81±0.06	7.85±0.60	7.97±0.07
	Final	6.58±1.14	6.78±0.11	7.06±0.11	7.25±0.01	7.19±0.00
	% change	-12.14	-12.17	-10.74	-7.60	-8.40
EC (µs)	Initial	5.87±0.09	5.78±0.12	5.39±0.40	5.89±0.58	5.41±0.21
	Final	2.26±0.05	2.09±0.00	3.46±0.05	4.11±0.00	4.99±0.01
	% change	-61.49	-65.05	-35.80	-30.22	-7.76
TDS mg/ml	Initial	3.91±0.64	4.87±0.16	4.25±0.05	5.57±1.37	5.56±1.40
	Final	1.73±0.34	1.59±0.00	2.26±0.25	2.52±0.28	2.29±0.00
	% change	-55.75	-67.35	-46.82	-54.67	-53.41
Ca g/kg	Initial	18.14±0.06	19.50±0.53	36.11±1.00	73.89±0.54	114.34±0.91
	Final	25.46±0.55	55.06±9.13	59.27±0.17	99.26±0.33	122.59±5.79
	% change	40.35	182.35	62.13	34.33	7.21
K(g/kg)	Initial	3.70±1.12	3.86±1.90	3.14±0.10	2.99±0.00	2.97±0.40
	Final	9.33±0.57	7.99±0.11	7.87±0.27	6.88±0.98	6.90±0.27
	% change	152.16	141.88	150.63	130.10	132.32
P (g/kg)	Initial	1.88±0.96	1.52±0.52	1.43±0.88	1.48±0.46	1.20±0.10
	Final	4.30±0.60	4.10±0.17	4.33±0.57	3.22±0.06	2.99±0.30
	% change	128.91	164.51	202.79	117.56	149.16
C:N g/kg	Initial	2.53±0.22	2.51±0.72	2.34±0.36	2.43±0.14	1.64±0.07
	Final	0.66±0.11	0.91±0.06	0.89±0.08	0.96±0.07	0.89±0.25
	% change	-73.91	-63.74	-63.63	-60.43	-45.73
N g/kg	Initial	17.08±0.94	16.99±1.00	16.29±0.61	16.84±1.09	16.78±0.08
	Final	27.38±1.40	26.17±1.48	25.36±0.54	25.61±2.19	21.89±1.35
	% change	60.30	54.03	55.67	52.07	30.45
OC g/kg	Initial	43.25±2.19	44.33±9.80	45.89±2.30	41.21±0.51	28.18±1.34
	Final	18.07±1.40	28.47±2.58	26.14±1.75	26.13±0.04	19.40±4.08
	% change	-58.21	-32.74	-43.03	-36.59	-32.15
Cu (mg/kg)	Initial	1.24±0.04	1.49±0.43	1.57±0.35	1.72±0.30	1.56±0.19
	Final	0.51±0.44	0.99±0.09	1.05±0.11	1.08±0.06	1.15±0.11
	% change	-86.29	-33.55	-33.12	-37.20	-26.28
Cr (mg/kg)	Initial	0.38±0.01	0.56±0.90	0.74±0.12	0.88±0.00	0.93±0.05
	Final	0.17±0.01	0.19±0.01	0.20±0.01	0.23±0.01	0.26±0.13
	% change	-.55.26	-66.07	-72.97	-73.86	-72.04
Zn (mg/kg)	Initial	2.63±0.12	2.59±0.51	2.79±0.16	2.84±0.26	2.92±0.23
	Final	0.53±0.00	1.37±0.29	1.58±0.04	1.66±0.17	1.60±0.11
	% change	-79.84	-38.99	-43.36	-41.54	-45.20
Fe (mg/kg)	Initial	0.80±0.06	1.90±0.34	2.81±0.37	2.85±0.12	2.96±0.06
	Final	0.40±0.08	0.17±0.28	0.58±0.39	0.78±0.00	2.08±0.13
	% change	-50	-91.05	-79.35	-72.63	-29.72

Weight in (g/kg) and (mg/kg) Significance level was determined by Tukey test $p \leq 0.0$*

Table 2 a. Initial and final physico-chemical parameters (mean ± SE) different proportion of textile sludge mixed with cow dung*

Parameters		V5	V6	V7	V8	V9	V10
pH	Initial	7.95±0.60	7.96±0.06	7.98±0.07	8.19±0.03	8.42±0.40	8.50±0.03
	Final	7.41±0.00	7.52±0.02	7.84±0.11	7.95±0.00	7.96±0.00	7.95±0.00
	% change	-6.20	-1.01	-.1.38	-2.95	-5.46	-.2.92
EC (µs)	Initial	6.20±0.51	7.35±0.03	7.21±0.18	7.65±0.00	7.42±0.39	7.96±0.09
	Final	5.87±0.56	7.18±0.00	7.04±0.06	7.14±0.12	7.17±0.29	7.86±0.23
	% change	-5.32	-2.31	-2.35	-6.66	-3.23	-1.25
TDS mg/ml	Initial	6.16±1.57	6.66±1.57	7.62±0.54	7.66±0.41	13.2±3.46	15.80±1.32
	Final	2.73±0.05	3.66±0.25	3.52±0.41	7.75±0.33	7.43±0.40	7.91±0.21
	% change	-55.68	-45.04	-53.80	-1.17	-43.71	-49.93
Ca g/kg	Initial	147.81±0.51	156.41±0.50	164.30±1.09	164.75±4.58	171.30±11.5	171.47±10.1
	Final	204.15±0.17	235.80±0.59	289.44±0.49	289.55±0.55	287.48±0.15	288.63±0.30
	% change	43.89	85.05	75.44	75.33	67.82	68.32
K(g/kg)	Initial	2.96±0.69	2.88±0.12	2.09±0.84	1.53±0.12	1.55±0.10	1.56±0.17
	Final	6.20±0.50	5.21±0.23	4.86±0.05	1.92±0.12	1.94±0.06	1.95±0.06
	% change	109.45	80.90	132.53	25.49	25.16	24.99
P (g/kg)	Initial	1.19±0.22	1.23±0.36	1.16±0.14	1.08±0.07	1.03±0.04	0.99±0.00
	Final	2.85±0.05	2.69±0.33	2.22±0.23	1.40±0.19	1.23±0.00	1.17±0.06
	% change	139.49	118.69	91.37	29.62	19.41	18.81
C:N g/kg	Initial	1.34±0.12	1.54±0.07	1.01±0.10	0.99±0.07	0.98±0.40	0.96±0.03
	Final	0.72±0.06	0.69±0.04	0.94±0.06	0.92±0.12	0.91±0.07	0..89±0.10
	% change	-45.26	-55.19	-6.93	-7.07	-7.14	-10.10
N g/kg	Initial	14.40±0.52	13.77±0.58	13.40±1.21	9.15±0.45	9.52±0.42	9.40±1.22
	Final	18.84±1.10	17.36±0.35	15.01±0.00	12.61±0.58	12.34±0.58	11.77±1.41
	% change	30.83	26.07	12.01	37.81	29.62	25.21
OC g/kg	Initial	19.40±0.86	21.24±0.54	13.61±0.40	12.76±0.40	11.65±0.16	12.43±0.18
	Final	13.98±0.00	12.52±0.15	12.63±0.61	10.48±0.06	9.62±0.32	9.41±0.61
	% change	-27.93	-41.05	-.7.20	-17.86	-17.42	-24.29
Cu (mg/kg)	Initial	1.85±0.22	2.29±0.32	2.34±0.40	2.50±0.34	2.60±0.56	2.37±0.47
	Final	1.37±0.01	1.58±0.00	1.68±0.00	1.77±0.02	1.90±0.01	1.87±0.01
	% change	-25.94	-31.00	-32.80	-29.2	-26.92	-21.09
Cr (mg/kg)	Initial	0.99±0.01	0.99±0.01	0.96±0.05	1.14±0.06	1.40±0.41	1.44±0.50
	Final	0.30±0.08	0.42±0.11	0.62±0.22	0.82±0.21	0.81±0.22	0.93±0.38
	% change	-69.69	-57.57	-35.41	-28.07	-28.07	-35.41
Zn (mg/kg)	Initial	3.36±0.46	3.38±0.39	3.40±0.40	3.80±0.51	3.85±0.61	3.88±0.32
	Final	1.65±0.03	1.77±0.00	1.82±0.11	1.87±0.02	2.00±0.11	2.37±0.54
	% change	-52.31	-47.63	-46.47	-50.78	-48.05	-30.29
Fe (mg/kg)	Initial	2.96±0.06	3.06±0.40	3.25±0.55	4.34±0.12	4.38±0.24	4.53±0.39
	Final	2.41±0.00	2.74±0.04	2.89±0.00	4.04±0.01	4.08±0.02	4.09±0.00
	% change	-18.58	-10.45	-11.07	-6.91	-6.84	-9.71

Weight in (g/kg) and (mg/kg) Significance level was determined by Tukey test $p \leq 0.05$

Results reveal the significant increase ($p \leq 0.05$) of phosphorus content after inoculation of earthworm. V2 showed highest increase in with 242% and V9 showed a minimum increase of 19.41%. The increase in P is possibly due to activities of P-solubilizing bacteria and enzymatic activities of earthworm gut (Garg et al 2005; Suthar 2012). The level of P in the final product indicates that the vermistabilized sludge is ready as soil amendment material.

The C: N ratio is an important indicator of vermicompost maturity. In this study the C: N ratio of vermicompost material was significantly low than initial substrates. Decline in C: N ratio in this study was due to higher loss of carbon through microbial respiration in the form of CO2 along with an increase in nitrogen and stabilization of waste by the action of *E. eugeniae*. Similar results were observed by Bhat et al. (2014). Earlier studies suggest that a C: N ratio below 20 is an indicative of acceptable maturity, while a ratio of 15 or

lower is being preferred for agronomic use of composts. The vermicompost obtained in this study showed C: N ratio within the preferable limits as described by Morais and Queda (2003).

Table 3. Root length of *Allium cepa* (mean±SE) exposed to pre-compost Extract and post-compost (100%) textile sludge

Concentration (%)	Pre compost Root length(cm)	Post compost Root length(cm)
PC	2.80±0.52	2.80±0.52
NC	8.30±0.36	8.30±0.36
10%	6.70±0.55	8.50±0.61
20%	5.63±0.55	8.06±1.00
30%	4.86±0.23	7.00±1.00
40%	4.33±0.57	6.80±0.72
50%	3.99±0.00	6.33±0.57
60%	3.73±0.15	6.76±1.04
70%	3.96±0.05	6.60±1.03
80%	3.33±0.57	5.33±1.36**
90%	3.66±0.41	5.43±0.45*
100%	3.06±1.00	4.60±0.45**

NC = negative control (distilled water), PC =positive control (Fresh ST), Signification level was determined by t- test *p≤0.01. **p≤0.05

The nitrogen content was significantly increased (p≤0.05) in all feed mixture at end of the experiment. N Maximum increase was observed in V2 (61.65%) and minimum was observed in V6 (12.01%). Generally body secretions of earthworm (excreta, mucus) add nitrogen in substrate if earthworms were inoculated in organic wastes for longer periods. Earthworms also alter the microclimatic conditions of vermireactors which consequently promote microbial populations responsible for nitrogen enrichment (Suthar et al. 2012). Hence in the present study the combination of the organic waste CD and textile sludge acted as good energy stuff for nitrogen fixing bacteria enhanced by *E. eugeniae*.

Organic carbon in the final product was significantly reduced when compared with the initial feed mixtures. The maximum reduction in OC was observed in V2 (21.12%) and minimum reduction was observed in V10 (2.06%). Reduction may be due to combined action of earthworm and microorganisms where the loss for organic carbon from the initial waste is in the form of release of CO2 by respiration of earthworms. Kaviraj and Sharma (2003) reported 45% loss of carbon during vermicomposting of municipality and industrial wastes. Similarly, Prakash and Karmegam (2010) reported reduction of OC in vermicomposting of sugar industry waste. Vermicomposting brings about significant reduced in TOC level of the waste property and accelerates waste

Table 3. Root length of *Allium cepa* (mean±SE) exposed to pre-compost Extract and post-compost (100%) textile sludge

Concentration	Mitotic index in pre-vermicompost[a]	Mitotic index in post-vermicompost[b]
NC	12.46±2.73	12.46±2.73
PC	2.06±0.11	2.06±0.11
10 % 3 h	7.16±0.05	10.33±0.57
6 h	7.66±0.57	10.63±1.09
20 % 3 h	7.53±0.57	9.73±20.70
6 h	7.33±0.41	9.33±1.52
30 % 3 h	6.40±0.69	8.83±1.25
6 h	7.86±0.61	9.56±2.18**
40 % 3 h	5.66±1.03	6.66±1.15*
6 h	6.06±1.81	6.93±1.00*
50 % 3 h	5.66±1.30	6.40±2.16**
6 h	6.65±1.44	7.60±0.87**
60% 3 h	4.66±0.83	4.86±0.64**
6 h	4.46±0.41	5.73±0.30**
70% 3 h	3.90±0.23	4.46±0.23**
6 h	4.20±0.20	4.80±0.20**
80% 3 h	3.36±0.70	4.20±0.20**
6 h	3.73±0.61	4.86±0.83**
90% 3 h	3.60±0.34	4.20±0.50**
6 h	3.80±0.11	4.40±0.34**
100% 3 h	2.40±0.40	2.80±0.52**
6 h	2.60±0.69	3.06±0.80**

NC Negative control (distilled water); PC= Positive control (fresh ST); aFrom each group 3000-5000 cells were scored to determine MI; bFrom each group 4000-6500 cells were scored to determine MI; The level of significance was determined by t- test:*p≤0.01 **p≤0.05

stabilization process (Singh and Suthar 2012; Hait and Tare 2011).

The sodium content was significantly increased (p≤0.05) in all feed mixture with maximum increase in V3 (77.31%) and minimum in V9 (16.37%). Our results are in corroboration with Singh et al. (2010).The heavy metal analysis reveals Cu, Cr, Zn and Fe decreased from initial feed mixture to final product of vermicomposting (Table 2 and 2 a). Reduction in the heavy metal concentrations could be related to discharge of these cations by excess water drainage (Garg etal. 2003). Genotoxic effects of textile sludge on meristematic cells of A.cepa were estimated on the basis of mitotic index and chromosomal aberrations.The root length was observed in different concentration of pre and post vermicomposting of textile sludge along with negative and positive controls after five days of exposure.

The average root length of onion in the positive and negative controls was (2.80±0.52) and (8.30±0.36)

respectively. The increase in root length was observed in post-vermicompost as compared to initial textile sludge extract. The maximum root length was observed at 10% (8.50±0.61), 20% (8.06±1.00) and 30% (7.00±1.00) concentration of post–vermicompost while minimum root length was observed at the 100% TS (4.60±0.45) post-vermicompost (Table. 3). By observing the results it can be concluded that vermistabilization reduce toxic effect of textile sludge. In the A.cepa test, inhibition of root development and the appearance of stunted roots indicate retardation of growth and cytotoxicity while root wilting explain toxicity (Sartaj et al 2014).

The total number of dividing cells in the cell cycle is the characteristics of Mitotic Index (MI) and in this study the MI increased as the concentration of the TS increased. (Table 4). The MI was maximum during 6 h exposure on 10%, 20%, 30% post-vermicompost extract. It is observed that the lesser the concentration of the textile sludge the lesser was the impact on the roots. Higher the concentration of TS showed higher abnormalities on root meristem cells. MI decrease in the A. cepa test has been reported by Jiang and Liu (2000); Jain et al.

Table 5. Different Chromosomal Aberration and nuclear abnormalities in the root meristem cells of Allium cepa exposed to pre-vermicompost and post-vermicompost textile sludge extract*.

Chromosomal Aberration	Negative control No .of aberrant cells	Positive control No. of aberrant cells	10% No .of aberrant cells		20% No of aberrant cells		30% No .of aberrant cells		40% No .of aberrant Cells	
			3h	6 h	3h	6h	3h	6h	3h	6h
	Different Chromosomal Aberration and nuclear abnormalities in pre-vermicompost									
C- Mitosis	0	23	5	5	6	7	9	9	11	11
Delayed anaphase	2	10	3	4	4	6	6	6	8	8
Stickiness	1	17	3	3	3	7	9	8	10	11
Chromosomal loss	1	21	2	5	2	1	3	5	6	7
Multipolarity	1	8	1	2	1	2	4	5	4	4
Vagrant	1	9	2	3	4	4	5	7	2	4
Chromatin bridge	5	23	5	6	5	6	8	8	10	12
Chromosomal breakage	3	22	4	5	6	6	7	9	6	6
Micronuclei	0	4	0	0	2	1	1	1	3	3
Polyploidy	0	5	1	1	1	1	1	2	3	2
Stars	0	0	0	0	1	1	0	0	0	0
Total aberration	14	151	26	28	36	37	56	60	65	68
Percent aberration (%)	2.8	30.2	5.2	5.6	7.2	7.4	11.2	12	13	13.6
	Different Chromosomal Aberration and nuclear abnormalities in post-vermicompost									
C- Mitosis	0	23	0	1	1	2	3	5	6	6
Delayed anaphase	2	10	1	2	2	2	3	4	5	7
Stickiness	1	17	1	2	2	2	4	4	5	7
Chromosomal loss	1	21	1	1	2	1	3	3	5	6
Multipolarity	1	8	1	1	1	1	1	1	1	1
Vagrant/s	1	9	2	1	1	1	3	2	2	4
Chromatin bridge	5	23	2	2	2	5	4	5	4	3
Chromosomal breakage	3	22	2	2	2	3	3	4	6	6
Micronuclei	0	4	1	1	2	2	2	2	1	3
Polyploidy	0	5	0	0	1	1	1	1	1	1
Stars	0	0	0	0	0	0	0	0	0	0
Total aberration	14	151	10	13	16	18	27	30	35	41
Percent aberration %	2.8	30.2	2	2.6	3.2	3.6	5.4	6	7	8.2

*Out of 500 cells examined

(2004) and Sartaj et al. (2014) and they have correlated the decrease of MI to exposure with heavy metals in the feed mixture. The significantly less MI of all the treatment groups when compared to the negative controls reflects an alteration in chromosomes due to the adverse effect of substances in the TS on the growth and development of exposed cells.

The chromosomal aberrations were studied by using A. cepa root meristem cells exposure to pre- and post-

vermicompost TS extract. (Table 5 and 5a) Results reveal that the levels of aberration in concentration-dependent as the post-compost showed significantly reduced aberration. The aberration percentage was highest in the pre-compost of V10 (100% TS) with 28.8 percent and it reduced to 24 percent after vermistabilization. Abnormalities such as C-mitosis, delayed anaphase, stickiness, chromosomal loss, multipolarity, vagrant, chromatin bridge, chromosomal breakage, micronuclei, polyploidy, and stars were observed. This is corroboration with the findings Dixit and Nerle (1985); Joshi and Singh (1989); Qian (2004) and Sartaj et al (2014) reported that the increase in the concentration of various effluents increased the aberration rate. It is evident from the results that the raw textile sludge has Genotoxicity potential and the proposed vermistabilization has the potential to mitigate the effect that arises through land filling or dumping of textile industry sludge.

Table 5a. Different Chromosomal Aberration and nuclear abnormalities in the root meristem cells of Allium cepa exposed to pre-vermicompost and post-vermicompost textile sludge extract*.

Chromosomal Aberration	50% No .of aberrant cells		60% No. of aberrant cells	70% No .of aberrant cells			80% No .of aberrant cells		90% No .of aberrant cells		100% No .of aberrant Cells	
	3h	6 h	3h	6 h	3h	6h	3h	6h	3h	6h	3h	6h
	Different Chromosomal Aberration and nuclear abnormalities in pre-vermicompost											
C- Mitosis	13	14	15	16	18	17	19	19	21	21	20	20
Delayed anaphase	10	11	10	12	10	11	11	12	12	13	13	15
Stickiness	11	11	11	12	14	15	16	16	12	14	15	15
Chromosomal loss	9	10	12	12	14	16	16	17	19	19	20	21
Multipolarity	5	6	5	6	3	3	4	7	7	6	8	8
Vagrant	6	7	9	9	11	12	8	7	10	11	6	8
Chromatin bridge	12	13	13	17	19	18	20	20	20	21	24	20
Chromosomal breakage	7	8	11	11	15	16	21	22	23	24	22	22
Micronuclei	2	2	4	4	3	3	4	5	6	7	6	7
Polyploidy	2	2	3	4	3	5	4	4	5	5	7	7
Stars	0	0	1	3	1	1	0	0	0	0	0	0
Total aberration	77	84	95	106	114	117	123	129	135	138	139	144
Percent aberration (%)	15.4	16.8	19	21.2	22.8	23.4	24.6	25.8	27	27.6	27.8	28.8
	Different Chromosomal Aberration and nuclear abnormalities in post-vermicompost											
C- Mitosis	5	6	8	10	12	14	15	16	18	20	20	23
Delayed anaphase	9	10	9	10	8	9	10	9	10	11	10	12
Stickiness	4	6	4	8	9	9	10	12	12	14	13	15
Chromosomal loss	4	7	10	10	10	10	12	14	13	15	17	18
Multipolarity	1	1	1	1	2	2	1	3	2	1	1	2
Vagrant/s	3	5	2	5	6	8	8	9	10	13	12	12
Chromatin bridge	6	8	10	9	11	13	12	17	20	18	21	20
Chromosomal breakage	5	7	8	9	10	12	13	12	16	17	20	15
Micronuclei	1	1	1	2	4	3	3	3	4	4	2	2
Polyploidy	1	1	2	2	0	1	0	0	1	1	1	1
Stars	0	0	0	0	0	0	0	0	0	0	0	0
Total aberration	39	44	51	66	72	81	83	92	106	118	113	120
Percent aberration %	7.8	8.8	10.2	13.2	14.4	16.2	16.6	18.4	21.2	23.6	22.6	24

* Out of 500 cells examined

CONCLUSION

The use of textile sludge as raw material in the vermistabilization system can potentially help to convert the waste into a value added final product and the preeminent growth and fecundity of Eudrilus eugeniae in CD 70: TS 30 is an evident for it. The study revealed that vermicompost produced from *E. eugeniae* possessed higher nutrient content, lower C/N ratio, and lower electrical conductivity. Further the genotoxicity study clearly indicate that the vermitechnology reduced the toxicity potential of raw TS. It also reflects the potential

of the earthworm *E. eugeniae* to convert raw textile sludge into nutrient- rich manure and play a major role in solid waste management.

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Design and Prediction of ADMET Properties of Drugs for Measles Using *In-Silico* Approaches

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ABSTRACT

Measles virus phosphoprotein tetramerization domain contains ligand named Acetate ion, (acetate, $C_2H_3O_2$). It was identified as a Drug Target protein. Its protein structure (PDB format) was downloaded from the Protein Data Bank. Ligand Acetate ion, (acetate, $C_2H_3O_2$) was obtained for further analysis. Compounds were selected from available drug libraries such as PubChem, DrugBank, ChemSpider etc. physical and chemical properties were tabulated in order to compare similarity score. Furthermore they were screened and docked with Acetate ion, (acetate, $C_2H_3O_2$) using PyRx-virtual screening software for Structure based drug design (SBDD). 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 13 compounds as potential leads in our dry lab experiments. These chemical compounds can be further investigated in wet lab experiments with a view to finding effective drugs against Measles.

KEY WORDS: ACETATE ION, ABSORPTION, DISTRIBUTION, METABOLISM, EXCRETION AND TOXICITY, ADMET, MEASLES, PYRX.

INTRODUCTION

Measles infection is occurred by a virus from the paramyxovirus family. These viruses are small parasitic microbes. Once you got infected, the virus attacked host cells and utilizes cellular components to complete its life cycle(Taylor et. al., 2002; Kayeet al., 2001). The respiratory tract is initially infected by the virus. However, it ultimately increases to other parts of the body through the blood stream. There are twenty four recognized genetic types of measles, although only six are at present circulating(Aickin, et al., 1994). Measles is spread through droplets from the mouth, nose or throat of infected persons. Initial symptoms can be observed after 10–12 days of infection. It includes bloodshot eyes, high fever, a runny nose, and tiny white spots on the inside of the mouth. A few days later, a rash expands, starting on the face and upper neck and slowly spreading downwards, (Naim, 2015).

Measles virus, that is a single-stranded, negative-sense RNA virus belongs to the Mononegavirales order which includes a number of human pathogens such as Ebola, Nipah, and Hendra viruses whose genome is encapsidated by multiple copies of the viral nucleoprotein (N). This N-RNA complex is the template for transcription and replication by the viral polymerase complex contains the large protein (L) that carries the RNA-dependent

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RNA polymerase activity and the phosphoprotein (P), the polymerase cofactor (Communieet al., 2013). The phosphoprotein of measles virus is a modular protein contains an essentially disordered N-terminal domain and of a C-terminal moiety (PCT) made up of alternating disordered and globular regions(Johansson et al.,2003).

In measles virus, during genome replication, synthesis of viral RNA and encapsidation by nucleoprotein are associated. Nucleoprotein also has the ability to self-assemble on cellular RNA to form nucleocapsid resembling particles in the absence of viral RNA and viral proteins. The association of phosphoprotein with the soluble, monomeric form of Nucleoprotein avoids it from binding to cellular RNA. The nucleoprotein – phosphoprotein (N-P) complex is the substrate employed by the polymerase to initiate encapsidation of genomic RNA. Nucleoprotein forms complexes with phosphoprotein and with the phosphoprotein - large protein (P-L) complex during transcription and, during replication too (Johansson et al.,2003).

Measles was an unavoidable disease during the human advancement with considerable level of morbidity and mortality. The seriousness of measles infection was to a great extent contained by the advancement of a live attenuated antibody that was brought into the vaccination programs. However, all efforts to eliminate the disease failed and continued to result in major deaths yearly. The expansion of molecular biology techniques permitted the rescue of measles virus from cDNA that facilitated vital insights into a variety of aspects of the biology of the virus and its pathogenesis. Consequently these technologies aided the development of novel vaccine candidates that stimulate immunity against measles and other pathogens. Depending on the promising prospective, the utilization of measles virus as a recombinant vaccine and a therapeutic vector is concentrated (Naim, 2015).

Measles is a viral infection that begins in the respiratory system. It stays a significant cause of death amongst young children all over the world, in spite of having, the accessibility of a safe and effective vaccine. According to the World Health Organization (WHO), there were almost 1,10,000 worldwide deaths happened due to measles in 2017 and majority of them were children under the age of 5. Measles was a predictable infection during the human development with considerable degree of morbidity and mortality. The severity of measles virus infection was mostly contained by the improvement of a live attenuated vaccine that was commenced into the Vaccination Plans. Still, all efforts to eliminate the disease are unsuccessful and it sustained to yearly result in significant deaths (Naim, 2015).

Vaccine of Measles has been found years ago. The disease was remained under control due to vaccination program for many years. Worldwide measles deaths have diminished by 84 percent worldwide as of late — from 550,100 deaths in 2000 to 89,780 out of 2016 — measles is as yet regular in many developing nations, especially in parts of Africa and Asia. However, the cases of measles are being reported in recent years. Undoubtedly the death ratio has been decreased still huge number of cases is reported. Measles is a very infectious viral illness. It remains a significant reason for death among youthful kids all around, in spite of the accessibility of a protected and successful antibody. An expected 7 million individuals were influenced by measles in 2016 (https://www.who.int/immunization/diseases/measles/en/, 2019).

Measles vaccine was replaced by mixing up measles, mumps, and rubella vaccine in 1988, with seekingthe elimination all three diseases from the UK. As the implementation of the vaccine, coverage has enhanced steadily with 93% of children now being vaccinated by 2 years of age. Though the overall reduction in the incidence of measles, outbreaks in older children have recently been reported, suggesting that a two dose strategy may be needed to make sure eradication(Brownet al., 1994).Evaluations of vaccination programs are generally based on the theory that vaccines have an impact only against specific diseases. This theory may not be true for measles vaccine. Current studies designate that vaccines may have significant non-specific effects as girls receiving high titre measles vaccines were established to have reduced long term survival compared with recipients of standard titre vaccines. Secondly, studies of standard titre measles vaccine have reported a better than expected reduction in mortality in areas with high mortality. Since these observations implies that measles immunization may have a non-specific, useful effect (Aaby et al., 1995).

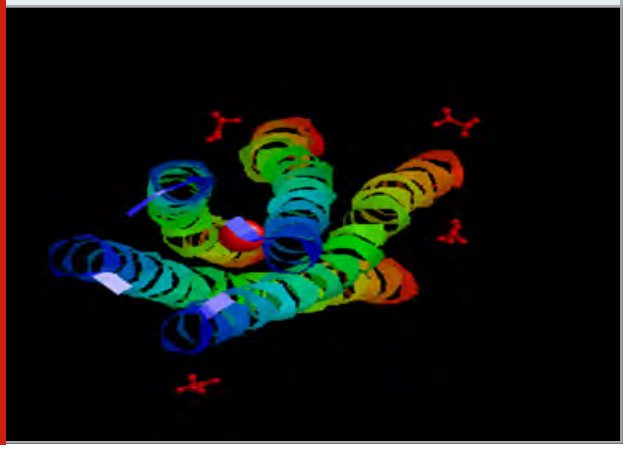
To design the drug with the help of the computer is a specialized branch that uses computational approaches to create drug-receptor interactions (Bissantzet al., 2000).These types of methods are greatly dependent on the tools of bioinformatics, applications and databases. Many of the promising drugs fail in clinical trials after many years of research. The failure 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 also be assumed with help of bioinformatics software and tools. The wide range of bioinformatics tools and web indexes (search engines) are available for this work (Bissantz et al., 2000; Iskaret al., 2011). Once potential lead molecules have been identified the next step is to find their structural and ADMET properties. This commonly consists of the arrangement of changes in accordance with the essential and secondary structure of the compound. The protocol can be enhanced by using software that explores related compounds to the lead candidate (Songet al., 2009).

MATERIAL AND METHODS

Target Identification: Phosphoprotein tetramerization domain of Measles Virus, which is responsible for

the induced folding of the C-terminal Domain of the Nucleoprotein (figure 1) was taken as target protein(Johansson et al., 2003).

Figure 1: Measles virus phosphoprotein tetramerization domain



The structure file of protein with PDB ID 4BHV was downloaded from Protein Data Bank. The experimental details are, Resolution[Å]: 2.1, R-Value: 0.232 (obs.) and R-Free:0.242.

Drug Library: Compounds having related structures and properties with Acetate ion, (acetate, C2H3O2) were obtained and screened using online chemical databaseswizPubChem, DrugBank, KEGG, ChemSpider etc.(Table 1) (Dias et al., 2008; Fernando et al., 2008).

Docking: Docking was performed on the structure filessuch as .sdf/.mol/.pdb of the compounds usingPyRx docking program with a view to obtaining binding energy(Table 2).

ADMET: Docked chemical substances were tested using QSAR database for ADMET properties such as Carcinogenicity, Lethal concentration, Mutagenicity, In vitro tests, Health End points etc. Thirteen compounds lastly considered as drug target candidates since they accomplish most of the characteristics(Table 3).

RESULTS AND DISCUSSION

In the present study 80 chemical compounds were analyzed as drug target candidate for Measles virus phosphoprotein tetramerization domain. Physical and chemical properties including pKa value, Molecular weight(MW), State, Polarizibility, Melting and Boiling Point were obtained from DrugBank, PubChem, ChemSpider etc. online databases with a view to understanding the essentiality of the compound as a drug target(Table 1)(Dias et al., 2008). pKa value of a weak acid varies between -2 to 12. pKa values more than twelve area unit are considered to be alkaline drug and lower than -2 area units are considered to be a strong acid. The ability of a molecule to be polar or non polar is indicated by Polarizability, which is reliant upon the charge distribution and affects the bond formation in a chemical compound.The standard range of binding energy is between -3 to -9 Kcal/mol.Docking results indicate that lower binding energy illustrate the higher affinity of the drug target candidate(Table 2)(Modiset al., 2003).

Table 1. Physical and Chemical Properties of Chemical Compounds

Drug Accession Number	Log P	State	pKa	Polarizability	Molecular Weight (g/mol)	Boiling Point	Melting Point
CID 176	-0.12	Solid	4.54	5.34 Å3	60.052	117.9 °C	16.6 °C
CID 757	-1	Solid	3.53	6.2 Å3	76.0514	NA	79.5 °C
CID 760	-0.59	Solid	3.3	5.35 Å3	74.0355	NA	98 °C
CID 763	NA	NA	NA	NA	117.108	NA	NA
CID6116	0.24	Solid	4.54	4.96 Å3	158.166	NA	> 160 °C
CID 6144	5.01	NA	NA	NA	380.171	NA	300 deg C
CID 7997	NA	NA	NA	NA	102.133	214.9° F	-93 °C
CID 8471	NA	liquid	NA	NA	101.193	192.7° F	174.5° F
CID 82741	NA	NA	NA	NA	133.19	NA	NA
CID 101751	NA	NA	NA	NA	104.016	NA	NA
CID 517045	NA	NA	NA	NA	82.034	NA	328°C
CID 3474584	NA	NA	NA	NA	65.984	NA	NA
CID 6917684	NA	NA	NA	NA	472.5	NA	NA

Each drug has to fulfill its ADMET properties such as Mutagenicity, Toxicity, In-vitro test and Carcinogenicity etc. in order to prove their potentiality as a drug. These properties are analyzed by confirmative tests such as Ames test, Food and Drug Administration's Center for Drug Evaluation and Research (FDA- CDER) properties

on Rat and Mouse,Skin irritation, Skin sensitization, Respiratory sensitization, Lethal Concentration, Lethal Dose in human, and Hypoxanthine-guannine phosphoribosyltransferase (HGPRT) test(Yang and Chen, 2004).Negative results of Skin sensitization, Skin irritation, Respiratory sensitization, Ames test and Hypoxanthine-guannine phosphoribosyltransferase (HGPRT) tests denote that the molecules can be taken into consideration for drug designing.

Table 2. Showing Binding Energy of Chemical Compounds

Drug Accession Number	Binding energy kca/mol
CID176	-5.01
CID757	-5.29
CID760	-5.14
CID763	-4.76
CID6116	-5.01
CID6144	-4.44
CID7997	-6.51
CID8471	-5.98
CID82741	-5.01
CID101751	-5.01
CID517045	-5.01
CID3474584	-5.01
CID6917684	-5.28

CID6917684 shows positive Respiratory sensitization; hence it requires modifications in the structure. The result of the Ames testproves that the drug is mutagenic(Wadood et al., 2013; Williamson, 2014). CID82741compound requires structural modifications as it gives positive Ames test.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 determine Carcinogenicity(Wadood et al., 2013; Williamson, 2014).No compound indicates positive Carcinogenicity(Table 3).

Lethal dose (LD)is taken into consideration to notify the short term poisoning potential of the material. Lethal concentration (LC)denotes the concentration of the chemical. This toxicity is dependentupon Lethal concentration of the drug. If therange of Lethal concentration is 100 – 1000mg/L that shows moderate toxicity of compound and based on that concentration of Lethal dose is set as 0.5-5 gm/kg. If Lethal concentration range is 10 -100 mg/L then it shows high toxicity of the substance and therefore Lethal dose must be 5-50 mg / kg. If Lethal concentration ranges between 1000 – 10000 mg/L then it showsminute toxicity of the compounds and Lethal dose is set as 5 – 15 gm/ kg(Wadood et al., 2013; Williamson, 2014). Mentioned all thirteen compounds possese Lethal concentration range 100 – 1000 mg/L as a result they are sensibly toxic and their Lethal dose should be kept 0.5 – 5 gm/Kg.

Table 1. Physical and Chemical Properties of Chemical Compounds

Drug Accession Number	Health End Point			Mutagenicity Ames test (Salmonella)	In Vitro Tests HGPRT	Carcinogenicity
	Severe skin irritation	Skin sensitization	Respiratory sensitization			
CID176	NEG	NEG	NEG	NEG	NEG	NEG
CID757	NEG	NEG	NEG	NEG	NEG	NEG
CID760	NEG	NEG	NEG	NEG	NEG	NEG
CID763	NEG	NEG	NEG	NEG	NEG	NEG
CID6116	NEG	NEG	NEG	NEG	NEG	NEG
CID6144	NEG	NEG	NEG	NEG	NEG	NEG
CID7997	NEG	NEG	NEG	NEG	NEG	NEG
CID8471	NEG	NEG	NEG	NEG	NEG	NEG
CID82741	NEG	NEG	NEG	POS	NEG	NEG
CID101751	NEG	NEG	NEG	NEG	NEG	NEG
CID517045	NEG	NEG	NEG	NEG	NEG	NEG
CID3474584	NEG	NEG	NEG	NEG	NEG	NEG
CID6917684	NEG	NEG	POS	NEG	NEG	NEG

(* POS = POSITIVE) (* NEG =NEGATIVE)

We validate below chemical compounds as potential lead molecules for Measles virus phosphoprotein tetramerization domainafter assessing physical and chemical properties, docking and ADMET analysis of all the screened chemical compounds,. ACETIC ACID – (CID176), GLYCOLIC ACID – (CID757), GLYOXYLIC ACID – (CID760), GLYCOCYAMINE – (CID763), CALCIUM ACETATE – (CID6116), SODIUM EDETATE – (CID6144), PROPYL ACETATE – (CID7997), TRIETHYLAMINE – (CID8471), TETRAMETHYLAMMONIUM ACETATE – (CID82741), DISODIUM ETHENE-1,1-DIOLATE – (CID101751), SODIUM ACETATE – (CID517045), LITHIUM ACETATE – (CID3474584), AMETANTRONE ACETATE – (CID6917684).

Figure 2: Showing the Docking Pose of ACETIC ACID (CID176) with Measles virus phosphoprotein tetramerization domain

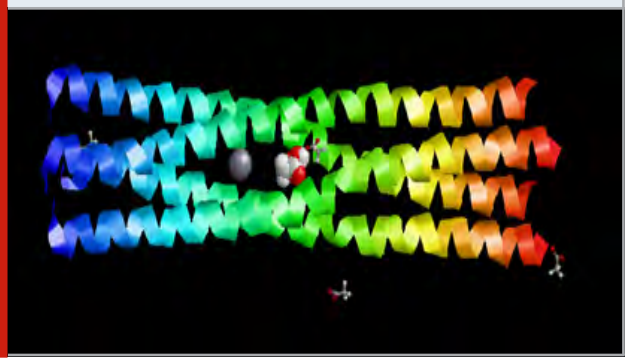


Figure 3: Showing the Docking Pose of GLYCOLIC ACID (CID757)with Measles virus phosphoprotein tetramerization domain

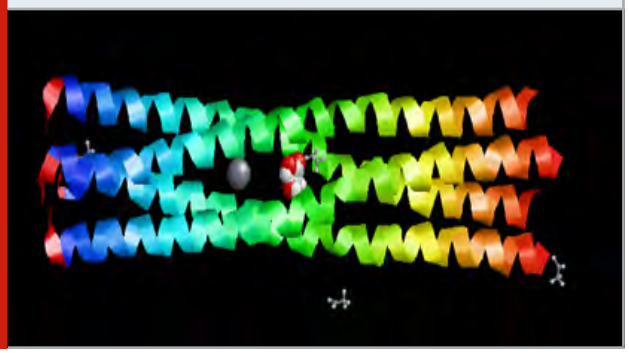


Figure 4: Showing the Docking Pose of GLYOXYLIC ACID (CID760) with Measles virus phosphoprotein tetramerization domain

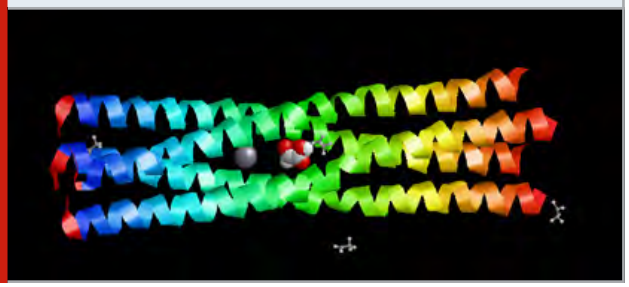


Figure 5: Showing the Docking Pose ofGLYCOCYAMINE (CID763) with Measles virus phosphoprotein tetramerization domain

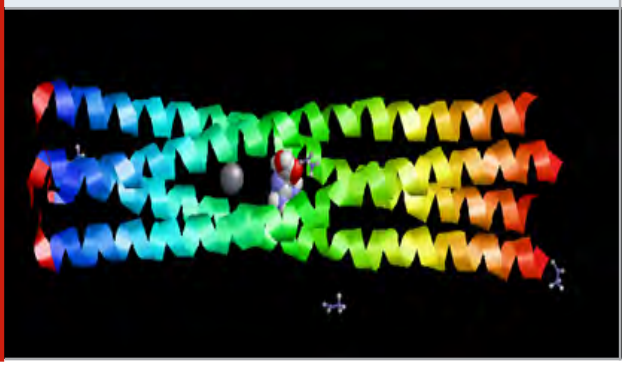


Figure 6: Showing the Docking Pose ofCALCIUM ACETATE (CID6116) with Measles virus phosphoprotein tetramerization domain

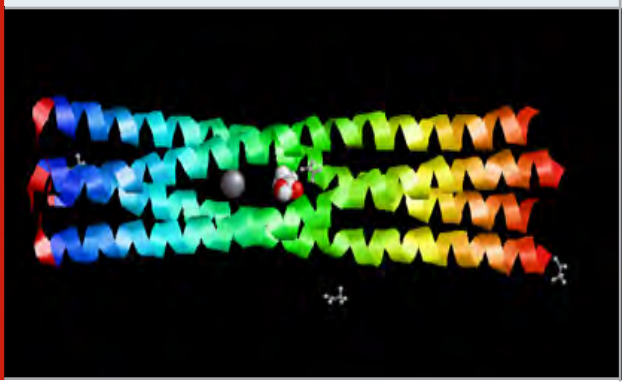
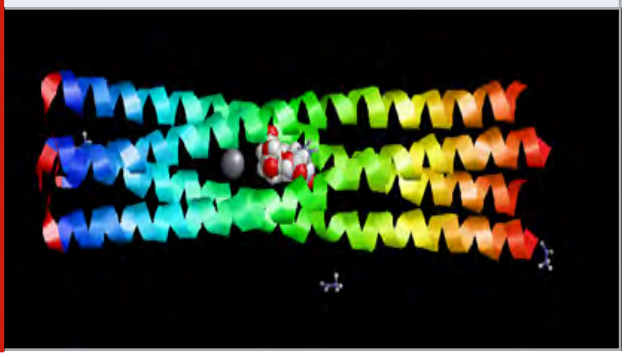


Figure 7: Showing the Docking Pose ofSODIUM EDETATE (CID6144)with Measles virus phosphoprotein tetramerization domain



Further we attempted to find out nature of the obtained compounds. These compounds include naturally occurring plant/ animal, metabolites or secondary metabolites, human metabolites, metabolite intermediates and synthetic compounds in order to understand the possible role of nutraceuticals intervention. Here none of the compounds are natural compounds. Acetic acid, glycolic acid, glyoxylic acid are intermediate metabolites, glycocyamine is a human metabolite whereas calcium acetate, sodium edetate, propyl acetate, triethylamine, tetramethylammonium acetate, sodium acetate , lithium

acetate, ametantrone acetate disodium ethene-1,1-diolate are synthetic compounds. Glycocyamine plays a role as a human metabolite, involved in metabolic pathways of amino acids (https://www.drugbank.ca/drugs ; https://pubchem.ncbi.nlm.nih.gov/).

Figure 8: Showing the Docking Pose of PROPYL ACETATE (CID7997)with Measles virus phosphoprotein tetramerization domain

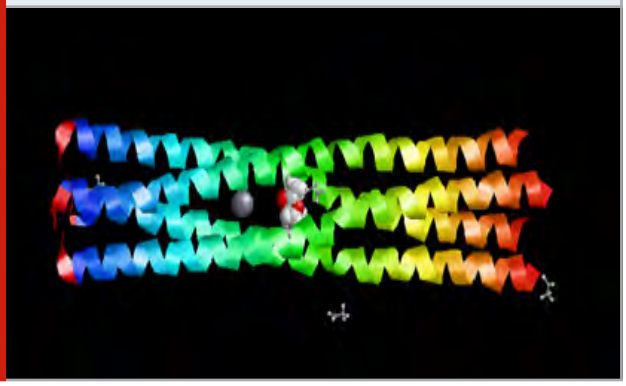


Figure 9: Showing the Docking Pose of TRIETHYLAMINE (CID8471)with Measles virus phosphoprotein tetramerization domain

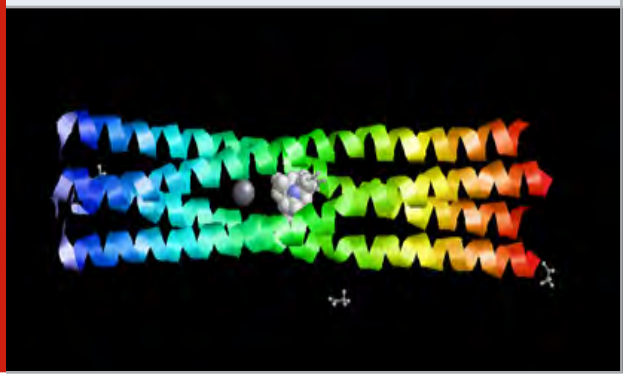


Figure 10: Showing the Docking Pose of TETRAMETHYL AMMONIUM ACETATE (CID82741) with Measles virus phosphoprotein tetramerization domain

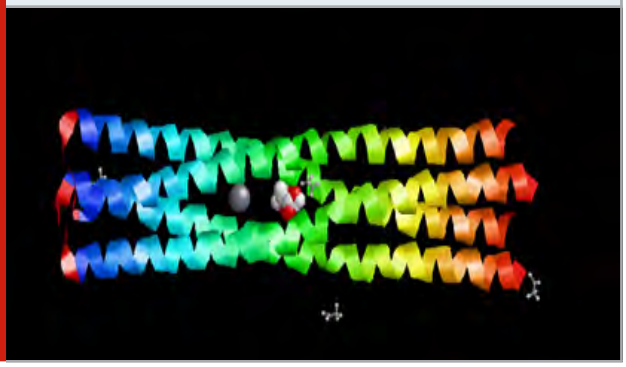


Figure 11: Showing the Docking Pose of DISODIUM ETHENE-1,1-DIOLATE (CID101751)with Measles virus phosphoprotein tetramerization domain

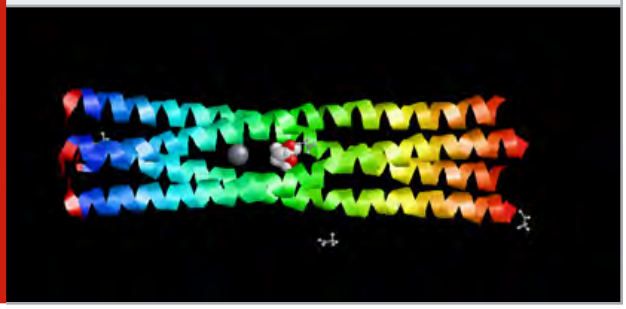


Figure 12: Showing the Docking Pose of SODIUM ACETATE (CID517045) with Measles virus phosphoprotein tetramerization domain

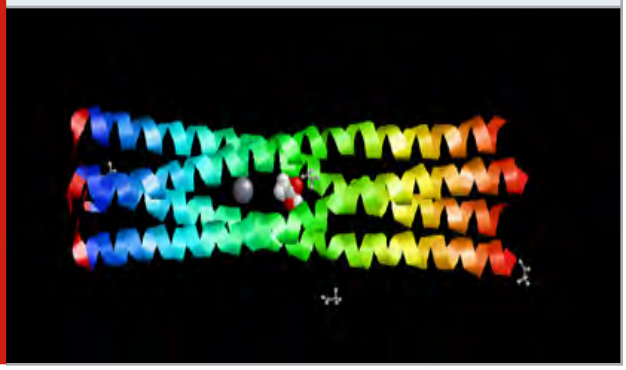


Figure 13: Showing the Docking Pose of LITHIUM ACETATE (CID3474584)with Measles virus phosphoprotein tetramerization domain

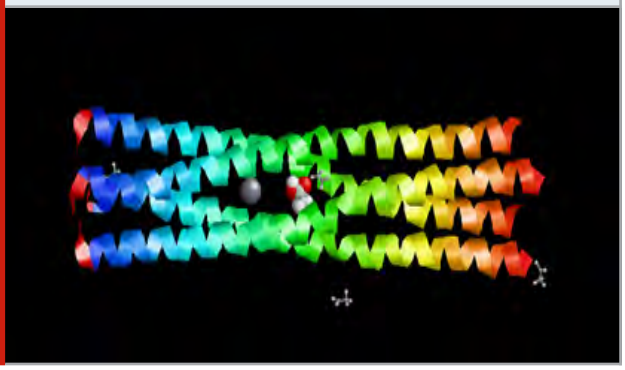
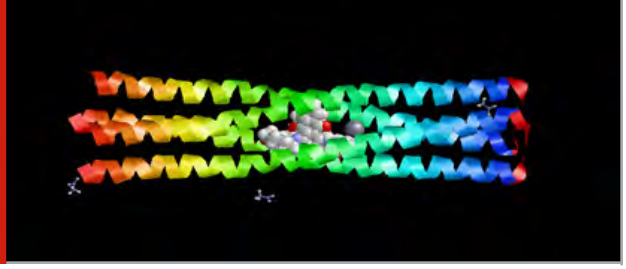


Figure 14: Showing the Docking Pose of AMETANTRONE ACETATE (CID6917684) with Measles virus phosphoprotein tetramerization domain



CONCLUSION

Measles virus phosphoprotein tetramerization domain contains ligand named Acetate ion, (acetate, C2H3O2). It was considered as a Drug Target protein. Its protein structure (PDB format) was attained from the Protein Data Bank. Ligand Acetate ion, (acetate, C2H3O2) was considered for investigation. 80 compounds were selected from available online chemical libraries and they were screened and docked with Acetate ion, (acetate, C2H3O2) using PyRx virtual screening software. Upon ADMET analysis using QSAR database, 13 compounds were identified as potential leads. These thirteen chemical compounds can be further characterized and examined in wet lab for the development of new drug for Measles.

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Extraction and Evaluation of *Salvadora persica* Bark Extract for its Antioxidant and antimicrobial Activity

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ABSTRACT

Natural medicines from plants are one of the options to avoid drug resistance and toxic effects of synthetic drugs. The objective of this study was to find out the new resources of bioactive phytochemical constituents. Dried crude powder of *Salvadora persica* bark was used for extraction in different solvents. Modified maceration method was used in extraction of *Salvadora persica* bark and highest % yield was found to be 1.96 in methanol. Extracts were found to be soluble in different pH buffer system i.e. PBS 6.4, PBS 7.4 and 0.1N HCl. pH value of extract was found to be near 7 so no irritation effect will cause at application site. Heavy metal and microbial load was not found to be on extract. Phytochemical screening of extract was reported the presence of various phytochemical constituents such as alkaloids, glycosides, tannins, Flavonoids, proteins, carbohydrates, sterol and terpenoids. H_2O_2 and DPPH scavenging methods were performed and confirmed antioxidant activity of extract.Various in-vitro methods were used to evaluate the bioactivity of extract. DPPH and H_2O_2 scavenging methods were confirmed the antioxidant activity of extract while disc diffusion method was confirmed for antimicrobial activity of the *Salvadora persica* bark extract. Outcomes of the present study confirmed that the antioxidant and antimicrobial potential of extract is very high. Antioxidant activity was found to be significant and more than standard drug (ascorbic acid) while antimicrobial activity was found to be less than the standard drug (ciprofloxacin). In future the *Salvadora persica* bark extract may prove as new milestone in the formulation of natural antioxidants and antimicrobial agents.

KEY WORDS: *SALVADORA PERSICA* BARK; NATURAL ANTIOXIDANT, NATURAL ANTIMICROBIAL; λ MAX VALIDATION, MICROBIAL LOAD.

INTRODUCTION

Plants are the friend of humans and have helped them to fulfill their daily needs. Herbals from nature have served us as an important source of various phytochemical constituents, particularly from the rich Indian treasure of medicinal plants for curing and healing of many diseases. Phytochemical constituents have been obtained from different parts of plants as leaves, stems, bark, root, flower, fruits and seeds. Different parts of plant are used as cosmetics, food, flavor, fuel and medicines (Bargah., 2015). The toothbrush tree, *Salvadora persica* L belongs to Salvadoraceae family and is also known as 'Miswak' or Pilu. It is evergreen shrub about 400-600 cm in height with a short trunk. It has green leaves, red to brick red fruits and brown bark. Leaves are sub-succulent, curvaceous, elliptic, 1-10cm long and 1-3cm wide. Flowers are small, greenish white and about 10cm long. Petals are 1-3 mm long, (Chabane et al., 2017 Malviya et al 2019).

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The bark of *Salvadora persica* is slightly rough, grayish brown on main stem, paler elsewhere. Research studies have revealed many phytochemical constituents to be present in its bark such as caffeine, theobromine, trigonelline, ursolic acid, oleanolic acid and trimethylamine (Anthoney et al., 2015; Abeer et al., 2011). Various studies have reported several activities of *Salvadora persica* bark including anti-gonorrhea (Shokeen et al., 2009), tonic, and stimulant in low fever, ascarifuge, gastric troubles (Verma et al., 2009). Other studies have reported its bark as vesicant and stimulant, to relieve cough and used as purgative, (Mathur et al., 2015). Bark was used to treat sores (Anthoney et al., 2015). Ursolic acid and oleanolic acid was extracted from *Salvadora persica* bark. Ursolic acid inhibited tumorigenesis, tumor promotion, invasion, metastasis, angiogenesis and induction of tumor cell differentiation. Oleanolic acid has hepato-protective, antistomach ulcer, hypoglycemic, anti-hyperlipidemic, anti-hypertensive, cardio-tonic, anti-dysrhythmic, anti-aggregation of blood platelet, anti-cancer, protection of renal toxicity, anti-inflammatory, anti-microbial and anti-fertility activities with low toxicity (Abeer et al., 2011, Malviya et al 2019).

MATERIAL AND METHODS

Material: *Salvadora persica* bark was collected from Shiddhbaba Ashram Ghanghauli Aligarh, India. Dried bark was powdered using electric granulating machine. Plant was authenticated by school of biotechnology, Gautam Buddha University (state government university) Greater Noida, India. Chemicals; Chloroform, Ethyl Acetate, Methanol, Ethanol was provided by research lab of SMAS Department, Galgotias University.

Method of extraction of plant material: Modified fractional maceration method was used to extract the plant material. Dried coarse powder of stem was placed in round bottom flask (RBF). Required amount of selected solvent were poured in RBF. RBF kept for 24 hours with occasionally shaking. Strained off the liquid part and repeated it three times. Liquid part was filter and concentrate. Remaining part of powder was treated with different solvent and repeats the process (Kumar et al., 2014).

Determination of % yield of extract: % Yield = Practical Yield * 100 / Theoretical Yield Solubility studies of extract:*Salvadora persica* bark extracts were shaken in different solvent and observed their solubility (Malviya., 2011). Determination of pH of extract:1 gram of *Salvadora persica* bark extract was dissolved in 100ml distilled water and determined pH using digital pH meter (Malviya., 2011).

Phytochemical studies of the extract: Phytochemical assays were done to reveal the pharmacological active substances in extract using test methods described previously (Peach et al., 1955). Phytochemical substances were evaluated using various methods such as Dragendorff test, Wagner test, Hager's test, Legal test, Ferric chloride test, Froth formation test, Zinc Hydrochloride reduction test, Biuret test, Molish's test, and Libermann-Burchard test.

Determination of microbial load in extract: Microbial load of extract was determined using modified spread method previously described (Mamun et al., 2014). 1000µg/ml of extract solutions was prepared. Sterile plates of nutrients agar were prepared and extract solution spread on them. Prepared plates were incubated in incubator for 48 hours. Microbial colonies formed on agar plates were counted using colony counter in CFU (colony forming unit). Limit test for heavy metals of extract:Limit test for heavy metals (lead, arsenic) were performed as 'Indian Pharmacopoeial' procedure (Indian Pharmacopoeia., 1996).

Determination and validation of λmax of extract: The solutions of different concentration of extract (SPBEC, SPBEEA, SPBEM, SPBEE and SPBEW) were prepared using buffer solution of 0.1 N HCL, phosphate buffer pH 6.8, phosphate buffer pH 7.4. Wavelengths of extract were recorded at absorbance less than one at the range of 200-800nm TID (Three times a day) for six days, (Malviya et al., 2019).

DPPH radicals scavenging activity of extract: DPPH radical scavenging assay (Verma et al., 2012) was used to evaluate the antioxidant potential of extract. 0.1mM DPPH solution was prepared in methanol and kept in dark place. Extract solution of different concentration (20, 40, 60, 80, 100µg/ml) were prepared in methanol and sonicated to obtained complete solubility. Ascorbic acid solutions were used as standard drug solution. Extract and ascorbic acid solutions were mixed in DPPH solution separately and allowed to stand for 30 minutes. Absorbance was recorded at 517nm using Shimadzu spectrophotometer. Methanol was used as control solution. Percentage DPPH scavenging was calculated using following equation.

DPPH scavenged (%) = [(Abs control – Abs extract) / Abs control] x 100
Abs control - is the absorbance of the control reaction
Abs extract - is the absorbance in the presence of the sample of the extracts

H_2O_2 scavenging activity of extract: H_2O_2 scavenging assay (Jayaprakash et al., 2004) was used to evaluate the antioxidant activity of extract. Extract of different concentration (20, 40, 60, 80, 100 µg/ml) were prepared. 40mM solution of H2O2 was prepared in phosphate buffer solution pH 7.4. Extract of different concentration (5ml) and H_2O_2 solution (5ml) was mixed in 10 ml volumetric flask. Absorbance was recorded at 230nm using Shimadzu spectrophotometer. Phosphate buffer solution pH 7.4 was used as control solution. Percentage H_2O_2 scavenging was calculated using following equation.

% H_2O_2 scavenging ability of extract = [(Abs control –

Abs extract)/Abs control] x 100

Abs control - is the absorbance of the blank solution
Abs extract - is the absorbance in the presence of the sample of the extracts.

Antimicrobial activity of extract: Disc diffusion method was used to determine antimicrobial potential of extract. Test microbes gram positive (Bacillus subtilis) and gram negative (Escherichia coli) was obtained from Department of Medical Lab Technology, School of Medical and Allied Sciences, Galgotias University, India. in this method, Petri dishes were washed and sterile using autoclave. These sterile petri dishes were used to prepare MH agar plates. Microbial solution was spread on surface of agar plate using cotton. Extract and ciprofloxacin solution contain Disc were applied on microbial surface of MH agar plate. Agar plates were incubated in incubator for 24 hours. Antimicrobial potential of extract was inhibited the growth of microbes. Microbial free area was measured in millimeter (Shubha et al., 2010).

FTIR analysis of extract: FTIR analysis (Kumar et al., 2015) is considered as most important tool for identifying the types of functional group present in sample. The wavelength absorbed light is recorded in spectrum. By interpreting the data of wavelength, chemical bond or possible functional group can be determined. Dried powders of different solvent extract of *Salvadora persica* bark was used to FTIR analysis. 10mg of dried extract was encapsulated in 90mg of KBr pellet to prepared sample discs. Sample of extract was loaded to Shimadzu FTIR spectroscope with the range of 400-4000cm-1 with resolution of 4cm-1.

RESULTS AND DISCUSSION

Percentage yield of extract was affected by various factors such as solvent, temperature, pH, tome and composition of plant material. In this research, *Salvadora persica* bark extract was obtained using different solvent such as chloroform, ethyl acetate, methanol, ethanol and water. The obtained comparative results were reported in table 1.

Table 1. % yield of extract

Extract	% yield
SPBEC	0.50
SPBEEA	0.20
SPBEM	1.96
SPBEE	1.81
SPBEW	1.05

Solubility is a most important factor that affects the therapeutic effect of drugs. Solubility of different extract of *Salvadora persica* bark were observed in cool and hot water, 0.1N HCl, phosphate buffer solution pH 6.8 and 7.4. SPBEC and SPBEEA were found to be insoluble in cool water and partial soluble in hot water. All extracts were found to be soluble in 0.1N HCl, phosphate buffer solution pH 6.8 and 7.4. Obtained results were listed in table 2.

Table 2. Solubility study of Salvadora persica leaves extract

	SPBEC	SPBEEA	SPBEM	SPBEE	SPBEW
Cool water (25°C)	IN	IN	S	S	S
Hot water (40°C)	PS	PS	S	S	S
0.1N HCL	S	S	S	S	S
Phosphate buffer pH 6.8	S	S	S	S	S
Phosphate buffer pH 7.4	S	S	S	S	S

S: Soluble, PS: Partial soluble, IN: insoluble

Prepared 1% solution of extracts were used to determine pH and found to be near neutral as listed in table 3. Based on these results, it may claim that when extract will use in formulation shows no irritation effect.

Table 3. pH of extract

1% solution of extract	pH
SPBEC	7.0±0.2
SPBEEA	7.0±0.1
SPBEM	6.8±0.2
SPBEE	6.6±0.1
SPBEW	6.9±0.2

Phytochemical assay revealed many bioactive substances present in the extract as listed in table 4.

No microbial colony was found on agar plates. Absence of microbes in extract might be due to use of chloroform, ethyl acetate, methanol and ethanol during the fractional maceration process. Limit tests were confirmed the absence of heavy metal (lead, arsenic) in extract. Procedure discussed in the present manuscript provides an accurate and convenient method to determine and validate the λmax of unknown sample. The different concentrations of extract in different buffer solution were obtained and recorded in table 5.

DPPH scavenging method was confirmed the antioxidant potential of extract. Percentage inhibitions of DPPH and comparative potential of extract and ascorbic acid were presented in figure 1 and calculated IC50 values were listed in table 6.

H_2O_2 scavenging method was used to determined antioxidant activity of *Salvadora persica* stem extracts (SPBEC, SPBEEA, SPBEM, SPBEE and SPBEW). Obtained percentage inhibition and comparative assessment was

showed in figure 2 and calculated IC50 presented in table 7.

Obtained result were confirmed the antimicrobial activity of extract. Comparative of result of extract and ciprofloxacin was presented in table 8.

Table 4. Phytochemical Screening of *Salvadora persica* bark extract

	Test performed	SPBEC	SPBEEA	SPBEM	SPBEE	SPBEW
Alkaloids	Dragendorff test, Wagner test, Hager's test	-	-	+	+	+
Glycosides	Legal test	-	-	+	+	+
Tannins	Ferric chloride test	-	-	+	+	+
Saponins	Froth formation test	-	-	+	+	+
Flavonoids	Zinc Hydrochloride reduction test	+	-	+	+	+
Proteins	Biuret test	-	-	+	+	+
Carbohydrates	Molish's test	-	-	+	+	+
Sterols and Terpenoids	Libermann-burchard test	+	-	+	+	+

Table 5. Determination and validation of λmax of extract

Extracted part of plant	Solvent used in extraction	Buffer solution used for dilution	Day 1			Day 2			Day 3			Day 4			Day 5			Day 6		
			9:30 am	12:30 pm	4:30 pm	9:30 am	12:30pm	4:30 pm	9:30 am	12:30 pm	4:30 pm	9:30 am	12:30 pm	4:30 pm	9:30 am	12:30 pm	4:30 pm	9:30 am	12:30 pm	4:30 pm
Bark	Chloroform	0.1 N HCL	670.0 ±3.5	670.0 ±1.0	670.0 ±0.0	676.0 ±2.5	676.0 ±2.0	676.0 ±1.5	768.0 ±5.0	768.0 ±2.0	768.0 ±0.0	723.0 ±4.0	723.0 ±5.0	723.0 ±2.5	748.5 ±6.0	748.5 ±5.5	748.5 ±3.5	792.5 ±5.0	780.0 ±2.0	780.0 ±1.0
		PBS pH 6.8	670.0 ±0.0	670.0 ±0.0	670.0 ±1.0	673.0 ±2.0	673.0 ±0.0	673.0 ±0.0	678.0 ±2.0	678.0 ±0.0	678.0 ±0.0	672.5 ±0.5	672.5 ±0.5	672.5 ±1.5	673.0 ±0.0	673.0 ±0.0	673.0 ±0.0	673.0 ±0.0	673.0 ±0.0	673.0 ±0.0
		PBS pH 7.4	691.5 ±5.0	691.5 ±3.5	691.5 ±0.5	676.0 ±2.0	674.0 ±0.0	674.0 ±0.0	670.0 ±0.0	670.0 ±0.0	670.0 ±0.0	748.5 ±2.5	748.5 ±1.5	748.5 ±0.5	536.5 ±4.5	536.5 ±2.5	536.5 ±2.5	651.0 ±0.0	651.0 ±1.0	651.0 ±2.0
	Ethyl acetate	0.1 N HCL	204.5 ±1.5	204.5 ±0.5	204.5 ±1.5	204.5 ±0.5	204.5 ±0.5	204.5 ±0.5	205.5 ±0.5	205.5 ±1.5	205.5 ±0.5	205.5 ±0.5	205.5 ±1.5	205.5 ±0.5	205.5 ±1.5	205.5 ±0.5	205.5 ±0.5	204.5 ±0.5	204.5 ±0.5	204.5 ±1.5
		PBS pH 6.8	674.0 ±0.0	674.0 ±0.0	674.0 ±0.0	676.0 ±0.0	676.0 ±0.0	676.0 ±0.0	674.0 ±0.0	674.0 ±0.0	674.0 ±0.0	676.0 ±0.0	676.0 ±0.0	676.0 ±0.0	676.0 ±0.0	676.0 ±0.0	676.0 ±0.0	670.0 ±0.0	670.0 ±0.0	670.0 ±0.0
		PBS pH 7.4	212.5 ±0.5	213.0 ±1.0	213.0 ±1.0	211.5 ±0.5	211.0 ±1.5	211.0 ±1.0	211.5 ±0.5	212.0 ±1.5	212.0 ±1.5	211.5 ±0.5	212.0 ±1.0	212.0 ±1.0	213.0 ±0.0	213.0 ±1.0	213.0 ±1.0	211.0 ±1.0	211.0 ±1.0	211.0 ±1.0
	Methanol	0.1 N HCL	272.5 ±1.5	272.5 ±1.5	272.0 ±1.0	272.5 ±1.5	272.5 ±1.5	272.5 ±1.5	272.5 ±1.5	272.0 ±1.0	272.0 ±1.0	273.0 ±0.0	273.0 ±0.0	273.0 ±0.0	278.0 ±5.0	276.0 ±1.0	276.0 ±0.0	273.0 ±0.0	273.0 ±0.0	273.0 ±0.0
		PBS pH 6.8	272.5 ±0.5	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	272.5 ±0.5	272.5 ±0.5	272.0 ±0.0	271.0 ±1.0	271.0 ±1.0	271.0 ±1.0	271.5 ±0.5	271.0 ±1.0	271.0 ±1.0	271.5 ±0.5	271.0 ±1.0	271.0 ±1.0
		PBS pH 7.4	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	274.0 ±1.0	274.0 ±2.0	274.0 ±1.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0
	Ethanol	0.1 N HCL	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	270.5 ±0.5	271.0 ±1.0	271.0 ±1.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	271.0 ±1.0	271.0 ±1.0	271.0 ±1.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0
		PBS pH 6.8	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	271.5 ±0.5	272.0 ±0.0	272.0 ±0.0
		PBS pH 7.4	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	274.5 ±1.5	273.0 ±1.0	273.0 ±1.0	272.5 ±0.0	272.0 ±0.0	272.0 ±0.0
	Water	0.1 N HCL	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	271.5 ±0.5	271.0 ±1.0	271.0 ±1.0	272.5 ±0.5	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	273.5 ±1.5	273.0 ±1.0	273.0 ±1.0	271.5 ±0.5	272.0 ±0.0	272.0 ±0.0
		PBS pH 6.8	271.5 ±1.5	271.0 ±1.0	271.0 ±1.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	270.5 ±1.5	271.0 ±1.0	271.0 ±1.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	268.0 ±3.0	269.0 ±2.0	269.0 ±2.5	268.0 ±2.0	268.0 ±0.0	268.0 ±0.0
		PBS pH 7.4	272.0 ±0.5	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	272.0 ±0.0	270.5 ±1.5	271.0 ±1.0	271.0 ±1.0	271.0 ±1.0	271.0 ±1.0	271.0 ±1.0	272.5 ±1.5	272.0 ±0.0	272.0 ±0.0	271.0 ±0.0	271.0 ±0.0	271.0 ±0.0

Figure 1: Comparative assessment of antioxidant potential (using DPPH scavenging model) of SPBEC: Salvadora persica bark extract in chloroform, SPBEEA: Salvadora persica bark extract in ethyl acetate, SPBEM: Salvadora persica bark extract in methanol, SPBEE: Salvadora persica bark extract in ethanol, SPBEW: *Salvadora persica* bark extract in water, AA: Ascorbic acid.

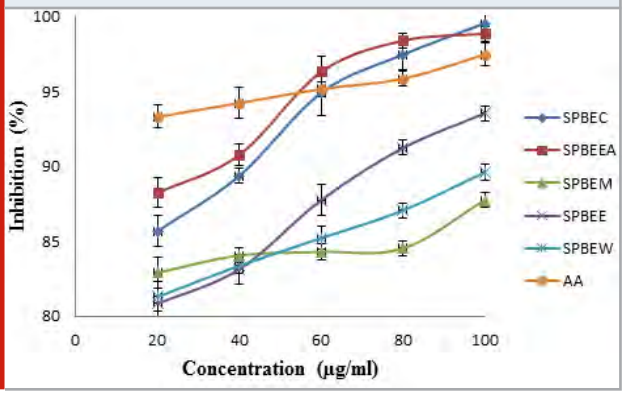


Table 6. IC50 of S. persica bark

Sample	DPPH assay IC-50(µg/ml)
SPBEC	192.05
SPBEEA	248.88
SPBEM	633.00
SPBEE	163.17
SPBEW	289.20
AA	861.42

Figure 4: FTIR of SPBEEA

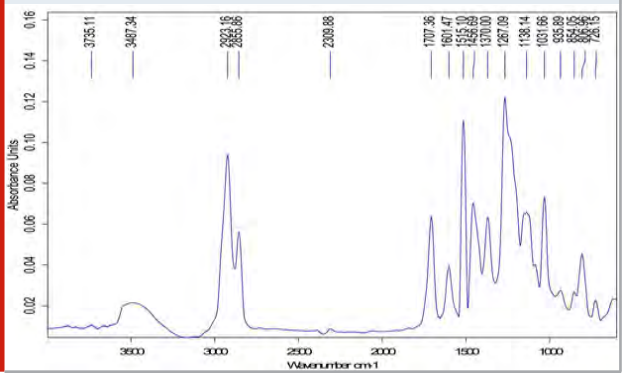


Figure 2: Comparative assessment of antioxidant potential of extract using H_2O_2 scavenging model.

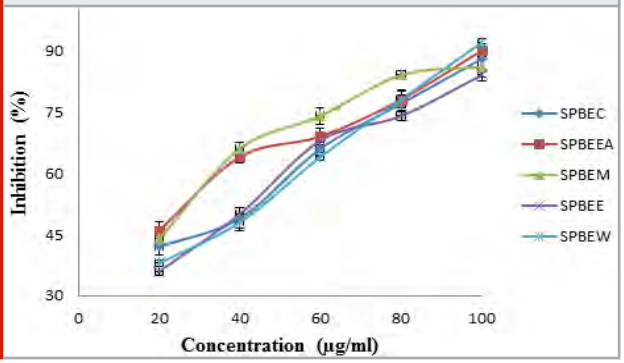
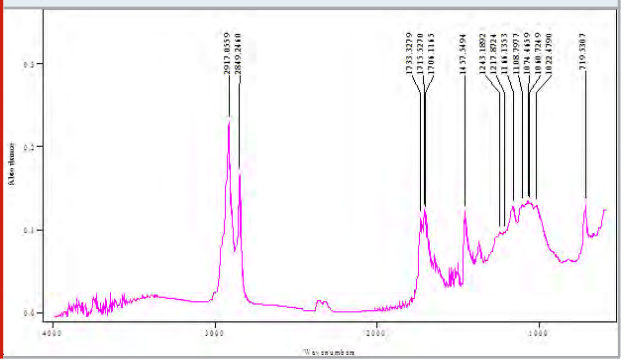


Table 7. IC50 of *S. persica* stem

Extract	H_2O_2 assay IC50 (µg/ml)
SPBEC	36.00
SPBEEA	21.96
SPBEM	19.21
SPBEE	39.33
SPBEW	39.71

Figure 3: FTIR of SPBEC



FTIR spectra of *Salvadora persica* bark extracts (SPBEC, SPBEEA, SPBEM, SPBEE and SPBEW) were measured and presented in figures 3 to 7. Recorded wavelengths and the probable functional groups (obtained by FTIR analysis) were obtained in the extracts were reported. By interpreting the data and find possible functional groups were listed in table 9,10,11,12,13.

Table 8. Antimicrobial activity of extract

Bacteria type	Zone of inhibition (mm)											
	SPBEC (µg/ml)		SPBEEA (µg/ml)		SPBEM (µg/ml)		SPBEE (µg/ml)		SPBEW (µg/ml)		Ciprofloxacin (µg/ml)	
	100	200	100	200	100	200	100	200	100	200	100	200
E. Coli	3	6	2	5	3	7	3	6	4	6	6	18
B. Subtilis	3	5	3	6	4	6	4	7	3	4	5	16

Figure 5: FTIR of SPBEM

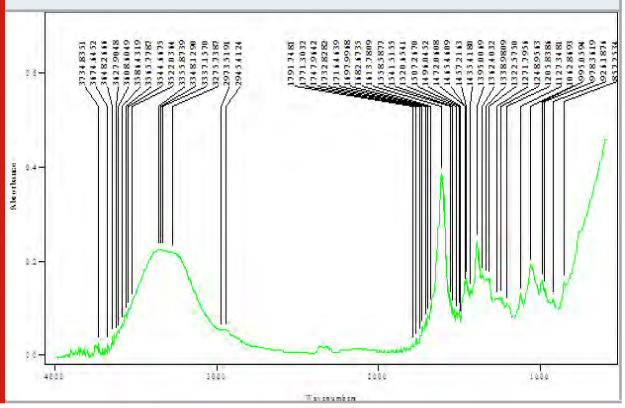


Figure 6: FTIR of SPBEE

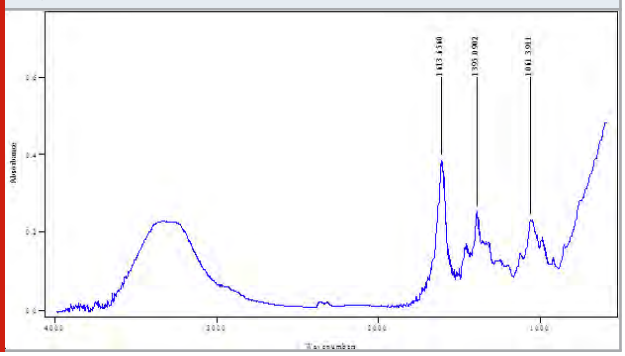


Figure 7: FTIR of SPBEW

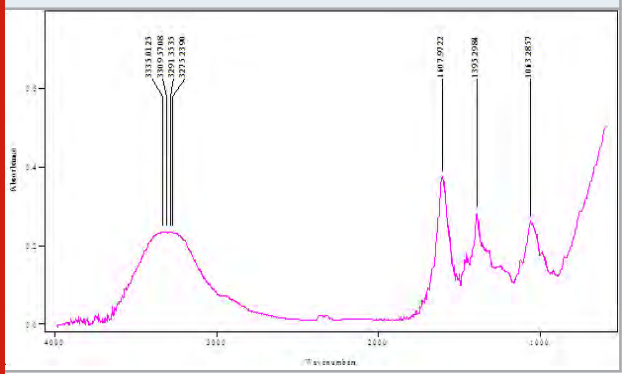


Table 9. Functional group in SPBEC

Peak value (cm^{-1})	Group presence
2917	C,H
1733	C=O, C=N
1166	O-H

Table 10. Functional group in SPBEEA

Peak value (cm^{-1})	Group presence
2923	C-H
1707	C=O, C=N
1456	C=C
1370	N-O
1267	C-O
1138	O-H
935	C-H

Table 11. Functional group in SPBEM:

Peak value (cm^{-1})	Group presence
3337	O-H
1613	C≡C
1472	C=C
1395	C-H
1248	C-O
1062	O-H
995	C-H

Table 12. Functional group in SPBEE

Peak value (cm^{-1})	Group presence
1613	C-C, C=O
1395	N-O, C-H
1061	N-H

Table 13. Functional group in SPBEW

Peak value (cm^{-1})	Group presence
3335	O-H
1607	C=O
1395	N-O
1063	N-H

CONCLUSION

Modified maceration method was used in extraction of *Salvadora persica* bark and highest % yield was found to be 1.96 in methanol. Extracts were found to be soluble in different pH buffer system i.e. PBS 6.4, PBS 7.4 and 0.1N HCl. pH value of extract was found to be near 7 so no irritation effect will cause at application site. Heavy metal and microbial load was not found to be on extract. Phytochemical screening of extract was reported the presence of various phytochemical constituents such as alkaloids, glycosides, tannins, Flavonoids, proteins, carbohydrates, sterol and terpenoids. H2O2 and DPPH scavenging methods were performed and confirmed

antioxidant activity of extract. Disc diffusion method was confirmed antimicrobial response of extract. FTIR analysis of extract was used to detect characteristic peak range and the presence of possible functional groups.

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Machine Learning Model for Vaccine Development: A Perspective

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ABSTRACT

A vaccine is a hope to prevent disease while training the immune system to produce antibodies against pathogens. As it is safe, easy in use, and having no side effects, it is used for the cure of many diseases. Vaccines may be of different types, like subunit vaccines, attenuated vaccines, DNA vaccines, etc. The process of vaccine development is taking a long way, needs highly sophisticated labs, clinical trials, and much more. This whole process will take a long time to develop a vaccine. And then manufacturing in the specific environment will again time is taken to reach to the market. While clinical trials of vaccines sometimes failed to produce the desired results. So to improve these trial methods and making vaccine production successful. Here in this paper, it is tried to propose machine learning methods i.e. classification, clustering, association (mainly used algorithms) in different stages of vaccine development clinical trials. Because machine learning techniques are soft, time-consuming, and help to achieve a particular target with great sensitivity and accuracy. It is hoped that if machine learning methods are followed in a proper way time will be saved and vaccine designing will be done in less time with great accuracy, sensitivity, and specificity.

KEY WORDS: CLINICAL, DISEASE IMMUNE SYSTEM, MACHINE LEARNING, VACCINE.

INTRODUCTION

A biological preparation that provides active acquired immunity to a particular disease is called vaccine. Typically it contains an agent that resembles a disease-causing microorganism. It is seen that vaccines are made from weakened or killed forms of the microbe, its toxins, or one of its surface proteins. When a vaccine is administered to any person by injection is referred as vaccination. These vaccinations have some effects on our body, which is called Immunization. Some 80 percent of the world's infants are adequately immunized against six important diseases: measles, tetanus, pertussis diphtheria, tuberculosis, and polio. This is a remarkable achievement which protects children life at an early age (Tomic et al., 2019).Vaccines based on viral vectors (tools that deliver genetic material into cells) offer strong immune response, which are based on recombinant proteins for other diseases; these vaccine candidates have an advantage of large scale production capacity, (Tung et al., 2020).

Hence we can say that the vaccine stimulates the immune system so that it can recognize the disease and protect us from the future infections (i.e. provide immunity to the infection). Since vaccine is cost effective and it will reach many of the lives. So it is necessary that the vaccine delivery is also done in a proper way. Its tremendous effect

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and reach will soon eradicate global disease burden. The best example till date is poliomyelitis. Importance of Vaccines (Kallarup 2014) are noted as: a) Accelerate immunologic response, b) It has no side effects, c) Easy in use, d) Prevents from disease, e) Develops fast antibody against pathogens, f) Immunity is achieved with minimum doses, g) Mass production is available, h) Stable in storage conditions for long period of time. There are some vaccines which induces better immunity than any natural infection (Kallarup 2014, Chaudhary 2019):

Table 1. Modes of action of vaccines with infection caused are detailed below

Accine type	Infection Cured	Method of vaccine formation	Working of vaccine	References
Viral vector based (i.e. adenovirusas vector)	Alzheimer's disease, malaria,HIV	It uses chemically weakened virus to transport pieces of pathogen. The genes in such vaccines are usually antigen coding surface proteins from the pathogenic organism.		
Live, attenuated	Measles, mumps, rubella (MMR combined vaccine) Varicella (chickenpox) Influenza (nasal spray) Rotavirus Zoster (shingles) Yellow fever	A virus targeted for use in a vaccine may be grown through—"passaged" through—upwards of 200 different embryos or cell cultures.	It will unable to replicate and produce good immune response in future	Plotkin (2013); Plotkin (2018)
Inactivated/Killed	Polio (IPV) Hepatitis A	This can be made by inactivating a pathogen, typically using heat or chemicals such as formaldehyde or formalin. This destroys the pathogen's ability to replicate, but keeps it "intact" so that the immune system can still recognize it.	tend to provide a shorter length of protection than live vaccines are more likely to require boosters to create long-term immunity	Plotkin (2018) Plotkin (2018)
Toxoid (inactivated toxin) (Toxoids is considered killed or inactivated vaccines)	Diphtheria, tetanus (part of DTaP combined immunization) Rabies	It produces (tetanospasmin) neurotoxin. Immunizations for this type of pathogen can be made by inactivating the toxin that causes disease symptoms. This can be done via treatment with a chemical such as formalin, or by using heat or other methods.	Long term immunity, Used as a combination.	Plotkin (2018) Angsantikul et al (2018).
Subunit/ conjugate	Hepatitis B Influenza (injection) Haemophilus influenza type b (Hib)	One way of formation is both subunit and conjugate vaccines containing only pieces	Conjugate vaccines are sed to create a more u powerful, combined immune response	Carvelho 2010, Tomic et al 2019

	Pertussis (part of DTaP combined immunization) Pneumococcal Meningococcal Human papillomavirus (HPV)	of the pathogens they protect against. Another way is genetic engineering.		
Recombinant vaccines	Influenza, Rabies, Hepatitis B and other diseases	Researchers identify the region in virus DNA that is not necessary for replication. Hence this region is used for vaccine. Researchers put one or more genes code for immunogen of other pathogens.	Such modified virus is injected into a person's body; the immunogen is expressed and able to generate immune response.	Carvelho (2010), Tomic et al., 2019
DNA Vaccines	Parasitic diseases like malaria etc.	It consists of DNA coding for a particular antigen.	DNA itself insert into particular cells which then produce the antigen from the infectious agent and able to produce immune response.	Carvelho (2010)

- *Human papillomavirus* (HPV) vaccine – the specific protein is highly pure so the immunity will increase.
- Tetanus vaccine – it can prevent tetanus, an infection caused by Clostridium tetanii bacteria. This vaccine lowers the disease effect.
- Haemophilus influenzae type b (Hib) vaccine – Hib vaccines are of two types: The Hib vaccine protects children and adults from Hib disease. The DTaP-IPV/Hib vaccine protects babies' ages 2 through 18 months from Hib disease, tetanus, diphtheria, whooping cough and polio. The vaccine links sugar coated polysaccharide to a helper protein that creates a better immune response than would occur naturally.
- Pneumococcal vaccine – working of Pneumococcal vaccine is same as Haemophilus influenzae vaccine.
- Adjuvant: Adjuvant is derived from Latin word adjuvare meaning to help or aid. In immunology it is defined as any substance that acts to accelerate, prolong or enhance antigen-specific immune responses when used in combination with specific vaccine antigens". Adjutants enhance immunity to vaccines and immune response. Hence it is used in humans. For example, measles, mumps, rubella, vermicelli, rotavirus (Choudhry et al., 2019).

New vaccine types are currently developed by researchers and they also try to improve current approaches for vaccine delivery. DNA vaccines and Recombinant vector vaccines are in progressing stages because DNA vaccines are easy and inexpensive and provide long term immunity whereas recombinant vector vaccines making the immune system to fight germs. For example, inhaled vaccines are used usually in the form of nasal spray in some cases of influenza (Carvalho 2010).

Reverse vaccinology: It is improved by Bioinformatics and first used against Serogroup B meningococcus. Here Bioinformatics tools are used to screen entire genome of pathogens to determine whether the protein is good vaccine target (Bowman 2011). With the development of genomics in vaccine development it is needed to study whole genotype to phenotype and environment exposure. It is said that it is the future. Reverse vaccinology are improving the process of vaccine development by classification methods like Support vector machines (Bowman 2011).

Stages of Vaccine Development and Testing: Usually, vaccine development and testing follow a certain standard set of steps. The first stages are exploratory in nature. Regulation and oversight increase as the candidate vaccine makes its way through the process. First Steps: Study of Animal and laboratory Exploratory Stage: The basic need is to study laboratory where animal experiments are carried out. This will take time 2-4 years. The scientists of fully funded academic and government institutes are identifying natural or synthetic antigens that will help in disease prevention and treatment. These antigens can be virus-like particles, weakened viruses or bacteria, or it can be any other substances derived from pathogens.

Pre-Clinical Stage: In this stage it is very important to study tissue-culture and cell culture for assessing the candidate vaccine safety. The immunogenicity or ability to provoke an immune response is also very important criteria for finding vaccine response in animal of study. Basically animal subjects for study include mice and monkeys. These studies help the scientists to start a safe

dose for humans and also administering the vaccine in a safe way. The efficiency/ efficacy of vaccine are also very difficult task for scientists. Many candidate vaccines are not able to produce desired immune response. This often takes 1-2 years.

Any private company which wants to work on vaccines submits an application for an Investigational New Drug (IND) to the Food and Drug Administration of their country (The Central Drugs Standard Control Organization (CDSCO) is the national regulatory body for Indian pharmaceuticals and medical devices). It is the responsibility of sponsors of private company that they describes the manufacturing and testing processes, all the laboratory reports and proposal of study of vaccines in subjects. This should also include clinical trials. If the proposal of vaccine clinical trials are approved, the vaccine will have three phases of testing.

Next Steps: Clinical Studies with Human Subjects- this is one of the most important step in vaccine designing and administration. Phase I Vaccine Trials: Human intervention to vaccines involves small group of adults basically nearly 20-80 which can be subjects. Gradually come down to the age of each category of age, if vaccines are for children. This phase clearly indicates whether the particular vaccine will be used or not. In this phase, candidate vaccine safety is utmost important with its immune response in the subjects. All the participants of the study are carefully monitored and the laboratory conditions are fully controlled carefully. This phase success rate will decide the next stage.

Phase II Vaccine Trials: Phase 2 testing includes larger group of individual participates, sometimes may be hundred. There are some people which are at great risk of acquiring the disease. This may be randomized but in a well controlled way. This phase success will proves the safety, immunogenicity of candidate vaccine, proposed doses, schedule of immunizations, and method of delivery (Plotkin 2008).

Phase III Vaccine Trials: When phase II trials are successful, candidate vaccines move on to larger trials which involves thousands of people. Again these tests are randomized and double blind. The assessment of vaccine safety in a large group of people is most significant goal of phase III trials. In this, side effects of vaccines are also studied.

Vaccine efficacy includes: a) is candidate vaccine is able to prevent disease. b) Is candidate vaccine provides prevention against pathogen infection. c) Is candidate vaccine is able to produce immune response and antibody production against pathogen.

Approval and Licensure is another step: As phase III trial is completed successfully, the vaccine developer needs to have license. The license providing agency will inspect the manufacturer unit where the vaccine will be made and approval for vaccine label. The agency continuously monitors the production, potency, safety and purity of vaccines.Post-Licensure Monitoring of Vaccines & phase IV trial: This includes vaccine adverse event reporting system and the vaccine safety data link. Concern companies conduct studies after vaccine is released. They continuously monitor the vaccine safety, efficacy and other potential uses (Plotkin 2008).

Since it is very important for success of vaccine trials because it involves lot of efforts, finance and animal used under study could go through pains. To make these vaccine trials more effective and successful machine learning and artificial intelligence are come into existence. Because these techniques are cost effective, efficient, and time consuming. This will help scientist to make vaccine trials a success.

MATERIAL AND METHODS

Machine Learning: Machine learning is an emerging field which reaches everywhere. Not only in industries and solving big problems it will be one of the biggest challenging applications of artificial intelligence. It provides systems the ability to automatically learn and improve from experience without being explicitly programmed (Samuel 1959). Machine learning focuses on the development of computer programs that can access data and use it learn for themselves. There are algorithms that learn from the data as provided to train and this will create machine learning model that can perform a given task without any specific instructions. These models are used to make predictions or classifying images and emails. These techniques are nowadays widely adopted in every field i.e. identifying spam mails, diagnose diseases from X-rays, crop yield prediction, and in future it will help in driving cars (Tang 2019).

This technology has already shown its potential. Here in this paper it is tried to implement the machine learning algorithms to improve the approaches in vaccine development with great sensitivity and accuracy. Machine Learning is classified into three categories at a high level depending on the nature of the learning system (Han & Kamber2010):

1. Supervised learning: Machine gets labeled inputs and their desired outputs. The goal is to learn a general rule to map inputs to the output. That is classification algorithms.
2. Semi supervised learning: in this small amount of labeled data with a large amount unlabeled data during training.
3. Unsupervised learning: Machine gets inputs without desired outputs; the goal is to find structure in inputs. That is clustering and association rule.
4. Reinforcement learning: In this algorithm interacts with a dynamic environment, and it must perform a certain goal without any guidance.

Working of machine learning algorithm: In machine learning, algorithm works by learning

strategy to map input to output without being explicitly programmed.

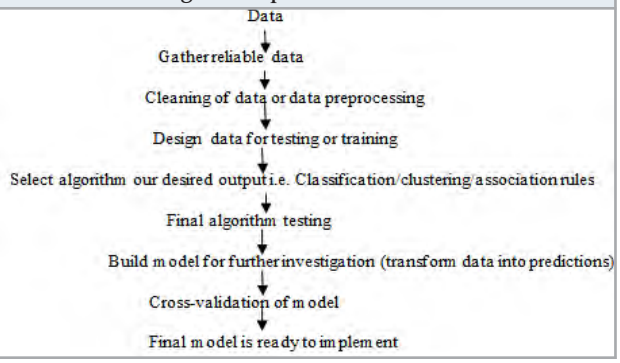
[A] Prediction and classification: Classification is a data mining method that assigns items in a collection to target categories or classes. The goal of classification is to accurately predict the target class for each case in the data. For example, a classification model could be used to identify loan applicants as low, medium, or high credit risks. Nowadays classification techniques are best way to use in health sector for predicting disease based on symptoms data and treatment (Dubey 2016; Dubey 2018). There are many classification algorithms i.e. J48 decision tree, random forest, Naïve bayes (Han and Kamber 2010) etc.

[B] Clustering: It is the assignment of a set of observations into subsets i.e. called clusters, so that observations in the same cluster are similar in some character (Parasian and Silitonga 2017). One of the best clustering algorithms is k-means clustering.

[C] Association rules: Association rules are generated which will be based on hidden patterns, correlations and other insights depends on data (http:link.springer.com//2february2020;Dubey 2014).

For implementing machine learning techniques, it is necessary to understand the biological data for vaccine development. Here is presented a schematic flow of working of machine learning technique.

Figure 1: A general flow diagram for implementing machine learning techniques.



RESULT AND DISCUSSION

If machine learning is applied to each of these above mentioned vaccine development stages it is suggested to save time and results obtained will be of great accuracy and efficiency.

A proposed way of implementation of machine learning in vaccine development: In this paper, author is tried to propose how biomedical scientists can implement different machine learning techniques: classification, clustering, association in different phases of vaccine development. The comparative chart of these techniques is given in table2.

To develop our model for vaccine, first requirement is– Data cleaning or preprocessing: There would be no noise or repetition in the data. There is need to remove all incorrect records.

Data testing and training: Training set is one on which we train and try to fit model according to parameters whereas test data is used for assessment of performance of model. Training data's output is ready to model but test data are unseen, only used for making predictions. There are many algorithms for data training and testing.

Cross validation of model: It is also called model assessment. In validation automatic computer check is done which ensure that the data entered for training is sensible and reasonable. All these methods involve high computation having mathematical and computer science background. For maximum efficiency of algorithms it is needed that it should minimize resource usage. Different machine learning methods need time, computing power, accuracy, space complexity etc. Hence which algorithm works better depends upon the type of data, goal of experiment and measure of efficiency of algorithm. In this paper author is taking the example of malaria and try to explain the role of machine learning algorithms in different stages of vaccine development (as proposed in table 2). Mostly decision tree, clustering and association rules are important to find the suitability in vaccine development.

Decision tree in vaccine development: The decision tree classifier is a simple and widely used classification technique. It poses a series of carefully crafted questions about the attributes of the test record. Each time it receives an answer according to follow-up asked questions, until a conclusion about the class label of the record is achieved. The following figure 1 shows an example decision tree for predicting whether the Antigen is specific and sensitive for disease i.e. malaria that can be use for vaccine development (as shown in table2). In the decision tree, the root and internal nodes contain attribute test conditions to separate records that have different characteristics. The entire terminal node is assigned a class label Yes or No. If the yes is found in testing the antigen then particular antigen is used for vaccine trial or development. Like this we can further move for experiments saving lot of time.

(b) Clustering (Han & Kamber 2010) in vaccine development: Here K-means clustering algorithm example is used to show how clustering is implemented (as given in table 2). All the antigens specific for malaria are grouped into small sub-groups /clusters according to their specificity i.e. more, medium, less. Now the k-means algorithm work as follows:

Specify number of clusters K.

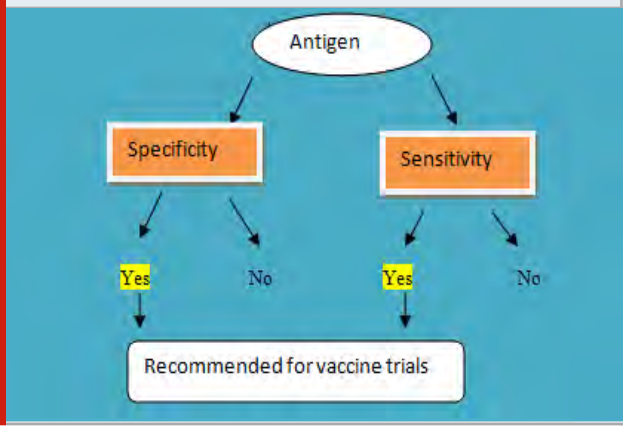
(i) The dataset is shuffled and centre is initialized. Now K data points are randomly selected.
(ii) Without changing centre, iterations are same.
(iii) Compute the sum of the squared distance between data points and all centroids.

(iv) Assign each data point to the closest cluster (centroid).
(v) Compute the centroids for the clusters by taking the average of the all data points that belong to each cluster. This will require mathematical and computation power.

Table 2. Proposed machine learning approaches in different pha ses of vaccine candidate development

Vaccine development PHASE I	Classification	Clustering	Association
1)Laboratory and animal studies			
[a]Exploratory stage	Specific antigens are classified on the basis of sensitivity and specificity.	Disease specific antigens are clustered in one group and used as per requirement	Host specific antigen associations are studied.
[b]Preclinical stage	Tissue culture or cell culture systems are classified as per their subject like monkeys or mice etc.	Animal vaccinate and target pathogen are clustered.	Candidate specific vaccines are in progress if association between candidate vaccine and target is studied perfectly.
PHASE II Clinical studies with human subjects			
Vaccine trial I	Several criteria of candidate vaccines are classified w.r.t. the type and extent of immune response.	Clustering of experimental group for particular injected participants with pathogens.	Association of participants i.e. mice etc with their pathogens is fully understandable for success of vaccine trials.
Vaccine trial II	Classify group that acquire the disease on certain parameters: i.e. (i)Vaccine safety, (ii)Immunogenicity, (iii)Proposed doses, (iv)Schedule of immunization and method of delivery	Cluster the group that work for immunization which produces immune response.	Association rule help to check particular vaccine safety to allergy and immunogenicity to disease. Schedule of immunization with age. Method of delivery of vaccine to humans.
Vaccine trial III	Classify vaccine efficacy on the basis of (i)disease prevention (ii)prevention from infected pathogen (iii)antibodies production	Cluster group of vaccines that proves better response while testing	Make associations of vaccines with disease prevention, vaccine inhibit pathogen prevention, vaccine able to produce antibody against infection.

Figure 2: Decision tree for particular antigen recommendation for vaccine trial



$$\text{Total distances} = \sum_{j=1}^{k} \sum_{i=1}^{n} \left\| x_i^{(j)} - c_j \right\|^2$$

Where k= numbers of clusters, n=number of points belonging to cluster j, cj=centroid of cluster j

(vi)Now the new centroid of each cluster is made by calculating the mean of all points assigned to that cluster.

(vii) Repeat from step 2 until we reach the required centroid which no longer moves. This antigen is more specific for disease (malaria the example taken) under study.

(c) Association rules (Han & Kamber 2010) in vaccine development: In case of malaria, it is tried to deduce the different pattern relations from the available data. Suppose a person having symptoms like malaria. So it is needed to understand two parameters: support and confidence for association rule study (as given in table 2).

Support: This indicates how frequently the if/then relationship appears in the database. This means the symptoms are supporting to having malaria or not based on if/then rule.

Confidence: It shows about the number of times these relationships have been found to be true. This means how the particular symptom is really associated with malaria.

For an association rule X--> Y, the support of the rule is denoted as sup(X-> Y) and is the number of transactions where XUY appears divided by the total number of transactions. The confidence is the number of transactions where XUY appears divided by the number of transactions where X appears. In our case, X- disease symptoms and Y is particular disease like malaria.

These are the different machine learning models that can be used according to the desired goal. These are achieved by implementing in a proper way in the path of vaccine development.

CONCLUSION

If classification and association rule mining techniques are implemented, it is possible to develop vaccine for infectious diseases where vaccine trials are not giving desired results. One most important factor to be studied for vaccine development is genotype to phenotype and their interactions with environment will surly give complete advantage to use machine learning methods in vaccine development. Machine learning and Artificial intelligence are the emerging technologies which paved the way for healthy way of living. The need of this modernized world is to develop more on these techniques and use the friendly nature of them. Robotic process automation is also emerging for making all things automatic and in a controlled manner. In future the method of immunization will be more simpler to administer, will provide long-lasting immune response and most importantly vaccines will survive in transport without any refrigeration. Lethal diseases like HIV/AIDS vaccines will also come into existence.

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On the Diversity and Abundance of Avian Species from Grassland and Wetland Areas of an Industrial Zone of Tropical Maharashtra

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ABSTRACT

India is a very rapidly growing country, with respect to its economy, world recognition, infrastrcture and industrilization. These surfacing industries need a large area for the operation and shouldn't near the human settelments, generally the city outskirts grasslands are choosen for convinience. This leads to loss of well settled grassland habitat or a wetland and therefore many species fostering in these habitas also get affected due to this. This study is selected because there is a lack of data concerning the avian diversity and their abundance associated to the grasslands and wetlands of wide spread Maharashtra Industrial Development Co-operation (MIDC) areas specifically. The study area is a richly diversified, with patches of grassland and wetland in all over the MIDC area. Hence this attempt has been made to know the present status of the avian fauna and its diversity associated with the grasslands and wetlands in MIDC area near Amravati a city having tropical climate . The study was carried out in November 2018 to February 2019 for four Months and monthly four visits were made in the study area. Line transect and belt transect methods of quantification was used for grassland bird study and point transect was used for wetland birds. Total 221 species were reported in the study area, out of which 126 birds were observed in grassland area and 95 species were recorded from the wetlands. In grassland a total of 1473 individuals from all the species were reported and from wetland 909 individuals were reported. The diversity index, species evenness, relative density, species abundance for grassland were 3.96, 0.81, 0-5.43 and 80 whereas 3.87, 0.85, 0-7.7 and 70 for wetland respectively. These entire parameters exhibit high avian faunal existence in MIDC area of Amravati. This indicates that the MIDC grasslands and wetlands provide a very rich avian diversity. The study showed a preliminary data regarding avian diversity and abundance in MIDC area of Amravati. But it would be useful for the study of environmental impacts of industrialization on bird population and diversity

KEY WORDS: AVIAN SPECIES, DIVERSITY, ABUNDANCE, GRASSLAND, WETLAND AND MIDC AMRAVATI.

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INTRODUCTION

The Indian subcontinent, a part of vast Oriental biogeographic regions, is very rich in bio-diversity. India hosts 1300 species of birds out of the 9000 of the total birds in the world, constituting 13 percent of the total bird population and thus is an area of high avian diversity. Bird fauna of India represents 48 families out of the total 75 families in the world. Ali and Ripley (1987) considered 176 species endemic to Indian subcontinent. Grasslands, Wetlands, Bushlands, Forests provide appropriate dwelling places for these organisms. Out of 1300 species of Indian subcontinent (Grimmett et al., 2009) more than 577 species have been reported from Maharashtra State (Kasambe 2016). In Vidarbha, a total of 417 species has been reported (Anon, 2009) and overall Amravati district has 392 birds species (Wadatkar et al., 2016).

Total Forest cover in Indian geography is 21.54 percent, wetlands (under the Ramsar Convention, excluding rivers) has an area of 60 million hectare, grasslands or bushlands occupy nearly 24 percent of the geographical area in India. Moreover, increasing demands on these fragile grassland ecosystems from multiple quarters, notably agricultural intensification, infrastructure development, mining, quarrying, industrial and commercial development, invasive species, and what is more, the very neglect of the common property resources, have eroded much of the native grasslands in the subcontinent with faunal diversity. However the biodiversity in the grassland is not studied to a depth. Comparative studies of avian community composition in different habitat inculding Forests, Wetlands, Grasslands and even in Urban and Sub-urban area can improve our knowledge of general pattern and process that characterize bird species and communities. Birds that depend on grassland and scrub vegetation was experienced a greater decline than any other habitats. Habitat loss and degradation of winter foraging and breeding ground observed leading causes of this decline. (Mankadan 2014, West , 2016, Johnson et al., 2019).

Hence, the present study was carried out for the documentation of diversity, species richness, abundance and evenness of birds associated to the grasslands and wetlands and to know the present status of avian fauna in and around the MIDC area of Nandgaonpeth Amravati specifically.

MATERIAL AND METHODS

Maharashtra Industrial Development Co-operation (M.I.D.C) was established in 1962. MIDC manages its 289 industrial complexes spread over 66,000 hectares of land. Amravati zone (21°01'20" N and 77°51'41" E), with an elevation of 364 meters, is divided in two Parts Amravati MIDC Badnera old bypass and MIDC Nandgaonpeth (NP). It is an area of 2800 hectare, distributed in Commercial, Industrial and Residential zones. The complete 2800 hectare is not a plot able land, 1124 hectare land occupied by textile industrial stakeholders. Rest of the 2800 hectare of land is divided amongst other zones, roads, open spaces, grasslands, wetland and other amenities. The selected study area was 24.79 kms, including grasslands, bush lands forests and wetland. All these lands were connected by major road-ways; one of such was Amravati-Nagpur Expressway (National Highway No. 6).

Figure 1: Map showing the study area (Nandgaonpeth MIDC area), Amravati M.S

Figure 2: (A) Showing the grassland habitat and (B) Showing the wetland habitat in study area

(A)

(B)

The study was carried out in November 2018 to February 2019 (Four Months) in MIDC Nandgaonpeth, Amravati. (Fig.1and2). For this study, 5 zones were chosen in study area. Monthly four visits were made in the study area. Observations were made during 7:30 am to 10:00 am and 4:00 pm to 6:30 pm. Various Quadrates were selected from the study area. Line Transect and Belt Transect bird survey methods of quantification were used for Grassland bird study and Point transect was used for Wetland birds.

Observation and Identification was done using Nikon 8-16x40 mm Binocular. Photographic observations were taken by using Nikon D90 DSLR Camera with 70-300mm and 80-400mm Zoom lens and 18-105mm normal lens. Nikon P600 Point-shoot Camera with a fixed lens, Go pro Hero 5 action camera was also used. Coordinates of the study area were recorded by GPS Garmin-60. Map of the study area was created using Arch Map 10.5 and

Arch GIS software. Birds were identified and listed with the help of available resources, books and checklist (Ali S., and Ripley (1987), Grimmet et al., (2000). Rasmussen, P.C. and Anderton, P.C., (2012) and Checklist of Birds of Amravati District, Wadatkar et al.,(2016).

Data Analysis: All the recorded species were statistically analyzed using various parameters like Diversity Index (Shannon-Wiener's Index; H'), Species Evenness, Relative Density, Species Abundance and Richness.

Shannon-Wiener Index (1963): – The recorded number of Individuals of each Species were Statistically evaluated using Species Diversity index (H') {Shannon-Wiener Index},The values ranging between 0, indicating Low community complexity and 4 and above indicating High community complexity.

$$H' = -\sum_{i=1}^{s} p_i \ln p_i$$

Relative Density: –

$$\text{Relative Density (RD)} = \frac{\text{Number of individual of species}}{\text{Total number of individual of all species}} \times 100$$

Relative Density (RD) = (Number of individual of species)/ (Total number of individual of all species) ×100

Abundance:– It is the number of birds of particular species as a percentage of total bird population of a given area.

$$\text{Abundance} = \frac{\text{Total number of individuals in all sampling units}}{\text{Total number of sampling units of occurance}}$$

Evenness: – It is a measure of relative abundance of different species, it is calculated by using,

$$\text{Evenness} = \frac{H}{H_{max}}$$

Where, H=Shannons Diversity Index, and H_{max}= maximum diversity possible.

RICHNESS: – The number of species per sample is a measure of Richness. The more the species present in the sample, the "richer" the sample. Margalef's index was used as a simple measure of species richness.

Margalef's index=(S-1/In N

Where S=total number of Species, N=total number of individuals in the sample and In= natural logarithm.

RESULTS AND DISCUSSION

Total 221 species were reported in the study area, out of which 126 birds were observed in Grassland area and 95 species were recorded from the Wetlands. In Grassland a total of 1473 individuals from all the bird species were reported and from Wetland 909 individuals were reported. The study area is a richly diversified, with patches of grassland and wetland in all over the MIDC area.(Table 1&2).In all 59 Families of birds were observed in the course of study. Out of which 35 families belong to the grassland birds, and 24 families are of wetland birds. In the families of grassland birds, maximum 14 Species were recorded from, Accipitridae family and in Wetland bird's maximum 15 Species were recorded from two families, Scolopacidae and Anatidae each. (Fig.3&4).Accipitridae family includes (Raptors) Black kite, Eurasian Sparrow-hawk, Short-toed Snake Eagle, Harriers, Booted Eagle etc. while Scolopacidae family include (Waders) Sandpipers, Snipes, Spotted Redshank, Common Greenshank, Godwit, Stint, etc. Indian Silver bill recorded the maximum number of individuals (80) of a Grassland Species, and Black-winged Stilt recorded the maximum number of individuals (70) from Wetlands.

Recorded species were also assorted according to the IUCN's list of Threatened species (2018) categorized. In grassland 122 species belong to Least Concerned and 4 species belong to Near-Threatened (Fig. 5). In wetland 85 species belongs to LC, 8 species were of NT status and 2 species belonged to VU (Fig 6). All the recorded species were statistically analyzed using various parameters like Diversity Index (Shannon-Wiener's Index; H') and Species Evenness , Relative Density, Species Abundance and species Richness. The diversity index, species evenness, relative density, species abundance for Grassland were 3.96, 0.81, 0-5.43,80 and 17.13 whereas 3.87,0.85,0-7.7,70 and 13.79 for wetland respectively. (Table no.3).

All the recorded species were categorized according to their presence in the study area. In grassland resident (R) constitutes 96 species, winter visitors (W) includes 23 species, breeding migrants (BM) includes 4 species and passage visitor (PV) 3 species (Fig. 7).Whereas in wetland, resident (R) constitutes 47 species, Winter visitors (W) constitutes 36 species, resident migrant (RM) include 4 species, breeding migrant (BM) include 2 Species and passage visitor (PV) include 3 species.(Fig. 8).

The extensive grasslands in the region have a very rich fauna of birds, especially grassland birds. They have large patches of steppe grasslands which have places for Feeding and Roosting and hence, it is a preferred habitat for various migratory and resident birds. Birds like Munias, Larks, Pipits, Baya weavers, Sparrows, Coursers, Partridges and Quails were amongst the most occurring in the wide spread grassland. Apart from these grassland dwellers this area hosts a wide-ranging raptor population. Many migratory birds recite here for definite period of time, various Falcons, Harriers, Eagles, Kites and Hawks were recorded during the course of study.In the Wetland region; Storks, Sandpipers, Plovers, Ducks, Water hens, and many other waders were recorded. The wetland patches every-year hosts a wide range of migratory birds

in huge numbers, Bar-headed Geese every-year come and rest here during their migration in 100 of numbers. Apart from these, little waders including Stints, Plovers and Sandpipers also turn up in large numbers.

Table 1. Checklist of Grassland Avian Fauna from MIDC area(NP), Amravati

Sr no	Common Name	Scientific Name	Family	ST	IUCN status
1	Grey Francolin	*Francolinus pondicerianus*	PHASIANIDAE (8)	R	LC
2	Painted Francolin	*Francolinus pictus*		R	LC
3	Common Quail	*Coturnix coturnix*		W	LC
4	Rain Quail	*Coturnix coromandelica*		R	LC
5	Jungle Bush Quail	*Perdicula asiatica*		R	LC
6	Rock Bush Quail	*Perdicula argoondah*		R	LC
7	Barred Buttonquail	*Turnix suscitator*		R	LC
8	Small Buttonquail	*Turnix sylvaticus*		R	LC
9	Eurasian wryneck	*Jynx torquilla*	PICIDAE (4)	W	LC
10	Common Flameback	*Dinopium javanense*		R	LC
11	Yellow-crowned Woodpecker	*Dendrocopos mahrattensis*		R	LC
12	White-naped Woodpecker	*Chrysocolaptes festivus*		R	LC
13	Coppersmith Barbet	*Megalaima haemacephala*	MEGALAIMIDAE (1)	R	LC
14	Common Hoopoe	*Upupa epops*	UPUPIDAE (1)	R	LC
15	Indian Roller	*Coracias benghalensis*	CORACIIDAE (2)	R	LC
16	European Roller	*Coracias garrulus*		PV	NT
17	Green Bee-eater	*Merops orientalis*	MEROPIDAE (2)	R	LC
18	Pied Cuckoo	*Clamator jacobinus*	CUCULIDAE (6)	BM	LC
19	Common Hawk Cuckoo	*Hierococcyx varius*		R	LC
20	Indian Cuckoo	*Cuculus micropterus*		R	LC
21	Eurasian Cuckoo	*Cuculus canorus*		BM	LC
22	Grey-bellied Cuckoo	*Cacomantis passerinus*		BM	LC
23	Asian Koel	*Eudynamys scolopaceus*		R	LC
24	Southern Coucal	*Centropus sinensis*	CENTROPODIDAE (1)	R	LC
25	Alexandrine Parakeet	*Psittacula eupatria*	PSITTACIDAE (3)	R	NT
26	Rose-ringed Parakeet	*Psittacula krameri*		R	LC
27	Plum-headed Parakeet	*Psittacula cyanocephala*		R	LC
28	Little Swift	*Apus affinis*	APODIDAE (2)	R	LC
29	Asian Palm Swift	*Cypsiurus balasiensis*		R	LC
30	Common Barn Owl	*Tyto alba*	TYTONIDAE (1)	R	LC
31	Eurasian Eagle Owl	*Bubo bubo*	STRIGIDAE (4)	R	LC
32	Spotted Owlet	*Athene brama*		R	LC
33	Brown Hawk-Owl	*Ninox scutulata*		R	LC
34	Short-eared Owl	*Asio flammeus*		W	LC
35	Indian Nightjar	*Caprimulgus asiaticus*	CAPRIMULGIDAE (2)	R	LC
36	Indian Jungle Nightjar	*Caprimulgus indicus*		R	LC
37	Rock Pigeon	*Columba livia*	COLUMBIDAE (6)	R	LC
38	Yellow-footed Green Pigeon	*Treronphoenicoptera*		R	LC
39	Eurasian Collard-Dove	*Streptopeliadecaocto*		R	LC
40	Red Collard-Dove	*Streptopelia tranquebarica*		R	LC
41	Spotted Dove	*Spilopelia chinensis*		R	LC
42	Laughing Dove	*Spilopelia senegalensis*		R	LC
43	Chestnut-bellied Sandgrouse	*Pterocles exustus*	PTEROCLIDAE (2)	R	LC
44	Painted Sandgrouse	*Pterocles indicus*		R	LC
45	Yellow-wattled Lapwing	*Vanellus malabaricus*	CHARADRIIDAE (2)	R	LC
46	Red-wattled Lapwing	*Vanellus indicus*		R	LC
47	Indian Courser	*Cursoriuscoro mandelicus*	GLAREOLIDAE (1)	R	LC
48	Black-shouldered Kite	*Elanus axillaris*	ACCIPITRIDAE (14)	R	LC

Continue Table 1

49	Black Kite	*Milvus migrans*		R	LC
50	Black-eared Kite	*Milvus migranslineatus*		W	LC
51	Shikra	*Accipiter badius*		R	LC
52	Eurasian Sparrow Hawk	*Accipiter nisus*		W	LC
53	Booted Eagle	*Hieraaetus pennatus*		W	LC
54	Eurasian Marsh Harrier	*Circus aeruginosus*		W	LC
55	Short-toed Snake Eagle	*Circaetus gallicus*		R	LC
56	Pallid Harrier	*Circus macrourus*		W	NT
57	Pied Harrier	*Circus melanoleucos*		W	LC
58	Montagu's Harrier	*Crcus pygragus*		W	LC
59	Crested Serpent Eagle	*Spilornis cheela*		R	LC
60	Oriental Honey Buzzard	*Pernis ptilorhynchus*		R	LC
61	White-eyed Buzzard	*Butastur teesa*		R	LC
62	Common Kestrel	*Falco tinnunculus*	FALCONIDAE (5)	R	LC
63	Lesser Kestrel	*Falco naumanni*		PV	LC
64	Eurasian Hobby	*Falco subbuteo*		W	LC
65	Red-necked Falcon	*Falco chicquera*		R	NT
66	Amur Falcon	*Falco amurensis*		PV	LC
67	Cattle Egret	*Bubulcus ibis*	ARDEIDAE (1)	R	LC
68	Bay-backed Shrike	*Lanius vittatus*	LANIIDAE (3)	R	LC
69	Long-tailed Shrike	*Lanius schach*		R	LC
70	Brown Shrike	*Lanius cristatus*		W	LC
71	Rufous Treepie	*Dendrocitta vagabunda*	CORVIDAE (7)	R	LC
72	House Crow	*Corvus splendens*		R	LC
73	Small Minivet	*Pericrocotus cinnamomeus*		R	LC
74	Black Drongo	*Dicrurus macrocercus*		R	LC
75	White-browed Fantail	*Rhipidura aureola*		R	LC
76	Asian Paradise-flycatcher	*Terpsiphone paradisi*		R	LC
77	Common Iora	*Aegithina tiphia*		R	LC
78	Oriental Magpie Robin	*Copsychus saularis*	MUSCICAPIDAE (8)	R	LC
79	Desert Wheatear	*Oenanthe desertii*		W	LC
80	Orange-headed Thrush	*Zoothera citrina*		R	LC
81	Indian Robin	*Saxicoloides fulicatus*		R	LC
82	Black Redstart	*Phoenicurus ochruros*		W	LC
83	Indian Chat	*Cercomela fusca*		R	LC
84	Common Stonechat	*Saxicola torquata*		W	LC
85	Pied Bushchat	*Saxicola caprata*		R	LC
86	Brahminy Starling	*Sturnia pagodarum*	STURNIDAE (5)	R	LC
87	Rosy Starling	*Sturnia roseus*		W	LC
88	Asian Pied Starling	*Gracupica contra*		R	LC
89	Common Myna	*Acridotheres tristis*		R	LC
90	Chestnut-tailed Starling	*Sturnia malabarica*		W	LC
91	Dusky Craig Martin	*Ptyonoprogne concolor*	HIRUNDINIDAE (4)	R	LC
92	Grey-throated Martin	*Riparia chinensis*		R	LC
93	Barn Swallow	*Hirundo rustica*		W	LC
94	Wire-tailed Swallow	*Hirundo smithii*		R	LC
95	Red-vented Bulbul	*Pycnonotus cafer*	PYCNONOTIDAE (1)	R	LC
96	Zitting Cisticola	*Cisticola juncidis*	CISTICOLIDAE (1) SYLVIIDAE (5)	R	LC
97	Jungle Prinia	*Prinia sylvatica*		R	LC
98	Plain Prinia	*Prinia inornate*		R	LC
99	Ashy Prinia	*Prinia socialis*		R	LC
100	Blyth's Reed Warbler	*Acrocephalus dumetorum*		R	LC
101	Lesser Whitethroat	*Sylvia curruca*		W	LC
102	Common Tailor Bird	*Orthotomus sutorius*		R	LC
103	Yellow-eyed Babbler	*Chrysomma sinense*		R	LC
104	Large Grey Babbler	*Turdoides malcolmi*		R	LC

Continue Table 1

105	Jungle Babbler	*Turdoides striatus*		R	LC
106	Common Babbler	*Turdoides caudatus*		R	LC
107	Indian Bush Lark	*Mirafra erythroptera*		R	LC
108	Ashy-crowned Sparrow Lark	*Eremopterix griseus*	ALAUDIDAE (6)	R	LC
109	Sykes's Lark	*Galerida deva*		R	LC
110	Singing Bushlark	*Mirafra cantillllans*		W	LC
111	Rufous-tailed Lark	*Ammomanes phoenicura*		R	LC
112	Greater Short-toed Lark	*Calandrella brachydactyla*		W	LC
113	Purple-rumped Sunbird	*Leptocomazeylonica*	NECTARINIDAE (2)	R	LC
114	Purple Sunbird	*Cinnyris asiaticus*		R	LC
115	Paddyfield Pipit	*Anthus rufulus*	PASSERIDAE (8)	R	LC
116	Tawny Pipit	*Anthus campestris*		W	LC
117	House Sparrow	*Passer domesticus*		R	LC
118	Chestnut-shouldered Petronia	*Petronia xanthocollis*		R	LC
119	Baya Weaver	*Ploceus philippinus*		R	LC
120	Red Avadavat	*Amandava amandava*		R	LC
121	Indian Silverbill	*Euodice malabarica*		R	LC
122	Scaly-breasted Munia	*Lonchura punctulata*		R	LC
123	Indian Pitta	*Pitta brachyura*	PITTIDAE (1)	BM	LC
124	Asian paradise Flycatcher	*Terpsiphone paradisi*	MONARCHIDAE (1)	R	LC
125	Grey-necked Bunting	*Emberiza buchanani*	FRINGILLIDAE (1)	W	LC
126	Oriental White-eye	*Zosterops palpebrosus*	ZOSTEROPIDAE	R	LC

R=Widespread Resident; W=Widespread Winter Visitor; PV=Passage Visitor; RM=Resident Migrant and BM=Breeding Migrant.

IUCN's list of Threatened species (2018), categorized as Least Concerned (LC), Near Threatened (NT) and Vulnerable (VU).

In grassland total of 91% were Least concerned and 9% were Near Threatened species (European Roller, Alexandrine Parakeet, Pallid Harrier, Red-necked Falcon). Similarly, in wetland 90% was Least Concerned, 8% were Near Threatened (Ferruginous Pochard, Black-tailed Godwit, Curlew Sandpiper, River Tern, Darter, Black-headed Ibis, Painted Stork and Great Stone Curlew) and 2% were vulnerable species (Common Pochard and Wooly-necked Stork). Also, the Avian diversity was classified according their status, 96 Resident species, 23 Winter visitors species,4 Breeding Migrants species and 3 Passage visitor species in grassland patches and in Wetland, out of 95 Species, 47 Resident species, 36 Winter visitor species, 4 Resident Migrants species, 2 Breeding Migrants and 3 Passage visitors species.

The Grasslands hosts a wide variety of avian life. Large patches of land provide space for Roosting and a rich prey base too. Most occurring Family was the Accipitridae, which includes mostly Raptors. The preference of this type of habitats by these birds indicates their presence in the particular area for Prey base hunting, and Roosting purposes. However, the number of Individuals or Frequency of their sightings is very insignificant. Loss of habitat could be one reason leading to this. One of such very rare Raptor species visiting MIDC grassland Amravati, is the Amur Falcon *Falco amurensis*. The Amur Falcon is a fascinating migratory raptor. Every year, they migrate west through India and across the Arabian Sea to Southern Africa. Because of the long journey, stopover sites are important for these birds to maintain energy level. Amravati lies in their migratory Flyway, although it is very rare to sight the bird in the vast grasslands around the city. Also, a very rich number of Larks, Sliver Munia, Red Avadavat and Scaly-breasted Munia were also observed. This could be because, these species are particularly granivores and the habitat provides a lot of different grasses and seeds dispersed all over the place, which is the specific diet for them.

Family Scolopacidae and Anatidae, recorded the maximum number of species in the Wetlands. Snipes, Sandpipers, Shanks, Stints and Ducks and Goose, constitute this family. Bar-headed Goose *Anser indicus* another prominent winter visitor observed in the wetlands of the study area. Their number is fairly enough and each year they arrive in flocks of 70-100 individuals. Their abundant number is due to the Food preference they exhibit. Bar-headed Goose prefers small lush green grasses around the water bodies. Another winter sojourning bird is the Painted Stork *Mycteria leucocephala*, is a member of Stork family, which is found in the wetlands, and prefer Fishes as major food item. Hence, their presence in the wetlands of study area is justified. Wading across the banks of the water body these birds constantly search for small fishes, snails etc. Apart from these, the wetlands host a variety of other ducks, migrating from right from the colder regions like Siberia, Russia to Africa, for various reasons.

Table 2. Checklist of Wetland Avian Fauna associated to MIDC area (N.P), Amravati

Sr no	Common Name	Scientific Name	Family	ST	IUCN status
1	Lesser Whistling Duck	*Dendro cygnajavanica*	DENDROCYGNDIAE (1)	R	LC
2	Bar-headed Goose	*Anser indicus*	ANATIDAE (15)	W	LC
3	Northern Pintail	*Anas acuta*		W	LC
4	Common Teal	*Anas crecca*		W	LC
5	Red-crested Pochard	*Rhedonessa rufina*		W	LC
6	Common Pochard	*Aythya ferina*		W	VU
7	Indian Spot-billed Duck	*Anas poecilorhyncha*		R	LC
8	Gadwall	*Mareca strepera*		W	LC
9	Garganey	*Anas querquedula*		W	LC
10	Tufted Duck	*Aythya fuligula*		W	LC
11	Northern Shoveller	*Anas clypeata*		W	LC
12	Eurasian Wigeon	*Anas penelope*		W	LC
13	Ruddy (Brahminy) Duck	*Tadorna ferruginea*		W	LC
14	Comb Duck (Knob-billed)	*Sarkidiornis melanotos*		R	LC
15	Ferruginous Pochard	*Aythya nyroca*		W	NT
16	Cotton Pigmy goose	*Nettapus coromandelianus*		R	LC
17	Common Kingfisher	*Alcedo atthis*	ALCEDINIDAE (1)	R	LC
18	White-throated Kingfisher	*Halcyon smyrnensis*	HALCYONIDAE (1)	R	LC
19	Pied Kingfisher	*Ceryle rudis*	CERYLIDAE (1)	R	LC
20	White-breasted Waterhen	*Amanrornis phoenicurus*	RALLIDAE (3)	R	LC
21	Purple Swamphen	*Porphyrio porphyrio*		R	LC
22	Common Coot	*Fulica atra*		R	LC
23	Black-tailed Godwit	*Limosa limosa*	SCOLOPACIDAE (15)	W	NT
24	Pintail Snipe	*Gallinago stenura*		W	LC
25	Common Snipe	*Gallinago gallinago*		W	LC
26	Jack Snipe	*Lymnocryptes minimus*		W	LC
27	Common Greenshank	*Tringa nebularia*		W	LC
28	Spotted Redshank	*Tringa erythropus*		W	LC
29	Green Sandpiper	*Tringa ochropus*		W	LC
30	Common Sandpiper	*Actitis hypoleucos*		W	LC
31	Wood Sandpiper	*Tringa glareola*		W	LC
32	Marsh Sandpiper	*Tringa stagnatilis*		W	LC
33	Little Stint	*Calidris minuta*		W	LC
34	Temminck's Stint	*Calidris temminckii*		W	LC
35	Curlew Sandpiper	*Calidris ferruginea*		PV	NT
36	Ruff	*Philomachus pugnax*		W	LC
37	Pied Avocet	*Recurvirostra avosetta*		PV	LC
38	Greater-painted Snipe	*Rostratula benghalensis*	ROSTRATULIDAE (1)	R	LC
39	Pheasant-tailed Jacana	*Hydrophasianus chirurgus*	JACANIDAE (2)	R	LC
40	Bronze-winged Jacana	*Metopidius indicus*		R	LC
41	Black-winged Stilt	*Himantopus himantopus*	CHARADRIIDAE (4)	RM	LC
42	Little-ringed Plover	*Charadrius dubius*		W	LC
43	Kentish Plover	*Charadrius alexandrinus*		BM	LC
44	Yellow-wattled Lapwing	*Vanellus malabaricus*		R	LC
45	Red-wattled Lapwing	*Vanellus indicus*	GLAREOLIDAE (3)	R	LC
46	Small Pratincole	*Glareola lactea*		R	LC
47	Collard Pratincole	*Glareola pratincola*		PV	LC
48	Brown-headed Gull	*Larus brunnicephalus*	LARIDAE (5)	W	LC
49	River Tern	*Sterna aurantia*		RM	NT
50	Little Tern	*Sterna albifrons*		BM	LC
51	Whiskered Tern	*Chlidonias hybrida*		W	LC

Continue Table 2

52	Gull-billed Tern	*Gelochelidon nilotica*		PV	LC
53	Little Grebe	*Tachybaptus ruficollis*	PODICIPEDIDAE (1)	R	LC
54	Darter	*Anhinga melanogaster*	ANHINGIDAE (1)	R	NT
55	Little Cormorant	*Phalacrocorax niger*	PHALACROCORACIDAE (3)	R	LC
56	Indian Cormorant	*Phalacrocorax fuscicollis*		R	LC
53	Little Grebe	*Tachybaptus ruficollis*	PODICIPEDIDAE (1)	R	LC
54	Darter	*Anhinga melanogaster*	ANHINGIDAE (1)	R	NT
55	Little Cormorant	*Phalacrocorax niger*	PHALACROCORACIDAE (3)	R	LC
56	Indian Cormorant	*Phalacrocorax fuscicollis*		R	LC
57	Great Cormorant	*Phalacrocorax carbo*		R	LC
58	Little Egret	*Egretta garzetta*	ARDEIDAE (10)	R	LC
59	Great Egret	*Casmerodius albus*		R	LC
60	Intermediate Egret	*Mesophoyx intermedia*		R	LC
61	Cattle Egret	*Bubulcus ibis*		R	LC
62	Grey Heron	*Ardea cinerea*		R	LC
63	Purple Heron	*Ardea purpurea*		R	LC
64	Indian Pond Heron	*Ardeola grayii*		R	LC
65	Little Green Heron	*Butorides striatus*		R	LC
66	Yellow Bittern	*Ixobrychus sinensis*		R	LC
67	Black Bittern	*Ixobrychus flavicollis*		R	LC
68	Black-headed Ibis	*Threskiornis melanocephalus*	PHOENICOP TERIDAE (4)	R	NT
69	Black Ibis	*Pseudibis papillosa*		R	LC
70	Eurasian Spoonbill	*Platalea leucorodia*		RM	LC
71	Glossy Ibis	*Plegadis falcinellus*		W	LC
72	Painted Stork	*Myeteria leucocephala*	CICONIIDAE (4)	RM	NT
73	Asian Openbill	*Anastomus oscitans*		W	LC
74	Wooly-necked Stork	*Ciconia episcopus*		R	V
75	Black Stork	*Ciconia nigra*		W	LC
76	White Wagtail	*Motacilla alba*	PASSERIDAE (6)	W	LC
77	White-browed Wagtail	*Motacilla maderaspatensis*		R	LC
78	Citrine Wagtail	*Motacilla citreola*		W	LC
79	Yellow Wagtail	*Motacilla flava*		W	LC
80	Grey Wagtail	*Mptacilla cinereal*		W	LC
81	Paddy-field Pipit	*Anthus rufulus*		R	LC
82	Sykes's Lark	*Galerida deva*	ALAUDIDAE (2)	R	LC
83	Ashy-crowned Sparrow Lark	*Eremopterix grisea*		R	LC
84	Dusky-craig Martin	*Hirundo concolor*	HIRUNDINIDAE (7)	R	LC
85	Grey-throated Martin	*Riparia chinensis*		R	LC
86	Barn Swallow	*Hirundo rustica*		W	LC
87	Wire-tailed Swallow	*Hirundo smithii*		R	LC
88	Red-rumped Swallow	*Hirundo daurica*		R	LC
89	Streak-throated Swallow	*Hirundo fluvicola*		R	LC
90	Pale Sand Martin	*Riparia diluta*		W	LC
91	Clamorous Reed Warbler	*Acrocephalus stentoreus*	SYLVIIDAE (2)	W	LC
92	Booted Warbler	*Iduna caligata*		R	LC
93	Indian Stone-Curlew	*Burhinus indicus*	BRUHINIDAE (2)	R	LC
94	Great Stone Curlew	*Esacu srecurvirostris*		R	NT
95	Osprey	*Pandion haliaetus*	PANDIONIDAE (1)	R	LC

Recorded species from grasslands and Wetlands of MIDC (N.P.), Amravati were statistically analyzed using various parameters like Diversity Index (Shannon-Wiener's Index; H'), Species Evenness, Relative Density, Species Abundance and Richness.

For Grassland bird Species:

H=3.936

H(max)= ln(N)= 4.836

Evenness=H/H(max)=0.813, (here H=3.936)

Shannon's Diversity Index (H)=3.963 and Evenness= 0.813

For Wetland bird Species:

H=3.874

H(max)= ln (N)= 4.553

Evenness= H/H(max)= 0.850, (here H=3.874)

Shannon's Diversity Index (H)=3.874 and Evenness=0.850

Table 3. Summary of Data Analysisarea(NP), Amravati

Observations	Grassland bird Species	Wetland bird Species
Total Individuals	1473	909
Total Species	126	95
Species Richness	17.13	13.79
Shannon's Diversity Index(H')	3.963	3.874
Evenness	0.813	0.850
Relative Density	0 - 5.431	0 – 7.700
Abundance	80 Min=1 Max=80	70 Min=1 Max=70

Figure 3: Graph showing family wise species number of birds associated to Grassland

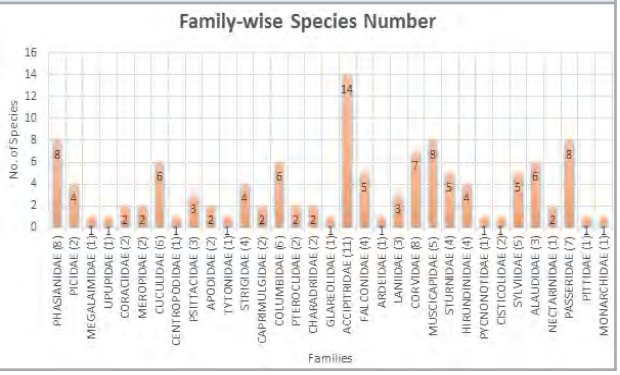
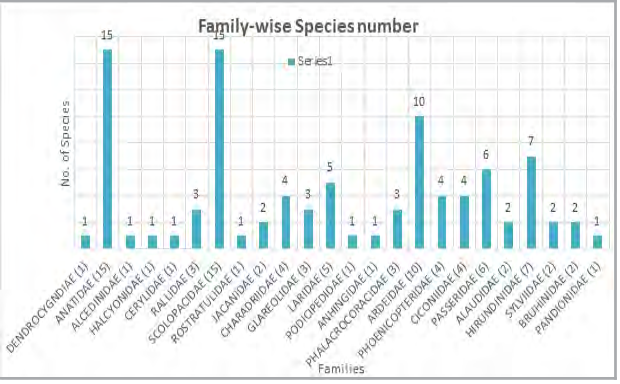


Figure 4: Graph showing family wise species number of birds associated to Wetland



From the report of birds of Banni Grassland Gujrat submitted by the Koladiya ,et.al (2011) . The grassland species of birds almost similar to our observations done in MIDC area, mostly the Raptor family was prominently seen at both the places i.e., Red-necked Falcon, Eurasian Hobby, Pallid and Pied Harriers, Eagles and Kites. Water birds like Red-crested Pochard, Common Pochard, Gadwall etc. were recorded from both the areas.

Figure 5: Graph showing IUCN status of birds in Grassland

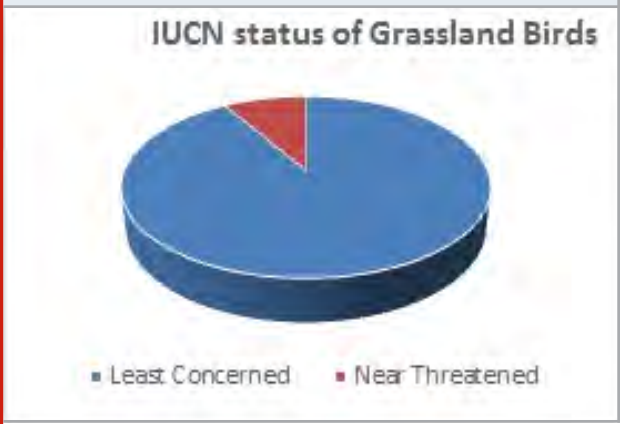


Figure 6: Graph showing IUCN status of birds in Wetland

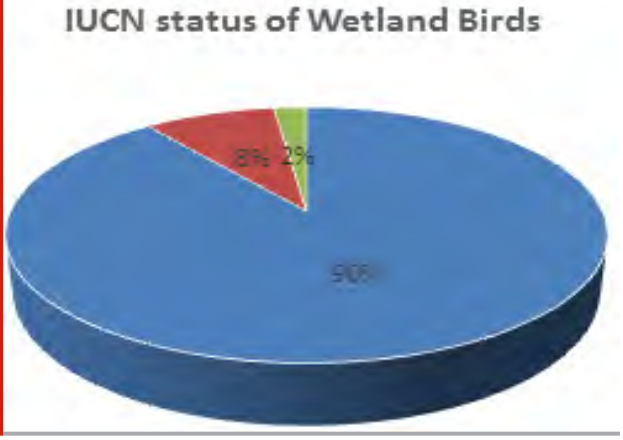


Figure 7: Graph showing percentage of (R) and (M) birds in Grassland

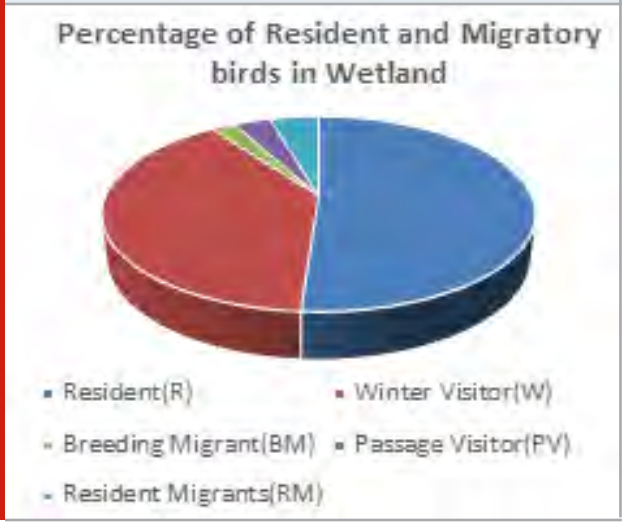
This depicts the species diversity and richness of the grasslands and wetland associated to MIDC Amravati.

Short-eared Owl *Asio flammeus*, one of the rarest-winter migratory bird also showed its presence in the study

area. this could be a positive sign, as their presence in the area marks the potential for new species to migrate here. Another unforeseen sighting from MIHAN Nagpur, a Lesser Florican female *Sypheo tidesindica* was recently recorded. It is endemic to Indian subcontinent and is declared as 'Endangered' species by the IUCN. MIHAN is an under developing Industrial Cargo-Hub, which was previously an extensive farm land. Due to relocation of the farms and conversion of left-over undeveloped areas as Grasslands, it could have established as a potential Habitat for Lesser Florican and hence, noted its presence. This could help in 'Species Restoration' of these birds in a newer developing grassland habitat might be leads to increasing their number, as the whole Vidarbha region was once their natural habitat. Apart from all the negative wreck, this takeover by MIDC authority of the farm lands, converting the unutilized part to grasslands, is causing their (Short-eared Owl, Lesser Florican), positive comeback too.

On statistically analyzing the observed avian diversity, it shows the ambit, a bit in control. But the rising industrial cover and anthropogenic pressure could lead to drastic effects on these species. Upon calculating the Relative Density of Grassland birds, the range was from (0 - 5.431) and Wetland Relative Density range was (0 – 7.700). Highest Abundance was 40 in grassland birds and 70 for wetland birds. The Species Diversity index was calculated as 3.963 for Grassland and 3.874 for Wetland birds. (Table 3). According to Shannon and Weiner (1963), if the value of Diversity is ranging between 0 indicates Low community complexity and 4 and above indicating High community complexity. All these parameters exhibit a fair presence of faunal diversity in MIDC area Amravati, but at the same time it showcases an Alarming-signal for future conservation measures.

Figure 8: Graph showing percentage of (R) and (M) birds in Wetland.



With such rich faunal diversity of Birds, the rising Industrialization has started to exhibit its consequences. Every year the number of visiting birds is on a fall, loss of Habitat could be one of the major causes of this loss. Loss of Roosting grounds, open prairies, leading to insufficient food and water supply to birds are also contributing majorly. Factors like construction of road-ways, involvement of domestic animals for grazing, disturbance by stray dogs, heavy transportation activities through grassland patches, Clamorous noises from the nearby factories also leads the neighboring species to choose another area, water shortage due to uncontrolled usage for commercial and residential are also associated to loss of bird diversity from grasslands. Regrettably, the inimitable biodiversity of the Grasslands remains poorly documented in our region and hence, this study could provide a base-line data and could be helpful in future studies.

CONCLUSION

In all 395 species of birds recorded from Amravati District, out of which 221 species were observed only in MIDC area of Nandgaonpeth Amravati. This is almost 50% of the entire Amravati district bird's species observed till now. This study also presented the Species Diversity Shannon-Wiener index, Species Abundance, Relative Density, Evenness and Richness of the avian species in MIDC area. These entire parameters exhibit high avian faunal existence in MIDC area of Amravati. This shows that the MIDC grasslands and wetlands provide a very rich fauna to the avian diversity, and would be a potential habitat for many others, like Short-eared Owl and Lesser Florican. However, MIDC of Nandgaonpeth Amravati was established in 2012 and since then developmental works started to bloom. This region has since been growing industrially at the cost of loss of Habitat. Once this region could have hosted a varied variety of Avian Fauna, but Industrialization has affected on their existence as well on their numbers. Hence, in this alarming situation this study showed a preliminary documentation of avian fauna in MIDC area, even though this is just a Base-line data but it would be of great use for future avian studies. Also, this study would be useful for establishing the environmental impacts of industrialization of MIDC area on bird diversity. There is critical demand for continuous study in this field.

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Investigations on the Processing and Production of Herbal Tea from Pakalana *Telosma cordata*, Flowers using Blanching and Drying

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ABSTRACT

Tea is one of the most popularly consumed beverages worldwide. Pakalana (*Telosma cordata* Merrill) or Thien Ly flower as a valuable crop in different rural areas of Vietnam. It has a great amount of phytochemical components beneficial for human health. It can be converted into herbal tea like a healthy drink. There is not any literature mentioned to tea processing from the Pakalana (*Telosma cordata* Merrill) flower. Therefore objective of this study focused on the effect of citric acid concentration (0.05, 0.1, 0.15, 0.2, 0.25%) in blanching; vacuum drying temperature (40°C, 45°C, 50°C, 55, 60°C) and pressure (-0.2, -0.4, -0.6, -0.8, -1.0 bar) to total phenolic content (mg GAE/g), antioxidant activity (μM Fe^{2+}/g) and organoleptic attribute (sensory score) of the dried pakalana flower tea. Results showed that pakalana flower should be blanched in hot water 95°C at 5 seconds in the present of citric acid 0.2% and then being dried by vacuum dryer at 50°C, -0.8 bar for 6 hours to get the final moisture content below 10.0% available for preservation. Pakalana flower could be utilized as an appropriate material for processing one kind of functional food.

KEY WORDS: PAKALANA FLOWER, BLANCHING, VACUUM DRYING, HERBAL TEA, TOTAL PHENOLIC, ANTIOXIDANT ACTIVITY.

INTRODUCTION

The genus Telosma includes three species, *Telosma cordata*, T. pallida and T. puberula (Michele and Manop, 2019). *Telosma cordata* or Thien ly is grown as a valuable crop in different rural areas of Vietnam. It is a perennial vine propagating from rooted stem cuttings and growing on trellises of various kinds (Dau et al., 2008). Pakalana (*Telosma cordata* Merrill) flower was cultivated as floral fragrance plant (Widodo, 2014). The flowers are very fragrant, peduncle 0.5-1.5 cm, puberulent (Shakun and Shivani, 2019). This plant was popular for its flower as vegetable with great antimicrobial property (Krasaekoopt and Kongkarnchanatip, 2005). The leaves are good source of essential oils as well as exhibit many medicinal properties for ailments such as wound, scaby, ulcer, headache. Flower buds have normally been consumed stir-fried or boiled. They were used in cooking and medicinally to treat conjunctivitis. At 21oC and 24oC, inflorescence initiation of pakalana flower occured after 3 weeks and the clusters grew to 6 mm in another 2 weeks (Criley 1995). Geraniol, beta-ionone, dihydro-beta-ionone, dihydro-beta-ionol, and cis-and trans-theaspirane were major volatile components of Pakalana (*Telosma cordata*

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Merrill) flower (Arai et al., 1993). Extract of pakalana (*Telosma cordata* Merrill) flower has a great antidiabetic potential (Lilibeth and Edna, 2014).

Edible flowers have valuable phytochemical constituents with abundant antioxidant activities. Phenolics are a substantial and diverse group of phytochemicals (Ngoitaku et al., 2016). Phenolics are responsible for antioxidant characteristics related to their capabilities to scavenge free radicals, break radical chain reactions and chelate metals. They are correlated with a reduced risk of cardiovascular disease and cancers (Huang et al., 2010). Herbal teas are infusions made from fruits, leaves, flowers, roots and stems of plants and are intended for oral aqueous consumption (Malongane et al., 2018). Herbal teas contribute extremely strong taste, relaxation, rejuvenation with different therapeutic and energizing benefits (Chandini, 2014; Joubert et al., 2017; Florence et al., 2020; Quang and Hoang, 2020).

Production of tea has been broadly investigated on variety of materials such as chamomile, ginger, ginseng, peppermint, cinnamon, roselle etc. However, the research on production of edible flower tea from pakalana flower has been rarely reported. Therefore purpose of this study focused on the citric acid concentration in blanching and vacuum drying temperature and pressure to total maintain the phenolic content, antioxidant capacity and organoleptic property of pakalana flower, to be used as tea a popular functional herbal drink.

MATERIAL AND METHODS

Pakalana (*Telosma cordata* Merrill) flowers were collected from Soc Trang province, Vietnam. After collecting, they must be quickly conveyed to laboratory for experiments. The fresh edible flowers were cleaned under tap water to remove foreign matters; the petals were separated and kept at room temperature to drain. Petals were blanched and dried under different conditions. Chemical substances and reagents such as Folin-Ciocalteu reagent, sodium carbonate, gallic acid standard, acetate buffer, TPTZ , HCl, FeCl. $_6H_2O$ were all analytical grade supplied from Rainbow Trading Co. Ltd., Vietnam.

Effect of citric acid concentration in blanching to total phenolic content (mg GAE/g), antioxidant activity (µM Fe2+/g) and organoleptic attribute (sensory score) in the dried pakalana (*Telosma cordata* Merrill) flower tea: Raw pakalana (*Telosma cordata* Merrill) flowers were blanched in citric acid solution (0.05, 0.1, 0.15, 0.2, 0.25%) at 95°C, 5 seconds. Then they were dripped and dried by vacuum dryer at 40°C, -0.2 bar for 6 hours by vacuum dryer. Then they were analyzed total phenolic content (mg GAE/g), antioxidant activity (µM Fe^{2+}/g), organoleptic attribute (sensory score) to choose the best citric acid concentration in blanching.

Effect of vacuum drying temperature and pressure to total phenolic content (mg GAE/g), antioxidant activity (µM Fe2+/g) and organoleptic attribute (sensory score) in the dried pakalana (Telosma cordata Merrill) flower tea: Raw *Pakalana* (*Telosma cordata* Merrill) flowers were blanched in hot water with 0.2% citric acid at 95oC in 5 seconds. Then these samples would be dripped and dried under vacuum dryer at different temperature (40°C, 45°C, 50°C, 55°C, 60°C) and different pressure (-0.2, -0.4, -0.6, -0.8, -1.0 bar) for 6 hours. All samples were analyzed total phenolic content (mg GAE/g), antioxidant activity (µM $Fe2^+$/g), organoleptic attribute (sensory score) to select the optimal drying condition.

Phytochemical, sensory analysis: The total phenolic content (TPC, mg GAE/g) was estimated as gallic acid equivalents by the Folin–Ciocalteu reagent method (Djeridane et al., 2006). An extract of 1 mL from Pakalana was mixed with 5 mL of Folin-Ciocalteu reagent in a test tube and kept at ambient temperature. After 10 min, 4 mL of 7.0% (w/v) Na_2CO_3 and the mixture was left for reaction at ambient temperature in a dark place for 30 minutes. The absorbance of the reacted mixture was then measured at 765 nm using a spectrophotometer. The total phenolic content of the extracts was expressed as mg gallic acid equivalent (mg GAE) based on the standard curve of gallic acid solutions.

The antioxidant activity (FRAP, µM Fe^{2+}/g) was measured by ferric-reducing antioxidant power (Benzie and Strain, 1996). The FRAP reagent was prepared by mixing acetate buffer (250 mM, pH 3.5), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). The reagent (3.400 µL) and sample solutions (100 µL) were added to each well and mixed thoroughly. The absorbance was taken at 593 nm after 30 minutes. Organoleptic attribute (sensory score) of *pakalana* (*Telosma cordata* Merrill) flower was assessed by a group of 11 panelists using the 9-point Hedonic scale.

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

RESULTS AND DISCUSSION

Effect of citric acid concentration in blanching to total phenolic content (mg GAE/g), antioxidant activity (µM Fe2+/g) and organoleptic attribute (sensory score) in the dried pakalana (Telosma cordata Merrill) flower tea: In our reseach, raw pakalana (*Telosma cordata* Merrill) flowers were blanched in citric acid solution (0.05, 0.1, 0.15, 0.2, 0.25%) at 95°C, 5 seconds. Then they were dripped and dried by vacuum dryer at 40°C, -0.2 bar for 6 hours by vacuum dryer. Then they were analyzed total phenolic content (mg GAE/g), antioxidant activity (µM $Fe2^+$/g), organoleptic attribute (sensory score) to validate the appropriate blanching condition. Results were mentioned in figure 1-3. From figure 1-3, the pakalana (*Telosma cordata* Merrill) flower should be blanched at 95°C in 5 seconds in the presence of 0.2% citric acid to maintain the most total phenolic content, antioxidant

capacity and organoleptic attribute in the dried pakalana (*Telosma cordata* Merrill) flower tea.

Figure 1: Effect of citric acid concentration (%) in blanching to total phenolic content (mg GAE/g) in the dried pakalana (*Telosma cordata* Merrill) flower tea

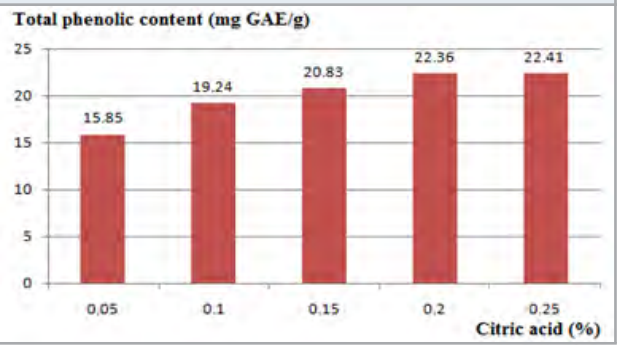


Figure 2: Effect of citric acid concentration (%) in blanching to antioxidant activity (µM Fe2+/g) in the dried pakalana (*Telosma cordata* Merrill) flower tea

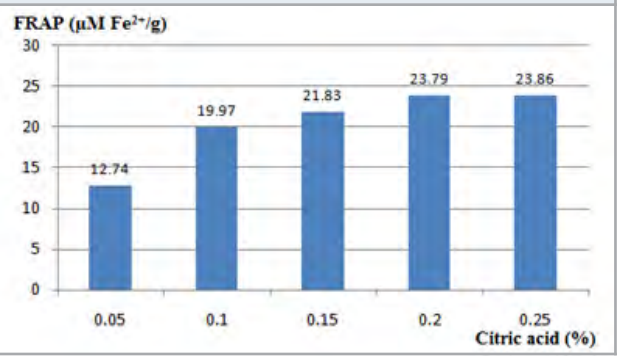
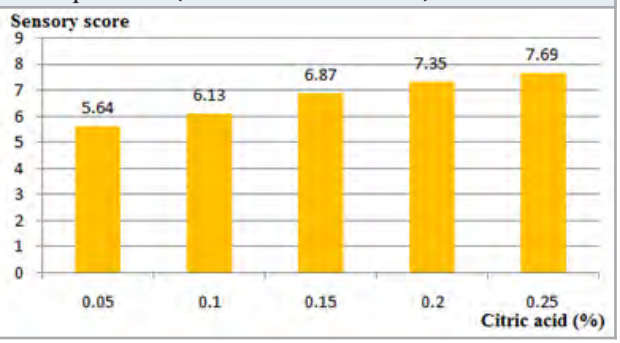


Figure 3: Effect of citric acid concentration (%) in blanching to organoleptic attribute (sensory score) in the dried pakalana (*Telosma cordata* Merrill) flower tea



Flower is an important part of plant containing natural antioxidants such as phenolic acids, flavonoids, anthocyanin and many other phenolic compounds (Kaur et al., 2006). Phenolic compounds are the most important phytochemicals in pakalana (*Telosma cordata* Merrill) flower which flavonoids and anthocyanins are the most common groups (Sarepoua et al., 2013). The application of citric acid in the blanching medium resulted in higher total phenolic retention and antioxidant activity than the usage of water only. This result was similar to other findings in blanching white saffron (Pujimulyani et al. 2010), corn (Randhir et al., 2008), wheat (Cheng et al., 2006). It was presumed that in blanching process, the antioxidants in the form of glycoside were hydrolized into aglikon and sugar (Yue and Xu, 2008). The blanching of pakalana flower increased antioxidant activity. Phenolic compounds could breakdown during the blanching. The increased of antioxidant activity in the blanching treatment could be explained due to heating which caused antioxidant compound to be extracted more easily (Pujimulyani et al. 2010).

According to Montinee et al. (2017), the aqueous extract of *Telosma* flower had the highest total phenolic concentration (991.3 mg GAE/100g). It had the highest activities as a DPPH radical scavenger (252.5 mg/L) and as an inhibitor of lipid peroxidation. For anti-lipid peroxidation, the IC50 (the half-maximal inhibition concentration) value of the aqueous *Telosma* flower extract was 1539.93 mg/L. Flavonoids are the bioactive phytochemical constituents which make the plant resistant to the attack of microbes, insects and also protect the animals against various diseases (Guevara et al., 2000; Silva et al., 2002; Nessa et al., 2012).

Flavonoids possess strong antioxidant activity and free radical-scavenging capacity and inhibit protein glycation (Silva et al., 2002; Matsuda et al., 2003; Wu et al., 2005). Blanching is one of the most important pre-treatment step in processing of various herbal tea products. The main purpose of blanching is to inactivate enzymes that cause negative effects on the product quality. High temperature in short time blanching is normally prefered to avoid the occurence of degradable reactions during the storage. Unfortunately blanching also creates changes in cellular structure and composition (Vina et al., 2007).

Effect of vacuum drying temperature and pressure to total phenolic content (mg GAE/g), antioxidant activity (µM Fe2+/g) and organoleptic attribute (sensory score) in the dried pakalana (Telosma cordata Merrill) flower tea: In the present research, raw pakalana (*Telosma cordata* Merrill) flowers were blanched in hot water with 0.2% citric acid at 95oC in 5 seconds. Then these samples would be dried by vacuum dryer at different temperature (40°C, 45°C, 50°C, 55, 60°C) and different pressure (-0.2, -0.4, -0.6, -0.8, -1.0 bar). All samples were analyzed total phenolic content (mg GAE/g), antioxidant activity (µM Fe^{2+}/g), organoleptic attribute (sensory score) to validate the appropriate drying temperature and pressure. Results were mentioned in figure 4-9. From figure 4-9, the pakalana (*Telosma cordata* Merrill) flower should be dried at 50°C, -0.8 bar to maintain the most total phenolic content (mg GAE/g), antioxidant activity (µM Fe^{2+}/g) and organoleptic attribute in the dried pakalana (*Telosma cordata* Merrill) flower tea. These could be explained

by faster inactivation of oxidase enzyme (polyphenol oxidase) at higher temperature (Lim and Murtijaya, 2007; Saifullah et al., 2019).

Figure 4: Effect of drying temperature (°C) at -0.2 bar pressure to total phenolic content (mg GAE/g) in the dried pakalana (*Telosma cordata* Merrill) flower tea

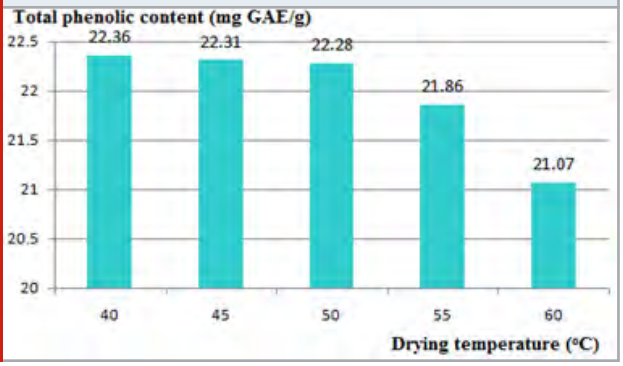


Figure 5: Effect of drying temperature (°C) at -0.2 bar pressure to antioxidant activity (µM Fe2+/g) in the dried pakalana (*Telosma cordata* Merrill) flower tea

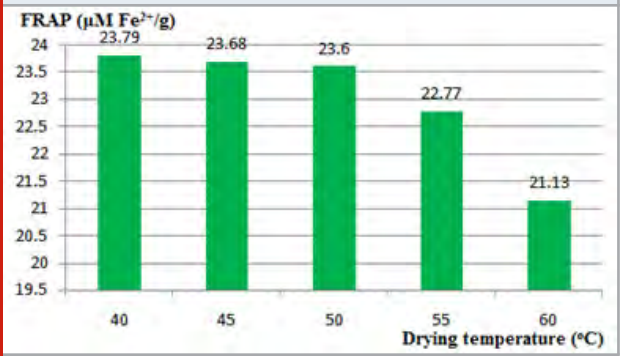
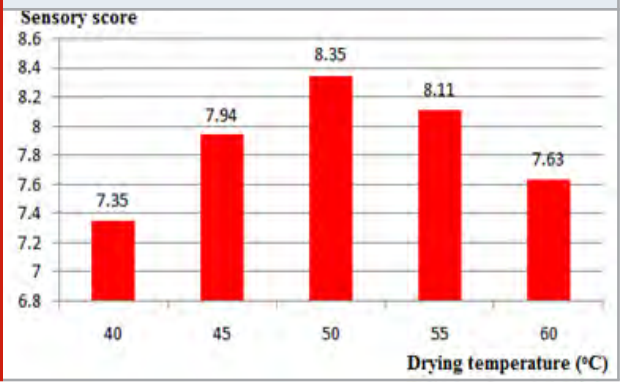


Figure 6: Effect of drying temperature (°C) at -0.2 bar pressure to organoleptic attribute (sensory score) in the dried pakalana (*Telosma cordata* Merrill) flower tea



Total phenolic content loss caused by enzymatic reactions could be minimized by blanching before vacuum drying. Drying is generally identified as a process of water removal and decreasing of herbal moisture content in

Figure 7: Effect of drying pressure (bar) at 50°C to total phenolic content (mg GAE/g) in the dried pakalana (*Telosma cordata* Merrill) flower tea

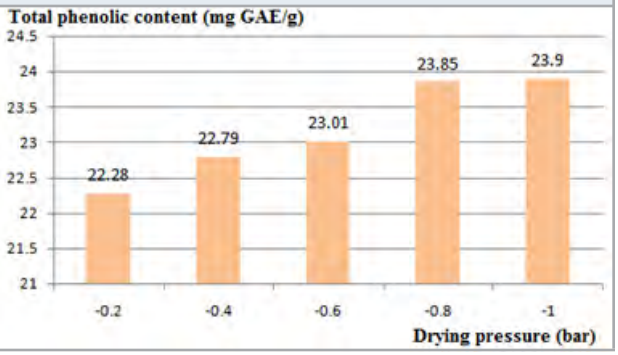


Figure 8: Effect of drying pressure (bar) at 50oC to antioxidant activity (µM Fe2⁺/g) in the dried pakalana (*Telosma cordata* Merrill) flower tea

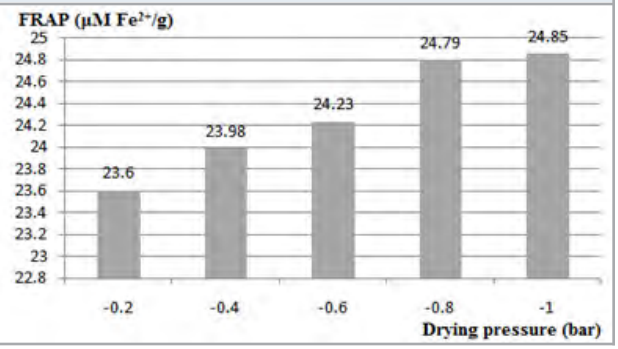
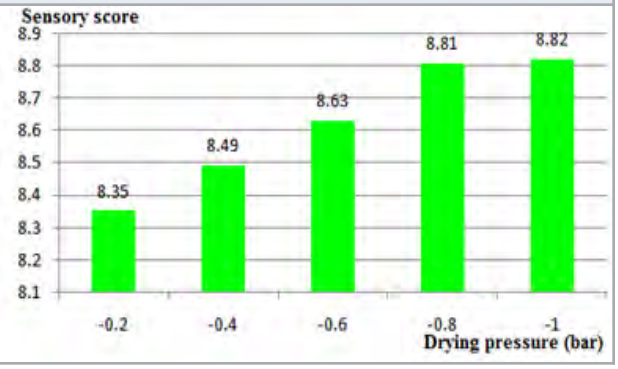


Figure 9: Effect of drying pressure (bar) at 50°C to organoleptic attribute (sensory score) in the dried pakalana (*Telosma cordata* Merrill) flower tea



limiting microbial and enzymatic activity, consequently maintaining product stability (Taha et al., 2015). Vacuum drying is a popular strategy utilized for dehydration of various herbal plants, especially matrices having thermal sensitive components (Methakhup et al., 2005).

In drying period, metabolically active plants lose moisture slowly. Polyphenol oxidases are not able to degrade phenolic components in dehydration. Degradation of phytochemicals could be related to thermal process only (Ismail et al., 2004). Drying process resulted in a depletion

of naturally occurring antioxidants was mentioned in raw plant materials (Tomaino et al., 2005). Thermal process might create significant loss in antioxidants, deactivate enzymes and degrade phytochemicals (Taha et al., 2015). Volatile compounds are key contributors to herbal tea flavour (Gong et al., 2017). The drying at 80 ^{0}C produced the dried Roselle with the highest retention of total phenolic content (Quang and Hoang, 2020).

CONCLUSION

Pakalana flower has attracted a great attention due to a variety of its bioactive phytochemical constituents. In this reseach, we have successfully investigated some major technical parameters of citric acid concentration in blanching and vacuum drying conditions influencing to processing of pakalana (Telosma cordata Merrill) flower into herbal tea. Phenolic component and antioxidant capacity could be stronly affected during the blanching and drying. Diversification of valuable pakalana (Telosma cordata Merrill) flower into healthy herbal tea could enhance its agricultural commercial value.

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Taxonomy of Medically Important Bacterial Species Through Intelligent Neural Network: A Soft-Computing Based Approach

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ABSTRACT

Bacteria are important disease causing agents in human and subhuman vertebrates. This study demonstrated a unique method to identify the bacteria up to their species level on the basis of key biochemical properties. A soft-computing-based Intelligent Neural Network (INN) method has been proposed to classify the pathogenic bacteria such that *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia, Shigella dysenteriae, Salmonella typhimurium, Proteus vulgaris, Pseudomonas aeruginosa, Alcaligenes faecalis, Staphylococcus aureus, Lactococcus lactis, Micrococcus luteus, Corynebacterium xerosis*, and *Bacillus cereus* with maximum accuracy. The methodology comprised two layered fully connected neurons to build the INN model and it is trained by using input vector having binary encoded values, from biochemical properties of taken bacterial set. Common biochemical properties of pathogenic bacteria have been taken for this study and a tabulation has been prepared by encoding the properties into zero('0') for which species does not react positive and the properties encode into one('1') where the species react positive. In this study the proposed INN method has been trained through forward and backward propagation by optimizing the Sum Squared Error of the network. To get the best outcome, the proposed method has been trained four times by varying epochs and learning rate, and the Sum Squared Error(SSE) has been reduced each time and finally it reached in '0.001165' which is the best regarding classification of these bacterial set. This study would help laboratory users about the pathogenic bacteria to identify them in a fast and easy way

KEY WORDS: TAXONOMY, PATHOGENIC, SOFT-COMPUTING, SGD, INN, SUM SQUARED ERROR.

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INTRODUCTION

Nowadays it is challenging to determine the exact bacterial strain causing a specific, unidentified disease. However, the precise identification of bacteria is imperative in the treatment of resultant diseases. Bacteria are omnipresent, inhabiting water, soil, plants and also in other living organisms. The intestinal tracts of humans and animals are particularly rich, including pathogenic bacteria, such as those belonging to the Enterobacteriaceae family; most of the members of this family are gram-negative, non-spore forming facultatively anaerobes (Brisse et al., 2017; David and Paterson, 2012).

In this paper we have focused on disease causing bacterial species such as *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia, Shigella dysenteriae, Salmonella typhimurium, Proteus vulgaris, Pseudomonas aeruginosa, Alcaligenes faecalis, Staphylococcus aureus, Lactococcus lactis, Micrococcus luteus*, *Corynebacterium xerosis*, and *Bacillus cereus* and their proper classification using the Intelligent Neural Network.

Escherichia coli is a free-living pathogen which is mainly found in human intestines. It is responsible for many infections, such as those of the urinary tract and colon as reported by (Meredith and Ulrich, 2013). Within the *E. coli* species, the strain E.0157 is particularly dangerous due to its production of the Shiga toxin. Within more vulnerable populations, such as children and the elderly, infection by this strain can be devastating, causing renal failure, hemolytic anemias, organ failure, and mental disequilibrium as reported by (Davis, 2018).

Less severe but still significantly, *E.coli* 0157 can cause chronic diarrhoea and dehydration. Chronic diarrhoea and dehydration from *E. coli* develop through the ingestion of contaminated food. The identification of the offending strain becomes possible when it is isolated from an infected patient's stool or blood. *Enterobacter aerogenes*, also known as *Aerobacter aerogenes*, is a gram-negative, rod-shaped bacterium of the Enterobacteriaceae family. It is ubiquitous in the environment and generally present in fresh water, soil, vegetables and the excrement of human and animals.

It most commonly infects vulnerable populations such as young children, the elderly, and patients in the ICU. *E. aerogenes* is transmitted as nosocomial infections, as well as within the community. It can infect numerous organ systems such as the central nervous system, colon, urinary tract, skin, and blood. *E. aerogenes* can be the cause of meningitis if the bacteria can reach into the cerebrospinal fluid (Khan, 2004; McGrath ,2017).

Despite its low motility rate (10.2%), hospital environments can also induce its growth and transmission. However, *E. aerogenes* often acts in association with other pathogens. It is generally easily treatable with antimicrobials. *Klebsiella pneumonia* is another common pathogen that warrants discussion. It is a gram-negative, non-motile, and rod-shaped bacterium belonging to the Enterobacteriaceae family (Amako et al., 1988). Often hospital-acquired, it is the cause of numerous infections from pneumonias to urinary tract and intra-abdominal infections (Amako et al.,1988; Ashurst and Dawson, 2019).

It is also a frequent respiratory pathogen which causes pneumonia by some symptoms like hemoptysis, and high fever. The prevalence of *K. pneumoniae* in community-acquired pneumonia has been reduced; however its prevalence in causing other pathology, such as liver abscess and meningitis, has been increased. *K. pneumoniae* is usually amenable to routine antimicrobial treatment. However, it is notable that antibiotic-resistant strains are emerging, which may present a challenge to treatment (Ashurst and Dawson 2019).

Shigella dysenteriae is one of the main species of *Shigella*. Being a member of the Enterobacteriaceae family, it is a small, gram-negative, non-motile, non-spore forming bacterium (Niyogi, 2005). It often resides in contaminated food and water, passing between hosts via either ingestion of the above or through fecal-oral contact. Houseflies have also been found to be a notable medium for infection by *S. dysenteriae*. It is responsible for bloody diarrhea, fever, abdominal pain, and stomach cramps in human (Keusch et al., 2011).

S. dysenteriae can be life-threatening, potentially causing Shigellosis, which is especially prevalent in developing countries. The Shiga toxin generated by this bacterium is responsible for Shigellosis. Beyond the acute danger of Shigellosis, less mild but chronic effects include malabsorption, growth retardation, and recurrent infections, which are especially prominent in already malnourished children (Niyogi, 2005). *Salmonella typhimurium* is gram-negative, rod shaped bacterium generally found in the human intestinal lumen (Keusch et al., 2011; Patel and McCormick, 2014). Its medical significance lies in its ability to cause typhoid fever (Gart et al., 2016 Ashurst and Woodbury, 2019).

Proteus vulgaris is also gram-negative, rod shaped bacterium, which is widely spread in the environment and mainly found in the human gastrointestinal tract, water, soil, and excrement. As opportunistic pathogen, it is the 3rd most common cause of hospital-acquired infections (Bahashwan and Shafey, 2013). *P.vulgaris* is a motile, chemoheterotroph, and non-spore forming genera with various modes of transmission (Bahashwan and Shafey, 2013; Broeck and Herter, 1911). It has urease enzymes that allow it to produce ammonia in bulk, which is responsible for treating bladder and kidney stones. *P. vulgaris* is often amenable to treatment with antibiotics and bio-field treatment (Broeck and Herter, 1911; Braton et al., 2015).

Pseudomonas aeruginosa is a gram-negative, rod-shaped opportunistic pathogen and a member of the Enterobacteriaceae family. It is the cause of chronic infections in the urinary tract, skin (burn or surgical wounds), and respiratory tract of humans

(Dzvova et al., 2016). It is ubiquitous in nature under distinct environmental conditions. P.aeruginosa is widespread in nature, soil, and water. This bacterium is well-grown in between 25^{0}C to 37^{0}C temperature (Bai F et al., 2015). *P.aeruginosa* is considered as hospital-acquired infections, particularly in immunocompromised patients. Antibiotics are required to treat this bacterial infection (Bai F et al., 2015; Cafasso, 2016).

Alcaligenes faecalis is a gram-negative, rod shaped motile, and aerobic pathogen (Mohammadizad and Salehizadeh, 2009), generally found in soil and water, as well as laboratory settings. *A.faecalis* is an opportunistic pathogen, which most often infects through liquid-based media such as nebulizers, respirators, and lavage of fluids (Mohammadizad and Salehizadeh , 2009). It can be found in the blood, urine, and feces. A more specific but notable manifestation in humans is the corneal ulcer. In addition to fluids, *A. faecalis* has been known to contaminate medical devices as well.

S.aureus is a gram-positive, facultatively anaerobic, naturally unencapsulated coccus. *S.aureus* is responsible for infections in newborn infants, breastfeeding women, and those with chronic disease such as vascular disease, lung disease, cancer and diabetes (Foster, 1996). This bacterium is a notable cause of sepsis through bacteremia. Infections due to *S.aureus* can be transmitted from one person to another always. Antibiotics are imperative in treating any infections caused by *S.aureus*. *Lactococcus lactis* is a gram-positive, lactic acid-producing bacterium massively used in cheese, buttermilk, pickled vegetable productions and other types of fermented products. *L.lactis* is occasionally pathogenic, with few cases of disease reported. *L.lactis* is considered as pathogenic bacteria; some cases of diseases have been reported like infective endocarditis in adults and in children (Georgountzos et al. 2018).

It was first found in green plants and had the capability to grow on various sugars. *L.lactis* can boost the immune system to strive to recover allergies, hypertension, and it has more beneficial effects on the skin, and IBD (In et al., 2017). *Micrococcus luteus* is a gram-positive coccus bacterium, about 0.5-3.5 micrometer in size, that can be found in water, soil, dust, and skin. M. luteus thrives in high oxygen environments, contributing to its prevalence in the respiratory tract and mucosal linings of the upper pharynx of humans (Rakhashiya et al., 2015). M.luteus can also grow in other environments, such as those with high salt concentrations, or little water at a 370C temperature (Krishnaveni and Umadevi, 2013).

Corynebacterium xerosis is a gram-positive, rod-shaped pathogen that can be found in skin and mucous membranes of humans. *C.xerosis* has been shown in normal skin flora, conjunctivas, and nasopharynx and most recently it has been isolated from vaginal swabs. Over the last two decades it has been recognized as a significant pathogen to humans, known to cause septicemia, endocarditis, pneumonia, osteomyelitis, septic arthritis, meningitis and ventriculitis especially in surgical patients or vulnerable populations like the immunocompromised or neonates (Cattani et al., 2000).

Bacillus cereus is a gram-positive, spore-forming, rod-shaped, motile pathogen. *B.cereus* is generally found in soil as well as a variety of foods. Its spore-forming characteristics help it to survive in severe environments where temperature is high or low. Its toxin production is the cause of gastrointestinal symptoms such as vomiting and diarrhea (Gherardi, 2016). In addition to its role in GI pathology, *B.cereus* can infect some surgical equipments and cardiac equipments in situ such as pacemakers, prosthetic mitral valves (Gherardi, 2016).

These thirteen bacteria display a broad diversity in significance within human pathology, highlighting the value of precise identification, often through phenotypic and biochemical characterization. Currently, bacterial taxonomy is based on the polyphasic approach comprising phenotypic, biochemical, serotypic and molecular methods. Soft computation is a new dimension in the field of bacterial taxonomy; here neural networking has been used as a soft computing-based classifier for the taxonomy of medically important pathogenic bacteria. An Intelligent Neural Network (INN) is a mathematical model for information processing, which is inspired by the neural system of the human brain (Hassankashi, 2019).

Now, INNs have become part of a technological revolution which have been focused on concrete decision making such as face recognition, gene identification and so on. We are now in the digital universe where many complex operations are executed through programmable intelligent electronic devices. Neural network technology is extensively used in both practical fields such as voice recognition and finger print recognition and academic fields such as pattern matching, data analytics, and programmable pathological equipment development and so on. In this study, INNs have been applied as a soft computing-based classifier in the taxonomy of pathogenic bacterial species.

We reviewed some significant literature regarding the classification of bacteria and their species. Huang et al. in their study applied, supervised and unsupervised machine learning techniques have been applied in eighteen classes of bacteria. Most of the bacterial species are known as disease-causing agents of humans. Three different algorithms were used in to classify species based on colony morphology. The supervised classification method comprising traditional and special Convolution Neural Network(CNN) has been applied. On the other side, Auto encoder has been included in the unsupervised method to classify bacteria. The deep neural network architecture of seven layers has been used and accuracy up to 90% has been achieved for distinguishing each bacterial species.

Manzoor et al.(2014) have introduced a new method for classification of bacterial strains of the same species. In this study, they applied the method on 40 bacterial

strains, which are generally acquired in hospital. The proposed method based on a neural networks algorithm was applied to detect mutations and genetic variations in the bacterial strains. Principal Component Analysis (PCA), Discriminant Function Analysis (DFA) and the Probabilistic Neural Networks (PNN) algorithms were explored in a sensory perception system for recognition of a pattern in bacterial species from contaminated water (Carrillo and Durán, 2019).

Most early studies explored the role of NN in the analysis of colony morphology to detect mutations and genetic variation in bacteria. In the above studies all the authors have done their works through pattern recognition techniques, which focused on extracting the features applying on image processing from bacterial colony images. However, there is scanty literature available for the more advanced use of NN using biochemical properties to characterize and identify bacterial species. During the present study, the Neural Network based Intelligent Neural Network method has been explored in the taxonomy of medically important bacterial species through their fundamental biochemical properties.

MATERIAL AND METHODS

In this section, it has been discussed how the Intelligent Neural Network (INN) has been designed to classify different bacterial species belonging to their respective common pathogenic group. The cultural and biochemical characteristics of unknown organisms are separated and these characteristics have been encoded into a binary value. These values are fed into a trained INN to precisely identify and classify organisms.

In this method, two-layered intelligent neural networks have been used and the training process has been done through both forward and backward propagation. In the forward pass, the following function has been used where the synaptic weights of neurons would be unchanged throughout the network, and the signal would be computed for each neuron (Haykin, 1999; Padhy and Simon, 2015). The function generated output for neuron *j* as

$$y_j(n) = \Psi(\vartheta_j(n) + Bias) \quad (1.1)$$

Where, n was the number of iterations and $\vartheta_j(n)$ was the calculated output at the local field of neuron *j*, defined as

$$\vartheta_j(n) = \sum_{i=1}^{m} w_{ji}(n) y_i(n) \quad (1.2)$$

In the above equation, m implied a total number of input signals from neuron i to neuron j with $w_j(n)$ connecting synaptic weights and $y_i(n)$ was the output of neuron i to the input in neuron *j*. Many hidden layers were used to design the proposed network model where the first hidden neuron in the network indicated by the value 1 in m in equation (1.2) and referred as y_j(n) was output of input node x_i(n) where i was indicated the first input terminal of the INN. The activation function in equation (1.1) was a Logistic function defined as:

$$\Psi_j(\vartheta_j(n) + Bias) = \frac{1}{1 + \exp(-(\vartheta_j(n) + Bias))}$$

$$\forall \ -\infty < (\vartheta_j(n) + Bias) < \infty \quad (1.3)$$

This was a sigmoidal nonlinearity function that generated an output on the induced local field of neuron j and Bias. The output amplitude of neuron *j* lied inside the range $0 \le y_j \le 1$ for a nonlinearity activation function (Haykin, 1999). This function was used at each level in all neurons with the same Bias to compute the output. The backward pass was the next phase of this method, where the output from the last neuron *j* was compared with the actual desired output and measured the amount of error signal generated by neuron *j* at iteration n, defined as

$$e_j(n) = d_j(n) - y_j(n) \quad (2.1)$$

where d_j(n) was the desired output and y_j(n) was the actual output from neuron j at iteration n.

Cost function: In general, cost function depends on error energy generated by the network model at each level of outcome. The error energy was calculated for neuron *j* is $\frac{1}{2}e_j^2(n)$ and the total error energy comes from all neurons in the output layer of the network as $\xi(n)$ (Haykin, 1999; Padhy and Simon, 2015) defined by:.

$$\xi(n) = \frac{1}{2}\sum_{j \in C} e_j^2(n) \quad (2.2)$$

In this equation, C refers to the set of all neurons in the last layer of the network. In this study, 13 patterns have been used to train this network model, so that the average squared error energy will be calculated by: 1) adding all the error energy obtained in the equation (2.2) and then 2) dividing that sum by the number of patterns used in the network (Haykin, 1999), which is defined as:

$$\xi_{av} = \frac{1}{N}\sum_{n=1}^{N} \xi(n) \quad (2.3)$$

where N implies the number of patterns, which is 13 in this equation and ξ_{av} is the cost function used to measure the learning performance. Next, the error obtained from each neuron's outcome was reduced, which was possible by updating weight vectors through

the backward pass in each iteration. Weight vectors in this INN model were updated by the stochastic gradient descent (SGD) technique (Haykin, 1999). The small correction in synaptic weight vector w_{ji} by Δw_{ji} is defined as:

$$\xi(n) = \frac{1}{2}\sum_{j \in C} e_j^2(n) \tag{2.4}$$

where n is the learning-rate assigned in this network as 0.001 and is unchanged throughout the training process. The back-propagation algorithm uses partial derivatives to correct weight vectors at all layers in the network, which happens through the chain rule of calculus, shown as:

$$\frac{\partial \xi(n)}{\partial w_{ji}(n)} = \frac{\partial \xi(n)}{\partial e_j(n)} \frac{\partial e_j(n)}{\partial y_j(n)} \frac{\partial y_j(n)}{\partial v_j(n)} \frac{\partial v_j(n)}{\partial w_{ji}(n)} \tag{2.5}$$

2.2 Proposed Method:

Step 1: Bacterial features were extracted from Table-2 and used as the input vector x after encoding the features into 0 and 1 as in the table-3.

Step 2: Random values (-1 to +1) were initialized in two weight vectors: W1 and W2 respectively, and learning rate was initialized in η with number of epochs ρ. The desired output was initialized in

Proposed Algorithm

Step 1: Bacterial features were extracted from Table-2 and used as the input vector X after encoding the features into 0 and 1 as in the table-3.

OE:=OY - Y
Dlo3:=(2-OY)*OY*OE
Tx3:= Transpose of (OH)
Dld3:=Dot product of (Dlo3,Tx3)
W2:=W2 - η*Dld3
Twx2:=Transpose of (W2)
Dld2:= Dot product of (Twx1,Dlo3)
Dlo1:=(2 - OH)*OH*Dld2
Tx:=Transpose of(X)
Dld1:= Dot product of (Dlo1,Tx)
W1:=W1 - η*Dld1
i:=i+1

Step 4: At the end of the training process, the weight vectors W1 and W2 were saved in two different files DB1 and DB2 respectively.

Step 5: To predict bacteria in the Table-2, a small set of features was applied from input vector X into trained INN model-

Lx:=Sub set of (X)
TW1:=Load data from (DB1)
TW2:=Load data from (DB2)
XO:= sigmoid(Dot product of (TW1,Lx)+1)
PO:= sigmoid(Dot product of (TW2,XO)+1)
PO, the predicted output, that showed individual probability as a result of all labeled bacteria.

Biochemical characteristics of organisms taken into present consideration: In the laboratory test the strains of Escherichia coli were shown as positive or negative for curd, gas, and reduction in litmus milk reaction and only positive for methyl red and negative for H_2S production, urease activity and citrate reaction.

Strains of *Enterobacter aerogenes* were positive for acid reaction and citrate utilization and negative for H_2S production, methyl red reaction and urease activity.

Strains of *Klebsiella pneumonia* were positive for acid and gas production in litmus milk reaction, methyl red test, urease activity and citrate utilization and negative for H_2S production. Strains of *Shigella dysenteriae* were negative for urease activity, citrate utilization and H_2S production and positive for methyl red test and alkaline reaction. Strains of *Salmonella typhimurium* are positive for alkaline reaction, methyl red, H_2S production and citrate utilization and negative for urease activity. Strains of Proteus vulgaris are positive for all features taken into consideration in this study such as litmus milk reaction, methyl red, H_2S production, and urease activity but are positive or negative for citrate utilization. Strains of *Pseudomonas aeruginosa* are positive for rapid peptonization reaction and citrate utilization and negative for methyl red, H_2S production, and urease activity.

Strains of *Alcaligenes faecalis* are positive for alkaline reaction and negative for methyl red, H_2S production, and urease activity but are positive or negative for citrate utilization. Strains of Staphylococcus aureus are negative for citrate test, H_2S production, and urease activity and positive for acid reaction and methyl red. Strains of *Lactococcus lactis* are positive for litmus milk reaction and methyl red and negative for citrate test, H_2S production, and urease activity. Strains of *Micrococcus luteus* are positive for litmus milk reaction and urease activity and negative for all remaining features such as H_2S production, methyl red, and citrate test. Strains of Corynebacterium xerosis are positive for litmus milk reaction and negative for H_2S production, methyl red, urease activity and citrate test. Strains of Bacillus cereus are negative for H_2S production, methyl red test, urease activity and citrate utilization and positive for peptonization reaction (Cappuccino and Sherman, 2009).

Data Representation: In this study, all biochemical characteristics of thirteen bacterial species have been encoded by binary value 0 and 1. Each biochemical characteristic of a particular species shows the reaction on use of some specified reagents (Cappuccino and Sherman 2014). The biochemical reaction has been shown in the form of symbols -, +, and ± and also some meaningful words which are

used to indicate the proper reactions. These symbolic representations of reactions were encoded in 0 and 1 where, 1 indicate positive and 0 indicate negative effect of biochemical activity.

RESULTS AND DISCUSSION

The INN algorithm has been proposed for the purpose of training and predicting the established disease-causing bacterial species separately in the classification model. The model is feasible on the Table-1 of thirteen bacterial species which are responsible for causing human disease. This classification model has been used to distinguish all these bacterial species efficiently. A database of thirteen bacterial species has been taken from Bergey's manual of Systematic Bacteriology where the biochemical characteristics like Litmus Milk Reaction, H2S production, MR reaction, Urease activity and Citrate utilization are taken as input for the INN. Biochemical properties are decoded into the vector. The vector is then translated into binary values and applied to the INN for training.

Table 1. An overview of thirteen disease causing organism and their cultural characteristics [Source: Cappuccino and Sherman, 2014].

Bacterial Species	Shape/Gram Stain(+ / -)	Agar Slant Cultural features	Responsible for
E.coli	Rod /-	White, moist, h glistening growt	Infection in the urinary tract, traveler's diarrhea, renal failure, anemia, dehydration; organ failure, and mental disequilibrium
E. aerogenes	Rod /-	Abundant, thick, white, glistening growth	Nosocomial infections and meningitis
K.pneumoniae	Rod/ -	Slimy, white, somewhat translucent, raised growth	Pneumonia, liver abscess, and meningitis
S.dysenteriae	Rod/ -	Thin, even, grayish growth	Shigellosis, diarrhea, fever, abdominal pain, and stomach cramps
S.typhimurium	Rod /-	Thin, even, grayish growth	Fever, acute intestinal inflammation, and diarrhea
P.vulgaris	Rod /-	Thin, blue-gray, spreading growth	Hospital-acquired infections
P.aeruginosa	Rod /-	Abundant, thin, white growth, with medium turning green	Hospital acquired severe infections
A. faecalis	Rod*/ -	Thin, white, spreading, viscous growth	Corneal ulcer in human eye
S.aureus	Cocci/ +	Abundant, opaque, golden growth	Vascular disease, cancer, lung disease, and diabetes
L.lactis	Cocci/+	Thin, even growth	Infective endocarditis in adults and in children
M.luteus	Cocci /+	Soft, smooth, yellow growth	Impaired resistance in patients and colonizing the surface of heart valves
C.xerosis	Rod/ +	Grayish, granular, limited growth	Septicemia, peritonitis, endocarditis, pleura pneumonia, osteomyelitis, septic arthritis, meningitis
B.cereus	Rod /+	Abundant, opaque, white waxy growth	Diarrhea, nausea, and vomiting

The proposed model has been trained in four different ways by varying epochs and learning rate. The error convergence plot of bacterial taxonomy has been accomplished by using INN model (Fig 1-4). First training has been plotted and shown in Figure 1, where as the Sum Squared Error (SSE) is 0.180615 on applied learning rate 0.01 and 10000 epochs but in comparison with same learning rate and the epochs has been increased up to 100000 to train this model in 2nd time where SSE is 0.011125 a better training, that as shown in figure 2. In the 3rd training process which is mentioned in the Table-4 where learning rate 0.015 has been initiated with 100000 epochs to train the INN model and the sum squared error generated is 0.010414 which is shown in Figure 3.

It has been indicated that the increase of epochs can reduce the SSE, so that the network has been trained 4th time which as in the Table-4 by initiating 1000000 epochs with learning rate 0.015 and has generated the SSE 0.001165 which is depicted in Figure 4. Moreover, the lower error-trained (SSE 0.001165) INN model has performed best regarding the recognition of bacterial species with an accuracy of 99.95%. We used an Intel core i5 processor with 2.6GHz frequency to train our proposed model. In every training period, we have noticed the time consumption that has increased due to the increment of epochs. 10000 epochs, with a learning rate of 0.0 1 took 2 seconds; 100000 epochs with a learning rate of 0.015 took 9 seconds and thus the others combinations of epochs and learning rate has been taken less time. The training period comprising 1000000 epochs with a learning rate of 0.015 took 105 seconds. The difference between seconds and minutes in the practical setting is an acceptable sacrifice if it will significantly improve accuracy. Our focus was first and foremost on the outcome of accuracy, as contrast to the time taken to reach it. The prediction accuracy was calculated by the relation of confusion matrix as

Table 2: Disease causing organisms and their biochemical reactions [Source: Cappuccino and Sherman, 2014.]

Bacterial Species	Litmus Milk Reaction	H_2S Production	MR reaction	Urease activity	Citrate
E.coli	Acid, curd±, reduction±	-	+	-	-
E. aerogenes	Acid	-	-	-	+
K.pneumoniae	Acid, gas, curd±	-	±	+	+
S.dysenteriae	Alkaline	-	+	-	-
S.typhimurium	Alkaline	+	+	-	+
P.vulgaris	Alkaline	+	+	+	±
P.aeruginosa	Rapid peptonization	-	-	-	+
A. faecalis	Alkaline	-	-	-	±
S.aureus	Acid, reduction ±	-	+	-	-
L.lactis	Acid, rapid reduction with curd	-	+	-	-
M.luteus	Alkaline	-	-	+	-
C.xerosis	Alkaline	-	-	-	-
B.cereus	Peptonization	-	-	-	-

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN}$$

Where TN and TP refer to true negative and true positive respectively and their sum was 12.9928 for applying learning rate 0.015 with 1000000 epochs; on the other side FP and FN refer to false positive and false negative respectively and their sum was 0.2606 for same learning rate and epochs. The overall prediction accuracy was 98.03%. It has been calculated on the confusion matrix and it is considered as best predictions result. The error convergence plots of four training processes has been figured out from 1- 4 respectively, according to applied learning rate and epochs into the INN-

To compare the accuracy of the actual outcome with the predicted outcome, two plots have been depicted. A 3-D plot of Figure 5 shows that the accuracy in overall predictions is 97.05% when the model is trained with 10000 epochs and 0.01 learning rate. Raising the number of epochs and using optimum learning rate marginally increased accuracy each training period. Eventually, the best result was achieved by using 1000000 epochs with 0.015 learning rate, where as accuracy was 98.03%, as shown in figure 6.

In the peruse of two figures 5 and 6 respectively, a clear perception is achieved that the plane of Figure 5 exhibits some small bars respect to the tall bars when 1st train cycle is used for separating of thirteen bacterial species. The average prediction rate was 97.05% for all thirteen bacterial species where SSE was 0.180615. In that case, the small altitude bars have been obtained which has indicated that no bacterial species will be predicted properly but in case of Figure 6 the average prediction rate was 98.03% for all bacterial species where SSE was 0.001165 and there was no small altitude bars in the plane which indicated that all the bacterial species have been predicted properly with high accuracy.

Table 3. Input vector of binary value [1= positive, 0=negative] for training to the proposed INN model of thirteen pathogenic bacteria.

Bacterial Species	Litmus Milk Reaction									H_2S Production		MR reaction			Urease activity		Citrate		
	Acid	curd+	reduction+	gas	gas+	Alkaline	Rapid peptonization	Rapid reduction with curd	Peptonization	-	+	-	+	+	-	+	-	+	+
E.coli	1	1	1	0	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0
E. aerogenes	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0
K.pneumoniae	1	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
S.dysenteriae	0	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0
S.typhimurium	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0
P.vulgaris	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	1
P.aeruginosa	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0
A. faecalis	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	1
S.aureus	1	0	1	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0
L.lactis	1	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0
M.luteus	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	1	0	0
C.xerosis	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	1	0	0
B.cereus	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0

Table 4. Comparison of four training processes through INN model

Training No.	Model	Learning rate	Epochs	SSE
1	INN	0.01	10000	0.180615
2	INN	0.01	100000	0.011125
3	INN	0.015	10000	0.010414
4	INN	0.015	100000	0.001165

Figure 1: Error Convergence plot with SSE 0.180615

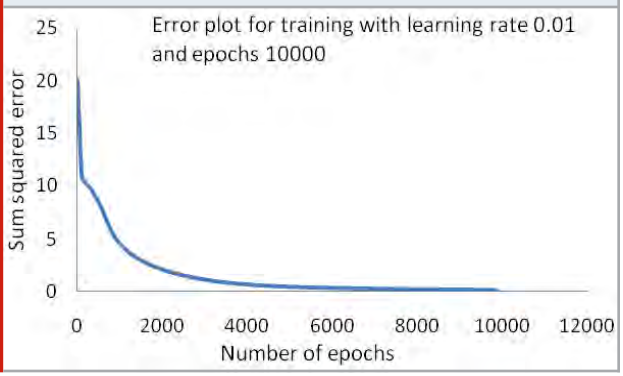


Figure 2: Error Convergence plot with SSE 0.011125

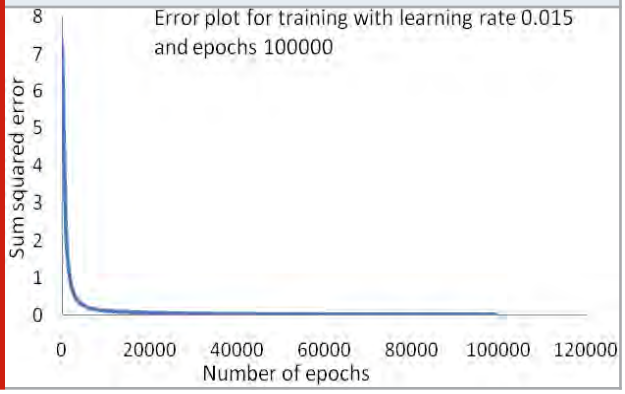


Figure 3: Error Convergence plot with SSE .010414

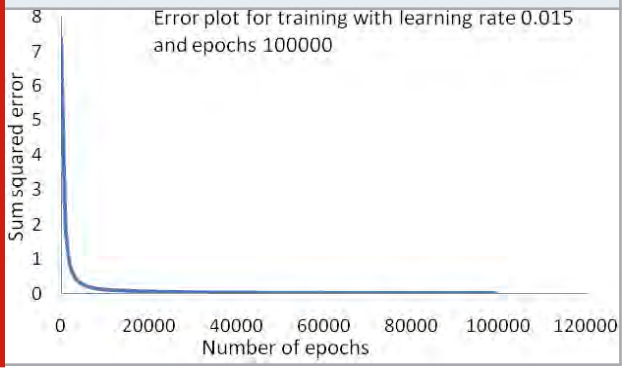


Figure 4: Error Convergence plot with SSE0.001165

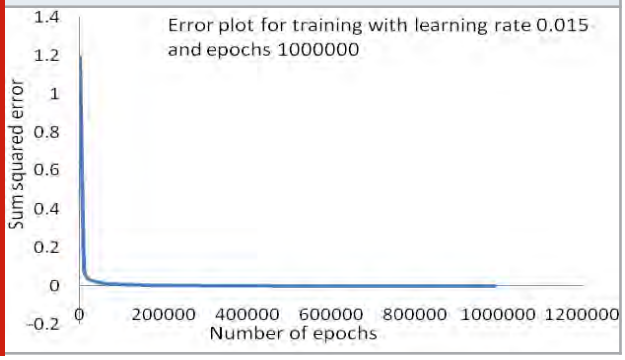


Figure 5: Accuracy plot of predicted bacterial species, where 10000 epochs with 0.01 learning rate have been used to train the INN.

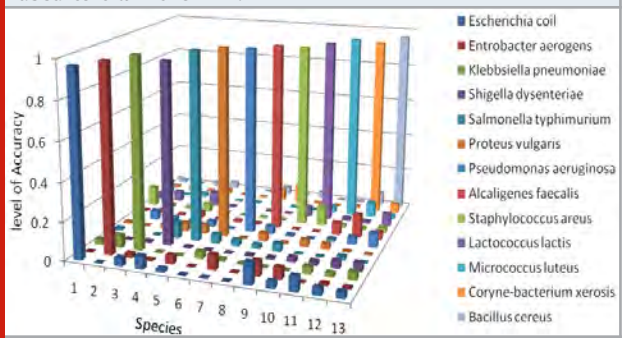
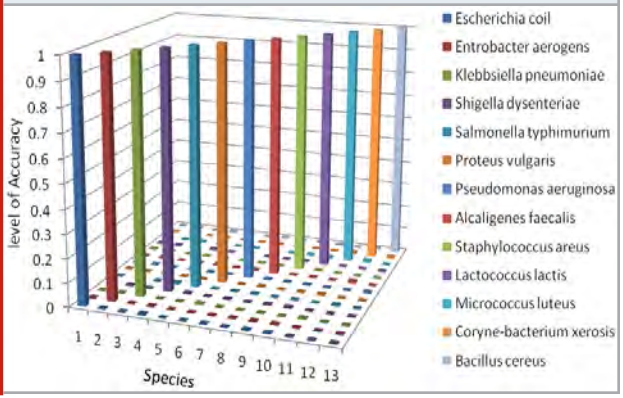


Figure 6: Accuracy plot of predicted bacterial species, where 1000000 epochs with 0.015 learning rate have been used to train the INN.



CONCLUSION

A two-layered INN model has been proposed for distinguishing disease-causing bacteria species based on biochemical properties such as Litmus Milk Reaction, H2S production, MR Reaction, Urease Activity, and Citrate utilization test. This model would help the researchers to identify the bacterial pathogens having fundamental biochemical properties. The proposed soft computing-based INN model is best suited for the classification of these medically important bacterial species.

Conflicts of Interests: The authors have no conflict of interest to declare.

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Brain Tumour Segmentation Using Residual Neural Network

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ABSTRACT

The uncontrolled growth of cells in the brain causes brain tumour, there are two different types of tumours : malignant and benign. Malignant brings the cancerous type and spreads to other regions. At present, processing and developing of medical images is an important field. It categorises many types of imaging methods. Some of them are Magnetic Resonance Imaging (MRI), X- rays and computed Tomography scans (CT scans) and so forth. The important goal of medical image processing is to identify accurate and meaningful information using images with the minimum error possible.MRI has high resolution and better quality images compared with other imaging techniques. So that, this technique is used to detect cancer tissues and to scan any part of the body. In this paper, neural network method is used for segmenting the brain tumours. The images were pre-processed for removal of noise and contrast-enhanced for better image. Accuracy, PSNR, Sensitivity were parameters calculated for the analysis of image quality.

KEY WORDS: BRAIN TUMOUR, MAGNETIC RESONANCE IMAGING (MRI), X- RAYS, COMPUTED TOMOGRAPHY SCANS (CT SCANS), MALIGNANT AND BENIGN TUMOUR, IMAGE PROCESSING.

INTRODUCTION

The uncontrolled growth of cancerous cells in the brain causes brain tumours. Tumours are classified as two types, they are malignant and benign. Malignant brings the cancerous type and spreads to other regions of the brain. The gliomas and meningioma are the examples for benign tumour and astrocytma and glioblastomas are high level tumours classified as malignant type. According to the World Health Organization (Loius et al., 2018 WHO,2018), brain tumours are graded from grade I to grade IV to classify them as benign or malignant type. Usually, benign tumours fall under grade I and grade II and Grade III to grade IV glioma are classified as malignant type. All types of brain tumour produce symptoms like seizures, headache, problem with vision and hearing. Early diagnosis and treatment of tumour is very important, Due to the complex structure of the brain, detection and extraction of tumour is very important. There is a revolutionary development in the field of Medical Image analysis and computer vision within the past two decades. CT scans and MRI are used for the diagnosis of brain tumours,(Szil´agyi,.et al., 2018, Akter et al., 2018, Al-Ashwal et al., 2018).

This advance technology in the field of medical image processing can be used for segmentation of brain tumours. Brain tumour segmentation can be done by computer-aided systems for further medical analysis. Based on region, contrast, texture and colour the image is divided

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and it is called as segmentation. In this residual neural network method is used for segmentation of brain tumours, (Srilatha et al, 2019).

Brain MRI images with tumours like glioblastoma, cerebral metastasis, medulloblastoma were considered for segmentation. Mostly images are treated as two-dimensional signals and for manipulation processing techniques are applied. MATLAB, WINGRASS, and ArcGIS, MathCAD are the image-based analysis research software's used to process images and to make an analysis on it, Kilic, et al, (2018).

A multi-paradigm numerical computing environment and proprietary programming language are the product of works in MATLAB. When images are as twodimensional data, this matrix laboratory is a useful tool for making analysis over images. Medical image processing is able to view the internal structure of the hidden organs in the body which are covered by skins and bones to predict the exact cause of the disease for further treatment procedure. The biological image is obtained from radiology (Ferlay et al, 2008), which uses technologies like X-ray, radiography, magnetic resonance imaging medical ultrasonography and endoscopy.

Srilatha. et al, (2019) and Al-Ashwal et al (2018) have proposed a system for early detection of brain tumour with image segmentation technique. The system uses a K-means clustering technique integrated with Fuzzy C means algorithm for brain MRI segmentation. They use two further segmentation such as threshold and level set segmentation to make an accurate brain tumour diagnosis. The system provides improved accuracy in various datasets.

Louis et al (20018) have proposed research on two-stage authenticated technique for detection of brain tumour. Right now, proposed system is a validated plan for the detection of a brain tumour, called a watershed matched methodology.The segmentation of brain tumour area was done through classification approach of the watershed algorithm. In addition, scale invariant feature transform technique was also used to extract feature regions and matching divided area of brain tumour with an actual picture.

Kilic, et al, (2018) have carried research on the detection and visualization of the tissues of the brain. Detect the dimension of the tumour of the brain image, different stages of the brain can be determined. The step may be cancerous or noncancerous. To perceive the size of the brain, a tumour of the brain can be recognized utilizing a k means clustering algorithm dependent on the detachment and morphological procedure. The morphological method used for the extraction of the features of the parts of the body through MRI scanning. Experimental analysis on calculating the extracted features of the portion of the brain disease.

Akter et al, (2018) have proposed a novel distributed picture segmentation technique for magnetic resonance brain pictures. In this technique, the different conversion is called a contour transformation that is the through canny edge detection. In this research work, the distribution techniqueacquired for the improved method on contour constants through the canny edge detection approach. Experimental analysis was done on improvement of contour let conversion through the segmentation process using canny edge detection technique.

Melissa and Srilatha, (2016) presented a novel fuzzy c mean clustering approach that is also known as possible fuzzy c mean clustering technique. They separate of the database through middle free fuzzy c mean clustering into fuzzy c means clustering technique. The proposed method is less prone to interference. In this research, it was analysed that the proposed method helps in the segmentation of the magnetic resonance imaging brain pictures along with intervention.

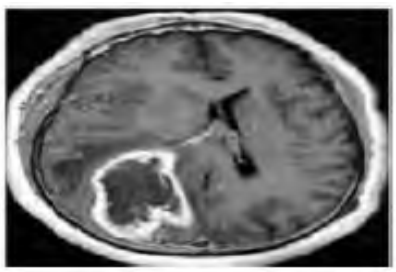
Szil´agyi et al., (2011, 2018) proposed segmentation of brain tumour from MRI using fast marching method. This method derives the gradients based on weights of each pixel in an image. The segmented image is done by watershed algorithm. This method acquires accuracy of 98.83%. Hamamci et al, (2012) and Akers et al, (1978) had proposed a method of segmentation of brain tumour using watershed technique and self-organizing maps. In this paper the brain images were skull stripped to eliminate the non-cerebral region, stationary wavelet transform (SWT) was used for feature extraction .then the tumour region was segmented using the watershed algorithm. This method combined with self-organizing maps achieved a segmentation accuracy of 95.93%.

MATERIAL AND METHODS

The Block diagram shown in figure 1 represents the methodology for brain tumour segmentation. In the proposed work, The images were initially considered and tumour part is detected. Before segmenting the image, image is pre-processed by using gray scale, noise removal and CLAHE enhancement techniques. It is followed by the segmentation residual neural network technique and the performance analysis like accuracy, sensitivity and PSNR were calculated.

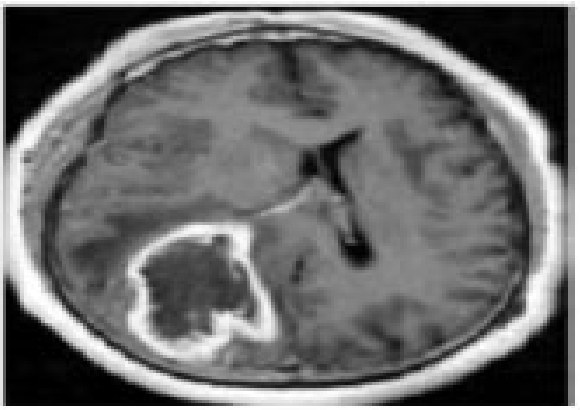
Data Search: Select an input image from data base or folder using Matlab. The selected input may be a colour image or binary image FIGURE.1.

Figure 1: Brain Tumour Input Image



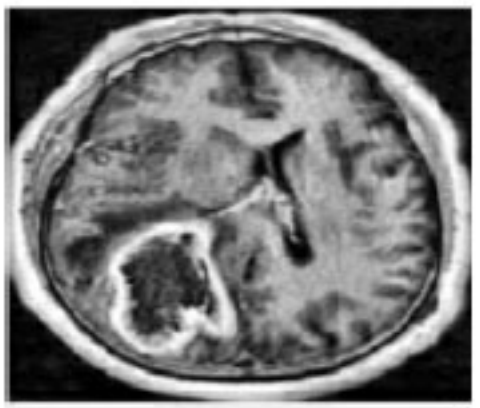
Pre-Processing: The MRI images are altered by the bias field distortion. This makes the intensity of the same tissues vary across the image. Pre processing mainly focus on removing the noise present in the selected image. Conversion of the selected image is to gray scale representation is an important process in the technique. In gray scale the 3D image is converted to 2D image for fast process. The pictures were resized to 512×512 pixels.

Figure 2: Brain Tumour Pre-processing Image



Removal of Noise In pre-processing noise is to be created in considered image. To remove that noise we are using Median filter. For digital image processing this Median filter is widely used because, It preserves the edges while removing noise, under certain conditions. The idea of using median filter is to run through the single entry by entry, replacing each entry with the median of neighbouring entries. patterns of the neighbours is known as "Window", which slides entry by entry over the entire signal for 1D signals, the most obvious window is just the few preceding and following entries, where for 2D data the window must include all entries within a given radius shown in FIGURE.2.

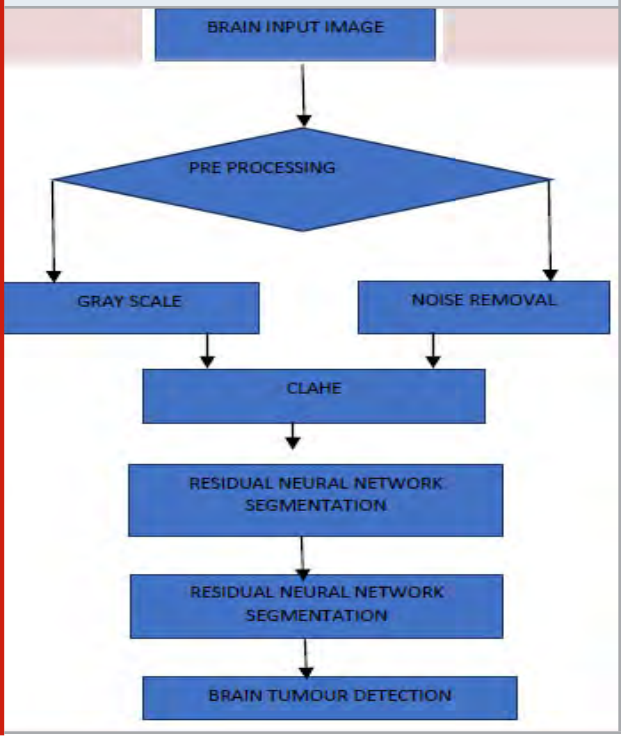
Figure 3: Brain Tumour CLAHE Image



CLAHE (Contrast Limited Adaptive Histogram Equalization): In Contrast Limited Adaptive Histogram Equalization is utilized to enhance the contrast of the image. CLAHE works on little districts in the picture, called tiles, instead of the whole picture. Each tile's difference is upgraded, with the goal that the histogram of the yield area around matches the histogram indicated by the 'Appropriation' parameter. The neighbouring tiles are then joined utilizing bilinear shown in FIGURE.3.

Residual Neural Network Segmentation: A Residual Neural Network is nothing but Artificial neural network of a kind that builds on constructs know from pyramidal cells in the cerebral cortex. This Neural Network work by utilizing skip connections, or shortcuts to jump over some layers. Extra weight matrix might be utilized to become familiar with the skip weights, these models are well known as Highway Nets.

Figure 4: Flow Chart of Proposed System



In the context of residual neural networks, a non-residual neural network may be described as aplain network. In Residual Neural network the are three main layers they are Input Layer: The functionality of these layers are , where the images first entered into input layer after doing all function process like conversion of image in to grey scale, filtering, CLAHE for improve resolution of the image. Hidden Layer: After the image passes into next layer called hidden layer, the segmentation process done under this layer. The hidden layer consists many processing techniques. These processes can be done layer by layer Input layer: This image is put in this layer.

Binary Convolution layer: it comprises in including a sign or a picture with piece to get highlight maps. Thus,

a section in a component map is connected to the past layer through the loads of the parts. The loads of the parts are adjust during the preparation stage by back proliferation, so as to upgrade certain attributes of the info. Since the bits are shared among all units of the same feature maps, Convolutional layers have fewer loads to prepare than thick FC layers, making simpler to prepare and less inclined to over fitting. In addition, since a similar portion is convolved over the whole picture, a similar element is recognized freely of the area interpretation in variance.

Activation Function: it is liable for Non- Linearly transforming the information.

Rectifier linear units (ReLU) defined as,

$$f(x) = \max(0, x)$$

Figure 5: Brain Tumour Identification and Detection Image (a-d)

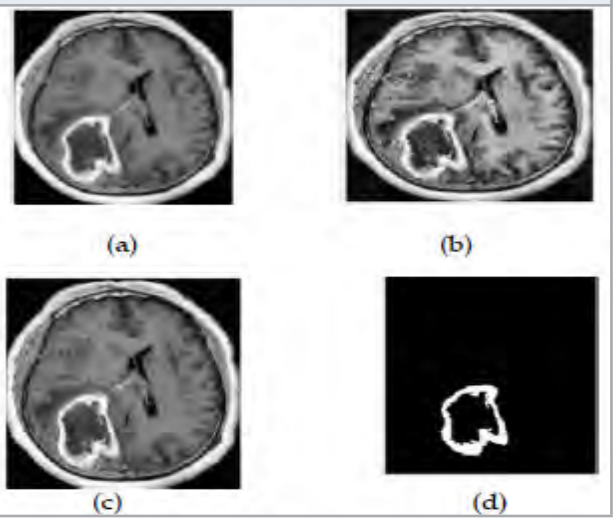
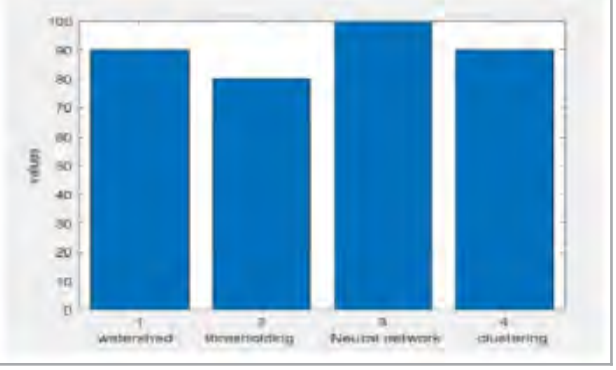


Figure 6: Brain Tumour comparison with various methods Accuracy bar chart



Were found to accomplish preferred outcomes over the more traditional sigmoid, or hyperbolic digression capacities, and accelerate preparing. Be that as it may, forcing a steady 0 can impede the angle streaming and subsequent modification of the loads. We adapt to these confinements utilizing a variation called flawed rectifier direct unit (LReLU) that presents a little incline on the negative piece of the capacity. This capacity is characterized as Where α is the brokenness parameter. In the last FC layer, we utilize delicate max. Pooling: It consolidates spatially close by features in the component maps. This combination of possibly redundant features makes the representation more compact and invariant to small image changes, such as insignificant details; it also decreases the computational load of the next stages. The common pooling techniques to use maxpooling or average-pooling. In max-pooling the maximum pixel is taken into account and remaining all is removed.

Training: To train the RNN the loss function must be minimized, but it is highly nonlinear. We utilize Stochastic Slope Drop as a streamlining calculation, which makes strides relatively to the negative of the angle toward neighbourhood minima. All things considered, in districts of low ebb and flow it tends to be moderate.

Table 1. Performance evaluation of segmentation

Analysis	Thresholding	Clustering	Watershed	RNN
Accuracy	79.99	89.91	89.99	97.91
Sensitivity	0.11	0.05	0.14	0.01
Precision	0.144	0.137	0.147	0.130
Specificity	0.117	0.054	0.140	0.017

In this way, we utilized Nesterov's Quickened Force to quicken the calculation in those areas. The force ν is kept consistent, while the learning rate ε was straight diminished, after every age. We consider an age as a total ignore all the preparation tests. Residual layer: In this based on their weights the image pixels is skipped. If layers are not necessary we can skip and reduce the time of execution. This is very important layer in this segmentation process shown in FIGURE.4.

Figure 4: Block diagram of Proposed Methodology The process technique layers in the hidden layer are repeated until the image is extracted perfectly. Output Layer: The segmented image is shown is the output layer. The tumour detected image is compared with all the existing methods.

RESULTS AND DISCUSSION

In this paper Neural Network Method is used for segmentation of brain tumour by considering the gradient weight of each pixel. The MRI images of brain are taken from radiopedia. The MRI images of the brain with Glioblastoma, cerebral metastasis and meningioma are considered for research purpose. The performance analysis is calculated based on the segmented image. The calculated values are to be compared with existing methods.

FIUGURE.5. (a)input image(b)filtered image(c) enhanced image with seed point(d)Tumour segmented and extracted image.

This process mainly focused to correct the intensity of t he image and removing the noise of the image. The above figures shows that how the segmented image is extracted from the input image. The filtered image removes the noise from the original image and enhanced image for the resolution of image .The RGB colour image for displaying the segmentation part of the tumour image. This RGB colour model used for MRI scanned images to display the result of the tumour. Bar Chart and Comparison Graph shown in Fig.6.

The above graph shows that accuracy values in the existing methods and the proposed methods and specificity has been calculated and compared with other methods. The highest and lowest accuracy values of the tumour images are displayed. The comparision of accuracy, sensitivity and Specificity values. The accuracy and specificity can be calculated by following equations.

Accuracy=(TP+TN)/(TP+TN+FP+FN)

Specificity=TN/(TN+FN)

Sensitivity=TP/(TP+FN)

The below TABLE.1. shows that comparison of existing and proposed methods for different parameters. It also displays high accuracy values and specificity values to identify tumour.

CONCLUSION

The proposed system is used to detect brain tumour using residual neural network segmentation technique. The proposed method produces exact location of the tumour in the image. To get an MRI input image a GUI is created pre-processed which includes conversion of gray scale filtering of image using median filter to remove noise enhancement can be done using CLAHE for resolution of image and segmentation using residual neural network technique. Then the results were analysed. The median filter and CLAHE also employed to get clear image of brain affected by tumour. The residual neural network segmentation used for layerization of every pixel of images. The accuracy is analysed in terms of PSNR, sensitivity and specificity. This segmentation reduces the error rate of MRI brain image. The number of layers used in this neural network is less and executes fast. The accuracy values also high compared to other techniques.

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Formulation and *In–vitro* Evaluation of Transdermal Patches of Anti–Arthritic Ayurvedic Medicinal Plants

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ABSTRACT

The allopathic system of medicine includes two conventional lines of treatment for rheumatoid arthritis, which come along with certain side effects. Hence, turning to safe, effective and time-tested Ayurvedic herbal drug formulation would be a preferable option. With this view transdermal films incorporating herbal drug components such as boswellic acid (*Boswellia serrata*) and Aloe gel (*Aloe barbadensis*) was envisioned. Transdermal patches of herbal extracts were prepared by solvent casting method. The patches were optimized on the basis of physicochemical evaluation such as thickness, folding endurance, physical appearance, uniformity of weight, moisture content, moisture uptake, pH and in vitro drug release, Dissolution studies. The graphs obtained for the average absorbance release with respect to time through transdermal film indicate drug release occurred at a constant rate. The skin irritation study done on wister rats skin showed that the formulation does not produce irritation to the skin. Overall, present study assures a novel approach in execution of transdermal delivery technology in the field of herbals.

KEY WORDS: BOSWELLIC ACID, ALOE GEL, HERBAL EXTRACT, TDDS, IN – VITRO DRUG RELEASE STUDY.

INTRODUCTION

The transdermal patch is a medicated adhesive patch that is placed on the skin to deliver a specific dose of medication through the skin and into the bloodstream (Tanwar et al., 2016). Transdermal drug delivery system (TDDS) has been an increased interest in the drug administration via the skin for both local therapeutic effects on diseased skin (topical delivery) as well as for systemic delivery of drugs. The skin as a site of drug delivery has a number of significant advantages over many other routes of drug administration, including the ability to avoid problems of gastric irritation, pH and emptying rate effects, avoid hepatic first-pass metabolism thereby increasing the bioavailability of drug, reduce the risk of systemic side effects by minimizing plasma concentrations compared to oral therapy (Dulan et al., 2016; Lakhani et al., 2015; Kharia et al., 2019).

The purpose of this research work was to Formulation and evaluation of transdermal drug delivery system of ayurvedic drug incorporating *boswellic* acid and aloe gel using various polymers such as PVP and Ethyl cellulose by solvent casting technique for improvement of bioavailability of drug and reducing toxic effects. Boswellic acid, a constituent of *Boswellia serrata* (Family-Burseraceae) showed anti-inflammatory and anti-rheumatic activities along with anti-pyretic effect with no ulcerogenic effect and well tolerated in as high a dose as 2 gm/kg (p.o) in mice (Umar et al.,2014). It improves

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blood supply to joints and restores integrity of vessels obliterated by spasm of internal damage (Chaodhary et al.,2015). *B. serrata* (*Sallai guggul*) shows its superiority over conventional drugs as it is a plant product being used since ages and is absolutely free from any toxic and side effects (Bhardwaj,2016).

Aloe barbadensis is a member of the lily family, and is a succulent plant that contains a lot of liquid in its think, spiny leaves. *Aloe vera* has found to be helpful in curing arthritis symptoms mainly inflammation and pain without side effects. Its anti-oxidant properties regulate expression of cyclooxygenase-2 (inhibition of this enzyme helps relieve pain and inflammation. The study reported significant inhibition of inflammation in arthritic models on using Aloe vera extracts (Khemkaran et al.,2011,Tiwari et al.,2018; Kar and Bera, 2018 (Sethi,2020).

MATERIAL AND METHODS

Herbal extract powders of *Boswellia serrata* and *Aloe barbadensis* are obtained as an gift sample from Sane Guruji Hospital and Ayurvedic Medical Store, Hadapsar, Pune. Ethyl cellulose, Polyvinylpyrrolidone (PVP), Propylene glycol were purchased from research lab centre, Pune. The laboratory chemicals other than mentioned above used in the study were of analytical reagents grade, and several types of equipment employed in the formulation of herbal patches were magnetic stirrer, Petri dish, ultrasonic cleaner, vernier caliper, electronic balance, pH meter, hot water bath, ultraviolet-visible spectrophotometer, tray dryer and hot air oven and dissolution apparatus etc.

Ethyl cellulose, PVP was used as the skeletal type polymer material of preparation. Propylene glycol as penetration enhancer and plasticizer. PVP (1g) and ethyl cellulose (1g) were weighed in requisite ratios and mixed in 50ml solvent containing distilled water : methanol (1:1 ratio) . Stirred the mixture over a hot water bath until dissolved. After the mixture was cooled down to 25°c, the drug AB + BS 500mg in the ratio of 50:50, 70:30, 30:70 and 100:100 was added. After that, propylene glycol (0.5ml), glycerol (0.5ml) were added. The mixture was then poured into glass Petri dish and dried at room temperature for 24 hrs. The petri dish was left undisturbed at room temperature for one day. The patch was obtained intact by slowly lifting from the petri dish and transdermal patches were cut into length of 2 x 4 cm. The patch was collected and stored in desiccator until further use (Chauhan et al., 2018). The drugs (boswellic acid and Aloe gel) were selected on the basis that they produce synergistic action in suppressing inflammation and are time tested and proven safe.

The thickness of each patch was measured by using Screw gauge or Vernier caliper at five different positions of the patch and the average was calculated (Santosh et al.,2009). Patches sizes of 2 x 4 cm was cut. The weights of five patches were taken and the weight variation was calculated (Kumar et al.,2013).

A patch of 2 x 4cm was cut evenly and repeatedly folded at the same place till it brakes. The numbers of times the film was folded at the same place without breaking give the value of the folding endurance (Prabhakar et al.,2011; Jadhav et al.,2009).The pH of the film was found by using pH paper. The prepared films were weighed individually and kept in a desiccators containing fuse calcium chloride at room temperature for 24h. After 24h, the films were reweighed and determined the percentage moisture content from the mentioned formula (Murthy et al.,2001; Saxena et al.,2006).% Moisture content = (Initial weight – Final weight) / Initial weight x 100

The weighed films were kept in desiccators at room temperature for 24h containing saturated solution of potassium chloride in order to maintain 84% RH. After 24h, the films were reweighed and determined the percentage moisture uptake from the below mentioned formula (Mali et al.,2015; Darwhekar et al.,2011). % Moisture uptake = (Final weight – Initial weight) / Initial weight x 100

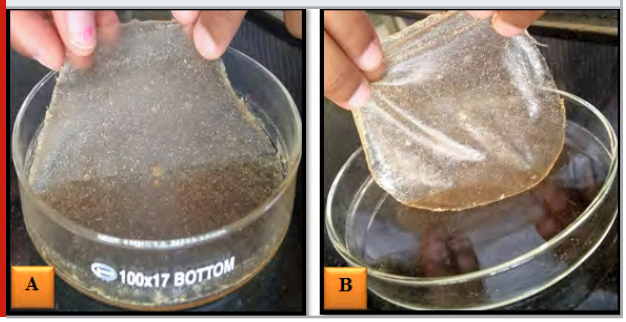
A specified area of patch was dissolved in a phosphate buffer solution. The content was stirred to dissolve the film. The content was transferred to a volumetric flask. The absorbance of the solution was measured at wavelength 253 nm and determines the drug content (Prajapati et al.,2011).For estimating the sensitivity of the formulation toward the skin, the formulated patches were applied to the hairless skin of the wistar rats for the duration of 24 hours and the skin inflammation effects were reported for all individual rats (totally 4 rats used) (Mohamad and Tabassum, 2012).

Figure 1: Formulation of Transdermal Patches Using Solvent Casting Method

By using USP type 1 dissolution test apparatus, Drug release from the prepared TD patch was studied using 8 station dissolution rate test apparatus (Labindia, DS 8000) employing a paddle stirrer at 50 rpm and at 37±1°C. Phosphate buffer of pH 7.4 (500 ml) was used as dissolution fluid. Samples of 5 ml of each were withdrawn at different time intervals over a period of 6 h. Each sample withdrawn was replaced with an equal amount of fresh dissolution medium. Samples were suitably diluted and assayed at 253 nm for herbal

transdermal patch using UV-visible spectrophotometer. The drug release was plotted on the graph as Time (min) on X- axis and Absorbance on Y – axis (Bhavani et al.,2016).

Figure 2: BS + AB Herbal Transdermal Patches



RESULTS AND DISCUSSION

The transdermal patches of *Boswellia serrata* and *Aloe barbadensis* were formulated successfully by solvent casting technique with an aim to improve the bioavailability of herbal drug in combination. The prepared film was found to be uniform, flexible, smooth and transparent as shown in Table 1 and figure 1and 2. The formulations were subjected to physical examination; films appeared to be slightly translucent suggesting that the drug was not completely solubilized rather dispersed/ suspended in the matrix (Shinde et al.,2008).

The results of thickness studies for different formulations are shown in Table 2. The above results obtained show that thickness of all the prepared formulations was found to be uniform (0.433 – 0.438 mm). It was found that the weights were uniform with respect to all prepared formulations and showed low standard deviation values. F4 formulation weight was found to be slightly increased to 0.2156 gms due to higher drug concentration present.

The results of folding endurance studies for different formulations are shown in Table 2. The above data show that the prepared formulations were found to be uniform with respect to folding endurance (12-17). The folding endurance number gives the mechanical property of the patches; high folding endurance number indicate that the patches have high mechanical property (Shirisha et al.,2017).

The results are shown in (Table 2). All the patches have around neutral pH 6. So it will not cause any kind of irritation to the skin.The moisture content in prepared formulation F1, F2, F3 and F4 was found to be increased in the order as follows: F1 > F2 > F3 > F4 (Table 3). The small moisture content in the formulations help them to remain stable and not being a completely dried and brittle film. Again, low moisture absorption protects the patches from microbial contamination (Jatav et al.,2013).

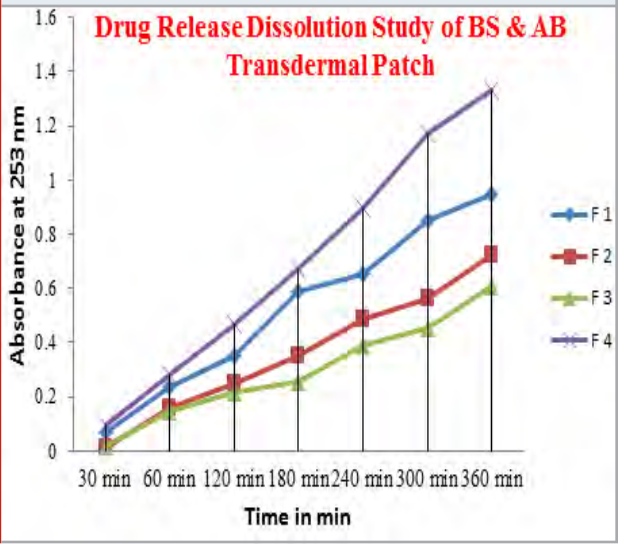
Moisture content and moisture uptake studies provide information regarding stability of the formulation (Layek et al.,2010). The percentage moisture uptake was found to be increased with increasing drug concentration Ratio in all the four formulations.i.e. F1 < F2 < F3 < F4 as shown in (Table 3).The percent drug content in BS + AB patches in various formulation ratio code was found in between 86.45% to 96.86 % in Table 3.

Table 1. Formulation Design

Ingredients	F 1	F 2	F 3	F 4
Ethyl cellulose	1000 mg	1000 mg	1000 mg	1000 mg
Polyvinylpy rrolidone (PVP)	1000 mg	1000 mg	1000 mg	1000 mg
Propylene glycol	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Glycerol	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Methanol : Water (1:1)	50 ml	50 ml	50 ml	50 ml
Drug BS + AB (mg)	50:50	70:30	30:70	100:100

The skin irritation study done on wister rats skin showed that the formulation does not produce irritation to the skin (Table 3). After 24 hours, no inflammation of the skin was observed in all the formulations. Hence all the prepared formulations passed the skin irritation test and supposed to be safe for human use also. The patches were found to be free of any skin irritation. Based on the above observations, it can be reasonably concluded that plasticizers have a significant influence on the mechanical properties of the transdermal patches (Bharkatiya et al.,2010).

Figure 3: Drug Release Profiles of BS + AB Transdermal Patches



In vitro studies of Patches F1, F2, F3 and F4 were carried out by using USP type 1 dissolution test apparatus (Table 4). As compared with other formulations, F4 shows the highest drug release profile, whereas lowest drug release

was expressed by the patch F3 at the end of 6 hours (Figure 3). From the results, it is clear that the best release profile was obtained with formulation F1 and F4 (containing 50:50 and 100:100 drug concentration). This may be due to the equal dug combination present in the formulations. The drug release was obtained in the following pattern: F4 > F1 > F2 > F3. The release of drugs from transdermal patch was found to be linear for the first 4 hours and attained a steady level upto 6 hours (Figure 3).

Table 2. Evaluation of Herbal Transdermal Patch of BS + AB

Formulation Code	Thickness (mm)	Weight Uniformity (gm)	Folding Endurance (n)	Surface pH
F 1	0.434 ± 0.02	0.1850 ± 0.002	15 ± 2.60	6
F 2	0.433 ± 0.01	0.1866 ± 0.002	12 ± 3.21	6
F 3	0.435 ± 0.01	0.1876 ± 0.003	13 ± 2.50	6
F 4	0.438 ± 0.03	0.2156 ±0.004	17 ± 1.52	6

Values are expressed as the Means ± SD, where (N = 3).

Table 3. Evaluation of Herbal Transdermal Patch of BS + AB

Formulation Code	% Moisture Content	% Moisture Uptake	Drug (%) Content	Skin Irritancy
F 1	3.96 ± 0.86	4.32 ± 0.56	86.45 ± 0.84	No irritation
F 2	2.67 ± 0.50	4.82 ± 0.60	89.54 ± 0.73	No irritation
F 3	2.48 ± 0.31	5.68 ± 0.77	92.41 ± 1.12	No irritation
F 4	2.31 ± 0.04	6.17± 0.17	96.86 ± 1.02	No irritation

Values are expressed as the Means ± SD, where (N = 3)

Table 4. *In-Vitro* Drug Release Study of Prepared Transdermal Patch of BS + AB+ AB

Formulation Code	F 1	F 2	F 3	F 4
30 min	0.0681	0.0137	0.0154	0.0983
60 min	0.2386	0.1563	0.1436	0.2830
120 min	0.3546	0.2483	0.2169	0.4681
180 min	0.5878	0.3504	0.2586	0.6734
240 min	0.6533	0.4876	0.3894	0.8965
300 min	0.8493	0.5618	0.4531	1.1693
360 min	0.9450	0.7228	0.6108	1.3347

Values are expressed as the Means

Release studies also indicate that by using two different polymeric combinations such as ethyl cellulose: PVP (1:1) and propylene glycol as a plastisizer were considered as the best formulations for the sustain drug release through the Transdermal Drug Delivery System (TDDS). The incorporation of PVP was helpful for rapid dissolution of the surface hydrophilic drug which results in the formation of pores and thus leads to the decrease of mean diffusional path length of the drug molecules to permeate into dissolution medium and higher permeation rates (Prabhakara et al.,2010). The stability studies indicated that all the patches maintained good physicochemical properties and drug content after storing the patches in different storage conditions. Compatibility studies indicated that there was no interaction between the drug and polymers (Bhatnagar et al.,2010). While choosing polymer ratio, it was found that ethyl cellulose - PVP polymers are better suited than eudragit - PVP polymers for the development of transdermal patches (Kathe et al.,2017).

CONCLUSIONS

From the present study, it can be concluded that transdermal drug delivery system for *Boswellia serrata* + *Aloe barbadensis* with ethyl cellulose and PVP meet the ideal requirement for transdermal devices which can be a good way to bypass the extensive hepatic first – pass metabolism and increase bioavailability. BS + AB transdermal patches were provide sustained transdermal delivery for prolonged periods, hence suitable in the therapy of Rheumatoid Arthritis (RA). Through the present experimentation, it has been found that the drugs

of ayurvedic origin can be utilized in a better form with enhanced efficacy for incorporation in modern dosage form. This work is one of the first few attempts to utilize Ayurvedic drugs through TDDS. The present work can further be proceeding with in – vivo study on healthy Wister rats to evaluate anti – arthritic activity.

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Influence of Arbuscular Mycorrhizal fungal isolates on Biochemical Parameters in *Sorghum bicolor*

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ABSTRACT

This present study mainly focused to check the enhance level of various biochemical parameters after giving the treatment of Arbuscular Mycorrhizal (AM) inoculums. Rhizosphere soil of *Sorghum bicolour* collected from various location contain hyphae with root fragments, spore, vesicles which were used as a propagule for mass multiplication. In the continuation of the research soil were processed for getting AM species and root segment separately. Isolated and screened AM species were mass multiply by trap culture .The increase number of AM propagules use as an inoculums for pot culture of *Sorghum*. The AM Spore interacts with roots of Sorghum and make hyphal connection. AM colonization enhances the nutrient uptake from soil and provide to plant which directly. AM fungal association with roots of *Sorghum bicolor* is important for nutrient uptake, growth and biomass production. To study the impact of AM inoculants in Sorghum plant protein, sugar, carotenoid, chlorophyll, phenol, nitrogen and phosphate estimation was done . As a result of successful colonization *Sorghum* plant shows higher concentration in all biochemical parameter.colonization also help in accumulation of heavy metal and make plant disease resistant. After successful pot culturing plants were analysed for physiochemical characteristic. Without any hazardous effect AM endophyte can change the metabolism of host plant hence it could be considered as a good bio fertilizer tool

KEY WORDS: TRAP CULTURE, PHYSIOLOGICAL PARAMETER, AM FUNGAL ISOLATES.

INTRODUCTION

Mycorrhizae has a well-established mutual relationship between plant root and fungi found in association with roots of land plants. Mycorrhizae are naturally occurring soil borne fungi which can potentially increase nutrient availability, biomass and make plant stress resistant (Sun et al., 2018).Besides growth promotion the major role of Arbuscular Mycorrhizal (AM) fungi is provide protection against various pathogen (Jung et al., 2012). AM fungi are obligate symbiont that means they required host root for their growth and complete their lifecycle. Once they get successful association, the mycorrhizal fungi starts to form arbuscules, vesicles hyphae and spores. Among all spores and hyphae also found in rhizosphere soil. Arbuscules are branching pattern which provide the place to facilitate the nutrient exchange and also accumulate reserve nutrient. Vesicles are bulbous structure which contains reserve food as glycogen and fat granules. Mycorrhizal hyphae extends deeply in soil

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in search of water hence it maintain the water level of plant, (Symanchik 2018 Wipf et al., 2019 Montero et al 2019).

Many research have been done to explore the contribution of AM fungi in plant growth and development, plant safety, soil health. In this continuation this research study has been done to evaluate the significant changes in *Sorghum* when inoculated with AM species. *Sorghum* is scientifically known as *Sorghum bicolor* L. Moench,. It is also known as sweet sorghum which belongs to family Poaceae. *Sorghum* is cultivated all over the world for food, feed, forage, fuel, and ranks fifth among the major cereal crops in terms of production (FAOSTAT, 2015).*Sorghum* belongs to C4 grass family which has well adaption to grow in arid or semi-arid region and also has a fibrous root system thus it easily interact with AM fungi. Root associations with arbuscular mycorrhizal fungi enhance plant growth by increasing P uptake, N uptake (Gerdemann, 1964; Janos, 1987; Stribely, 1987,Montero et al 2019).

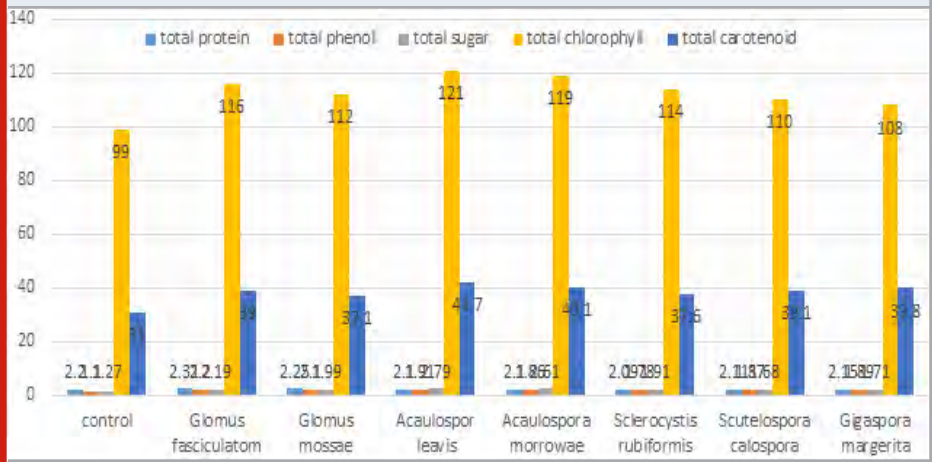
According to Cavagnaro et al. (2015) the nutrient loss from soil can be reduced by potential use of AM fungi due to increasing the absorption zone. It was analysed that *Sorghum* absorbed more P from soils when colonized with AMF than non mycorrhizal plants (Krishna and Bagyaraj, 1981). For preparing pot culture, sorghum plant treated with AM inoculums which were obtained from trap culture. These AM inoculums belong to genus *Glomus Acaulospora Scutellospora Gigaspora*. In the meanwhile studied the growth stages of development of mycorrhizal fungi such as infection, apprisoria formation, hyphae development, arbuscule, vesicles and spore formation. From the pot culture study also it has been compared and analysed the effects of AM fungal spore on the plant growth and physiological changes. Besides physiological changes this interaction also enhances root growth, leaf surface area. Besides these AM fungi plays a important role to maintain soil texture, improve quality of soil, nutrient cycling, hence it contribute to balance our ecosystem. AM inoculated *Sorghum* shows significant increase in all parameter as compare to control. Increased in photosynthetic rate, protein and sugar content, uptake of N P promote overall growth and yield of plant, (Montero et al 2019, Wipf et al 2019).

According to Amiri et al., (2017), increased concentration of N P Fe was found in Pelargonium graveolens. The present research describes the beneficial effect of AM fungi that can improve nutrient status by changing the host plants physiology and improving the biomass.

Table1. Effect of different Arbuscular mycorrhizal fungi on biochemical changes in Sorghum plant after one month

Treatment	Total protein mg/gm fresh wt.	Total phenol mg/gm fresh wt.	Total sugar mg/gm fresh wt.	Total chlorophyll mg/gm fresh wt.	Total carotenoid mg/gm fresh wt.
Control	2.2	1.10	1.27	99	31
Glomus fasciculatum	2.31	2.2	2.19	116	39
Glomus mossae	2.25	2.1	1.99	112	37.1
Acaulospora leavis	2.1	1.91	2.79	121	41.7
Acaulospora morrowae	2.1	1.86	2.61	119	40.1
Sclerocystis rubiformis	2.09	1.78	1.91	114	37.6
Scutellospora calospora	2.11	1.87	1.68	110	39.1
Gigaspora margarita	2.15	1.89	1.71	108	39.8

Figure 1



MATERIAL AND METHODS

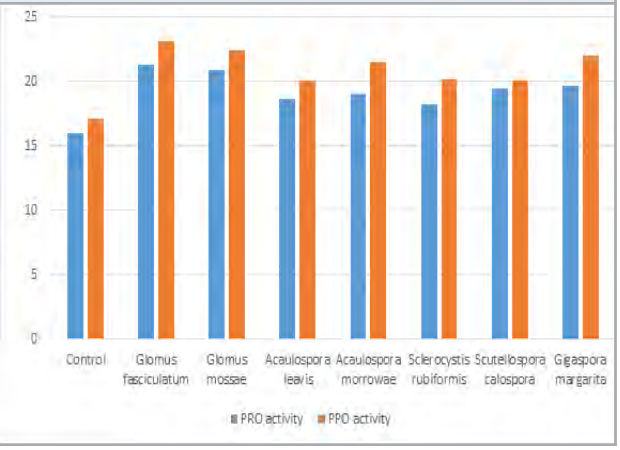
A field survey conducted at six sites near Jodhpur area. Rhizosphere soil along with root sample collected in three replicate. As rhizosphere soil considered to be a source of AM spores that's why AM spores were collected from rhizosphere soil. For the extraction of AM spore wet sieving and decanting technique has been carried out from soil sample. (Gerdemann and Nicolson1963). The isolated AM fungi were identified by key of Trappe and manual of identification of AM fungi of Trappe (1962) Schenck and Perez. (1987). In the present work root samples were collected from the field and were cleared and stained using trypan blue in lactophenol (Phillips and Hayman, (1970).) AM spore percentage and root colonization were evaluated by the gridline intersect method (Giovannetti and Mosse, (1980).).After identification AM spores *Glomus fasciculatum, Glomus mossae. Sclerocystis rubiformis, Acaulospora morrowae* and *Acaulospora leavis, Scutellospora calospora, Gigaspora margarita*, were seen mostly. These AM spores were transferred to various pot cultures with host plant *Cenchrus ciliaris* or *Sorghum bicolor* for mass multiplication .After one month when AM spores were germinate then infected root segments of host plant with hyphae, vesicles and spore used as inoculum for experimental plant i.e. *Sorghum*. These pot were kept in isolated condition. The pot which had no AM inoculum served as control. Plants root and rhizospheric soil were collected after 15days, 30 days, 45days, 60days,90 days for biochemical estimation.

Biochemical Determination-: The parameters studied include chlorophyll and carotenoid, proteins, total sugar, total phenol, peroxidase, polyphenol oxidase.Chlorophyll and carotenoid content in leaves was estimated using Arnons method (1949). The protein content in leaves of mycorrhizal inoculated and non-mycorrhizal *Sorghum* plant was estimated using Bradford(1976) method. The total sugar content was estimated in leaves of AM inoculated plant and controlplant using the method Mc. Cready et al,.(1950).The total amount of peroxidase enzyme was estimated from AM inoculated and control plants using modification method proposed by Putter (1974) and Malik and Singh (1980). The total amount of polyphenol oxidase enzyme in root was calculated from mycorrhiza inoculated and control plant using the method proposed by Esterbaner et al(1977) modified by Fujita et al. (1995). Total phenol content was estimated by Mahadevan's method (1975).

Table 2. Effect of different AM fungi on enzymatic changes in Sorghum plant After one month

Treatment	PRO activity (Units mg-1 protein)	PPO activity (ΔA420/ 100 mg fw)
Control	16	17.1
Glomus fasciculatum	21.33	23.1
Glomus mossae	20.91	22.4
Acaulospora laevis	18.6	20.1
Acaulospora morrowae	19.1	21.5
Sclerocystis rubiformis	18.2	20.2
Scutellospora calospora	19.5	20.11
Gigaspora margarita	19.7	22

Figure 2



RESULTS AND DISCUSSION

AM fungi association found to be most common relationship in nature which affect cultivated plants as well as the plants growing in natural ecosystems, thus it is most important finding in agriculture research field (Brundrest 2018). AM fungal association with *Sorghum* plant affects plant growth, nutrient content, and enzymatic activity. In present study result was based on comparison of mycorrhiza treated plant with control plant. Amount measured of chlorophyll and carotenoid from AM inoculated leaf significantly higher as compare to uninoculated control plant.. The highest concentration of chlorophyll were seen in *Acaulospora laevis* (121 mg/gm fresh wt.) and *Acaulospora morrowae* (119 mg/gm fresh wt.) while least were observed in *Gigaspora margarita* that is (108 mg/gm fresh wt.) inoculated *Sorghum* plant. Carotenoid content were observed higher in (41.7 mg/gm fresh wt.).The increased concentrations of these pigments directly affected the rate of photosynthesis and metabolism in host plants. By the experimental result of (Bhosale and Shinde 2011) the rate of photosynthesis was found to be higher in mycorrhizal treated plants compared to non- mycorrhizal control plants.

Chlorophyll and carotenoids are important plant pigment which plays significant role to accelerate the process of photosynthesis and biomass production. AMF can modified the nutritional value of grains in many agricultural crops and improve the production of carotenoids and certain volatile compounds Bona et al. (2017). When total sugar content of AM treated *Sorghum* plant compared to control plant then increase concentration in total sugar content was observed in *Acaulospora laevis* (2.79 mg/gm fresh wt.) inoculated

plant followed by *Acaulospora morrowae* (2.61mg/gm fresh wt.) treated plant.

Baslam et al (2011) used Mycorrhiza as a bioinoculant to test whether the plant shows enhanced accumulation in terms of chlorophyll, carotenoids, total soluble phenol, tocopherols, and various essential nutrients or not. Inoculated plants get better accumulation in contrast to control. Peroxidase activity was analysed for both control and AM inoculated plant which resulted that peroxidase activity is higher in fresh root weight of *Glomus fasciculatum* inoculated plants. Increasing amount of both enzymes that is Peroxidise and polyphenoloxidase positively correlated with increase in phosphorus concentration because in previous research resulted that AM fungi increases phosphate uptake which ultimately increase peroxidase and polyphenoloxidase activity of host plant. Peroxidase activity observed higher in *Glomus fasciculatum* that is 21.33 / mg fresh wt and polyphenoloxidase activity also in *Glomus fasciculatum* that is 23.1 / mg fresh wt. As a result of metabolic activity (ROS) species were formed as a by product thus it is necessary to detoxify reactive oxygen species (ROS) by the enzymes such as superoxide dismutase, catalase, peroxidase, and enzymatic level enhance significantly after mycorrhizal treatment, (Ahanger and Agarwal, 2017 Montero et al., (2019).

The phosphorus availability with in the soil is taken up with phosphate transporter located in the extra-radical hyphae of this fungus (Harrison and Buuren 1995). *Glomus fasciculatom* (2.2 mg/gm fresh wt.) and Glomus mossae (2.1 mg/gm fresh wt.) came out to be most active in enhancing phenolic accumulation. Total increase in phenol content may be due to an increased phosphorus level and PPO activity. Accumulation of phenol in AM plants which has been reported by (Covacevich and Berbara (2011).As concentration of PRO and PPO enzymes increase they oxidised phenol compound into Quinone or increase in phenyl propane i.e. lignin precursor which are well known for antimicrobial property. It is toxic for attacking pathogen so it is important content of plant defence mechanism.

The findings of Nisha and Kumar (2010) also support these results that higher levels of polyphenoloxidase and peroxidise are observed in mycorrhizal plants. They used seven different types of AM species, inoculated with *Wedilla* plant and observed increase concentration in enzyme as compare to non inoculated. Protein content in all AM inoculated plant resulted almost similar still The maximum protein content found in Glomus fasciculatum (2.31mg/gm fresh wt.) and *Glomus mossae* (2.25 mg/gm fresh wt.) inoculated plant. Hence this AM endophyte can improve the nutritive value as well as enzymatic value of host plant. Through the mycorrhizal symbiosis an increase in protein content in grains of chickpea has also been reported by Pellegrino and Bedini (2014).

The present investigation demonstrate that inoculation with *Acaulospora laevis*, *Acaulospora morrowae* and *Glomus fasciculatom* can enhance the enzymatic activity of *Sorghum* plant that is good for plant defence system. AM inoculated plant also enhance the accumulation of sugar, chlorophyll, protein which collectively enhance biomass and more highly proteinaceous grain. Occurrence of AM fungi have been reported almost all soil type (Bernaola, et al.,2018). Any of soil condition semi-arid, arid, humid it can perform vigorously and make a plant nutrient stable for biomass, defence stable for pathogenic attack. The obligatory relationship of plant and fungi are bidirectional because mycorrhizae provides nutritional benefits to the plant in exchange fungus receives carbon compound to complete its life cycle, (Wipf et al 2019). It is very helpful for soil health as well as plant development.

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Evaluation of Certain Technical Factors Affecting Commercial Production of Pickled Papaya from *Carica papaya* Fruit

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ABSTRACT

Papaya belongs to family Caricaceae scientifically known as Carica papaya Linn. It contains different phytochemical constituents including proteases, lycopenes, carotenoids, alkaloids, monoterpenoids, flavonoids, minerals and vitamins. Its pulp has a pleasant flavor. After ripening, this fruit softens immediately. Perishable nature of papaya fruit limits it from commercial distribution. One investigation surveyed a lactic fermentation from papaya fruit by penetrating on the efficacy of various variables such as blanching duration and temperature, sugar concentration, lactic fermentation duration to total phenolic, total flavonoid, organoleptic property of pickled papaya fruit. Results of ther present study showed that blanching raw papaya fruit in hot water, heated at 95oC for 20 seconds with 1.0% CaCl2, 7% sugar, in 15 days of lactic fermentation were adequate to achieve an overall acceptance of pickled papaya. Papaya pickle can be an ideal choice of our daily cuisine.

KEY WORDS: *PAPAYA FRUIT*, LACTIC FERMENTATION, SUGAR, BLANCHING, TOTAL PHENOLIC, TOTAL FLAVONOID, ORGANOLEPTIC.

INTRODUCTION

Papaya, *Carica papaya* fruit is a rich source of vitamins A and C. It also contains thiamine, riboflavin, calcium, iron, potassium, magnesium and sodium (Bari et al., 2006). Raw papaya is a good source of carbohydrates, vitamins and proteins, and the content decreases as it ripens. Raw papaya has a large amount functional constituents such as saponin, alkaloid, tannin, β-carotene, lycopene, anthocyanin, flavonoid and polyphenol beneficial for human health (Chukwuka et al., 2013; Pavithra et al., 2017).

These bioactive elements are responsible for the pharmacological properties useful in daily intake and alimentation. Papaya is considered as nutraceutical fruit due to its multifaceted medicinal properties (Mahendra et al., 2016). Papaya acts as an antioxidant, antimicrobial, anticarminative, anticancer, and has hepato-protective, immunological, and other therapeutic attributes. The seed and pulp of papaya have bacteriostatic effects against several enteropathogens, such as *Bacillus subtilus* and *E. coli*, (Saeed et al., 2014).

Lactic acid bacteria metabolizes the sugar elements of fruit into lactic acid, which decreases the pH of the pickled products to ensure shelf-life. Lower pH value limits the growth of spoilage flora and pathogenic bacteria. These bacteria enhance the human intestinal microbial balance

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and improve health by inhibiting the proliferation of pathogens such as Escherichia coli, Salmonella and Staphylococcus (Ohmomo et al. 2000, Ross et al. 2002). They are normally believed as probiotic, beneficial for our health and active in lowering the serum cholesterol level (Kaur et al. 2002). They also stimulate immune responses and prevent tumour formation by inhibiting carcinogenic compounds in the gastro-intestinal tract through reducing fecal bacteria enzyme activity (Nakphaichit et al. 2011) or breaking down certain enterotoxins (Bernardeau et al. 2006). The fermented vegetables or pickle products provided probiotics to the consumers (Chaiyavat Chaiyasut, 2018).

Papaya fruit is an underutilized agricultural product contaning high fermentable sugar composition ideal for lactic fermentation. One study compared total phenolic, total flavonoid, β-carotene, lycopene, ascorbic acid contents and antioxidant properties between fresh and pickled papaya. The pickling process of papaya caused a significant decrease in their antioxidant component and activity (Nurul and Asmah 2012). Hence in the presently undertaken study, the effect of various variables such as blanching duration and temperature, sugar concentration, fermentation duration to total phenolic, total flavonoid, organoleptic property of pickled papaya fruit have been examined from a commercial utitly point of view.

MATERIAL AND METHODS

Papaya fruits, Carica papaya were cultivated and harvested from Hau Giang province, Vietnam. They were cultivated following VietGAP, without using insecticides or herbicides to ensure food safety regulations. After harvesting, they were conveyed to laboratory as soon as possible for further experiments.

Effect of blanching temperature and duration to total phenolic, total flavonoid and organoleptic property of pickled papaya: Papaya fruits were peeled to remove green outer, sliced into pieces (0.5 cm in thickness) and pre-treated by thermal blanching in hot water containing 1.0% $CaCl_2$ with different duration and temperature (100°C in 10 seconds, 95°C in 20 seconds, 90°C in 30 seconds and 85oC in 40 seconds). Effectiveness of blanching duration and temperature in papaya fermentation was evaluated on value of total phenolic (mg/g), total flavonoid (mg/g) and organoleptic property (sensory score).

Effect of sugar concentration in fermentation to total phenolic, total flavonoid and organoleptic property of pickled papaya: Papaya fruits were fermented with different sugar concentration (3%, 5%, 7%, 9%). Effectiveness of sugar concentration in papaya fermentation was based on value of total phenolic (mg/g), total flavonoid (mg/g) and organoleptic property (sensory score).

Effect of fermentation duration to total phenolic, total flavonoid and organoleptic property of pickled papaya: Papaya were fermented with different fermentation time (5, 10, 15, 20 days). Effectiveness of fermentation duration in papaya fermentation was based on value of total phenolic (mg/g), total flavonoid (mg/g) and organoleptic property (sensory score).

Chemical, sensory evaluation and statistical analysis: Total phenolic (mg/g) were determined using Folin–Ciocalteu reagent as gallic acid equivalents (GAE). The spectrophotometer assay for the quantitative determination of flavonoid content (mg/g) was carried out as catechin equivalents. Sensory score of pickled product was assessed by a group of panelist using the 9-point Hedonic scale. Data were statistically summarized by Statgraphics Centurion XVI.

RESULTS AND DISCUSSION

Effect of blanching temperature and duration to total phenolic, total flavonoid and organoleptic property of pickled papaya: Blanching was a quick thermal treatment widely applied before processing to inactivate internal enzymes and to kill harmful microbial existing in raw material (Saltveit 2000). Popularly hot water blanching

Table 1. Showing the effect of variables: blanching, temperature and duration to total phenolic, total flavonoid and organoleptic contents of *Pickled papaya*

Blanching temperature and duration	Total phenolic content (mg/g)	Total flavonoid content (mg/g)	Sensory score
100oC, 10 seconds	71.15±0.03^b	21.33±0.00^b	4.52±0.00^b
95oC, 20 seconds	75.49±0.00^a	24.59±0.01^a	6.39±0.03^a
90oC, 30 seconds	67.82±0.01^c	18.48±0.03^c	3.24±0.01^c
85oC, 40 seconds	61.09±0.02^d	14.73±0.01^d	2.77±0.02^d

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

method was used in the food processing of fruits and vegetables (Prakash Kumar et al. 2018).

Papaya were peeled to remove green outer, sliced into pieces (0.5 cm in thickness) and pre-treated by blanching in water containing 1.0% CaCl2 with different duration and temperature (100oC in 10 seconds, 95oC in 20 seconds, 90oC in 30 seconds and 85oC in 40 seconds). Efficay of blanching duration and temperature in papaya fermentation was estimated on total phenolic (mg/g), total flavonoid (mg/g) and organoleptic property (sensory score). Results are elaborated in table 1. It's obviously noticed that blanching at 95oC in 20 seconds was optimal for papaya fermentation. So we used this value for further experiments.

Table 2. Effect of Sugar concentration (%) on total phenolic, total flavonoid, organoleptic contents of *Pickled papaya*

Sugar concentration (%)	Total phenolic content (mg/g)	Total flavonoid content (mg/g)	Sensory score
3	75.49±0.00^{c}	24.59±0.01^{c}	6.39±0.03^{c}
5	79.42±0.03^{b}	26.13±0.03^{b}	7.21±0.00^{b}
7	80.35±0.01^{a}	28.36±0.00^{a}	8.14±0.03^{a}
9	79.93±0.02^{ab}	27.58±0.01^{ab}	7.89±0.01^{ab}

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

Effect of sugar concentration in fermentation to total phenolic, total flavonoid and organoleptic property of pickled papaya: In the pickling industry, sugar has been popularly applied for lactic fermentation of various fruits and vegetables (Hudson et al 1985, Fleming et al., 1995; Mcfeeters et al., 1993). It is an essential ingredient to improve the preservative, technological and sensory quality of food (Brady 2002). It's one of the most widely additives for food preservation to increase product shelf-life by decreasing water activity. During pickling, sugar addition will affect the carbohydrate contents. Some vegetables are deficient in sugars and are liable to develop undesirable types of bacteria unless a small amount of sugar is added (Sultana et al., 2014).

Papaya were fermented in various sugar concentration (3%, 5%, 7%, 9%). Effectiveness of sugar concentration in papaya fermentation was based on total phenolic (mg/g), total flavonoid (mg/g) and organoleptic property (sensory score). Results were revealed in table 2. It's thoroughly realized that 7% sugar was adequate for papaya fermentation. So we decided to choose this parameter for further experiments.

Table 3. Effect of Fermentation duration (days) on the total phenolic content, total flavonoid content and sensory score of pickled papaya

Fermentation duration (days)	Total phenolic content (mg/g)	Total flavonoid content (mg/g)	Sensory score
5	80.35±0.01^{b}	28.36±0.00^{b}	8.14±0.03^{b}
10	81.46±0.03^{ab}	29.53±0.02^{ab}	8.45±0.01^{ab}
15	82.11±0.00^{a}	30.19±0.01^{a}	8.68±0.00^{a}
20	82.17±0.02^{a}	30.23±0.00^{a}	8.37±0.02^{ab}

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

Effect of fermentation duration to total phenolic, total flavonoid and organoleptic property of pickled papaya: Naturally occurred lactic acid bacteria from the raw material play an important role in lactic fermentation. Papaya were fermented with different fermentation time (5, 10, 15, 20 days). Efficacy of fermentation duration in papaya fermentation was based on value total phenolic (mg/g), total flavonoid (mg/g) and organoleptic property (sensory score). Results were mentioned in table 3. It's obviously revealed that 15 days of fermentation was adequate for papaya fermentation. So we selected this variable for application. The present results are quite different with one report. The pickling process of papaya caused a significant decrease in their antioxidant component and activity (Nurul and Asmah 2012).

CONCLUSION

The fermentation process of pickles is very simple and there is no need for specific equipment. Pickling is one of the most effective ways for fruit and vegetable preservation. Pickle is the good source of antioxidants, probiotics, vitamins, and minerals beneficial for our health. Lactic acid fermentation is believed as a simple and useful biotechnology to improve the safety, nutritional, sensory and stability attributes of papaya fruit. Pickle papaya is a good appetizers and adds to the palatability of the meal. It can also stimulate the flow of gastric juice and thus helps in digestion.

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Effect of Bacteriocin of *Paenibacillus polymyxa* on Biofilm Forming *Listeria monocytogenes* MTCC 657

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ABSTRACT

Anti-listerial bacteriocin producing bacteria were isolated and identified using 16S rRNA sequencing and the bacteriocin were purified by Amberlite XAD-16 absorption. The biofilm forming ability of *Listeria monocytogenes* MTCC 657 was assessed using tube method and CVB assay. Biofilm formation on substrates were also checked using different substrates. Polystyrene possessed strong biofilm formation of (0.185) *Listeria monocytogenes* MTCC 657. In biofilm eradication studies, the bacteriocin GRDS6 showed similar activity of Nisin on all the three substrates (polystyrene, glass and aluminum foil) at 45°C, 30°C, 4°C and -20°C. Based on CBD® assay, the activity of GRDS6 bacteriocin and Nisin were similar in both high (45°C) and freezing (-20°C) temperatures. This study shows that the GRDS6 bacteriocin is able to inhibit the biofilm formed by *Listeria monocytogenes* MTCC 657 under different temperatures and different incubation periods. The activity of GRDS6 bacteriocin is similar to activity of Nisin. So we recommend to use this bacteriocin in the food preservation industry at higher (45°C) to freezing temperature (-20°C). In the present study, toddy was used as a source for isolation of bacteriocin producing bacteria. The isolate we assayed for its bacteriocin production. *Listeria monocytogenes* MTCC 657 is able to produce biofilm which helps the microorganism to survive and grow for an extended period of time. Adhesive ability and biofilm formation ability of *Listeria monocytogenes* MTCC 657 on solid substrates (polystyrene, glass and aluminum foil) at different temperatures was determined and found to be higher on polystyrene at 72 h followed aluminum foil and glass. The biofilm eradication potential of GRDS6 bacteriocin and Nisin were evaluated by microtiter plate method and CBD® assay. Based on our results the activity of GRDS6 bacteriocin was similar to Nisin. The incubation temperature doesn't influence the activity of the bacteriocin, so we recommend this bacteriocin in food storage industry.

KEY WORDS: BACTERIOCIN, BIOFILM, *LISTERIA MONOCYTOGENES*, NISIN.

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INTRODUCTION

Microorganisms can exist in the environment as planktonic cells or on the surface in biofilms enclosed with in a matrix predominantly made up of polysaccharide material (Gandhi et al, 2007). The existence of these bacteria can be termed as biofilms; a microbially derived sessile community characterized by the cells that are irreversively attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substance that they have produced and with an altered phenotype with respect to growth rate and gene transcription (Donlan et al, 2002). The formation of biofilms by some pathogenic bacteria such as *Listeria monocytogenes* have been reported. Such biofilms could be continuous sources of contamination of food and medical products in contact with them. That may also lead to spoilage of foods or transmission of food borne diseases (Harvey et al, 2006, Shivangi et al 2020).

Listeria monocytogenes has been recognized as a major food borne pathogen with the ability to survive in various environmental conditions such as extreme cold conditions (Donnelly et al, 1997), at lower pH such as 3.6 in foods (Parish et al, 1989), in salt concentration up to 10% (Mc Clare et al, 1989), in the presence of surfactant sanitizers (Frank et al, 1990) and at high temperatures (Mc Carty et al, 1990). It has been demonstrated that *Listeria monocytogenes* can grow and form biofilms on several food processing surfaces including rubber, plastics, glass and stainless steel (Wong et al, 1998, Di Bonaventura et al, 2008). It is also found in fermented products (cheese, yogurt, fermented milk, sausages etc.) made from raw materials contaminated with the organism (Berry et al, 1999). The infection caused by *Listeria monocytogenes* (listeriosis) can lead to gastroenteritis, septicemia, perinatal infections, miscarriages, meningitis and meningo-encephalitis in immune-compromised individuals (Barbuddhe et al, 2012, Eliot et al, 2016 Gurgu et al 2019).

Listeria monocytogenes can adhere rapidly and firmly to inert surfaces commonly found in the food processing industry (Frank et al, 1990) with protection against various physical and chemical stress is conferred on when they adhere. The cellular mechanisms underlying microbial biofilm formation and behavior are beginning to be understood and are targets for novel specific intervention strategies to control problems caused by biofilm formation in these different fields, and in particular for the food processing environments. Biofilm of *Listeria monocytogenes* is much more resistant to environment stress and can stay for long time on the surface. Bacteria in the form of biofilm will be protected against disinfectant as the efficiency of disinfectant declines in the presence of biofilm; sanitation faces serious difficulties in food industries (Chapman et al, 2003). Investigation of bacteriocins which inhibit pathogens such as *Listeria monocytogenes* has become particularly attractive for the food industry. *Listeria monocytogenes* in ready to eat (RTE) food, is of special concern because it can adhere to abiotic surfaces in food processing creating a cellular mass that joins nutritious residues and other microorganisms forming biofilms (Regiane et al, 2008 Gurgu et al., 2019 Shivangi et al 2020).

The various bacteriocins have shown great diversity in their effects on bacterial species (Radler et al, 1990). Bacteriocins are defined as extracellular bioactive peptides or peptide complexes that are bactericidal, because of the combined action of the bacteriocin and the host autolysin (Martinez cuesta et al, 2000) or bacteriostatic against other species, usually closely related to the producer strain. The ability to produce bacteriocin is associated with the presence of a stable genetic factor in the cell and is composed of DNA (De Witt et al, 1965). Bacteriocins produced by lactic acid bacteria have attracted increasing attention since they are active in a nanomolecular range and have no toxicity. Bacteriocins and similar metabolites produced by certain group of bacteria like lactic acid bacteria (LAB), isolated from different types of food, have been known to inhibit the growth of *Listeria monocytogenes* (Colak et al, 2007 Gurgu et al., 2019 Shivangi et al 2020).

These bacteriocins can be considered as an alternative to the use of chemical preservatives (Zhu et al, 2005). Bacteriocins produced by Lactococcus species, especially Nisin are widely used in foods since the LAB have generally been regarded as safe (GRAS) organisms. Nisin is active especially at the lower pH values of many fruits and some vegetables (Jung et al, 1992). In the food industry, Nisin is obtained from the culturing of certain LAB present in natural sources such as milk, milk products, meat etc. and it can't be chemically synthesized. The anti-microbial activity of Nisin on planktonic cells of *Listeria monocytogenes* has been well identified (Buddee et al, 2000). Studies addressing the effect of Nisin producing strains on *Listeria monocytogenes* biofilms are less (Leriche et al, 1999). *Paenibacillus polymyxa* is a common soil bacterium which belongs to plant growth promoting (PGPR) rhizobacteria. (Ash et al, 1993). *Paenibacillus polymyxa* is widely distributed in the environment as well as food products (Gupta et al, 2014, Gurgu et al., 2019).

Several small peptide antibiotics, for e.g.: Polymyxin B produced by this organism (Kimura et al, 2009). Isolated from various sources the genus *Paenibacillus comprises* bacterial species relevant to humans and animals, plants and the environment. *Paenibacillus* derived anti-microbial substances also have application in medicines including polymyxins (bacteriocin like substance) and fusaricidins, which are non-ribosomal lipopeptides first isolated from strains of *Paenibacillus polymyxa* (Eliot et al, 2016). The main objective of this study is to find out the biofilm formation potential of Listeria monocytogenes on different substrates and its eradication using bacteriocin and Nisin by different methods like Micotitre plate method and Calgary biofim device method.

MATERIAL AND METHODS

Screening of bacteriocin producing bacteria: Toddy samples were collected from Palakkad district of Kerala, India. A tenfold dilution of the sample were serially diluted and plated in MRS agar (Man Rogosa De Sharpe Agar) using spread plate technique. The plates were incubated for 48 to 72h. Microorganism producing bacteriocin were identified using spot on lawn method in which *Listeria monocytogenes* MTCC 657 was used as test organism. *Listeria monocytogenes* (MTCC 657) were obtained from Microbial Type Culture Collection, Chandigarh, India. The cultures were maintained in MRS Agar (HiMedia) and Nutrient Agar (HiMedia) respectively. The isolate was identified based on morphological, biochemical and molecular (16S rRNA sequencing). For molecular identification the bacterial DNA was amplified with universal primers and send to Eurofins Scientific, Bangalore, (India) for genus identification. The sequence after identification were submitted to GenBank, NCBI.

Bacteriocin purification: The crude extract harvested from the isolate were purified using Amberlite XAD-16(HiMedia) adsorption by column chromatography. 5g of Amberlite XAD-16 were soaked in 50% isopropanol and stored at 4°C overnight. This was then washed repeatedly with distilled water to remove isopropanol. The cleaned Amberlite was added to 250mL of heat inactivated cell free supernatant of the isolate and incubated for 4h in a shaking incubator. This was then transferred to a chromatographic column for elution. The matrix was washed with 20mL of distilled water and 20mL of 40% Ethanol. The extract were eluted with 20mL of 70% isopropanol and followed by washing with 20mL of absolute ethanol. The eluted extract was evaporated to half its volume.

Nisin: 100mg of Nisin (HiMedia) was solubilized in 10mL of 0.02N HCl to give the concentration of 104 IU/mL (40 IU=1g). The solution was sterilized by filtration through 0.45mm filters and was stored at -4°C. (Mahdavi et al, 2007)

Biofilm formation studies: The objective of this study is to investigate the biofilm forming ability of *Listeria monocytogenes* MTCC 657 on abiotic surfaces such as stainless steel, glass and aluminum foil at different temperatures using microtiter plate assay in order to evaluate the reduction potential of Nisin and GRDS6. A parallel study was undertaken to determine the minimal biofilm eradication concentration (MBEC) of GRDS6 and Nisin (standard) against *Listeria monocytogenes* MTCC 657 biofilm using Calgary Biofilm Device (CBD®).

Tube method: *Listeria monocytogenes* MTCC 657 isolates were tested for biofilm formation by standard method (Christensen et al 1982). 5mL of Tryptic Soy Broth (TSB) in test tubes were inoculated with a loop full of microorganisms from overnight culture plates and incubated for 48h at 37°C. After incubation the contents were decanted and washed with Phosphate buffered saline (PBS pH 7.3) and kept for drying at room temperature. Then the tubes were stained with crystal violet solution (4%) and rotated for uniform staining and contents were decanted. The tubes were placed upside down for draining. The biofilm formation was observed when a visible film lined wall and bottom of the tubes.

Microtiter Plate Method (Christensen et al, 1985): The test organism were inoculated from overnight cultured Nutrient agar plates into 5mL of TSB broth and incubated 18h at 30° C and transferred (125 µL) to 5mL growth medium (TSB). Then the culture were vortexed for 1min and a volume of 100µL was transferred into the wells of a 96 well microtiter plate. Plates were covered with a tightly fitting lid and incubated at 30° C for 24h, 48h and 72h. Each plate included 12 control wells comprising of 100µL of un-inoculated growth medium. After incubation, the cultures were removed and cell densities were determined by measuring turbidity at 595nm. The plates were then washed three times with sterile distilled water to remove loosely bounded bacteria and dried at 30° C for 3min. 100µL of 95% alcohol was added to each well to destain the biofilm and the concentration of crystal violet was determined by measuring optical density (OD) at 595nm.

Biofilm formation on different substrates: Overnight culture of the test organism was grown in 100mL conical flask containing TSB at 30° C for 24h. One un-inoculated conical flask with growth medium kept for incubation as control. The abiotic substrates were washed with detergent, rinsed with sterile distilled water and air dried and placed into hot air oven at 75° C for 30min. After 24h incubation, sterile substrates were aseptically added to the grown broth culture and incubated at 30° C for 24h, 48h and 72h. At the end of each incubation period, a set of substrates were removed aseptically from the broth culture for biofilm quantification using Crystal violet binding assay (CVB) (Stepanovic et al, 2004). The substrates were then washed with 5mL of deionized water and the adherent bacteria were fixed with 250mL of Methanol. Each substrate was stained with crystal violet for 15min and the excess stain were removed under running tap water and air dried. The dye bound to the adherent cells were resolubilized with 2.5mL of 33% glacial acetic acid. The resolubilized liquid of each substrate were collected and absorbance was measured at 595nm in which un-inoculated control was set as blank using spectrophotometer (HACH) (Pawar et al 2005).

Biofilm eradication studies on *Listeria monocytogenes* MTCC 657 using GRD S6 and Nisin Microtiter Plate Method: The microtiter plate method was slightly modified in which abiotic substrates (polystyrene, glass and aluminum foil) were added into sterile 96 well microtiter plate and filled with 200 µL of bacterial culture. Un-inoculated broth was used as negative control. The plates were covered and incubated at different temperatures ranging 45°C, 30°C, 4°C and -20°C for 72h. After incubation the substrates were transferred into fresh and sterile microtiter plate and washed three times with 250µL of sterile phosphate saline. The plates were shaken vigorously to remove non-adherent

bacteria and added 200µL of different concentration of GRD S6 and Nisin to each well except control well and incubated for 1h. After incubation, the bacteriocins were removed and washed 5 times with sterile distilled water to remove loosely adherent bacteria, Nisin and GRD S6 (Mahdavi et al, 2007). The plates and substrates were stained for 5min with 200µL of 2% crystal violet per well. Excess stain was rinsed off and air dried. The dye bound to the adherent cells were resolubilized with 160µL of 33% glacial acetic acid and incubated for 15min at 30°C. The optical density was measured at 595nm using an ELISA reader. The reduction percentage of biofilm were calculated using the following formula (Harvey et al 2007, Stepanovic et al, 2000)

$$\text{Reduction Percent} = \frac{(\text{Control OD}_{595nm} - \text{Test OD}_{595nm})}{\text{Control OD}_{595nm}} \times 100$$

Calgary Biofilm Device® Method: *Listeria monocytogenes* MTCC657 biofilms were grown at different temperatures such as 45°C, 30°C, 4°C and -20°C to determine the biofilm eradication potential using Nisin and GRDS6 at different concentrations.

Step 1: Growing Listeria biofilm: The indicator strain inoculum were prepared (Harvey et al, 2007) and 200µL each were added to all the wells in microtiter plate closed with lid containing polystyrene pegs and incubated for 72h at 45°C, 30°C, 4°C and -20°C.

Step 2: Preparation of challenge plate: After incubation of 72h, a new microtiter plate was taken and two fold dilutions of GRDS6 and Nisin were prepared in TSB broth. Biofilm pegs were washed three times with sterile PBS. The pegs were then immersed in a fresh microtiter plate containing two fold dilutions of bacteriocins and incubated at 45°C, 30°C, 4°C and -20°C for 24h.

Step 3: Neutralization and recovery: To a new sterile microtiter plate, add 200µL of freshly prepared TSB broth to all the wells. The pegs from challenge plate were removed and rinsed twice with PBS. The pegs were then immersed into a new microtiter plate (recovery plate) and sonicated for 24h to disrupt the biofilm from the surface of the pegs. 200µL of neutralizing agent (Proteinase K) were added to the challenge plate and kept for 24h incubation.

Step 4: Determination of MIC and MBEC (Allan et al, 2011): To determine MIC values, the turbidity in the wells of the challenge plate was read at 650nm using ELISA reader. The MIC (OD650 < 0.1) is defined as the minimum concentration of bacteriocin that inhibits growth of the organism. To determine the MBEC values, the turbidity in the wells of recovery plate was read at 650nm. The MBEC is defined as the (OD650 < 0.1) minimum concentration of bacteriocin that inhibits growth of the organism and evidence of biofilm eradication.

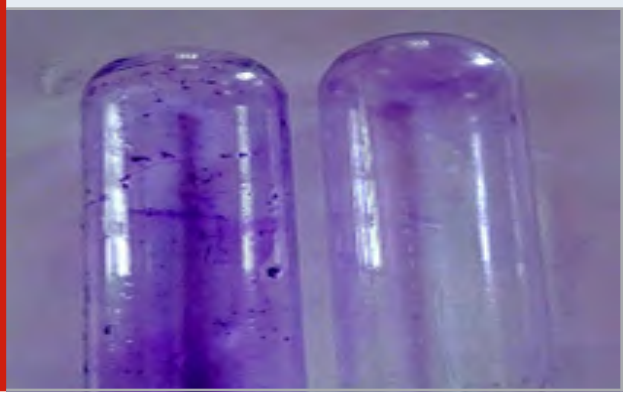
Statistical Analysis: All results were presented in mean ± standard deviation (Mean±SD). A one- way analysis of variance (ANOVA) was used to determine significant difference between mean for each surface and strains. Statistical significance were evaluated at $P<0.05$. Graphs were plotted using Microsoft Excel (Adetunji et al, 2011).

RESULTS AND DISCUSSION

Screening of bacteriocin producing bacteria: Toddy was selected as a source of bacteriocin producing bacteria. From more than 30 colonies screened for the antibacterial activity against *Listeria monocytogenes* MTCC 657, one colony were found to produce inhibitory zone and designated as GRDS6. The pure culture were maintained on MRS agar. The culture were found to be gram positive cocci by Gram's staining. The isolate showed negative results for catalase test, bile esculin hydrolysis and found to be Vancomycin resistant. The strain were next subjected to 16S rRNA sequencing and phylogenetic analysis. Based on 16S rRNA phylogeny, the bacterial strain showed 98% similarity with *Paenibacillus polymyxa*. The sequence was submitted to GenBank, NCBI and the accession number MH113816 was obtained.

Production and purification of GRDS6 bacteriocin: Anti-listerial bacteriocin production was carried out using production medium (TGE and Tween 80), which supported maximum bacteriocin production. Extraction of anti-listerial bacteriocin was done using Amberlite XAD-16 absorption, which is a non-ionic macro-reticular resin that absorbs and release ionic species through hydrophobic interactions (Zenguo et al, 2007). The anti listerial bacteriocin will selectively adhere to the amberlite resin which is applied to the crude cell free supernatant. This bounded bacteriocin were eluted from XAD-16 with 70% isopropyl alcohol by column chromatography method and the fraction was evaporated to half the volume and stored in 4°C.

Figure 1: The biofilm formation by *Listeria monocytogenes* MTCC657 by tube method, A- Test strain with crystal violet staining indicates biofilm formation, B- Control

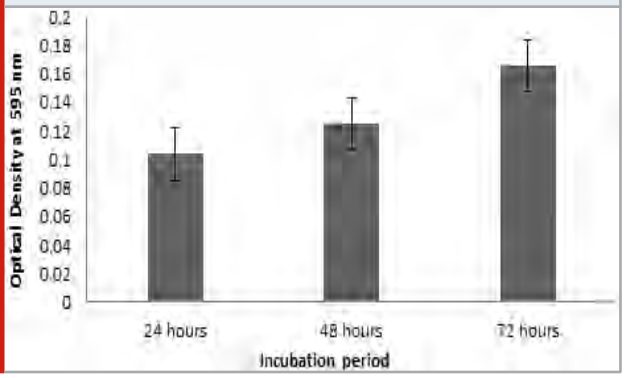


Biofilm formation studies: Primary studies to find out the biofilm formation ability of *Listeria monocytogenes* MTCC 657 was carried out by tube method and microtiter plate method.

Tube method: The tube method involves the adherence of *Listeria monocytogenes* MTCC 657 on the sides if the test tube through visual assessment. In our study, Listeria monocytogenes MTCC 657 showed positive result i.e. adherence of cells after crystal violet staining (Fig.1). This indicates that *Listeria monocytogenes* MTCC 657 is a strong biofilm producer. Studies shows that there are different strategies employed by microorganisms to produce biofilm and to understand the pathway. Saitou et al, (2009) reported that scientists has discovered that biofilm producing bacteria secrete certain chemicals that protect them from disinfectants and anti-microbial agents.

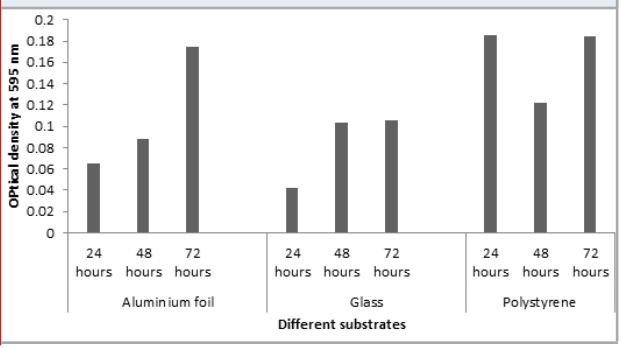
Microtitre plate method: Optical density of the *Listeria monocytogenes* MTCC 657 biofilm grown at 24, 48 and 72 h were observed at 595nm. Results showed that the strain was able to form biofilm at 24h itself. The optical density at 595nm ranged from 0.103 to 0.165 (Fig.2) after 72h. In all the three incubations, the biofilm formation seems to be increasing. In our study, there is an increase in biofilm formation with extension of incubation period (Adetunji et al, 2008). According to Foualdynezhad et al, (2013) biofilm formation as a complicated process influenced by factors such as time, temperature and growth medium.

Figure 2: Biofilm formation at different incubation period in crystal violet binding assay



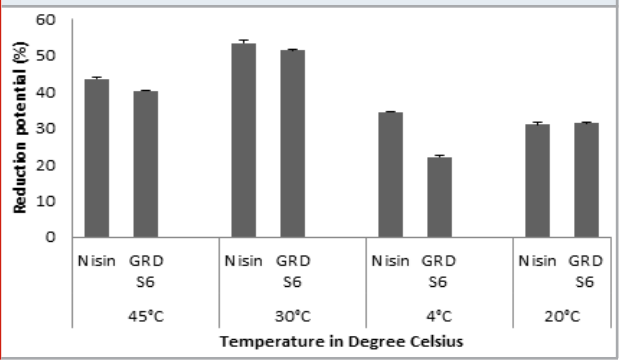
Biofilm formation on different substrates: In this study, we selected three abiotic surfaces such as polystyrene, glass and aluminum foil for different incubation periods. These substrates were selected because they are commonly used in food processing and preservation industry. The OD values at 595nm at 24 h showed that *Listeria monocytogenes* MTCC 657 formed more biofilm on polystyrene (0.185) followed by aluminum foil (0.064) and glass (0.042). Similar results were observed in 48h and 72h. From our results, it is clear that *Listeria monocytogenes* MTCC 657 has a high capacity of biofilm formation on these substrates. The absorbance values of biofilm adherence of *Listeria monocytogenes* MTCC 657 showed that polystyrene maintained higher biofilm formation than aluminum foil and glass (Fig.3). According to Treese et al, (2006), this may be due to the surface hydrophobicity in polystyrene and other physico-chemical properties compared to other surfaces.

Figure 3: Biofilm formation on different substrates at different incubation periods



Biofilm eradication studies on Listeria monocytogenes MTCC 657 using different bacteriocins: Microtiter Plate Method: The main objective of our study was to evaluate the biofilm forming ability of *Listeria monocytogenes* MTCC 657 on three different substrates commonly used in food industries (polystyrene, glass and aluminum foil) at different temperatures (24h, 48h and 72h) and to assess the biofilm eradication potential of Nisin and GRDS6 bacteriocin. All the values were expressed as Mean±SD. The results shows that GRDS6 bacteriocin has similar activity of Nisin. For polystyrene, the reduction potential (%) of GRDS6 were 40.26±0.071 (45°C), 51.47±0.025 (30°C), 22.17±0.035 (4°C) and 31.65±0.165 (-20°C) respectively. And for Nisin the results were 43.63±0.298 (45°C), 53.40±0.721 (30°C), 34.40±0.224 (4°C) and 31.06±0.539 (-20°C). The activity of GRDS6 was equivalent with the activity of Nisin on biofilm of Listeria monocytogenes MTCC 657 grown on polystyrene (Fig.4).

Figure 4: Y- axis represents the reduction potential (%) of *L. monocytogenes* MTCC 657 and X - axis represents the temperatures (°C)



The results we got for biofilm eradication by Nisin on glass were 88.27±0.375, 89.14±0.349, 94.3±0.349 and 86.6±0.388 and for GRDS6, the values were 91±0.558, 93.04±0.142, 87.20±1.395 and 81.36±1.029 for 45°C, 30°C, 4°C and -20°C. From this result we can relate the biofilm eradication potential of GRDS6 to Nisin (Fig.5).

For aluminum foil the results obtained as follows, 41.31±0.072 and 38.5±0.465 for 45°C, 42.28±0.149 and 36.59±1.518 for 30°C, 40.34±0.149 and 36.55±0.203 for

4°C, 41.84±0.084 and 17.46±0.204 for -20°C for GRDS6 and Nisin respectively (Fig.6). According to this result the reduction potential of GRDS6 and Nisin were relatable.

Figure 5: Y- axis represents the reduction potential (%) of *L. monocytogenes* MTCC 657 and X - axis represents the temperatures (°C)

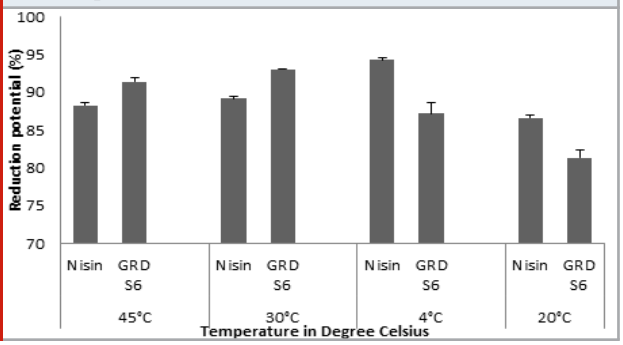
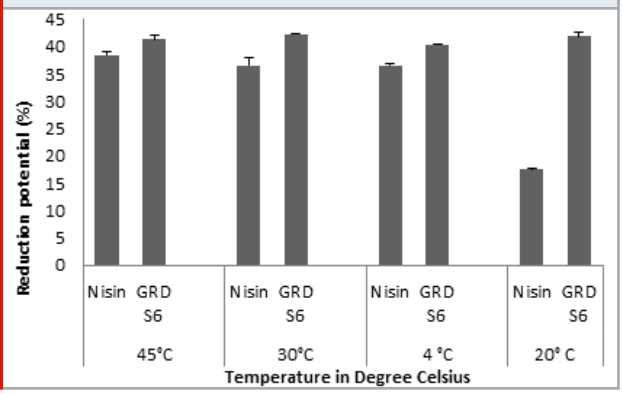


Figure 6: Y- axis represents the reduction potential (%) of *L. monocytogenes* MTCC 657 and X - axis represents the temperatures (°C)



Nisin was used as standard bacteriocin in our study as it is a proven bacteriocin and is commercially available. Nisin-containing packaging materials have potential applications to prevent growth of many foodborne pathogenic bacteria and spores of *Clostridium botulinum* and *Listeria monocytogenes* (Ray et al, 1992). It has been shown before that Listeria. monocyogenes will adhere to a wide variety of surfaces (Mafu et al, 1990). Many techniques have been developed to study bacterial attachment, some of which are based on counting the number of detached cells from the surface, such as standard plate counts and sonication (Green et al, 1993).

2Calgary Biofim Decice®: The results shows that the GRDS6 bacteriocin and Nisin are able to inhibit the biofilm formed by *Listeria monocytogenes* MTCC 657 irrespective of the temperatures. The results are depicted in the table as follows (Table.1, 2, 3, 4).

In 45°C, the biofilm formation is optimum and the biofilm eradication potential is low. Our results shows that, higher concentration of bacteriocin is needed for the eradication of biofilm for higher temperature (Table.1). There are studies which explains that the formation of biofilm by *Listeria monocytogenes* will increase with the time of incubation at higher temperature (Lee et al, 2003).

At 30°C, based on statistical data *Listeria monocytogenes* MTCC 657 showed significance difference (except 10 µg, 20µg/mL, 40 µg/mL and 80 µg/mL) among the activity of bacteriocins (Table.2) and therefore the activity of Nisin and GRDS6 bacteriocins are relatable.

Biofilm formation were observed at 4°C and the statistical results of MIC showed significant difference among GRDS6 and Nisin. In the case of MBEC, no significance difference were observed (except 160µg/mL).

Table 1. Susceptibility of *Listeria monocytogenes* MTCC 657 for Nisin and GRDS6 at 45°C

Conc.of bacteriocin µg/mL	MIC		MBEC	
	Nisin	GRDS6	Nisin	GRDS6
1280	0.073±0.003	0.076±0.001	0.072±0.002	0.067±0.001
640	0.075±0.003	0.073±0.002	0.076±0.001	0.067±0.003
320	0.077±0001	0073±0.004	0.081±0.003	0.065±0.001
160	0.081±0.002	0.074±0.009	0.087±0.001	0.066±0.001[a]
80	0.084±0.002	0.076±0.001	0.089±0.002	0.068±0.002
40	0.086±0.001	0.078±0.002[a]	0.093±0.001	0.072±0.001
20	0.089±0.002	0.082±0.002[a]	0.095±0.003	0.075±0.002
10	0.091±0.001	0.082±0.001[a]	0.098±0.001	0.082±0.002

The given values are the optical density of *L. monocytogenes* MTCC 657 biofilm obtained by measuring the turbidity at 650nm on Elisa plate reader and expressed as Mean ± SD at significance $p < 0.05$. a - the significant relationship between Nisin and GRDS6

Table 2. Susceptibility of *Listeria monocytogenes* MTCC 657 for Nisin and GRDS6 at 30°C

Conc.of bacteriocin µg/mL	MIC		MBEC	
	Nisin	GRDS6	Nisin	GRDS6
1280	0.061±0.002	0.064±0.001	0.057±0.002	0.063±0.001
640	0.065±0.001	0.064±0.002	0.056±0.002	0.063±0.002[a]
320	0.066±0.002	0.065±0.004	0.059±0.001	0.062±0.002[a]
160	0.071±0.001	0.067±0.002	0.061±0.003	0.067±0.004[a]
80	0.073±0.001	0.071±0.002[a]	0.065±0.002	0.070±0.001
40	0.078±0.003	0.073±0.001[a]	0.067±0.003	0.073±0.001
20	0.081±0.001	0.074±0.002[a]	0.071±0.002	0.076±0.002
10	0.082±0.002	0.080±0.001[a]	0.072±0.001	0.077±0.001

The given values are the optical density of *L. monocytogenes* MTCC 657 biofilm obtained by measuring the turbidity at 650nm on Elisa plate reader and expressed as Mean ± SD at significance $p < 0.05$. a - the significant relationship between Nisin and GRDS6

Table 3. Susceptibility of *Listeria monocytogenes* MTCC 657 for Nisin and GRDS6 at 4°C

Conc.of bacteriocin µg/mL	MIC		MBEC	
	Nisin	GRDS6	Nisin	GRDS6
1280	0.071±0.002	0.061±0.001	0.056±0.001	0.060±0.001
640	0.076±0.002	0.062±0.001	0.058±0.001	0.061±0.001
320	0.081±0.001	0.072±0.002	0.061±0.002	0.065±0.002
160	0.085±0.003	0.071±0.001	0.063±0.001	0.073±0.001[a]
80	0.089±0.002	0.076±0.001[a]	0.067±0.003	0.074±0.001
40	0.092±0.001	0.080±0.001[a]	0.071±0.001	0.076±0.001
20	0.094±0.003	0.084±0.002[a]	0.076±0.001	0.081±0.001
10	0.095±0.002	0.089±0.002	0.079±0.002	0.085±0.001

The given values are the optical density of *L. monocytogenes* MTCC 657 biofilm obtained by measuring the turbidity at 650nm on Elisa plate reader and expressed as Mean ± SD at significance $p < 0.05$. a - the significant relationship between Nisin and GRDS6

The biofilm formation and eradication at -20°C was observed. The MIC values showed no significane difference. MBEC also showed the same results. From this study we can conclude that GRDS6 bacteriocin is similar to Nisin. According to Hanene et al, (2013), the starvation and cold stress enhance the biofilm formation, surface hydrophobicity and modify the membrane lipopeptides of *Listeria monocytogenes*. The assessment of the anti-biofilm activities of antimicrobial agents is generally based on interference with quorum sensing, inhibition of adhesion, enhancement of dispersion, and various experimental and promising alternatives, such as the use of biofilm-specific antibodies, bacteriophage-based treatments, and the species-specific control of biofilms (Chen et al, 2011).

This study shows that the GRDS6 bacteriocin is able to inhibit the biofilm formed by *Listeria monocytogenes* MTCC 657 under different temperatures and different incubation periods. The activity of GRDS6 bacteriocin is similar to activity of Nisin. So we recommend to use this bacteriocin in the food preservation industry at higher (45°C) to freezing temperature (-20°C). In our study, toddy was used as a source for isolation of bacteriocin producing bacteria. The isolate we assayed for its bacteriocin production. *Listeria monocytogenes*

MTCC 657 is able to produce biofilm which helps the microorganism to survive and grow for an extended period of time. Adhesive ability and biofilm formation ability of *Listeria monocytogenes* MTCC 657 on solid substrates (polystyrene, glass and aluminum foil) at different temperatures was determined and found to be higher on polystyrene at 72 h followed aluminum foil and glass. The biofilm eradication potential of GRDS6 bacteriocin and Nisin were evaluated by microtiter plate method and CBD® assay. Based on our results the activity of GRDS6 bacteriocin was similar to Nisin. The incubation temperature doesn't influence the activity of the bacteriocin, so we recommend this bacteriocin in food storage industry.

Table 4. Susceptibility of *Listeria monocytogenes* MTCC 657 for Nisin and GRDS6 at -20°C

Conc. of bacteriocin µg/mL	MIC		MBEC	
	Nisin	GRDS6	Nisin	GRDS6
1280	0.053±0.001	0.050±0.002	0.056±0.001	0.062±0.001
640	0.055±0.003	0.058±0.001	0.059±0.002	0.061±0.003
320	0.061±0.002	0.055±0.001	0.061±0.001	0.064±0.001
160	0.063±0.001	0.061±0.001	0.063±0.002	0.065±0.001
80	0.069±0.002	0.065±0.003	0.065±0.003	0.067±0.002
40	0.071±0.002	0.069±0.001	0.068±0.001	0.069±0.003
20	0.076±0.003	0.071±0.001	0.072±0.002	0.072±0.001
10	0.081±0.002	0.070±0.002	0.078±0.001	0.074±0.001

The given values are the optical density of *L. monocytogenes* MTCC 657 biofilm obtained by measuring the turbidity at 650nm on Elisa plate reader and expressed as Mean ± SD at significance $p < 0.05$

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Conflicts of Interest: The authors declare no conflicts of interest.

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MammoTWEET: A Novel Approach for Digitalized Retrieval Report of Breast Cancer for Medical Professionals

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ABSTRACT

The study of the mammogram datasets of computer aided design to deploy it on the social networks for the decision-making retrieval report to medical professional is presented in this paper. The existing radiology diagnosis system to integrate with social network has investigated on it. The CAD prototypes of mammogram images have generated by MATLAB such as data acquisition, pre- processing, segmentation, feature extraction and classification. These prototypes deployed by IoT analytics platform (ThinkSpeak) service. This platform generated the instant visualization of live excitations/information which alters to web service. This IoT platform integrates with MATLAB. Hence, these retrieval reports communicate to medical professionals through Twitter/Twilio. The breast cancer is one of the major health diseases for human society. The prevention for the major stage of cancer from human body and exchange the analytical report on medical professions through the social media as like Twitter/ Twilio is delivered into this paper. These clinical trials provide the complete the clinical reports, which includes the statistical distribution of cancerous organ. These analytical reports on cancerous organ image has fixed for 81.25% of threshold value. Because Gaussian rules based Gabor filter adapted for pre-processing. Therefore, the smart phone based digitalized mammogram, which demonstrate the image retrieval, classification of trails and display toolset with covers the basic radiology function of today's medical requirements. These clinical reports fulfilments of medical professionals to analyse the quick suggestion

KEY WORDS: BREAST CANCER, IOT ANALYTICS, INSTANT VISUALIZATION, MAMMOTWEET.

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INTRODUCTION

Breast cancer is the second most common tumour in world and more prevalent in the women population, not only a women disease its present in men even. Since the origin of disease remains unclear, early detection and diagnosis is the optimal solution to prevent tumour progression and allow a successful medical intervention, protect lives and reduce cost. It is identified by using the x-ray mammography technique with the absence of signs. Tiny cancer cells are identified without any symptoms before undergoing x-ray (Brzakovic et al., 1990 Nassar et al., 2017, Sivaranjani 2019).

Early stage cancer is identified in mammography. In the early stage, pre-diagnosing tools are mandatory to identify the cancer cells. The mammography marks abnormal growth of cells exactly from the beginning point of cancer. Even tiny portion of abnormal tumours are also detected in this methodology. Breast cancer can occur in both genders, it can affect any parts in the body. Mainly it affects women, and severe stages it leads to death. Early detection of breast cancer increases the survival rate whereas late diagnosis results in patient to a critical stage and later to death. Digital Mammograms are the images of breast, which are used to find potential signs of breast cancer like tumours and abnormal changes in the skin (Siewertsz et al., 2016, Nassar et al., 2017, Sivaranjani 2019).

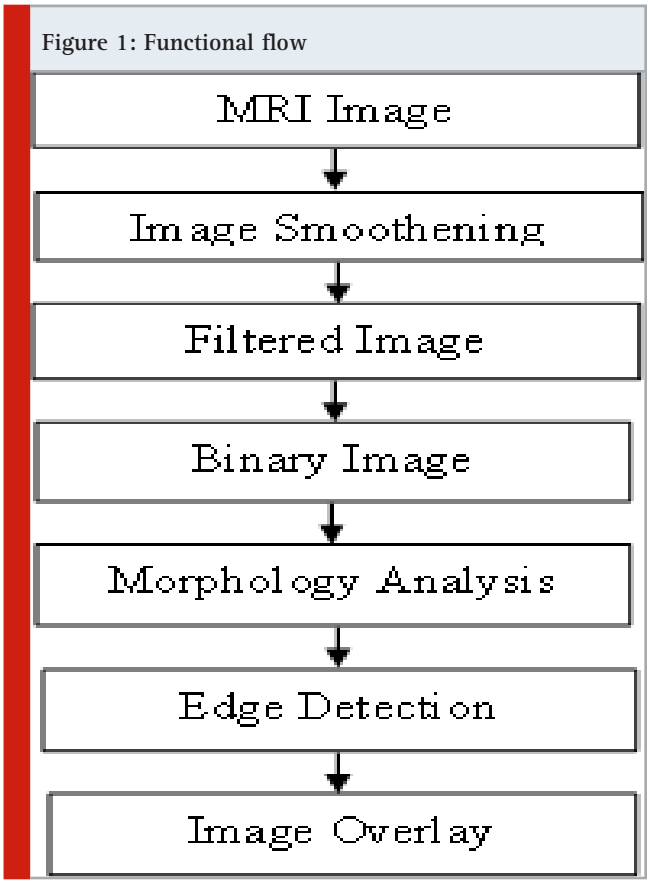
Based on the American Cancer Society (ACS) report, more number of women may affect breast cancer, considered the largest disease and it leads to death USA. Suffering from breast cancer usually increases with age. The cancer cells are initially found with X-ray Mammography and this technique is accepted as the suitable imaging technique to detect breast cancer. But the methodology is not producing better results for the patient with dense breasts and also it interpret patients to ionizing radiation. ACS recommended that a person with the family background or finding the symptoms related to breast cancer need to be checked every year, or at the age about 40, or every 10 years from they affected (Boyd et al., 2007). When viewing high risk of women, the overall detection rate of mammography is 36% and combined mammography and MRI detection rates 92.7% (Carkaci et al., 2011 Pradeep and Ribana 2018 Ribana and Pradeep 2018).

Automatic ultrasound imaging allows you to play image quality and reliability, and also eliminates user variability(Cheng et al., 2010). But it has some restrictions to use due to image resolution of scanners is limited (Kelly et al., 2010). Moreover, general practitioner should familiarize themselves with the interpretation of data sets. MRI process uses a larger range of magnets (3 to 5 Tesla) placed along with RF coils (Morris, 2003). MTI produces the 3D picture and locates the cancer cells. This method is too expensive because the Gadolinium is injected in the veins. So the person suffered from kidney disease should not undergo MRI scanning (Morris, 2003). Also, the person placed with Pacemaker or underwent any metal implant surgery are not recommended to take MRI (Morris, 2001).

MATERIAL AND METHODS

The worldwide researcher had authorized the Magnetic Resonance Imaging (MRI) for breast cancer diagnosis application. It is one of the drawback, which follows difficult to indiscriminate the normal cells from the cancerous organ (Nassar et al., 2017). Moreover, the existing methods classified the different kinds of analytical and numerical methods. The proposed methodology is implemented by following major four steps such as, Image Acquisition with display module, image transfer/storage, software development module and report retrieval module. The clinical images of mammogram collected from MIAS (Mammogram Image Analysis Database). These data sets translated into the matrix using 32 bit pixel depths such as 512 grey scale images. These image rescaled to smart phones. These data set can be varied from the colour brightness, quality of smart phone. These personal assistant devices connected with web servers via Internetworking Protocol (IP).

Figure 1: Functional flow



The PACS provide the image transferred to mobile phones over Transmission Control Protocol/Internetworking Protocols (TCP/IP). The proposed application implemented to DICOM receiver over HTTP (Hyper Text Transfer Protocol). Therefore, the web browser read the image of BMP format. It can be stored and delivered based on usage of network services such as, HSDPA (High

Speed Downloading Packet Access), EDGE, WiFi (Wireless Fidelity), Bluetooth, GPRS (General Packet Radio access Service), or any other external storage devices such as, removable memory card, pen drive and HDD (Hard derive disk).

The software development module consists of image processing and tumour detection with their classification units. The BMP image is stored in the Android operating system from mobile browser. The DICOM receiver offers the read and write operations for image files. It also capable of offered with web browsers for dynamic tasks such as, rescaling of images, panning image, User Interface (UI), Scalable Vector Graphics (SVG), HTML and interacting huge web pages and their applications. The tumour detection and their classification implemented by the CAD system. At instant, radiologist reviewed the clinical trials and their final report interconnected with medical professional via smart phones with encrypted key. They may be investigated the further medical suggestion. Therefore, the feedback reports interacted with patient through their own social media's accounts. The proposed system is able extended for continuous monitoring to patient, readiness for further medical processing and make awareness in risky factor of breast cancer.

Image processing is a tool for extracting information from a captured digitized image with some mathematical operations on it. Usually image is a two dimensional signal, while performing mathematical operations the useful information can be retrieved. To enhance the quality of MRI images proper denoising filter is to be used. Linear and nonlinear filtering techniques are commonly used on MRI image to increase the quality (Pradeep and Ribana 2018 Ribana and Pradeep 2018).

The Gaussian rule based Gabor filter is used to remove the noises. Little smoothing is done when the larger variance and for smaller variance smoothing is done with preserving edges. Contrast Limited Adaptive Histogram Equalization (CLAHE) algorithm is used to enhance the contrast in medical images. The algorithm is applied on smaller data region of images to improve contrast. The bilinear interpolation is incorporated to connect the neighbouring pixels (Ribana and Pradeep 2018). Sobel edge detection method is used for identifying the edges of tumours. Finally the tumours are detected with the help of Matlab, the identified cancer cells are tweeted to the doctors and care takers immediately to know the severity of the tumour. The flow diagram is depicted in figure 1.

ThingSpeak is mainly developed to visualize the data from IoT platform. It is also more helpful to pre-processing the data and analytics (Sivaranjani 2019). It a cloud based platform build on Ruby and helps the researchers and prototyping hobbyist to build their internet of things applications with wired or wireless connectivity. It collects the data from third-party resources and visualize instantly. The ThingSpeak helps the designers to run their applications from the cloud on time bases and it also update the received the information from cloud to Twitter or Twilio. Once the cancer cells are detected via the Matlab, the Thingspeak automatically tweet the information to the doctors and care takers.

Figure 2: The edge detected image and percentage of cancer cells occupied in the cancerous organ

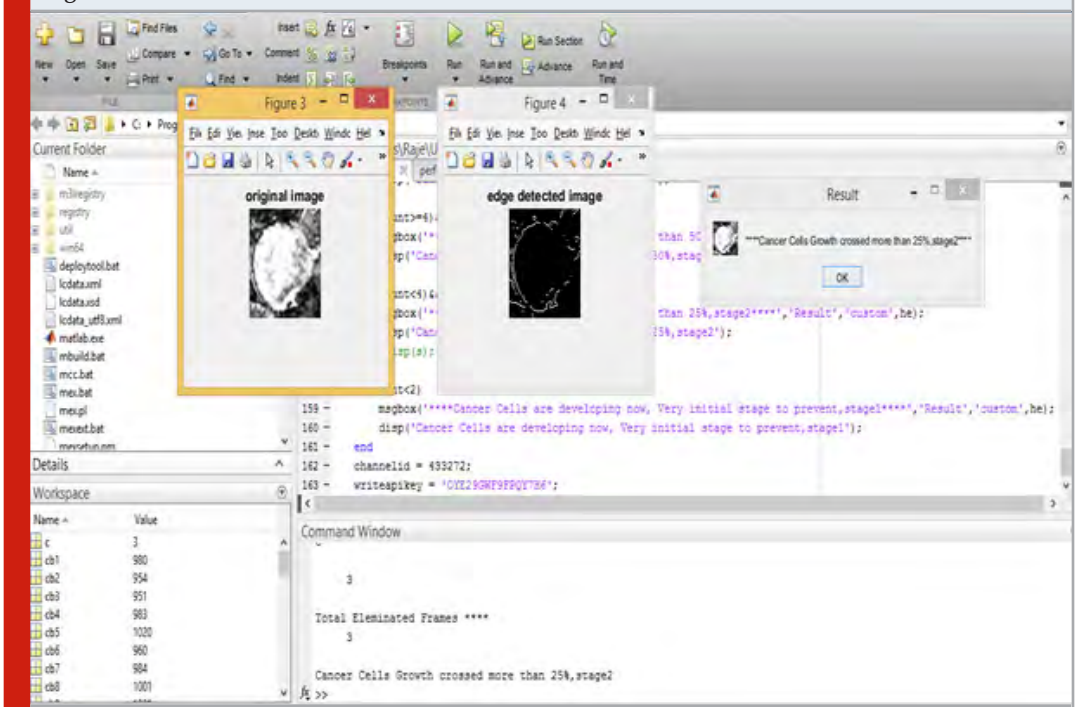
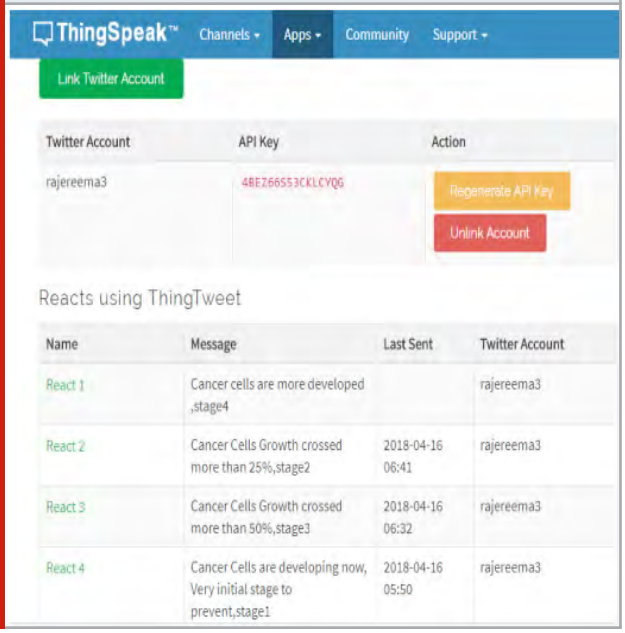


Figure 3: The numerical report simulates to Tweeter social media

Figure 4: Numerical analysed report from Thingspeak with Twitter/Twilio for percentage of the cancer cells with respect to different stages of the cancer



RESULTS AND DISCUSSION

In this proposed system is a mobile communication interacted with web application. Hence, it called as MammoTweet, which uses in the smart personal assistant device for radiologist, clinician and surgeon. It considers the less time computation, technical specification and ability of clinical reports. The MIAS datasets implemented with smart phones and Tweetspeak Apps. These clinical trials based on carcinogen death rate. Hence, it is considered as real time clinical trials. It is to prevent the cancer at early stage. It is to reduce the expenditure of clinical trials since, it provides depth analytical survey about the every trails. Moreover, it is preventing survive of 90% patient life time. Hence, it connected to eHealth care profession.

CONCLUSION

The breast cancer is one of the major health diseases for human society. The prevention for the major stage of cancer from human body and exchange the analytical report on medical professions through the social media as like Twitter/Twilio is delivered into this paper. These clinical trials provide the complete the clinical reports, which includes the statistical distribution of cancerous organ. These analytical reports on cancerous organ image has fixed for 81.25% of threshold value. Because Gaussian rules based Gabor filter adapted for pre-processing. Therefore, the smart phone based digitalized mammogram, which demonstrate the image retrieval, classification of trails and display toolset with covers the basic radiology function of today's medical requirements. These clinical reports fulfilments of medical professionals to analyse the quick suggestion.

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Phytochemical Screening and GC-MS Analysis of Flower Extract of *Dillenia indica*

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ABSTRACT

Dillenia indica is an important medicinal tree. Its leaf and bark extracts are being used as medicine due to the presence of several pharmaceutically important phytochemicals, but presence of phytochemicals in its flowers is still unexplored and there is utmost need to check medicinal efficacy of flower extract. Phytochemical screening of flower extract showed the presence of coumarins, steroids, saponins, flavonoids. GCMS study was done for the identification of bioactive compounds from the methanolic extract of flowers, which showed the presence of various phytochemicals such as Tetrabutyl Titanate, Fucoxanthin, Chromone, Beclomethasone, Betamethasone Acetate, Demecolcine etc. Most of these compounds have many pharmacological activities such as antibacterial, antimicrobial, cancer radiotherapy, cytotoxic activity, antioxidant etc., whereas some chemicals are of industrial importance. Some toxic chemicals were also reported. Present work shows pharmaceutical importance of flowers of *Dillenia indica*.

KEY WORDS: GCMS, PLANT EXTRACT, PHYTOCHEMICAL, SECONDARY METABOLITES.

INTRODUCTION

Dillenia indica is commonly known as Elephant Apple, belongs to family Dilleniacae. It is an ethnomedical plant and is being used for the treatment of severe disease (Shahin et al, 2015). Its stem bark, leaves, fruit and seed extracts show the presence of various active compounds such as polyphenols, tannins, alkaloids, steroids, saponins and flavonoids. Extracts of *Dillenia* have shown antileukemic, antioxidant, anti-inflammatory, antiproliferative, antidiabetic, hepatoprotective, antimicrobial and other pharmaceutically important activities (Gandhi & Mehta, 2013). Due to over exploitation, it is considered as rare plant in Egypt (Khalifa and Loutfy, 2006) and as an endangered plant in China (Qin et al, 2017). It is indigenous to Indonesia. It also occurs in Bhutan, Malaysia, China, Sri Lanka, Myanmar, Nepal, Philippines, Vietnam and Bangladesh. In India, it is distributed in Assam, North Bengal, Bihar, Orissa, Madhya Pradesh and Gujarat. However, no qualitative and quantitative studies were made for the extract of flowers. Present work shows GCMS study of floral extract of *Dillenia indica* for detailed information of phytochemicals.

Gas chromatography-mass spectrometry (GCMS) is widely used in pharmaceutical industries for the identification and quantification of secondary metabolites (Rukshana et al, 2017). It is considered sensitive and suitable method for the analysis of natural organic substances. The analysis provides the details of bioactive compounds

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present in the methanolic extract and retention time indicates the separation of compounds at different time interval. GCMS analysis of many plants has helped in the identification and characterization of phytochemicals present in their extracts (Socrates & Mohan, 2019; Eswaraiah et al. 2020).

Figure 1 (A): Tree of *D.indica* (B)Flower

MATERIAL AND METHODS

Flowers of *Dillenia indica* were collected from the plant growing in Ayurvedic garden, Dravyaguna, Institute of Medical Sciences, Banaras Hindu University, Varanasi (Fig.1).

Phytochemical Screening: Shade dried flowers were made into fine powder by mixer grinder. The powder of flower (5 gm) was added to 50 ml of distilled water and was boiled at 60° C for 10 min. Boiled extract was filtered with Whatman filter paper. Filtered solution was boiled till the formation of chocolate colored powder. This powdered plant extract was used for preliminary phytochemical screening. Aqueous plant extract was prepared by dissolving 25 mg of crude extract to 25 ml of double distilled water. Presence or absence of phytochemicals was observed by performing following tests with minor modifications: For the test of Coumarins (Rizk, 1982), aqueous plant extract (2 ml) was added to 3ml of 10% NaOH. The confirmation of coumarins was observed by the change in colour. Formation of yellow colour shows the presence of coumarins. To detect the presence of Saponins, foam test was done (Kumar et al, 2009). In this test, plant extract (2 ml) was mixed in 6 ml of DDW and was shaken vigorously.

Presence of foam is confirmation for saponins. To observe the presence of phenol (Gibbs, 1974) in plant extract, 2 ml of aqueous extract was added to 2 ml 5% of aqueous ferric Chloride (FeCl3). If deep blue or black color is observed, then it indicates the presence of phenol. Presence of Flavonoids was observed by Alkaline Reagent Test and 2 ml plant extract was taken and few drops of 1N NaOH were added. Presence of flavonoids will be observed if it turns to yellow color and becomes colorless after adding dilute HCl. For detection of Tannins (Braymer's Test, Ugochukwu et al, 2013), 2 ml extract was added to 3 ml 10% alcoholic ferric chloride solution. Formation of blue or greenish color will indicate the presence of tannins. Test for Quinones (Ramya et al., 2015) was done by treating 2 ml plant extract with 4 ml of dilute Sodium hydroxide. If blue green or red color is formed, it will show the presence of quinone. To see the presence of Phlobatannins (Precipitate Test, Auwal et al, 2014),

1ml of extract was treated with 2ml of 1% hydrochloric acid. Then mixture was boiled. Observation of red precipitation will confirm the presence of phlobatannins. In the test for Alkaloids (Mayer's Test, Auwal et al, 2014), 2ml of extract was added to 0.5 ml of Mayer's reagent. White creamy precipitate formation is indicative of the presence of alkaloids. Fehling solution test was done to observe the presence of carbohydrate. Mixture of equal volume of Fehling solution A and B was made. Extract (2 ml) was added to 2 ml of Fehling solution and then it was boiled in water bath for 30 minute. Red precipitate will indicate the presence of carbohydrate. For observing the presence of Anthocyanins (Paris and Moyse, 1969), 2 ml extract was treated with 2N Hydrochloric acid. If pink-red color appears, which turns into blue-violet after addition of Ammonia, then it shows the presence of anthocyanins. For detection of fatty acid (Ayoola, 2008), 1 ml of extract was mixed with 3 ml of ether. It was poured on the filter paper and was evaporated till the filter paper becomes dried. Transparent filter paper indicates the presence of fatty acid. For confirmation of Steroids (Salkowski reaction, Shear & Kramer, 1926), 1ml of extract was dissolved in 10 ml chloroform. Concentrated sulfuric acid was added by side of test tube wall, appearance of red and yellow color in the upper and lower layer, respectively along with green florescence indicates the presence of steroids.

Table 1. Phytochemical analysis of *D. indica* flower

S.N.	Phytochemicals	Flower
1.	Tannins	-
2.	Coumarins	+
3.	Flavonoids	+
4.	Steroids	+
5.	Anthocyanin	-
6.	Saponin	+
7.	Quinones	-
8.	Phlobatnins	-
9.	Fatty Acid	-
10.	Phenolics	-
11.	Alkaloids	-
12.	Carbohydrates	-

Preparation of extract and methodology of GCMS: Shade dried flowers were made into fine powder by mixer grinder. Flower powder was incubated with 25ml 95% methanol and kept for 72 hour. Methanolic extract was filtered by using Whatman filter No.1 (pore size 0.4 µm). GCMS analysis of filtered extract was done to get the details of bioactive compounds.Methanolic extract was injected in the port of the Gas chromatography (GC) device. Here the extract got vaporized and spectral peaks of various phytochemicals was recorded on chromatogram. GCMS analysis of methanolic extract of flower of the plant was performed using

a THERMOSCEINTIFIC Gas Chromatography-TRACE ULTRA VER: 1100. and mass spectrometry- TSQ Duo. The oven temperature was maintained at 220°C at a rate of 6°C/min and flow rate of carrier gas was adjusted at 1 ml/min. The column of the GC was TG-5MS. Different parameters of the column were as such- the length of the column: 30 m, the diameter: 0.25nm and the thickness of the film: 0.25µm. The GCMS programming were done as follows: Injector temperature 215°C,

Transfer line 218o C, oven temperature program 80-280°C with ramping 5°C per min, carrier gas: Helium at 1.5 mL/min, individual components were identified by NIST MS 2.0 structural library.The split sampling technique was used to inject the sample in the ratio of 1:10. The time elapsed between elution and injection was recorded as the "retention time".

Figure 2: GCMS analysis and Chromatogram of methanolic extract of D. indica Flower

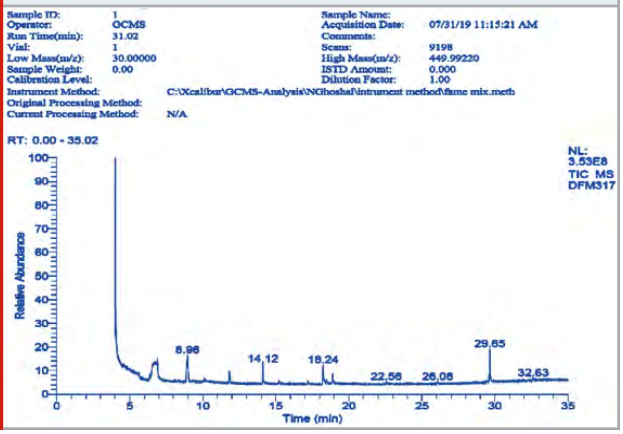
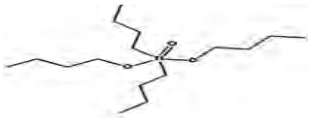
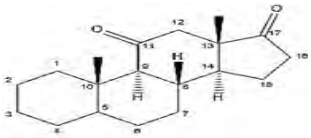
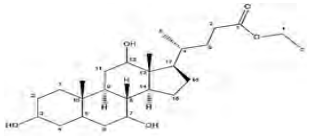
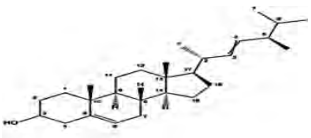
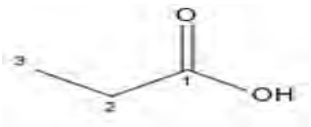
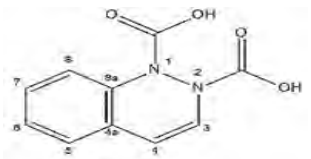
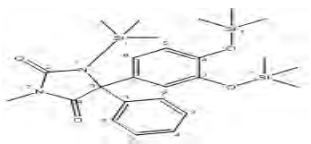
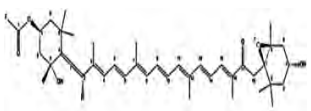
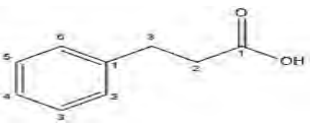


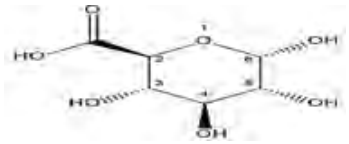
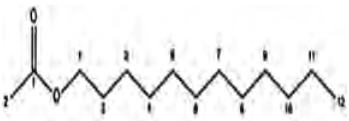
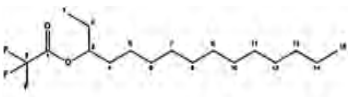
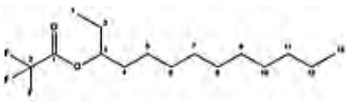
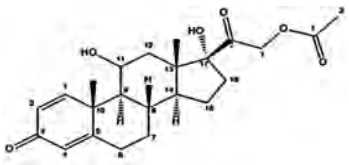
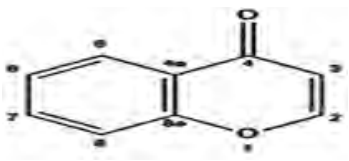
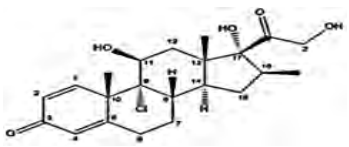
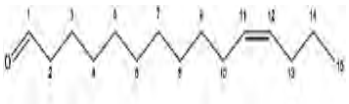
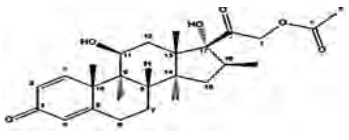
Table.2 Bioactive compounds present in methanolic extract of *D.indica* Flowers

S.N.	COMPOUND	M. W.	M. F.	%AREA	RT
1.	TETRABUTYL TITANATE	344.35	$C_{16}H_{40}O_4Ti$	0.35	4.35
2.	ANDROSTANE-11,17-DIONE	260.5	$C_{19}H_{32}$	0.07	6.21
3.	ETHYL ISO-ALLOCHOLATE	436.6	$C_{26}H_{44}O_5$	1.02	6.36
4.	ERGOSTA-5	394.6	$C_{28}H_{42}O$	1.02	6.36
5.	PROPANOIC ACID	74.08	C3H6O2	0.04	7.22
6.	1,2-CINNOLINEDICARBOXYLIC ACID	173.17	$C_{10}H_7NO_2$	0.04	7.22
7.	2,4-IMIDAZOLIDINEDIONE, 5--[3,4-BIS[(TRIMETHYLSILYL)OXY] PHENYL]-3-METHYL-5-PHENYL-1-(TRIMETHYLSILYL)	516	$C_{25}H_{40}N_2O_4Si_3$	0.05	7.29
8.	FUCOXANTHIN	658.91	$C_{42}H_{58}O_6$	0.09	7.36
9.	BENZENE PROPANOIC ACID	152.19	$C_9H_{12}O_2$	0.13	7.46
10.	α-D-GLUCOPYRANOSIDURONIC ACID	208.17	$C_7H_{12}O_7$	0.13	7.46
11.	LAURYL ACETATE	228.37	$C1_4H_{28}O_2$	0.47	8.34
12.	3-TRIFLUOROACETOXYPENTADECANE	324.4	$C_{17}H_{31}F_3O_2$	0.15	9.29
13.	3-TRIFLUOROACETOXYTRIDECANE	296.37	$C_{15}H_{27}F_3O_2$	0.15	9.29
14.	PREDNISOLONE ACETATE	402.5	$C_{23}H_{30}O_6$	0.19	11.21
15.	CHROMONE	146.14	$C_9H_6O_2$	0.19	11.21
16.	BECLOMETHASONE	408.9	$C_{22}H_{29}ClO_5$	0.06	11.34
17.	Z-11-PENTADECENAL	224.38	$C_{15}H_{28}O$	0.19	13.49
18.	BETAMETHASONE ACETATE	434.5	$C_{24}H_{31}FO_6$	0.21	16.49
19.	17-PENTATRIACONTENE	490.9	$C_{35}H_70$	0.1	20.27
20.	DEMECOLCINE	371.4	$C_{21}H_{25}NO_5$	0.1	23.06
21.	ACETIC ACID	60.052	CH_3COOH	0.13	23.28
22.	ISOXAZOLE	69.06	C_2H_3NO	0.02	26.22
23.	PHTHALIC ACID	166.14	$C_8H_6O_4$	6.67	29.65
24.	DIISOOCTYL PHTHALATE	390.55	$C_{24}H_{38}O_4$	6.67	29.65
25.	HEXA-T-BUTYLSELENATRISILETANE	505.90	$C_{24}H_{54}SeSi_3$	0.04	29.93
26.	NORMORPHINE	271.31	$C_{16}H_{17}NO_3$	0.05	29.98
27.	PROSTAGLANDIN	354.5	$C_{20}H_{34}O_5$	0.04	33.04

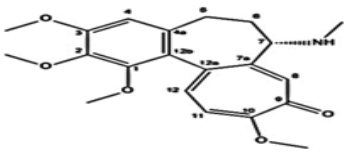
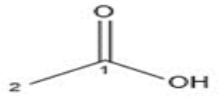
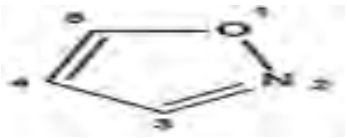
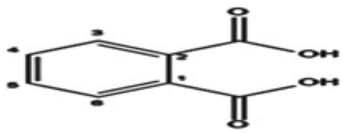
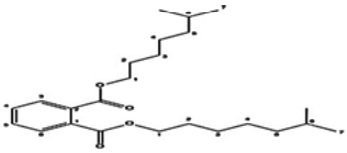
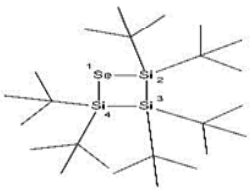
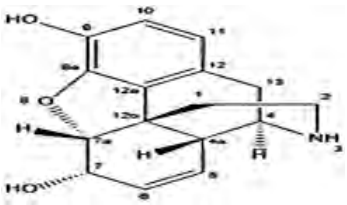
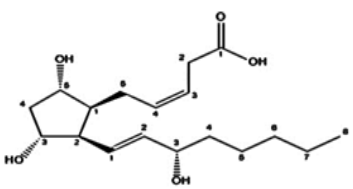
Table 3. Compound Structures with their Biological activities

S.N.	Compound Name	Biological Activity	Reference
1.	 TETRABUTYL TITANATE	Antibacterial	Zhang & Zhang,2014
2.	 ANDROSTANE-11,17-DIONE	Steroid	Hamalainen et al, 1991
3.	 ETHYL ISO-ALLOCHOLATE	Antimicrobial	Malathi & Ramaiah, 2017
4.	 ERGOSTA-5,22-DIEN-3-OL	Anti-tumor activity and immunomodul atory activity	Hussein et al, 2016
5.	 PROPANOIC ACID	Uses in the food industry but has recently found applications in the cosmetic, plastics and pharmaceutical industries.	Gonzalez- Garcia et al, 2017
6.	 1-,2-CINNOLINEDIC ARBOXYLIC ACID	Antitumor, antiviral, aestrogenic	Hassan et al, 2016
7.	 2,4-Imidazolidinedione, 5-[3,4-bis [(trimethylsilyl)oxy]phenyl]-3-methyl-5-phenyl-1-(trimethylsilyl)-	Used as modulator of Kv3.1 and/or Kv3.2 for the treatment of depression, schizophrenia, mood disorders, sleep disorders, etc.	Alvero et al, 2012
8.	 Fucoxanthin	Antioxidant, anti-obesity, anti-diabetic, anticancer, and antimicrobial	Karpinski & Adamczak, 2019
9.		Treatment of Polycystic kidney disease	Mei, 2006

Continue Table 2

	BENZENEPROPANOIC ACID		
10.	 α-D-GLUCOPYRANOSIDURONIC ACID	Antibacterial	Lou & Cassidy,2010
11.	 LAURYL ACETATE	Flavoring agents, skin permeation enhancer, Industrial use	Burkoth et al, 1996
12.	 3-TRIFLUROACETOXYPENTADECANE	Anti-nephrotoxic and antioxidant activities	Hussein et al, 2016
13.	 3-TRIFLUROACETOXYTRIDECANE	Uses in chemical industry Toxic to aquatic life, harmful to skin and eyes	Anonymous, 2018
14.	 PREDNISOLONE ACETATE	Intraocular anti-inflammatory	Stanley, 2008
15.	 CHROMONE	Asthma, Antimicrobial, Anticancer, anti-inflammatory, Antioxidant	Tawfik et al, 2014
16.	 BECLOMETHASONE	Uses in chronic asthma	Adams et al,2002
17.	 Z-11-PENTADECENAL	Bioleum (industrial)	Wang, 2013
18.	 BETAMETHASONE ACETATE	Dry eye control	Shokoohi-Rad et al (2018)
19	 17-PENTATRIACONTENE	Antioxidant, antitumour, antiviral, hypolipidemic	Enema et al, 2019

Continue Table 2

20.	 DEMECOLCINE	Chemical enucleant	Fernandes et al, 2007
21.	 ACETIC ACID	Effective disinfectant, wine & bakery industry	Togashi et al, 2004; Cortesia et al, 2014
22.	 ISOXAZOLE	Immunomodulatory	Zimecki et al, 2018
23.	 PHTHALIC ACID	Plasticizer, causes several human health hazards	Bang et al, 2011
24.	 DIISOOCTYL PHTHALATE	Plasticizer (industrial), Adhesive, Antiandrogenic activity	Saillenfait et al, 2013
25.	 HEXA-T-BUTYLSELENATRISILETANE	Use not reported in literature	
26.	 NORMORPHINE	Analgesic, neurotoxic	Oguri et al, 1989 Glare et al, 1990
27.	 PROSTAGLANDIN	Acute anterior uveitis, Abortifacient, Rheumatoid arthritis	Miller et al, 1973 Keirse,1992; Fattahi and Mirshafiey, 2012

The peak was measured from the base to the tip of the peak. Retention index of the compounds were identified by comparing the retention times and identification of each component was confirmed by the comparison of its retention index with data in the NIST library. Interpretation of Mass-Spectrum was carried out by using the database of the National Institute Standard and Technology (NIST) having more than 62,000 patterns. Spectrum of the known compound which are stored in NIST library was used to compare the spectrum of unknown component. The molecular weight, name, chemical structure and molecular formula of the components of the test materials were ascertained.

RESULTS AND DISCUSSION

GCMS is a technique that combines the separation of phytochemicals by gas chromatography and their detection by mass spectroscopy (Chauhan et al., 2014). Various parts of *Dillenia indica* i.e.fruit, leaf and bark have shown the presence of many primary and secondary metabolites (Barua et al, 2018). Present work shows preliminary screening of phytochemicals of aqueous flower extract of *Dillenia indica* and the presence of some very important phytochemicals such as coumarins, flavonoids, steroids and saponin were observed (table-1).

The peak in GCMS of methanolic extract of the flower of *Dillenia indica* showed the presence of the secondary phytochemical compounds like phenolic and fatty acids and other medicinally important bioactive compounds. GCMS analysis of methanolic flower extract showed significant presence of 27 phytochemicals (Fig. 2 and Table-2). The most abundant 27 compounds found in the methanolic extract of flowers were TetrabutylTitanate, Fucoxanthin, Ergosta-5, Lauryl Acetate, Beclomethasone, Betamethasone Acetate, Demecolcine,Androstane-11,17-Dione, Ethyl Iso-Allocholate, 1,2-Cinnolinedicarboxylic Acid, 2,4-Imidazolidinedione, Benzene Propanoic Acid, α-D-Glucopyranosiduronic Acid, Lauryl Acetate, 3-Trifluoroacetoxypentadecane, 3-Trifluoroacetoxytridecane, Prednisolone Acetate, Chromone, Z-11-Pentadecenal, 17-Pentatriacontene, Acetic Acid, Isoxazole, Phthalic Acid, Diisooctyl Phthalate, Hexa-T-Butylselenatrisiletane, Normorphin, Prostaglandin. The structures of above compounds have been mentioned in Table-3.

Many of above compounds are pharmaceutically important and their medicinal efficacies have been reported by many researchers as antibacterial, anti-inflammatory, and antiviral activities (Table 3). Some compounds show their industrial applications, whereas few compounds also show toxicity (Table 3). GCMS is an integrative technique for separation, identification and quantification of chemicals in a given sample (Leary et al, 2019). Quantification is an important step for data analysis and different softwares are being used for the calculation of retention time corresponding to specific peaks (Johnsen et al, 2017). The technique has great applications in pharmaceutical industries as it helps in the identification of bioactive compounds as well as any impurities present in the plant extract (Chauhan et al., 2014). In present study, several compounds have been identified from flower extract. Pharmaceutical activities of many compounds have been reported earlier by several researchers (Table- 3), which indicates potential of flower for the production of medicines. Therefore, like leaves and bark, flowers of *D. indica* too has significant medical efficacy. Uses of other parts of *Dillenia indica* as traditional medicines are already in practice. Now, there is need to use flower extract as well.

CONCLUSION

In the present investigation, 27 bioactive compounds have been identified from the methanolic extract of D. indica by GC-MS. The presence of various bioactive compounds in D. indica proved pharmaceutical and medicinal importance. However, further research is needed in order to analyze its bioactivity and toxicity profile.

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Conflict of interest: The authors declare no conflict of interest.

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Adaptation of an Indigenous Insect Cell Line DZNU-Bm-1 to Mitsuhashi and Maramorosch (MM) Culture Medium Supplemented With 3% Foetal Bovine Serum

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ABSTRACT

Although utility of insect cell lines for production of proteins of human interest has been scaled up from experiment-level to industrial production level, use of vertebrate sera is discouraged during industrial production of therapeutics. DZNU-Bm-1, a larval ovarian cell line is highly susceptible to BmNPV in MGM-448 medium supplemented with 10% Foetal Bovine Serum and can be used as IC-BEVS platform for foreign gene expression. The high cost of this medium is a serious bottleneck for use of DZNU-Bm-1 in production of therapeutics. This study reports reduction in serum dependence of this cell line and its successful adaptation to a low-cost Mitsuhashi and Maramorosch (MM) medium supplemented with 3% FBS to bring down the cost of maintaining the cell line. Adaptation of MM medium was achieved by decreasing the volume of original medium by 20% and increasing the volume of the new medium -to which the cell line was being adapted- by 20% at each passage. The initial cell density, final cell density, number of days taken to attain final cell density and population doubling time were noted at each passage. The cell line was slow to reach sufficient cell density during initial steps but showed reduced population doubling time during subsequent passages

KEY WORDS: DZNU-Bm-1; INSECT CELL LINE; ADAPTATION; POPULATION DOUBLING TIME.

INTRODUCTION

Insect cell lines have found application in a wide array of fields including cell biology, genetics, virology and agriculture. Insect cells have many advantages over mammalian cells in culture, being able to tolerate a broad range of environmental conditions, to proliferate and maintain physiological function in variable pH, temperature, and oxygen conditions (Akiyama et al., 2013). Their ability to grow without carbon dioxide supplementation has further simplified culture systems and has made them an economically viable alternative to mammalian cells (Irons et al., 2018). Insect cells exhibit many additional desirable characteristics like easy adaptation to suspension and serum-free culture as well as low media requirements. These qualities point to possibility of their large-scale culture (Neermann and Wagner, 1996; Donaldson and Shuler, 1998; Lynn, 2001; Ikonomou et al., 2003; Mitsuhashi and Goodwin, 2018).

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Recently, Rubio et al. (2019) have suggested that pursuing development of insect cell-based foods may lead to new sustainable food products thereby accelerating the field of cellular agriculture. Trager's (1935) success in using inorganic salts, maltose, digested egg albumin and *B. mori* haemolymph to maintain ovarian tissues of *B. mori* spawned a wave of experiments to formulate growth media for culture of insect cells. Mitsuhashi (1982, 1984) successfully modified Grace's medium several times for cultivation of different cell lines. Supplementation with vertebrate sera like foetal bovine serum (FBS) or other proteinaceous materials has been successfully used to get high growth rate (Weiss and Vaughn, 1986). Gradually with increase in interest of researchers in insect cell culture, many serum-free media have been developed (Hink, 1991; Donaldson et al., 1998). Caron et al. (1990) reported high growth rate by increasing total Pluronic F-68 concentration to 0.3% with IPL/41 serum-free medium. Lepidopteran cell lines are now being viewed as an attractive alternative to mammalian cell lines for biomanufacturing of proteins of human interest, biopesticides and vaccines (Kost et al., 2005; Drugmand et al., 2012; Airenne et al., 2013; Van Oers et al., 2015).

Over the years the utility of insect cell lines for production of proteins of human interest has been scaled up from experiment-level to industrial production level (Elias, 2007). Use of vertebrate sera is discouraged during industrial production of therapeutics because of inherent potential risk for transmission of infectious agents as well as the heterogeneity and lack of reliability (Grillberger et al., 2009). Insect cell lines that have high susceptibility to a baculovirus can be used to develop efficient Insect Cell-Baculovirus Expression Vector Systems (IC-BEVS) (Agathos, 2010). An efficient IC-BEVS production platform can be used for foreign gene expression, production of therapeutic compounds and vaccines. DZNU-Bm-1, a larval ovarian cell line has been shown to be highly susceptible to BmNPV in MGM-448 medium (Khurad et al., 2006) and can be used as IC-BEVS platform for foreign gene expression (Khurad et al., 2013).

However, the high cost of MGM-448 medium with supplementation of 10% Foetal Bovine Serum (FBS) is a serious bottleneck for its use in production of therapeutics or other proteins of interest. The present study describes successful reduction in the serum dependence of the cell line DZNU-Bm-1 by first adapting it to MGM 448 growth medium supplemented with 3% FBS and then to a low-cost Mitsuhashi and Maramorosch (MM) medium supplemented with 3% FBS to reduce the cost of maintaining this cell line.

MATERIAL AND METHODS

Culture Media: Modified Grace's medium MGM-448 (Mitsuhashi, 1984) and Mitsuhashi and Maramorosch medium (Mitsuhashi and Maramorosch, 1964) were prepared in laboratory. Modified Grace's Medium (MGM-448): MGM-448 is a complex medium composed of six salts, twenty amino acids, three sugars, four organic acids, ten vitamins and four additives, inosine, cytochrome-c, fetuin and bovine plasma albumin fraction-V. Five stock solutions viz. MGM O.S.A., GMA-salt-mix-A, GMA-salt-mix-B, GMA-Vita-mix-IA, GMA-Vita-mix-IIB were prepared as described by Mitsuhashi (1984).

In order to prepare 250 ml of complete MGM-448 medium, required quantities of respective stock solutions were mixed and the double distilled water was added to make up the volume. To this solution bovine plasma albumin fraction-V, fetuin, cytochrome- c, inosine were added. The medium was supplemented with required quantity (3% or 10%) of FBS. The pH was adjusted to 6.37-6.40 with saturated KOH. The medium was sterilized by passing through 0.2 µm pore size Millipore membrane filter using negative pressure through Sartorius filter unit. No antibiotics were added to the medium.

Mitsuhashi and Maramorosch medium (MM Medium): It is among the simplest insect tissue culture media. It is a mixture of salts used in Carlson's Balanced Salt Solution (CBSS), lactalbumin hydrolysate, TC-yeastolate and glucose supplemented with 3% Foetal Bovine Serum (FBS). For preparation of this medium, stock solution A and stock solution B of CBSS were prepared. 25 ml of each stock solution was mixed. The additives were added and the volume was made up to 250 ml by adding double distilled water. The medium was supplemented with 3% FBS. Sterilization was carried out by passing through 0.2 µm pore size Millipore membrane filters using negative pressure through Sartorius filter unit. No antibiotics were added to the medium.

Maintenance of cell line and subculturing: DZNU-Bm-1 a larval ovarian cell line from B. mori was kindly provided by Dr. A. M. Khurad. The cell line was being cultured in MGM-448 medium supplemented with 10% FBS. The cell line was maintained in the laboratory in glass tissue culture flasks, incubated at 25± 1^0C and passaged regularly. After some time, the cell line could be subcultured regularly by splitting the cultures in a ratio of 1:2 at an interval of 4-5 days.

Adaptation to MGM-448 medium supplemented with 3% FBS: The cell line was first adapted to MGM-448 supplemented with 3% FBS to reduce its serum dependence as described by Mitsuhashi and Grace (1969) through passaging of the cells. This was done by decreasing the volume of original medium (MGM-448 supplemented with 10% FBS) by 20% and increasing the volume of MGM-448 medium supplemented with 3% FBS by 20% at each passage. The initial cell density, final cell density, number of days taken to attain final cell density and population doubling time were noted at each passage.

Adaptation to MM medium supplemented with 3% FBS: The cell line was subsequently adapted to MM medium supplemented with 3% FBS. The volume of MGM-448 supplemented with 3% FBS was reduced while the volume of MM medium supplemented with 3% FBS

was increased through regular passaging. During each passage, the volume of original medium was reduced by 20% while the volume of MM medium supplemented with 3% FBS was increased by 20%. The process was repeated till MGM-448 supplemented with 3% FBS was completely replaced with MM medium supplemented with 3% FBS. The initial cell density, final cell density, number of days taken to attain final cell density and population doubling time were noted at each passage.

Population Doubling Time: Cell suspension was sampled from culture flasks at the time of seeding the culture and on attainment of final cell density. The cell suspension was allowed to flow in the chambers of Neaubauer haemocytometer chamber and cells were counted in four large corner squares each of which is divided into 16 small squares. The volume of one large square is 0.1 mm^3. Therefore, average cell count was multiplied by 104 to give number of cells per ml. The cell number was determined as an average of readings from two culture flasks. The population doubling time (PDT) was calculated using the exponential formula given by Hayflick (1973).

$$r = 3.32 (\text{Log } x_2/x_1)/t_2\text{-}t_1$$

where,
r = Multiplication rate,
x_1 = Initial cell number at selected time t_1,
x_2 = Final cell number at selected time t_2,

Generation time (g) = 1/r

Cell Morphology: The cell cultures were observed under MAGNUS INVI inverted phase contrast microscope and photographed regularly after they were adapted to MM medium supplemented with 3% FBS. Cell population comprised of different cell types. The cell size of each type was determined by ocular micrometer in the population taking about 50-100 readings. The relative percentage of each type in population was determined by counting different cell types in optical fields under the inverted microscope.

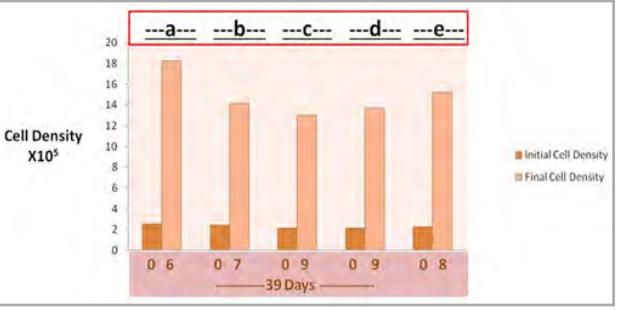
Karyotype Analysis: Karyotypic studies were carried out in healthy cell cultures during exponential cell growth phase after their successful adaptation to MM medium supplemented with 3% FBS. Demicolcine (Sigma) having a concentration of 50 µg/ml was added to culture flasks to arrest the cell division at metaphase. Final concentration of demicolcine in the culture flask was 1 µg/ml of medium. The cultures were allowed to stand at 25°C for 24-72 hours. The cells were harvested by centrifugation and the supernatant was removed. The pellet was resuspended in hypotonic solution (0.6% KCl) for 15-20 minutes. The cells were again centrifuged and the cell pellet was fixed in 50% glacial acetic acid for 10-15 minutes. The fixed cells were spread on glass slides and allowed to air dry. The slides were washed in distilled water, transferred to Acetorcein stain for 20 minutes, washed briefly in running tap water, air-dried for 12 hours, cleared in two changes of xylene and mounted in DPX. About 50 chromosome spreads were counted and spreads were photographed to determine the range of chromosomes.

Table 1. Population Doubling Time of DZNU-Bm-1 during adaptation from MGM-448 medium supplemented with 10% FBS (A) to MGM-448 medium supplemented with 3% FBS (B).

No. of Days after Seeding of Cultures	Step	Medium Composition	Population Doubling Time in Hours
06	a	80% A + 20% B	52
07	b	60% A + 40% B	68
09	c	40% A + 60% B	83
09	d	20% A + 80% B	81
08	e	100% B	70

(A)= MGM-448 medium supplemented with 10% FBS
(B)= MGM-448 medium supplemented with 3% FBS

Figure 1. Graph showing adaptation of DZNU-Bm-1 from MGM-448 medium supplemented with 10% FBS (A) to MGM-448 medium supplemented with 3% FBS (B). a– 80% A + 20% B; b- 60% A + 40% B; c- 40% A + 60% B; d– 20% A + 80% B; e– 100% B



RESULTS AND DISCUSSION

In the first stage of experiment, DZNU-Bm-1 cell line originally cultured in MGM-448 medium supplemented with 10% FBS was successfully adapted to MGM-448 medium supplemented with 3% FBS through several passages. The cell line took about 39 days for adaptation (Fig.1). DZNU-Bm-1 has been reported to take more than 10 months for adaptation to haemolymph-free MGM-448 (Bahekar, 2018). The cells remained freely suspended in culture medium. Cell clumping reported in earlier studies (Bahekar and Qureshi, 2013) was not observed. During step b of adaptation the cells showed reduced growth (Fig. 1). The growth of cells during step c and step d was slowest (Fig. 1). During each of these steps cells required 9 days to reach sufficient cell density for subculturing. The population doubling time was also highest during these steps showing a peak value of about 83 hours (Table 1). Belloncik et al. (1990) have reported slow

growth rate and other difficulties during adaptation. In the present study, by step e the population doubling time was reduced to about 70 hours. After further passages in the same medium population doubling time was reduced to about 49 hours.

The cell line was later adapted to more cost-effective MM culture medium supplemented with 3% FBS. The cell line was easily adapted to MM medium taking about 32 days for adaptation (Fig. 2). Growth of cells was slow during step b and c requiring about 7 days each for attaining sufficient cell density for subculture. Population doubling time was maximum (about 81 hours) during step b (Table 2). During next subcultures the cell line could be passaged by day 6 with population doubling time being reduced to about 54 hours. There was no significant change in cell morphology after adaptation to MM medium supplemented with 3% FBS. The population of DZNU-Bm-1 cell line is heterogeneous and can be differentiated into four cell types- large round, small round, giant cells and spindle shape - on the basis of their shape and size.

Table 2. Population Doubling Time of DZNU-Bm-1 during adaptation from MGM-448 medium supplemented with 3% FBS to MM medium supplemented with 3% FBS (B)

No. of Days after Seeding of cultures	Step designation	Medium composition	Population Doubling time in Hours
06	a	80% A + 20% B	48
07	b	60% A + 40% B	81
07	c	40% A + 60% B	70
06	d	20% A + 80% B	53
06	e	100% B	54

(A)= MGM-448 medium supplemented with 3% FBS
(B)= MM medium supplemented with 3% FBS

Figure 2: Graph showing adaptation of DZNU-Bm-1 from MGM-448 medium supplemented with 3% FBS (A) to MM medium supplemented with 3% FBS (B). a– 80% A + 20% B; b- 60% A + 40% B; c- 40% A + 60% B; d– 20% A + 80% B; e– 100% B

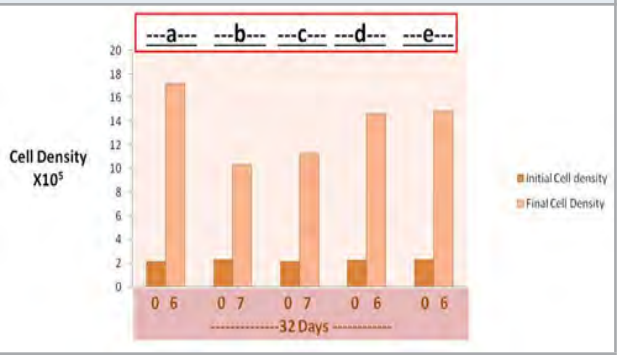
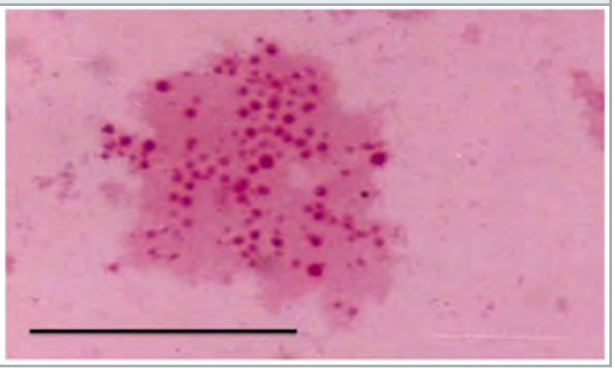


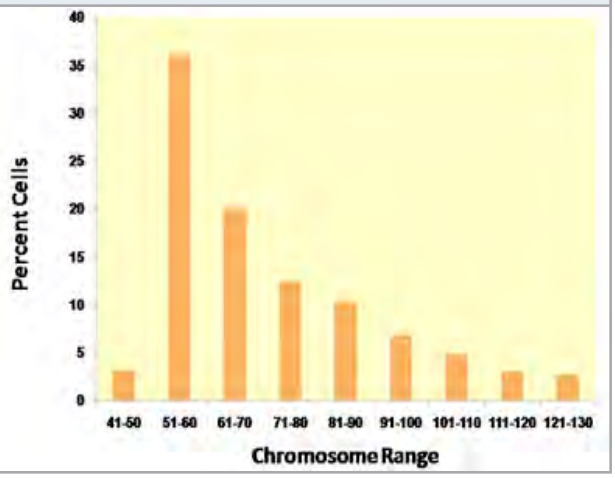
Figure 3: Chromosomes of DZNU-Bm-1 cell line grown in MM culture medium supplemented with 3% FBS. Bar= 30 µm



Large round cells: They are round, measuring about 31 ± 0.62 µm in diameter. Their relative percentage in the population is about 30.2%. Initially, they showed partial attachment to the surface of culture flask immediately after subculture.

Small round cells: The small round cells measure about 13.74 ± 0.16 µm. They form 53.4% of the total cell population.

Figure 4: Graph showing distribution of chromosome number in DZNU-Bm-1 cells.



Giant cells: These are very large round cells having a diameter of about 69 ± 3.22 µm. They form 4.7% of the total cell population.

Spindle-shape cells: These cells have protoplasmic processes on the sides giving them appearance of a spindle. Their size is about 51 ± 3.42 µm × 11 ± 0.51 µm. Their percentage in cell population is 11.7%.

The chromosome spreads of cells exhibited numerous microchromosomes that resembled the chromosomes of DZNU-Bm-12 cell line (Khurad et al., 2009). The chromosomes appeared as numerous dot-like, darkly stained bodies (Fig. 3). The diploid number of *B. mori* chromosomes has been known to be 56. The chromosome

number in the cell line ranged from 45 to 126 with a mode towards diploidy. A few cells also possessed more than 100 chromosomes indicating the presence of polyploid cells (Fig. 4). Grace (1967) has reported more than 100 chromosomes in the cells of B. mori ovarian cell line. Chromosome range of 35 to 150 in larval ovarian cell line and 60 to 180 in the pupal ovarian cell line of *B. mori* have also been reported (Sudeep et al., 2002).

CONCLUSION

In the present study DZNU-Bm-1 cell line being cultured in MGM-448 medium supplemented with 10% FBS was first adapted to MGM-448 medium supplemented with 3% FBS. The cell line was slow to reach sufficient cell density during initial steps but showed reduced population doubling time during subsequent passages. The cell line was then successfully adapted to cost effective MM medium supplemented with 3% FBS. The morphological and cultural characteristics as well as the chromosome range of the cells did not exhibit any significant change from those cultured in MGM-448 (10% FBS). DZNU-Bm-1 has earlier been shown to be able to produce foreign proteins. The current study establishes its reduced serum dependence and ability to grow in low-cost culture media raising hopes of adapting it to other serum-free media.

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Phytochemical Screening and Antibacterial Activity of Three Medicinal Plants – *Lawsonia inermis*, *Mangifera indica* and *Piper betel*

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ABSTRACT

Medicinal plants play a predominant role in our ecosystem due to the vast distribution of bioactive components such as alkaloids, phenols, tannins, flavanoids etc... within them and each with a specific function. A study on these bioactive compounds and their beneficial roles is of much interest and are of utmost importance these days due to their wide therapeutic properties. The present work has been carried out in order to investigate the presence of important bioactive components in different solvent extracts of *Lawsonia inermis L., Mangifera indica L.* and *Piper betel L.* leaf samples and thereby testing their antibacterial activity. Cold extraction method was employed for extraction of leaf samples using different solvents such as ethanol, chloroform, ethyl acetate and water (aqueous). The extracts were subjected to both qualitative and quantitative analysis to determine the presence and amount of bioactive compound in the sample. The extracts were also tested for their pathogen inhibitory activity (antibacterial activity) against both Gram +ve and Gram –ve bacteria such as *Staphylococcus aureus, Streptococcus mutans*, *Klebsiella pneumoniae, Salmonella typhi* and *Enterobacter spp*. Based on the results obtained ethanolic extract was found to be rich in majority of phytocomponents with increased bioactivity compared to other extracts. This further suggests that ethanolic extract of these samples could be used in future for isolation of various novel compounds for different pharmacological applications

KEY WORDS: ANTIBACTERIAL ACTIVITY, BIOACTIVE COMPONENTS, COLD EXTRACTION, MEDICINAL PLANTS, PHYTOCHEMICAL SCREENING, SOLVENT EXTRACTS.

INTRODUCTION

Nature has gifted us with enormous resources and one among them is the widely distributed, a therapeutically valuable source termed as "Medicinal Plants". These medicinal plants contain numerous beneficially active compounds that are highly under research worldwide for treatment of many diseases. India has a rich diversity of plant species and is known for its traditional system of medicine such as Siddha, Ayurveda and Unani (Pandey et al., 2013). Medicinal plants are used as a primary source in health care wherein all the parts such as root, leaf, flower, seed or even whole plant as such is used in the development of drug formulations. These natural or herbal drugs are widely preferred over synthetic medicines due to their easy availability, high efficiency and low toxicity (Patel DK.,2016). Phytochemicals are

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also referred to as "Secondary metabolites" and these are naturally occurring compounds found in plants which play an important role in protecting plant cells against various environmental conditions and apart from it they also impart health benefits to human beings in the form of dietary supplements and as medications (Nyamai et al., 2016). The nature and concentration of secondary compounds present also varies within plant species and each compound exhibits a specific activity. Based on their structure, solubility and biosynthetic pathway these compounds are classified into three groups: Phenolic compounds, Alkaloids and Terpenes. These compounds exert various biological activities and also participate in both the primary as well as secondary metabolism of plants (Irina Francesca Gonzalez Mera et al., 2019).

Some of the pharmaceutical applications of secondary metabolites involve antiviral, antitumor, antioxidant, immunosuppressant, anti-ageing, anti-inflammatory, anti-hyperglycemic activities etc (Vaishnavi and Demain., 2011).

Lawsonia inermis L. is a medicinal plant commonly referred to as "Henna" belonging to the family Lythraceae. They contain various bioactive components such as Lawsone, quinone, xanthone, gallic acid, triterpenoids which are found to exhibit anti-neoplastic, anti-inflammatory, anti-haemorrhagic, hypotensive activity etc. They are also used as astringents, in treating leprosy, nervous disorders, also for treating boils and burns (Ghodekar et al., 2019).

Mangifera indica L. commonly referred to as "Mango" belongs to the family Anacardiaceae. Some of the phytochemical components reported in the plant involve *Mangiferin, catechin, tannins, triterpenoids*. Different parts of the plant are used in the treatment of rheumatism, dysentery, haemorrhage, asthma and anaemia They also possess anti-inflammatory, antiparasitic, anti-helmintic, anti-allergic, antipyretic, anti-HIV and hepatoprotective properties, (Shah et al., 2010). *Piper betel L.* commonly referred to as "Betel/Paan" belongs to the family Piperaceae. It is grown extensively in India, Malaysia, Srilanka and Thailand....It is an evergreen herb which has been studied from past for its pharmacological activites and also used in many ayurvedic preparations. They are used as antiseptics, as healing agents, mouth freshener and are also reported to possess antimicrobial, antioxidant and antileishmanial properties, (Datta et al., 2011).

Due to the high prevalence and emergence of new dreadful diseases, there is a need for development of new drug formulations in order to overcome these circumstances. Plant based secondary metabolites has drawn the interest of many researchers worldwide due to their low toxicity, easy availability and minimal side effects compared to the synthetic ones. Plants contain abundant secondary compounds most of which are yet to be completely studied. This study deals with the phytochemical screening and antibacterial activity of different solvent extract of three important medicinal plants – L*awsonia inermis L., Mangifera indica L.* and *Piper betel L.*

MATERIAL AND METHODS

Sample Preparation: The leaf samples of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L. were collected from different localities. The collected samples were cleaned, washed thoroughly with distilled water and shade dried for about 3 weeks at room temperature. The samples were then powdered and stored in clean bottles until required.

Extraction & Determination of Percentage Yield: The powdered samples were extracted using solvents of different polarities and water (aqueous). For solvent extraction, about 10 grams of the powdered samples were kept immersed separately in 100ml of solvents such as ethanol, chloroform, ethyl acetate in the ratio of 1:10. The samples were covered and incubated in dark for about a week (Sivareddy et al., 2019).

For aqueous extraction, about 10 grams of the powdered samples were added to 100ml of double distilled water and heated on water bath for about 30mins (Al-Manhel and Niamah., 2015). The samples were filtered using whatmann no.1 filter paper and the filtrate obtained was concentrated or dried to yield the crude extracts. The percentage (%) yield of crude extracts obtained was calculated based on the formula: (Terblanche et al., 2017). Percentage yield (%) = Dry weight of extract (g) / Dry weight of sample (g) × 100

Qualitative analysis Phenol Estimation: Ethanol, Chloroform, Ethyl acetate and Aqueous (water) extracts of all the three samples were subjected to qualitative phytochemical analysis in order to identify the presence of bioactive components in the extracts. The analysis was carried out for about 23 different phytocomponents such as Phenol (Ferric chloride test), Tannins, Glycosides (Keller-Killani test), Cardiac glycoside, Alkaloid (Hagers test), Flavanoids (Lead acetate test), Terpenoids (Salkowski test), Diterpenes (Copper acetate test), Steroids, Phytosterol, Quinones, Saponin, Resin (Acetone water test), Coumarins (Sodium hydroxide test), Volatile oil, Fixed oil and fats (Stain test), Carboxylic acid, Phlobotannins, Carbohydrate (Benedicts and Fehlings test), Protein (Biuret and Xanthoproteic test), Aminoacid (Ninhydrin test), Starch (Iodine test) and Lipids according to the standard procedure (Godghate et al., 2012; George., 2017; Kumar et al., 2013).

Quantitative analysis: Phenol estimation: Phenol estimation was carried out according to Folin-Ciocalteau method. To 0.5 ml of extract, 2.5ml of 10% Folin-Ciocalteau reagent and 2.5ml of 7.5% Sodium carbonate was added. The samples were then incubated at 45° C for about 45 minutes and the absorbance was determined at 765nm using UV-Visible Spectrophotometer. Gallic acid was used as the standard. Samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure

was repeated for gallic acid standard and calibration line was constructed. Total phenolic content was expressed in terms of Gallic acid equivalent (mg GAE/g extract),(Singleton et al., 1999).

Tannin estimation: To 0.5ml of extract, 8ml of distilled water was added followed by the addition of 0.5ml FC reagent and 1ml Sodium carbonate. The samples were then incubated for about 30 mins at room temperature and absorbance was measured at 725nm using UV-Visible Spectrophotometer. Tannic acid was used as the standard. Samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for tannic acid standard and calibration line was constructed. Total tannin content was expressed as mg TAE/g extract, (Anbukkarasi et al., 2017).

Flavanoid estimation:10 gram of plant sample was extracted twice with 100ml of 80% methanol at room temperature. The whole solution was filtered and the filtrate obtained was transferred into a crucible and allowed to dry over water bath and weighed to a constant weight. Percentage flavanoid content was calculated using the formula: % Flavanoid content = Weight of dried extract / Weight of Sample × 100 (Bohm and Kocipai., 1994).

Alkaloid estimation: About 200ml of 10 % acetic acid in ethanol was added to 5gram of plant sample taken in a conical flask, covered and incubated for about 4 hours. It was filtered and the filtrate was concentrated on water bath to one quarter of original volume. Concentrated ammonium hydroxide was added drop wise to the extract until precipitation was complete. The solution was allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and filtered. The residue is alkaloid which was dried and weighed. Percentage alkaloid content was calculated using the formula: % Alkaloid content = Weight of alkaloid (residue) / Weight of sample × 100 (Harborne, 1973).

Saponin estimation: About 20% of aqueous ethanol was added to 5gram of plant sample taken in a conical flask and heated at 55°C with continuous stirring for about 4 hours. The residue was again extracted with 100ml of 20% ethanol and heated at 90 °C in water bath. The concentrate was transferred into a separating funnel and to that 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered and about 60ml of butanol was added and extracted twice with 5% sodium chloride. The remaining solution was heated on water bath and after evaporation the sample was dried to a constant weight. Percentage saponin content was calculated using the formula: % Saponin content = Weight of Saponin / Weight of sample × 100 (Obadoni and Ochuko., 2001).

Terpenoid estimation: 10 gram of the powdered sample was kept soaked in 100ml of ethanol for about 24 hours. It was filtered and the filtrate was extracted with 40ml petroleum ether using the separating funnel. Ether extract was seperated and allowed to dry. After drying the percentage terpenoid content was calculated using the formula: % Terpenoid content = Weight of terpenoid extract / Weight of Sample × 100 (Ferguson, 1956).

Glycoside estimation: About 10ml of Baljets reagent (95ml of picric acid + 5ml of aqueous sodium hydroxide) was added to 1ml of extract and the sample was incubated for about 1hour at room temperature. It was then diluted with 20ml distilled water and mixed well. The absorbance was measured at 495nm using spectrophotometer. The samples were prepared in triplicate and mean value of absorbance was obtained. The values are expressed in Mean ± Standard deviation, (El-Olemy et al., 1994).

Cardiac glycoside estimation: 1gram sample was taken and to that 2.5ml of 15% lead acetate was added and the mixture was filtered. To the filtrate 2ml chloroform was added and shaken vigorously. 3ml glacial acetic acid was added followed by the addition of 0.5ml of 5% ferric chloride and 0.05ml of concentrated sulphuric acid. The reaction mixture was incubated in dark for about 2hours and the absorbance was measured at 530nm. The samples were prepared in triplicate for each analysis and mean value of absorbance was obtained. The values are expressed in Mean ± Standard deviation, (Ugwoke et al., 2017).

Coumarin estimation: To 0.5ml of extract, 2ml of distilled water and 0.5ml of lead acetate solution was added and shaken. To this 7ml distilled water was added and mixed well. 2ml of this solution was taken in a test tube and to that 8ml of 0.1M Hydrochloric acid solution was added and incubated at room temperature for about 30 minutes and the absorbance was measured at 320nm using UV-Visible spectrophotometer. The samples were prepared in triplicate for each analysis and mean value of absorbance was obtained. The values are expressed in Mean ± Standard deviation, (Osorio and Martins., 2004).

Steroid estimation: About 0.5ml of extract was taken separately in a test tube and similarly different concentrations (20-100µg/ml) of standard were added to a series of test tubes labelled S1-S5. Cholesterol was used as the standard. To this 5ml of ferric chloride diluting reagent was added followed by the addition of 4ml of concentrated sulphuric acid. The reaction mixture was incubated at room temperature for about 30mins and the absorbance was measured at 540nm in colorimeter. Standard graph was plotted from which the steroid content was determined. The concentration of steroid was expressed in mg/100ml (Zak, 1954).

Analysis of Nutritional components Carbohydrate estimation: It was performed according to Phenol-sulphuric acid method. Glucose was used as the standard. Different concentrations of standard glucose (20-100µg/ml) and about 0.5ml of extract were taken separately into a series of test tube. Volume in all test tubes were made upto 1ml using distilled water.1ml phenol and 5ml of sulphuric acid was added, shaken for 10 minutes

and incubated in boiling water bath at 25 - 30°C for 20 minutes. Green colour developed was read at 490nm in colorimeter. Standard graph was plotted from which the carbohydrate content was determined. The concentration was expressed in mg/100ml, (Dubois et al., 1956).

Protein estimation: It was performed according to Lowry's method, Bovine serum albumin was used as the standard. Different concentrations of standard (BSA) (50-250µg/ml) and 0.5ml of extract were taken separately into a series of test tube. Volume was made upto 2.5ml in all test tubes with distilled water. 5ml of Reagent I (48ml of Reagent A- 2% sodium carbonate in 0.1N sodium hydroxide + 1ml of Reagent B – 1% Potassium sodium tartarate + 1ml of Reagent C – 0.5% Copper sulphate) was added to each tube and incubated for about 10minutes. After 10 minutes, 0.5ml of Reagent II (Folin - Ciocalteau in the ratio of 1:2) was added, mixed and incubated at room temperature for about 30minutes. Blue colour developed was read at 660nm in colorimeter. Standard graph was plotted from which the protein content was determined and concentration was expressed in mg/100ml (Lowry et al., 1951).

Aminoacid estimation: It was performed according to Ninhydrin method. Leucine was used as the standard. Different concentrations of standard Leucine (20-100µg/ml) and 0.5ml of extract was taken separately into a series of test tube. Volume was made upto 2ml in all tubes with distilled water. To this 2ml of ninhydrin reagent was added, vortexed and incubated in water bath for about 15 minutes and cooled. About 3ml of 50% ethanol was added to all tubes and mixed well. The absorbance was measured at 570nm in colorimeter. Standard graph was plotted from which the aminoacid content was determined and the concentration was expressed in mg/100ml. (Moore, S and Stein, W.H., 1948)

Lipid estimation: It was carried out according to Folch method. 1gram of sample was kept immersed in 20ml solvent mixture comprising Chloroform: methanol in the ratio of 2:1. It was agitated for about 15-20 minutes in shaker and the solution was filtered. About 0.9% sodium chloride solution was added to the filtrate and centrifuged at 2,000 rpm for 10 minutes. Upon centrifugation 2 layers got seperated wherein the upper phase is methanol while lower chloroform phase contains lipid. Lipid was collected, allowed to dry and weighed. Weight of lipid is expressed as mg lipid per 100mg of dry material, (Folch, 1957).

Antibacterial activity: The extracts were screened for their antibacterial activity against both Gram positive and Gram negative bacteria such as *Staphylococcus aureus*, *Streptococcus mutans*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Enterobacter spp* by means of Agar well diffusion method. About 150ml of Muller Hinton agar medium was prepared, poured into the petriplates and allowed to solidify. Once solidified the bacterial cultures were swabbed onto the agar medium using a sterile cotton swab .The wells were punctured using a sterile cork borer and the extracts were dispensed into each well using a micropipette. The petriplates were then incubated at 37°C for 24 hours and observed for the zone of inhibition. The diameter was measured in mm (Saif et al., 2017).

RESULTS AND DISCUSSION

Extraction: In this study, cold extraction method was employed for extraction of plant leaf samples (Figure

Figure 1: *Lawsonia inermis* L

Figure 2: *Mangifera indica* L

Figure 3: *Piper betel* L.

Figure 4: Extraction of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L. Using Different Solvents and Water

4A: ETHANOL EXTRACTS

4B: ETHYL ACETATE EXTRACTS

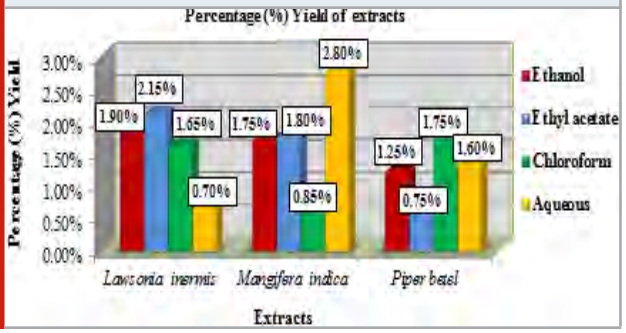
4C: CHLOROFORM EXTRACTS

4D: AQUEOUS EXTRACTS

1-3) using various solvents such as ethanol, ethyl acetate, chloroform and aqueous (Figure 4).This method of extraction is found to be advantageous over hot extraction method wherein the chance for loss of required volatile compounds in the plant is low.

Organic solvents are generally used for extraction of bioactive components from plant sample. However, among the solvents ethanol is most commonly used in medicinal preparations due to its safety and low toxic nature (Wendakoon et al., 2012). The choice of solvent used for extraction also varies depending on the type of compound to be isolated. Ethanol can be used for extraction of Polyphenol, flavanols, tannins, alkaloids, steroids etc...Water can extract compounds such as saponins, tannins, starch, polypeptides, while chloroform is used for extracting flavanoids and terpenoids (Pandey and Tripathi., 2014).

Figure 5: Shows Percentage yield of extracts in different solvents



Percentage Yield of extracts: The percentage yield of extracts obtained using different solvents like ethanol, chloroform, ethyl acetate and aqueous were calculated using the formula: % Yield = Dry weight of extract (g) / Dry weight of sample (g) × 100. *Lawsonia inermis* L. showed higher yield in ethyl acetate extract with 2.15% and lower yield in aqueous extract with 0.7%. *Mangifera indica* L. showed higher yield in aqueous extract with 2.8 % and lower yield in chloroform extract with 0.85%. Piper betel L. showed higher yield in chloroform extract with 1.75% and lower yield in ethyl acetate extract with 0.75% (Figure 5). Among the solvents ethanol showed considerably better % yield in all the three samples.

Table 1. Shows Qualitative Phytochemical Results for Ethanol (ET), Ethyl Acetate (EA), Chloroform (CH) and Aqueous (AQ) Extracts of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L.

S.no	Phytochemicals	Results											
		Lawsonia inermis L.				*Mangifera indica* L.				*Piper betel* L.			
		ET	EA	CH	AQ	ET	EA	CH	AQ	ET	EA	CH	AQ
1	Alkaloid	+	+	+	+	+	+	+	+	+	+	+	+
2	Phenol	+	-	-	+	+	+	-	+	+	-	-	+
3	Tannin	+	+	-	+	+	+	-	+	+	-	-	+
4	Flavanoid	+	+	-	+	+	+	+	+	+	+	+	+
5	Glycoside	+	+	+	+	+	+	+	+	+	+	+	+
6	Cardiac glycoside	+	+	+	+	+	+	+	+	+	+	+	+
7	Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+
8	Diterpenes	+	+	+	+	+	+	-	+	+	+	+	+
9	Coumarins	+	+	-	+	+	+	-	+	+	+	-	+
10	Lipids	+	+	+	+	+	+	+	+	+	+	+	+
11	Saponin	+	+	-	+	+	-	-	+	+	-	-	+
12	Steroid	+	+	+	+	+	+	+	+	+	+	+	+
13	Phytosterol	+	+	+	+	+	-	+	+	+	+	+	+
14	Quinones	+	-	-	+	+	-	-	-	+	+	+	+
15	Resin	+	-	+	-	+	-	+	+	-	-	+	+
16	Volatile oil	+	+	-	-	+	+	-	+	+	+	-	-
17	Fixed oil and Fats	+	+	+	-	+	+	+	-	+	+	+	-
18	Carboxylic acid	+	+	+	+	+	+	+	+	+	+	+	+
19	Phlobotannins	+	-	-	+	+	-	-	+	+	-	-	+
20	Carbohydrate	+	+	+	+	+	+	+	+	+	+	+	+
21	Protein	+	-	-	+	+	-	-	+	+	-	-	+
22	Aminoacid	+	-	-	+	+	-	-	+	+	-	-	+
23	Starch	-	+	+	-	-	+	+	-	-	-	-	-

A study by Mohammed et al., 2016 has reported the use of about 250ml of ethanol, chloroform and 500ml water for extraction of 50 grams of *Mangifera indica* L. leaf by percolation method. The % yield reported was 13.24% for aqueous extract, 6.76% for ethanolic extract and 2.46% for chloroform extract. The extract yield also varies depending upon the factors such as solvent choice, pH, solvent to sample

ratio, temperature, time and the technique followed (Terblanche et al., 2017).

Figure 6: Total Phenolic Content (mg GAE/g Extract)

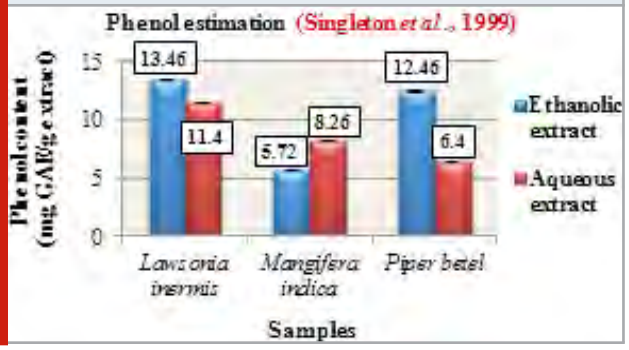


Figure 7: Total Tannin Content (mg TAE/ g extract)

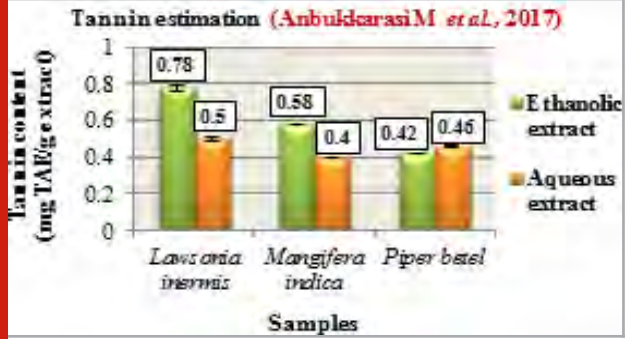


Figure 8: Flavanoid Estimation

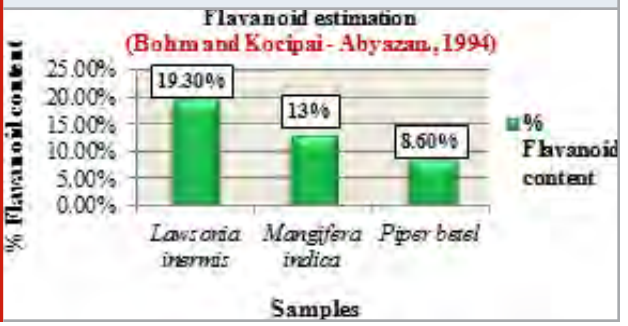
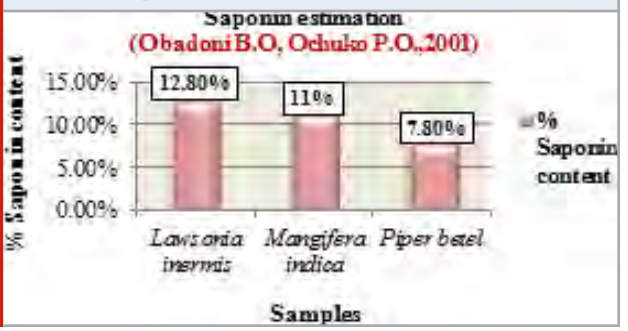


Figure 9: Saponin Estimation



Qualitative phytochemical analysis: Preliminary phytochemical screening was carried out for the extracts of all three samples and the results obtained were tabulated (Table 1). Among the extracts, ethanolic and aqueous extract of all the three samples showed positive result for majority of the phytochemicals such as phenol, tannin, flavanoid, alkaloid, saponin, terpenoids, diterpenes, glycoside, cardiac glycoside, coumarins, steroids, phlobotannins, carbohydrate, protein, aminoacid and lipids etc... compared to ethyl acetate and chloroform extracts. Similar results were found to have been reported by Sharma et al., (2018) for ethyl acetate extracts of *Lawsonia inermis* L. and by Forhad Uddin et al., (2015) for ethanol extract of *Piper betel* L.

Quantitative phytochemical analysis: Based on the qualitative results, ethanolic and aqueous extracts of all the three samples were chosen for further quantification studies. Phenol estimation was carried out according to Folin-Ciocalteau method and the total phenolic content is expressed in mg GAE/ g extract. The phenolic content was found to be higher in the ethanolic extracts of *Lawsonia inermis* L. with 13.46 mg GAE/g and *Piper betel* L. with 12.46 mg GAE/g while *Mangifera indica* L. showed higher phenolic content in its aqueous extract with 8.26 mg GAE/g. Comparatively among the three samples, *Lawsonia inermis* L. showed higher phenolic content in both its ethanolic and aqueous extracts followed by *Piper betel* L. and *Mangifera indica* L. (Figure 6). Phenols are reported to possess anti-cancer, antioxidant as well as antimutagenic properties. They are also used in the treatment of ailments such as cardiovascular problems, inflammation, stroke ,(Ozcan et al., 2014).

Tannin estimation was carried out according to Folins method and the total tannin content was expressed in mg TAE/ g extract. The tannin content was found to be higher in the ethanolic extracts of *Lawsonia inermis* L. with 0.78 mg TAE/g and *Mangifera indica* L. with 0.58 mg TAE/g while *Piper betel* L. showed higher tannin content in its aqueous extract with 0.46 mg TAE/g. Comparatively *Lawsonia inermis* L. showed higher tannin content in both its ethanolic and aqueous extracts followed by *Mangifera indica* L.and *Piper betel* L. (Figure 7). Tannins are polyphenolic compounds which are reported to possess antiviral, antiparasitic, anti-oxidant, anti-inflammatory activities. They are also used in the treatment of hemorrhoids, infections,skin ulcer, (Praveen and Upadhyaya., 2012).

The % Flavanoid content was determined based on the method by Bohm and Kocipai-Abyazan.,1994. Among the samples, *Lawsonia inermis* L. showed higher flavanoid content of 19.30% followed by *Mangifera indica* L. with 13% and *Piper betel* L. with 8.60% (Figure 8). Flavanoids are pharmacologically active compounds that are loaded with numerous health benefits and are found to be potential candidates in drug production with reported activities such as antioxidant, antiageing & anticancer properties, (Hayat et al., 2017). The % Saponin content was determined based on the method by Obadoni B.O and Ochuko P.O., 2001. Among the samples, *Lawsonia inermis* L. showed higher saponin content of 12.80% followed

by *Mangifera indica* L. with 11% and *Piper betel* L. with 7.80% (Figure 9). Saponins exhibit numerous medicinal properties and are widely used in the treatment of cancer, against microbial infections, as anti-inflammatory and hepatoprotective drugs. Apart from health care they are also used in industries for preparation of detergents, cosmetics, soap due to their surfactant properties (Moghimipour and Handali, 2015).

Figure 10: Alkaloid Estimation

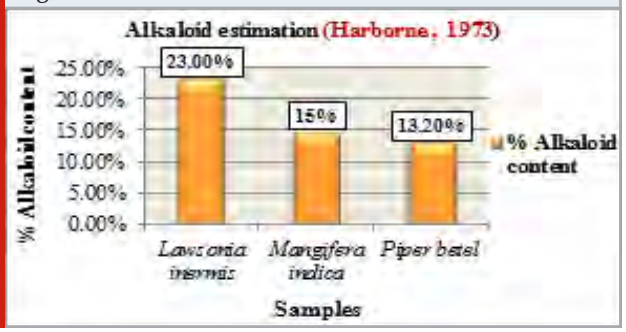
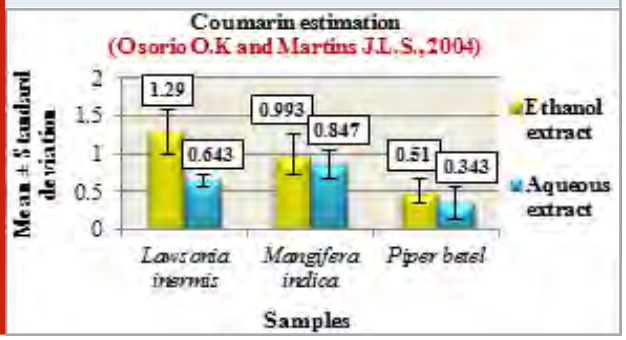


Figure 11: Coumarin Estimation



Alkaloid estimation was carried out according to the method given by Harborne., 1973. Comparatively *Lawsonia inermis* L. showed higher alkaloid content with 23% followed by *Mangifera indica* L. with 15% and *Piper betel* L. with 13.20% (Figure 10). Alkaloids play a protective role in plants against insects and herbivores due to their bitter tasting property. They exhibit antiarrhythmic, antihypertensive activities and are also used as analgesic & antimalarial drugs due to their stimulant properties, (Saxena et al., 2013).

Coumarin estimation was carried out based on the method by Osorio O.K and Martins J.LS., 2004. The results are expressed in Mean ± Standard deviation. The ethanolic extracts of all the three samples showed higher coumarin content compared to the aqueous extracts.Among the samples, *Lawsonia inermis* L. showed increased coumarin content followed by *Mangifera indica* L. and *Piper betel* L. (Figure 11). Coumarins are reported to possess anticancer, anti-aging as well as anticoagulant properties and are also used as enhancing agents in cosmetic products and as food additives, (Rohini K and Srikumar PS., 2014).

Figure 12: Glycoside Estimation

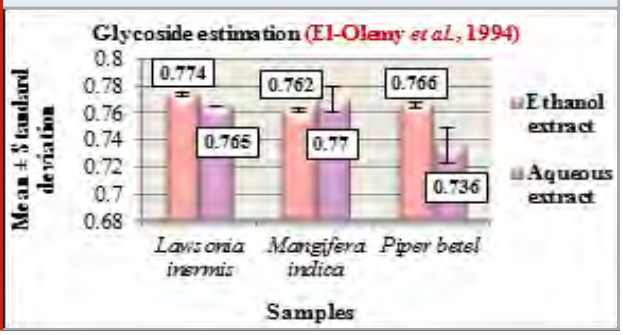


Figure 13: Cardiac Glycoside Estimation

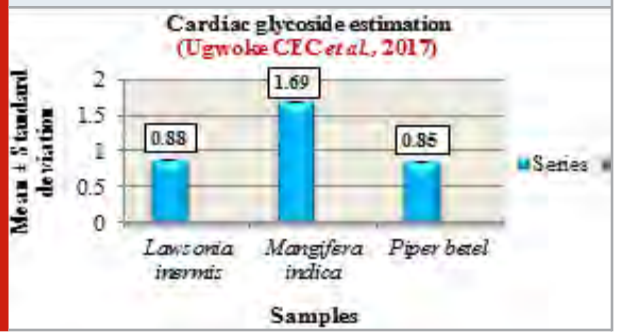


Figure 14: Steroid Estimation

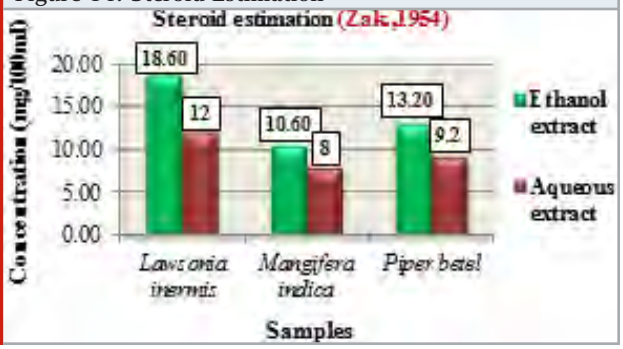
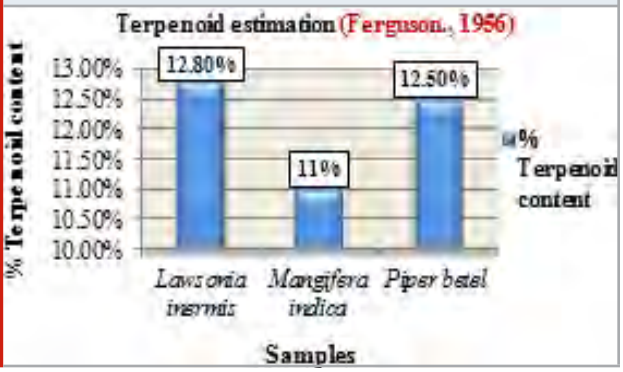


Figure 15: Terpenoid Estimation



Glycoside estimation was carried out according to Baljets method. The results are expressed in Mean ± Standard deviation. The glycoside content in the ethanolic and aqueous extracts of all the three samples were determined. Based on the estimation, *Lawsonia inermis*

L. and *Piper betel* L. showed higher glycoside content in the ethanolic extracts while *Mangifera indica* L. showed higher glycoside content in its aqueous extract (Figure 12). Glycosides play a predominant role in pharmaceutical industry due to their innate biocompatibility and are also used as analgesic, antipyretic, anti-inflammatory agents, (Saawarn et al., 2015).

Cardiac glycoside estimation was carried out according to method by Ugwoke CEC et al., 2017. The results are expressed in Mean ± Standard deviation. Among the samples, *Mangifera indica* L. showed higher cardiac glycoside content followed by *Lawsonia inermis* L. and *Piper betel* L. (Figure 13). Cardiac glycosides are compounds produced by plants in very small amounts. Cardiac glycoside such as peruvoside are used as a promising source in the control of cancer mainly ovarian cancer and leukemia (Patel., 2016). They are also used in the treatment of congestive heart failure (Morsy., 2017).

Steroid estimation was carried out according to method by Zak.,1954. The total steroid content was expressed in concentration(mg/100ml). *Lawsonia inermis* L. showed higher steroid content in its ethanolic extract with 18.60mg than aqueous extracts with 12mg. *Mangifera indica* L. also showed increased steroid content in its ethanolic extract with 10.60mg than aqueous extract with 8mg while *Piper betel* L. showed higher steroid content in its ethanolic extract with 13.20mg than aqueous extract with 9.2mg. Comparatively among the three samples, *Lawsonia inermis* L. showed higher steroid concentration followed by *Piper betel* L. and *Mangifera indica* L. (Figure 14).

Steroids exhibit different biological activities such as regulation of cell proliferation, tissue differentiation and in cell membrane stabilization. They are also utilized as carbon and energy sources by various bacteria and also participate in cellular signalling mechanisms (Cabezon et al., 2018). Terpenoid estimation was carried out based on method by Ferguson.,1956. Among the samples, *Lawsonia inermis* L. showed higher terpenoid content with 12.80% followed by *Piper betel* L. with 12.50% and *Mangifera indica* L. with 11% (Figure 15). Terpenoids are the largest class of compounds produced

Figure 16: Carbohydrate Estimation

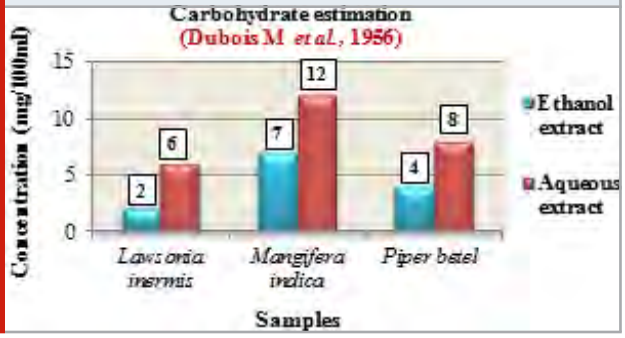
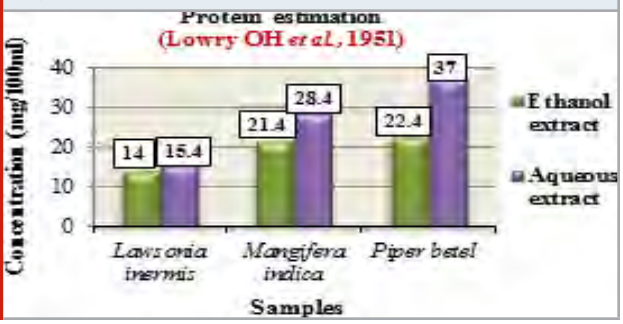


Figure 17: Protein Estimation

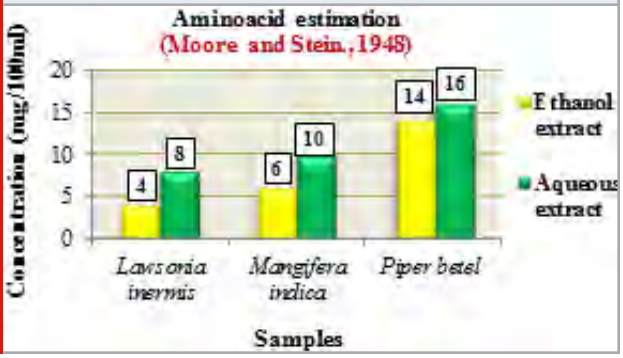


by plants. They exhibit antiviral, anti-inflammatory, antimicrobial, antioxidant activities and are also used as food additives and as fragrances in perfumes, (Priyanka P.Brahmkshatriya and Pathik S. Brahmkshatriya.,2013.

Analysis of nutritional components: Essential nutritional components such as carbohydrate, protein, aminoacid and lipid content in the ethanolic and aqueous extracts of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L. were determined.

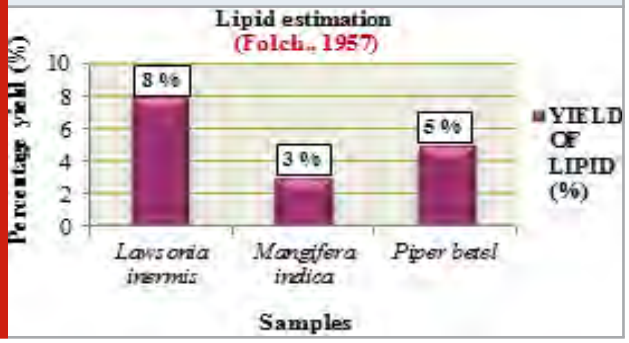
Carbohydrate estimation was carried out according to Phenol sulphuric acid method. The results were expressed in mg/100ml. Carbohydrate concentration was found to be higher in the aqueous extracts of all the three samples compared to that of the ethanolic extracts. Among the three samples, *Mangifera indica* L. showed higher carbohydrate content with 12mg in aqueous and 7mg in ethanolic extract followed by *Piper betel* L. with 8mg in aqueous and 4mg in ethanolic extract and *Lawsonia inermis* L. with 6mg in aqueous and 2mg in ethanolic extract (Figure 16). Carbohydrates are essential compounds produced by plants which serve as energy reserves and are widely used in pharmaceutical industry, textile, paper, food, agricultural as well as in chemical industries, (Bemiller J.N., 2004).

Figure 18: Aminoacid Estimation



Protein estimation was carried out according to Lowry's method. The results were expressed in mg/100ml. The aqueous extracts showed higher protein concentration than the ethanolic extracts in all the three samples. Comparatively among the three samples, *Piper betel* L.

Figure 19: Lipid Estimation



showed higher protein content with 37mg in aqueous and 22.4mg in ethanolic extracts followed by *Mangifera indica* L. with 28.4mg in aqueous and 21.4mg in ethanolic extract and *Lawsonia inermis* L. with 15.4mg in aqueous and 14mg in ethanolic extract (Figure 17). Proteins are organic compounds distributed in plant tissues and are widely used in clarification of wine, in vaccines, tissue engineering and controlled drug delivery (Wadhave et al., 2014).

Aminoacid estimation was carried out according to Ninhydrin method. Results were expressed in mg/100ml. The aqueous extracts showed higher aminoacid concentration than ethanolic extracts in all the three samples. Aminoacid content in *Lawsonia inermis* L. was found to be 8mg in aqueous and 4mg in ethanolic extracts. *Mangifera indica* L. had aminoacid content of about 10mg in the aqueous and 6mg in ethanolic extracts while *Piper betel* L. showed aminoacid content of 16mg in aqueous and 14mg in ethanolic extracts. Comparatively *Piper betel* L. showed higher aminoacid content followed by *Mangifera indica* L. and *Lawsonia inermis* L. (Figure 18). Amino acids play an important role in Pharma industry in the form of precursor in antibiotic production, in transfusion, in the treatment of heart failure, peptic ulcer etc. They are also utilized as flavor enhancers, food additives, in the manufacture of artificial sweetners (Ivanov et al.,2016).

Table 2: Antibacterial Activity of Ethanol (ET), Ethyl Acetate (EA), Chloroform (CH) and Aqueous (AQ) Extracts of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L

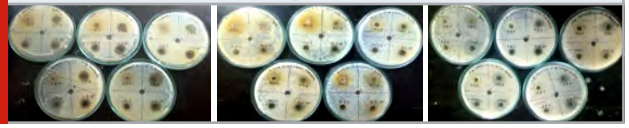
S.no	Organisms	Extracts – Zone of inhibition (in mm)											
		Lawsonia inermis L.				*Mangifera indica* L.				*Piper betel* L.			
		ET	EA	CH	AQ	ET	EA	CH	AQ	ET	EA	CH	AQ
1	*Staphylococcus aureus*	8	5	4	2	16	15	-	3	9	7	3	1
2	*Streptococcus mutans*	5	3	7	1	2	-	1	6	6	5	2	1
3	*Klebsiella pneumoniae*	6	3	3	3	3	5	2	5	6	3	2	1
4	*Salmonella typhi*	6	4	4	2	10	9	6	5	7	8	3	-
5	*Enterobacter spp.*	8	3	4	1	7	5	-	10	4	5	2	1

Lipid estimation was carried out according to method given by Folch, 1957. Among the samples, *Lawsonia inermis* L. showed higher lipid content of 8% followed by *Piper betel* L. with 5% and *Mangifera indica* L. with 3% (Figure 19). Lipids are widely utilized in the preparation of cosmetics, in paints and varnishes, in detergents, also in pharmaceuticals and diagnosis,(Alvarez and Rodriguez., 2000).

Antibacterial activity of extracts:

Antibacterial activity was determined for the ethanol, ethyl acetate, chloroform and aqueous extracts of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L. against 5 Pathogenic organisms such as *Staphylococcus aureus, Streptococcus mutans, Salmonella typhi, Klebsiella pneumoniae* and *Enterobacter spp* (Figure 20).

Figure 20: Antibacterial Activity of *Lawsonia Inermis* L., *Mangifera Indica* L. and *Piper Betel* L. Extracts



Among the three samples *Lawsonia inermis* L. showed good inhibition against all organisms followed by *Piper betel* L. and *Mangifera indica* L. The ethanolic extract from all the three samples were found to be more active in inhibiting bacterial pathogens while aqueous extracts from *Lawsonia inermis* L. & *Piper betel* L. and chloroform extract from *Mangifera indica* L. displayed less inhibition activity. Chloroform extract of *Mangifera indica* L. displayed no inhibition against *Staphylococcus aureus* and *Enterobacter spp* while ethyl acetate extracts didn't show any inhibition against *Streptococcus mutans.* In case of *Piper betel* L. only aqueous extract was found not to be active against *Salmonella typhi* (Table 2). Bangash et al., (2012) have also reported that ethanolic extract was the most effective against bacterial pathogens than chloroform extract which is least effective.

CONCLUSION

The present work deals with the phytochemical studies and antibacterial activity of leaf samples of Medicinal plants *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L. Phytochemical analysis is generally carried out in order to analyze the presence or absence as well to determine the quantity of a specific compound in the

plant extract. The nature and distribution of bioactive compound varies in different plant species based on their growth and environmental conditions. In this study phytochemical analysis (Qualitative & Quantitative study) was carried out for the leaf extracts of *Lawsonia inermis* L., *Mangifera indica* L. and *Piper betel* L. obtained using different solvents and their antibacterial activity was also determined against 5 bacterial pathogens such as *Staphylococcus aureus, Streptococcus mutans, Klebsiella pneumoniae, Salmonella typhi* and *Enterobacter spp*. Among the tested extracts, ethanolic extract showed good yield as well as increased activity against all bacterial pathogens and based on the results obtained further studies will be carried out in future on the isolation and purification of compounds for different pharmacological applications.

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Conflict of interest: None

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Antidiabetic and Antioxidant Activity of Hydro-alcoholic Extract of *Oxalis debilis* Kunth Leaves in Experimental Rats

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ABSTRACT

Ethnomedicinal survey documents the traditional usefulness of *Oxalis debilis* Kunth leaves in the management of diabetes mellitus in North-eastern region of India. This study screens the hydro-alcoholic extract (HAE) of leaves of *O. debilis* for antidiabetic activity in streptozotocin-induced diabetic rats along with antioxidant activity. The antidiabetic activity of HAE was evaluated in streptozotocin-induced diabetic rats at the doses of 250 and 500 mg/kg body weight. Results of antidiabetic activity study revealed that HAE of *O. debilis* leaves possesses significant hypoglycemic activity (100.52±14.94 and 78.79± 9.67 at 250 and 500 mg/kg, respectively) in diabetic rats (322.64± 11.86) as compared to normal control group (99.88 ± 2.58) after 21st day of treatment. The metformin (5 mg/kg) treated rats also showed significant reduction (76.99 ± 8.63) in plasma glucose level when compared to normal rats. HAE also exhibited promising antioxidant potential in experimental rats. Results indicated possible role of the HAE as herbal antioxidants in the prevention and/or treatment of oxidative stress induced diabetes. The phenolic/flavonoid contents of HAE having antioxidant potential might be responsible for antidiabetic property of *O. debilis* leaves.

KEY WORDS: ANTIDIABETIC, ANTIOXIDANT, *O. debilis*, OXIDATIVE STRESS, PLANT FLAVONOIDS.

INTRODUCTION

Despite the availability of hypoglycemic agents from synthetic sources, diabetes is still life-threatening because of limited therapeutic utility of existing drugs (Riaz et al., 2020). Traditional medicines derived from plants play a significant role in the management of diabetes mellitus (Junejo et al., 2018). WHO recommended the evaluation of traditional plant remedies used in the treatment of diabetes because they are effective with less or no toxicities as compared to synthetic oral hypoglycemic agents (Junejo et al., 2017). Many indigenous Indian medicinal plants have been found to be useful in the treatment of diabetes mellitus (Sekhin-Loodu et al., 2019).

Oxalis debilis Kunth (Oxalidaceae) is a tristylous species native to Southern America and is a member of the bulb-forming shrub and distributed widely throughout the world. It is abundantly found in the Brahmaputra valley region of India (Junejo et al., 2006). The traditional uses of herbs and their extracts have been to cure human ailments since ancient times. It has been used traditionally for the treatment of dysentery and diarrhea (Kumar et al., 2012). In modern literature, antioxidant, anticancer, anti-inflammatory, analgesic, antimicrobial, antiamoebic, antifungal, astringent, diuretic and febrifuge

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activities of this plant species have also been reported (Panda et al., 2016; Rehman et al., 2015).

Ethnobotanical study indicates that leaf decoction of *O. debilis* have been used by tribal people of North-eastern states of India for the management of diabetes. There are no scientific reports in modern literature on the antidiabetic efficacy of *O. debilis* leaves. The objective of our present study was to ascertain the scientific basis of using this particular plant species traditionally in the management of diabetes, using streptozotocin-induced diabetic rats. According to WHO guidelines (Junejo et al., 2020c), the extract of *O. debilis* leaves was prepared using hydro-alcoholic solvent and evaluated for the antidiabetic activity. It has been reported that traditional medicinal plants having antidiabetic activity possess antioxidant potential in experimental animals (Irudayaraj et al., 2012; Debasis et al., 2010). Moreover, since a biochemical relationship exists between diabetic hyperglycemia and cellular oxidative stress, the antidiabetic activity evaluation of the hydro-alcoholic extract (HAE) of *O. debilis* leaves was carried out along with the study of in vivo antioxidant activity.

MATERIAL AND METHODS

All chemicals and reagents used in the study were of analytical grade and were procured from Rankem, Mumbai and Himedia Laboratories Ltd., Mumbai. Streptozotocin (STZ) was procured from Sigma-Aldrich, Germany. Commercial reagent kits used for determination of biochemical parameters and enzymatic assays were purchased from SPAN Diagnostics Ltd., Surat (India).

Fresh leaves of *Oxalis debilis* were collected from forest areas of Dibrugarh district, Assam (India) during the month of December 2014. The plant species was identified and authenticated (BSI/ERC/2014/Plant identification/360, dt. 26.08.2014) at the Botanical Survey of India, Eastern Regional Centre, Botanical Survey of India, Eastern Regional Centre, Shillong (India). A voucher specimen (DU/PSC/HRB/B-11/2014) of the identified plant species was deposited in the Herbarium of the Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh.

Preparation of hydro-alcoholic extract (HAE): The air-dried leaves were coarsely powdered (Sieve no. 40) using a cutter mill, and 50 g of powdered leaves was used for the preparation of extract. Powdered leaves were extracted using sufficient quantity (400 ml) of ethanol-water mixture (7:3) by cold maceration for 24 h. The extraction was carried out successively thrice and the combined extract was then concentrated under reduced pressure to dryness in a rotary vacuum evaporator to obtain a thick semisolid-like paste. The crude extract was dried at -40 °C in a lyophilizer and the dried extract (dark brown colour) so obtained was stored in a desiccator until further use. The percentage yield of the dried hydro-alcoholic extract (HAE) was calculated per dry weight of powdered leaves.

Estimation of total phenolic and flavonoid contents (TPC and TFC): The total phenolic content of the HAE was evaluated following the Folin-Ciocalteu colorimetric method and results were expressed as mg of gallic acid equivalent (GE) per g of dry weight of the extract. The total flavonoid content was estimated using the aluminum chloride colorimetric method and results were expressed as mg of quercetin equivalent (QE) per g of dry weight of the extract (Junejo et al; 2020a; Chang et al., 2002). The total phenolic content was calculated from the calibration curve of gallic acid (20, 40, 60, 80, 100 µg/ml, 90% ethanol). The total flavonoid content was calculated from a calibration curve of quercetin (20, 40, 60, 80, 100 µg/ml, 90% ethanol). Results were obtained as mean ± SEM of three replicate studies.

Test animals: Healthy Wistar male albino rats (240–260 g) were maintained under standard environmental conditions (temperature 25±2 °C, relative humidity 50±5 %) with a 12 h light / dark cycle. They were fed on with normal laboratory chow pellet diet and drinking water was given ad libitum. Animals were allowed to acclimatize for 7 days before commencement of the experiment. The animals were used with the approval of the Institutional Animal Ethics Committee (Approval no. IAEC/DU/50 dt. 24.9.13) under guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India).

Acute oral toxicity study: Over-night fasted rats were randomly divided into six groups of six animals each. Rats of different groups were administered with increasing doses (250, 500, 1000, 2000 and 5000 mg/kg b.w.) of the HAE. One group was maintained as normal control and was given vehicle alone. The acute toxicity study was done as per OECD guideline-423 (Junejo et al., 2020b; Oliveira et al., 2008).

Oral glucose tolerane (OGT) test: This test was performed in overnight fasted normal rats according to the method reported by Junejo and co-workers (Junejo et al., 2014).

Hypoglycemic activity in STZ-induced diabetic rats: Diabetes was induced (Amira et al., 2016) in overnight fasted animals by a single intraperitoneal (i.p) injection of streptozotocin (STZ, 55 mg/kg b.w. in normal saline). The animals confirmed as diabetic (after 72 h of STZ injection) by the elevated plasma glucose levels (200-300 mg/dl) was used for the experiment. The animals were divided randomly into five groups of six rats in each group. Group I rats served as normal control and were given vehicle (0.5% CMC w/v in normal saline) alone. Group II rats served as diabetic control and were administered with vehicle alone. Group III and IV were treated with HAE at 250 mg/kg b.w. and 500 mg/kg b.w., respectively. Group V rats were received the standard drug, metformin hydrochloride (5 mg/kg b.w.). Treatments were given orally using a canula once daily for a period of 21 days. Blood was collected from the tail vein each time for the determination of glucose levels on

0, 7, 14 and 21 day. Blood glucose levels were measured by the GOD-POD method.

Liver and kidney function tests:The initial and final body weights were measured. Liver tissues were excised, blotted, weighed and stored at -70 °C for assay of glycogen content. Liver glycogen was estimated by the method of Carrol et al. (Carroll et al., 1956). Blood was collected by cardiac puncture in dry test tubes containing a mixture of potassium oxalate and sodium fluoride (1:3) and the serum was separated by centrifugation (2000 rpm, 10 min) for estimation of various biochemical parameters.

Serum insulin levels were measured by the microplate ELISA method using a commercial kit (SPAN Diagnostics Ltd.). Serum lipid profile was also estimated using commercially available kits (SPAN Diagnostics kit). Triglycerides (TG) and Total cholesterol (TC) were estimated by enzymatic methods (HDL (How density lipoprotein) cholesterol by phosphotungstate method and LDL (low density lipoprotein) cholesterol were calculated by Friedewald's formula (Friedewald et al., 1972).Serum was used to estimate glutamate oxaloacetate transaminase (GOT) glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP), total protein (TPR) and creatinine (CRTN). SGOT and SGPT were measured by UV kinetic method and ALKP was estimated by PNPP method. TPR was measured by Bradford Macro method (Bradford, 1976), while CRTN was by picrate method (Saligman et al., 1950).

In vivo antioxidant activity: On 21st day, all the groups of animals were anaesthetized using diethyl ether, liver was dissected out, washed with normal saline and one part was preserved in 10% formalin for histopathological studies. The other part of liver was homogenized by ice chilled Tris-HCl buffer and used for activities/levels of superoxide dismutase catalase, reduced glutathione (GSH), glutathione peroxidase (GPx), and malondialdehyde MDA. The malondialdehyde (MDA) production is a direct indicator of lipid peroxidation (LPO) process that was measured by TBA reaction using an ELISA reader (at 532 nm) (Minami and Yoshikawa, 1979; (Xu et al., 1997).

Histopathological studies: At the end of 21th day of treatment, the animals were fasted for 12 h, anaesthetized using diethyl ether and sacrificed by cervical dislocation. Pancreas was instantly dissected out, excised and rinsed in ice-cold saline solution. Tissue was processed further histopathological observations (Irudayaraj et al., 2012; Kumar et al., 2011; Ozbek et al., 2017).

Statistical analysis: Values are represented as mean ± SEM of three replicate studies. Statistical analysis was performed using the IBM SPSS 19.0 statistical software package, for Windows. Statistical differences at 5% level of probability ($p < 0.05$) between the groups were analyzed by one-way ANOVA followed by Student's t-test (Sabu et al., 2002).

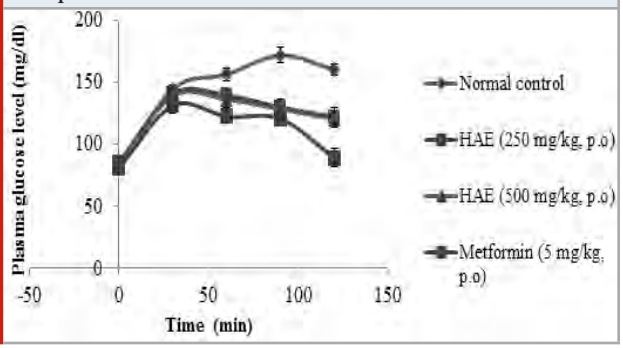
RESULTS AND DISCUSSION

TPC and TFC of HAE: The total phenolic content of the HAE, calculated from the calibration curve of gallic acid (R2 = 0.984), was 56.34 ± 2.09 mg gallic acid equivalent (GE) /g of dry wt. of HAE, and the total flavonoid content (R2 = 0.987), calculated from the calibration curve of quercetin was 45.22 ± 2.31 mg quercetin equivalent (QE) /g of dry wt. of HAE.

Acute toxicity study: No sign and symptoms of acute toxicity and mortality up to 2000 mg/kg body weight dose were observed during the whole experimental period. The body weight and food consumption were normal compared to vehicle treated rats. For further studies, the doses were fixed as 250 and 500 mg/kg body weight.

Effect of HAE on OGT test in normal rats:In OGT, HAE (250 & 500 mg/kg) showed significant ($p < 0.05$) reduction of glucose load (plasma glucose level) as compared to normal control group. The metformin (5 mg/kg) treated group also showed significant ($p < 0.05$) activity compared to normal control group (Fig. 1).

Figure 1: OGT test. Values are mean ± SEM of three replicate experiments. Activities of HAE and metformin (metformin) are statistically significant at $p < 0.05$, compared to normal control



In STZ-induced diabetic rats (B), the histopathology shows the presence of more shrinkage, increased necrosis and damaged β-cell, whereas, the diabetic treated (HAE) animals (C & D) shows increased number of islets, lesser degree of shrinkage and restoration of necrosis of β-cells of pancreas

Effect of HAE on blood glucose levels in diabetic rats: STZ-treated diabetic rats exhibited significant increase in the levels of blood glucose in comparison to normal rats. After treatment with HAE the blood glucose levels were significantly ($p<0.05$) reduced compared to the diabetic control rats at both the doses, viz. 250 & 500 mg/kg. The metformin (5 mg/kg) treated rats also showed significant ($p < 0.05$) reduction in plasma glucose level when compared to normal rats. Results of the effect of HAE on blood glucose levels in normal and diabetic rats are depicted in Table 1.

Table 1. Effect of HAE on blood glucose levels in diabetic rats

Group	Days			
	0 day	7th day	14th day	21st day
Normal control Diabetic control	94.39 ± 2.71	102.86 ± 3.51	103.49 ± 3.99	99.88 ± 2.58
STZ (55 mg/kg, i.p) Diabetic + HAE	266.72 ± 8.29	289.21 ± 9.57	298.12 ± 6.58	322.64± 11.86
(250 mg/kg, p.o) Diabetic + HAE	252.91± 8.34	216.55± 5.21*	133.72± 7.98*	100.52±14.94*
(500 mg/kg, p.o) Diabetic + Metformin	255.88 ± 5.61	200.61± 9.98*	103.68± 7.78*	78.79± 9.67*
(5 mg/kg, p.o)	264.56 ± 7.26	170.66 ± 8.95	116.89 ± 9.04	76.99 ± 8.63

Values indicate mean ± SEM (n = 6)
*p < 0.05, compared with normal control values

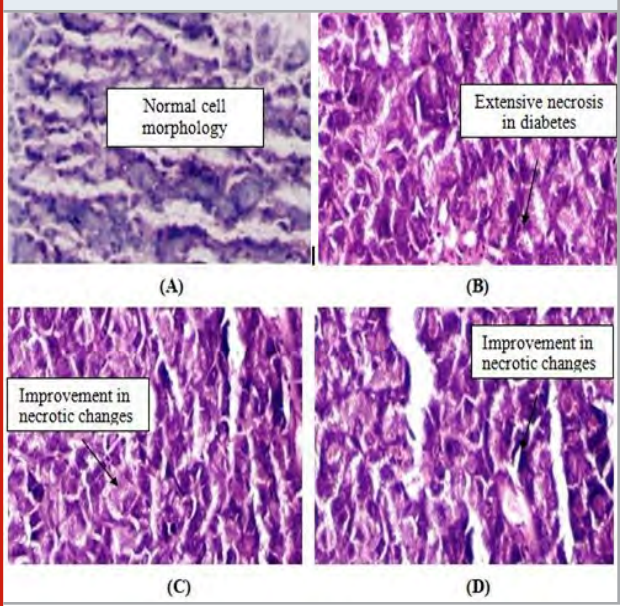
Effect of HAE on body weight, plasma insulin and liver glycogen in diabetic rats: Table 2 depicts the effect of HAE on body weight, levels of plasma insulin and liver glycogen in STZ-induced diabetic rats. In diabetic rats, the body weight, insulin level and glycogen content were significantly decreased. After 21 days of treatment with HAE at 250 & 500 mg/kg, the body weight was significantly ($p < 0.05$) increased, insulin level and glycogen content were also significantly ($p < 0.05$) increased as compared to diabetic rats. The activity of HAE was found less than that of metformin (5 mg/kg) treated group.

Effect of HAE on lipid profile in diabetic rats: The effect of HAE on lipid profile of diabetic rats is displayed in Table 3. In diabetic rats, the levels of triglycerides (TG), total cholesterol (TC), and low density lipoprotein (LDL) were significantly increased and high density lipoprotein (HDL) level was significantly decreased. In HAE (250 & 500 mg/kg) treated groups, the TG, TC and LDL levels activities were significantly ($p < 0.05$) reduced and the HDL level was significantly ($p < 0.05$) increased as compared to diabetic control rats, which is in turn comparable to metformin (5 mg/kg) treated group.

Effect of HAE on SGOT, SGPT, ALKP, TPR and CRTN in diabetic rats: There was a significant increase in activities of SGOT, SGPT and ALKP in diabetic rats. After treatment with HAE (250 and 500 mg/kg) the activities of SGOT, SGPT and ALKP activities were significantly ($p<0.05$) reduced as compared to diabetic control rats. A significant decrease in serum total protein (TPR) level and a significant increase in creatinine (CRTN) level were observed in diabetic rats. After treatment with HAE at 250 and 500 mg/kg doses for 21 days the TPR level was significantly increased ($p < 0.05$) and CRTN level was significantly ($p < 0.05$) decreased compared to diabetic control rats. Metormin (5 mg/kg) treated rats also showed significant effects on blood levels of SGOT, SGPT, ALKP, TPR and CRTN in diabetic rats (Table 4).

Effect of HAE on liver antioxidant enzymes and MDA: Table 5 displays the activities of SOD, CAT, GSH and GPx in normal and diabetic rats. In STZ-treated diabetic rats, the activities of SOD, CAT, GSH and GPx were significantly increased. There was a significant ($p < 0.05$) reduction in the activities of these antioxidant enzymes in diabetic rats as compared to normal rats. Metformin (5 mg/kg) also showed significant ($p < 0.05$) reduction of these enzymes. Increased levels of MDA, an indicator of LPO, in diabetic rats were significantly ($p < 0.05$) reduced after treatment with HAE (250 & 500 mg/kg) as compared to the normal rats.

Figure 2: Histology of pancreas of experimental rats after treatment with HAE, 500 mg/kg. (A) Normal control- (B) Diabetic control, (C) Diabetic treated with HAE (500 mg/kg), (D) Diabetic treated with metformin



In STZ-induced diabetic rats (B), the histopathology shows the presence of more shrinkage, increased necrosis and damaged β-cell, whereas, the diabetic treated (HAE)

animals (C & D) shows increased number of islets, lesser degree of shrinkage and restoration of necrosis of β-cells of pancreas

Histopathological observations: Histopathological studies of pancreas (Fig. 2) of STZ-treated diabetic rats exhibited reduction in the dimensions of islets, damaged β-cell population and extensive necrotic changes followed by fibrosis and atrophy (B). HAE (500 mg/kg) and metformin treated rats restored the necrotic and fibrotic changes and also increased the number and increased the size of the islets (C). In normal control group normal acini and normal cellular in the islets of Langerhans the pancreas were observed (A). The changes in pancreas morphology in metrformin treated group (D) are similar to HAE treated rats.

Table 2. Effect of HAE on body weight, plasma insulin and liver glycogen in diabetic rats

Group	Body weight in g		Plasma insulin (µU/ml)	Liver (mg/g tissue) glycogen
	0 day	21st day		
Normal control	200.50 ± 2.84	205.38± 11.49	15.31± 3.62	72.14 ± 2.23
Diabetic control STZ (55 mg/kg, i.p)	205.67 ± 4.88	130.38 ± 7.27	6.23 ± 6.45	32.18 ± 2.25
Diabetic + HAE (250 mg/kg, p.o)	200.82± 6.81	188.30±9.21*	9.89±5.33*	59.44±3.76*
Diabetic + HAE (500 mg/kg, p.o)	204.15± 5.29	193.96±9.75*	14.04±3.87*	67.75±4.38*
Diabetic+ Metformin (5 mg/kg, p.o)	206.66 ± 2.32	192.59 ± 8.30	15.65± 6.72	72.80 ± 6.67

Values indicate mean ± SEM (n = 6)
*p < 0.05, compared with normal control values

Hydro-alcoholic extract (HAE) of *O. debilis* leaves did not exhibit toxicities up to a dose of 2000 mg/kg b.w. in experimental animals which indicated high margin of safety of bioactive principles present in the extract. HAE treated rats lowered glucose level when compared to normal rats which indicated that the increased glucose tolerance in HAE treated rats was due to insulin secretion from β-cells and increased glucose utilization by the tissues. The HAE treated group exhibited significant reduction of fasting plasma glucose levels as compared to the diabetic control group. The possible mechanism by which HAE brought about its hypoglycemic action might be by improving glycaemic control mechanism and by increasing insulin secretion from regenerated β-cells of pancreas (Jangir and Jain, 2017).

It was further supported by histopathological observations which clearly revealed the presence of shrinkage, necrosis and damaged β-cell population in the endocrine region of pancreas in STZ-induced diabetic rats. The diabetic treated (HAE) animals showed increase in the number of islets, lesser degree of shrinkage and restoration of necrosis of β-cells of pancreas. Our finding is consistent with an earlier report by Irudayaraj and co-authors (Irudayaraj et al., 2012). Diabetic rats treated with HAE showed an improvement in body weight in comparison to the diabetic control rats and standard metformin treated rats, signifying the protective effect of HAE in controlling muscle wasting i.e., reversal of gluconeogenesis. Moreover, the ability of HAE to protect body weight loss might be the result of its ability to reduce hyperglycemia (Sudasinghe and Peiris, 2018).

Diabetic rats treated with HAE increased significantly the liver glycogen content as compared to the diabetic control, which could be due to increased insulin secretion. The significant increase in the glycogen levels of the HAE treated diabetic animals might be because of the reactivation of glycogen synthase system.Diabetic rats treated with HAE significantly improved serum TG and TC. The significant control of the levels of serum lipids in the HAE treated diabetic rats might be attributed to improvements in insulin levels. Significant lowering of LDL cholesterol and raise in HDL cholesterol were observed in treated diabetic rats. The HAE extract treated animals showed a weight loss, which probably be due to the lipid lowering activity of the extract or indirectly to the influence on various lipid regulation systems. Lipid lowering activity of the HAE may help in prevention of diabetic complications like atherosclerosis and ischaemic conditions (Junejo et al., 2018)

An increase in the activities of SGOT, SGPT and ALP in plasma of diabetic rats might be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream which was an indicator of the hepatotoxic effect of STZ (Sreedevi et al., 2020). Treating the diabetic rats with HAE reduced the activity of these enzymes compared to the diabetic control group. Reduction in plasma TPR was observed in diabetic rats, which might be due to the negative nitrogen balance, enhanced proteolysis and decreased protein synthesis (Reddy et al., 2017). The plasma protein level was improved in diabetic rats after treatment with HAE. Diabetic rats showed increased level of CRTN, whereas, a

significant reduction in the level of CRTN was observed in HAE treated diabetic rats indicated that the HAE prevented the progression of renal damage in diabetic rats (Attanayake et al., 2015).

Table 3. Effect of HAE on lipid profile in diabetic rats

Groups	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL(mg/dl)
Normal control	86.89 ±7.26	152.20 ± 6.56	38.29 ± 2.14	95.32 ± 4.92
Diabetic control STZ (55 mg/kg, i.p)	210.43± 6.84	270.83± 14.96	30.61 ± 2.60	199.33± 15.67
Diabetic + HAE (250 mg/kg, p.o)	145.71±5.92*	162.21 ±5.92*	40.27 ±5.67*	117.29±5.46*
Diabetic + HAE (500/kg, p.o)	133.21±4.54*	158.71± 6.91*	48.29± 5.67*	100.69± 8.59*
Diabetic + Metformin (5 mg/kg, p.o)	115.29± 3.79	146.59± 11.15	54.69 ± 3.28	78.89 ± 6.74

Values indicate mean ± SEM (n = 6)
*p < 0.05, compared with normal control values

Table 4. Effect of HAE on SGOT, SGPT, ALKP, TPR and CRTN in diabetic rats

Group	SGOT (U/L)	SGPT (U/L)	ALKP (U/L)	TPR (mg/dl)	CRTN (mg/dl)
Normal control	49.78± 6.69	46.89± 6.23	116.61±4.98	8.89± 0.46	0.436±0.026
Diabetic control STZ (55 mg/kg, i.p)	102.80±8.43	88.39 ± 4.59	320 ± 8.95	4.62± 0.92	0.826±0.037
Diabetic + HAE (250 mg/kg, p.o)	67.33± 5.35*	60.21± 4.32*	146.27±4.89*	6.95±0.31	0.646±0.04*
Diabetic + HAE (500 mg/kg, p.o)	62.04± 4.32*	56.73± 5.21*	138.72±7.29*	8.73±0.52*	0.498±0.06*
Diabetic + Metformin (5 mg/kg, p.o)	58.79 ± 7.61	53.91 ± 8.56	132 ± 6.52	8.98± 2.02	0.434±0.062

Values indicate mean±SEM (n = 6)
*p<0.05, compared with normal control values

The activities of enzymatic antioxidant (SOD, CAT, GSH and GPx) were increased to normal indicating the efficacy of HAE in attenuating the oxidative stress (OS) and eventual inhibition of LPO in diabetic liver. Decrease in MDA level indicated reduced rate of LPO in HAE treated diabetes. Mechanisms that contribute to increased OS in diabetes include non-enzymatic glycosylation, autooxidative glycosylation and metabolic stress (Jan et al., 2015). A marked increase in the concentration of TBARS and MDA were observed in STZ induced diabetic rats indicating the LPO of tissues under oxidative stress (Banerjee et al., 2017). Since the HAE significantly decreased TBARS levels as well as MDA in liver of diabetic rats indicating strong lipid peroxidation scavenging activity of the HAE as antioxidant agent. Some studies (Kadali et al., 2017) suggest that high molecular weight phenolic compounds including plant flavonoids comprising hydroxyl group and aromatic ring serve as potent free radical scavengers. It is now assumed that the antioxidant activity is responsible for the antidiabetic action of the HAE, and phenolic compounds and flavonoids present in the HAE may be involved in reducing underlying cellular OS and eventual hypoglycemic reactions (Sekhin-Loodu et al., 2019). Plants rich in phenolics, flavonoids and related substances, have antioxidant activity due to their redox properties, and as their free radicals scavenging ability is facilitated by hydroxyl groups (Junejo et al., 2000a). So, the determination of total phenolic and flavonoid contents could be used as a basis of assessing the antioxidant potential of plant extracts. It has been reported that antioxidant properties of plant derived phenolic compounds are brought about mainly via their radical scavenging activities (Junejo et al., 2018).

Table 5. Effect of HAE on SOD, CAT, GSH, GPx and MDA in normal and diabetic rats

Treatment	SOD (U/mg protein)	CAT (U/mg protein)	GSH (U/mg protein)	GPx (U/mg protein)	MDA (LPO) (U/mg protein)
Normal control	8.28± 0.06	72.16±4.32	16.20±0.48	23.12 ± 1.89	0.52 ± 0.12
Diabetic control	4.23± 0.08	42.60±3.69	6.20 ± 0.28	9.12 ± 0.48	0.82 ± 0.03
STZ (55 mg/kg, i.p) Diabetic + HAE	6.87± 0.07	61.67±3.11*	11.15±0.54	12.97±0.73*	0.69±0.04
(250 mg/kg, p.o) Diabetic + HAE (500 mg/kg, p.o)	7.98± 0.08	67.56±5.43*	14.11±0.75*	19.04±0.43*	0.58±0.07*
Diabetic+ Metformin (5 mg/kg, p.o)	8.51± 0.05	69.86±3.64	15.20±1.20	20.34 ± 0.82	0.50 ± 0.10

Values indicate mean ± SEM (n = 6)
*p < 0.05, compared with normal control values

CONCLUSION

The findings of our present investigation justify the traditional use of *O. debilis* leaves in ethnomedicine of Northeast India for the treatment of diabetes. The antioxidant activity of *O. debilis* leaves reported here in signifies the potential of the plant as herbal antioxidant with possible role in the prevention of oxidative stress induced diabetes and associated disease complications. Studies are in progress in our laboratory to isolate bioactive principles from the HAE extract of *O. debilis* leaves and further exploration of biochemical mechanisms involved in antidiabetic action of isolated compopunds. As this is the first report on the antioxidant activity of *O. debilis*, thorough phytochemical analyses need be executed in order to identify the possible antioxidant phenolic and flavonoid components having antidiabetic activity.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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An Insight into the Various Toxicological Effects of Nanoparticles on our Living Systems: A Review

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ABSTRACT

Nanotechnology has revolutionized the various sectors like diagnostics, medical imaging, electronics, biosensors, environment, production of innovative materials and devices. Application of nanotechnology in several sectors is owing to the unique physiochemical and optical properties of nano-materials compared to large size materials. Nano-materials due to their high surface area to volume ratio possess unique properties. Physiochemical properties of nano-materials mainly depend on the size, shape, composition and surfactant used. In global economic growth introduction to metallic nanoparticles has been rising. Despite of all the advantages in public and industrial sectors, nano-materials have raised alarm for workers, consumers and human habitat. A novel research domain is needed to counter the potential harmful effects of nanoparticles. Humans can be exposed to nano-materials either deliberately or accidently through various routes like inhalation, ingestion and intravenous. Nanomaterials due to their narrow size may easily penetrate deeper into the body. The interactions of nanoparticles with different bio-molecules and associated toxicity mainly depend on their size, shape, structure, surface area to volume ratio and surface characteristics.Though nanotechnology has become a promising field of science, a check has to be established in order to avoid cytotoxic effects of particles with dimension less than 100 nm. There is a gap in understanding and setting international standards for the toxic and environmental effects of nanoparticles. Here we will short down some adverse effects of nanoparticles coming in contact with viable cells.

KEY WORDS: NANOPARTICLES, NANOMATERIAL, TOXICITY, HUMAN HABITAT.

INTRODUCTION

Nanotechnology is a continuously growing field from last few decades. Nanotechnology deals with the materials at nano-scale. This includes synthesis, fabrication, characterization, and application of nano-materials. Nano-materials (NMs) have gained more attention owing to their different and improved physiochemical properties over their bulk counterparts. Synthesized nanomaterials may be nanoparticles, nanofibers, nanotubes. Particles which have at least one dimension in nanometer range are considered as nanoparticles (NPs) (Laurent et al., 2010). Nanotechnology owing to vast application potential has revolutionized a wide array of sectors like food industry, medicine, diagnostic, energy, agriculture, electronics and environment (Renn et al., 2006; Karn et al., 2005; Hougaard et al., 2015).

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Nanoparticles may be synthesized from metals, polymers, and different bio-molecules (polysaccharides, proteins, and nucleic acid) (Oberdorster et al., 2013). The most commonly synthesized nanoparticles are of silver (Ag), gold (Au), zinc oxide (ZnO), titanium dioxide (TiO2), silica (SiO2), and polymer (PNPs). NPs have already been exploited in various products like sun protecting creams, cosmetic products, food preservative, self-sterilizing surfaces, disinfectants, agriculture, textile, construction, energy and optics. A variety of NMs have also been employed to enhance the functionality and sensitivity of medical devices. Role of nanoparticles in healthcare and medicine is expanding day by day. Recently, nanoparticles are used for tissue engineering, encapsulation, drug and genes delivery (Linkov et al., 2008; Beck-Broichsitter et al., 2012). Due to various applications of nanoparticles in medicine, a new medicine field nanomedicine is emerging. Nanomedicines have greatly impacted human health with their significant contribution in diagnosis and treatment.

In vivo Fate of nanoparticles: Despite the continuous rise in the application of nanoparticles in daily routine life, it is imperative to think about the undesirable effects associated with nanoparticles. Nanoparticles due to their nano-size are able to cross the membrane barrier and can affect tissues and organs. An intensive research is required to study the toxic effect of nanoparticles on human health. Humans can be exposed to nanoparticles either intentionally or by chance through different routes like lungs, skin, oral, and injection. After absorption of nanoparticles in body, they either may accumulate in peripheral organs or may excrete out of the body (Oberdorster et al., 2013; Rinaldo et al., 2015). If nanoparticles enter into the body via ingestion, nanoparticles have to cross various barriers like the epithelial cells of gastrointestinal tract, the mucus barrier and the subepithelial tissue (Lundquist et al., 2016).

After their entry in to circulatory system, NPs can enter into different organs. It is already reported that particles can cross the blood–brain barrier, nuclei of cells and enter into the cells by crossing cell membrane (Oberdorster et al., 2004; Jiang et al., 2008). The various studies showed that NPs can exert undesirable effects in a range of animal organs (Lecoanet and Wiesner, 2004; Oberdorster, 2004; Polandet al., 2008). Nanoparticles may interact with different biomolecules and may leads to reactive oxygen species production, oxidative stress, DNA damage, protein mutation, endothelial dysfunction, impaired mitochondrial functioning and altered cell cycle regulation (Nel et al., 2006, Karlson et al., 2008 Accomasso et al., 2016).

However, particles toxicity is dependent on type of particles, size distribution, concentration of NPs, length of exposure time (Rozman et al., 2001, David and Wagner, 1998; Winder 2004). In 2009, a study has been conducted to determine the excretion pathways, absorption pathways, bio-distribution and interaction with cells (Morawska et al., 2009). Size distribution is key factor that mainly affects the entry and absorption of particles in respiratory tract (Bakand et al., 2012; Asgharian et al., 2006). Ultrafine particles can enter deeply into the alveolar region, from where the excretion may be difficult (Siegmann et al., 1999; Rozman et al., 2001; Witschi et al., 2001). Deeply penetrated particles take more time in elimination from lungs and are expected to cause severe adverse effects (Blank et al., 2009). Liver may absorb 30%–99% of nanoparticles from blood circulation which can cause serious toxicity at cellular level (Kan et al., 2008).

Toxicity associated with nanoparticles: Increased use of nanoparticles in industries and environmental applications has raised the concentration of NPs in ecosystem. These nanoparticles may be released either deliberately into the environment for treatment purpose or accidently from various products. The increased uses of NPs in various products has also contaminated the aquatic environment and imposed undesirable effects on aquatic organisms including algae, fish and daphnia (Navarro et al., 2008). In plants, aluminium oxide nanoparticles can lead to slight reduction in root growth. A significant adverse effect of Al-NPs on root enlargement in various plants like Zea mays, Glycine max, Brassica oleracea and Daucus carota was reported (Hegde et al., 2015). Nano encapsulates used in food, feed products and medical field, being more stable forms of encapsulates may increase the bioavailability of ingredients (Zhang et al. 2016). The nanoparticles can enter in to the body through various routes (Sufian et al., 2017; Palmeret et al., 2016). After entry into the animals, NPs may also translocate to secondary organs (Taghavi et al., 2013). The electron transport system of chloroplast and mitochondria are adversely affected by the NPs, by elevating the level of reactive oxygen species (ROS) (Tripathi et al., 2018).

Cytotoxicity of different nanoparticles like gold, silver NPs, Titanium oxide, carbon nanotubes, and quantum dots has been widely studied. Severity of toxicity of nano-particles may vary depending on the method of synthesis, particles size, exposure time, routes of entry. Nanoparticles of size less than 50 nm may enter into the cells and may affect the functioning of sub-cellular organelles (Geiser et al., 2005, Chithrani, 2010). Combustion-derived NPs, a main constituent of air pollution can adversely affect Central Nervous System, and cardiac functions (Donaldson et al., 2005; Donaldson et al., 2004). This is reported that several nanoparticles like titanium dioxide (Hamilton et al., 2009), zinc oxide (Cho et al., 2011), polystyrene (Lunooy et al., 2011), and poly-cation particles can damage the lysosome (Molinaro et al., 2013). Major consequences of lysosomal injury are the release of protons, and hydrolytic enzymes, which can cause oxidative stress, endoplasmic reticulum (ER) stress and mitochondrial dysfunction (Stern et al., 2012). Thus, the cytotoxic effect of silver NPs includes ROS production, glutathione reduction and mitochondrial dysfuntion (Hussain et al., 2005; Dubey et al., 2015). Metal nanoparticles deposition in liver may exert several toxic effects (Li et al., 2016; Chinde et al., 2017; Kreyling et al., 2018).

Deposited nanoparticles may cause structural changes, metabolic dysfunction and liver injury manifested in form of decrease of bilirubin level and increase of alkaline phosphatase (Sha et al., 2014; Magaye et al., 2014; Li et al., 2016). AgNP, TiO2 and AuNP can exert liver toxicity by causing hepatocyte necrosis, liver structure alteration and liver injury respectively, (Recordati et al., 2016; Matthias et al., 2012). NPs due to their nano size can cross the placenta and may impair brain development in fetus (Song et al., 2016). Nanoparticles can also affect food digestion and absorption of macro- and microelements from food components, which can lead to deficiency of vital elements (McClements et al., 2016, Suker et al., 2018).

In mice, TiO_2 NPs exhibited cytotoxicity and necrosis of liver cells and apoptosis, liver fibrosis and swelling of renal glomeruli were observed. TiO2 NPs deposition in the lungs was observed which may be due to blockage of blood vessels (Chen et al., 2009). Silver due to its antimicrobial activity is used in many products available in market. AgNPs of 10 nm size have also been reported to be cytotoxic to human lung cells (Gliga et al., 2014). Nanoparticles penetration in to the skin can also have cytotoxic effect on skin due to photo-activation of NPs when present at skin (Tsuji et al., 2006). Hair follicles and sweat glands present in skin may facilitate the entry of small-sized NPs into the skin (Teow et al., 2011). After penetration into the body, nanoparticles are majorly deposited in the brain such as the olfactory bulb and the hippocampus (Hong et al., 2015; Simkó and Mattsson, 2010; Bramini et al., 2014 Allen, 2016).

Studies have reported that inflammation or other signs of toxic effects were observed within 24hr of administration of TiO2 NPs. (Warheit et al., 2007). Some report suggest that some NPs enhance different level of stress and its leads to the generation of free radicals which would ultimately deteriorate the endothelial cell membrane (Sharma and Sharma, 2007). TiO2 and ZnO NPs used in sunscreen can penetrate skins, can stay within the human stratum corneum or can enter into some hair follicles (Schrand et al., 2010). The Guiana Pigs exposed to NPs of different sizes (less than 100 nm) confirmed a connection between skin exposure and tissue levels of Ag NPs (Park et al., 2011).

The rainbow trout respiratory system is negatively affected by carbon nanotubes (Templeton et al., 2006). Due to thin fibre-like constitution and insolubility in lungs, carbon nanotubes can cause severe harmful effects (Donaldsonans Poland, 2009). Various studies indicate that carbon nanotubes may support allergic immune responses, exacerbate airway inflammation (Cuiet al., 2005; Nygaard et al., 2009; Inoue et al., 2009). The interstitial inflammation and lesions in mice and rats are some of the adverse effects of single-walled carbon nanotubes (Lam et al., 2004). The multi-walled carbon nanotubes have also been reported to cause DNA damage in mouse embryonic stem cells (Zhu et al., 2007). It was reported that NPs can pass through the different organ systems without detected by normal phagocytic control system, and can also pass the blood-brain barrier (Chen and Boi, 2007). Ag-NPs show toxicity by generating roxidative stress, genotoxicity and cell apoptosis (Kahru and Dubourguier, 2010).

The cytotoxic effect of NPs varies with cell type and method of synthesis (Kong et al., 2011). Apoptosis and cell cycle inhibition was also observed in PC12 cells treated with TiO2 NPs (Wu et al., 2010). The interactions of NPs with cells and tissues are different due to their unique physicochemical environment (Aillonet al., 2009; Zhanget al., 2012). Many nanoparticles are photoactive, show high absorption coefficients and can act as catalysts (Colvin, 2003; Hristozov and Malsch, 2009). Glial activations and glial-neuronal interactions observed in neurotoxic effect may result in several subsequent effects (Li and Martin, 2017). Oxidative stress, cell apoptosis and autophagy, which affect the blood brain barrier function, may be the main cause of neurotoxicity. The entry of TiO2 nanoparticles in human body can have deleterious effect on brain function. Thus, the selection of effective delivery method for delivery of therapeutic drug to the brain is essential to treat neurodisorders (Huang et al., 2017 Zang and Gao, 2016).

NPs can also enter in to the reproductive tissues and then accumulate. The reproduction and embryonic development of mammals are adversely affected by the exposure of nanoparticles (De Jong and Borm, 2008). The AgNPs can significantly raise the intensity of nucleic acid damage in germ cells (Garcia et al., 2014). Inorganic nanoparticles wrapped up by bacterial cells, e.g., zinc oxide with E. coli cell wall can absorb CeO_2 nanoparticles (Thill et al., 2006). Studies have shown that the occurrence of various diseases related to reproductive organs like testicular cancer is increasing (Wang et al., 2018). Gold alloys may stimulate the inflammation of epididymis and adversely affect the sperm motility (Wang et al., 2018).

CONCLUSION

Nanoparticles have tremendous application in various sectors word-wide. Despite their vast application potential, the toxicity associated with their uses cannot be ignored. Exponentially increasing use industrial application of nano-materials has increased the possibility of interaction of nanoparticles with biological milieu. However, lots of reports have already been published related to toxicity of different nanoparticles. Still there is need for detailed study about the toxicity associated with different type of nanoparticles and mechanisms of their toxic effect. In vitro methods may be standardized and can be used in toxicity profiling of nanomaterials. However, various strategies have been developed to analyse the accidental human exposure to nanomaterials. Strict measures should be applied to avoid the risk of nanomaterials exposure to safeguard human health. In future, nanoparticles can also be modified to decrease their toxicity without affecting their properties.

The assessment of risks associated with nano-materials should be implemented as an essential part of synthesis and production processes.

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Optical Properties of Bismuth Oxy Chloride (BiOCl) Using Density Functional Theory

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ABSTRACT

BiOCl Crystal's electronic band structures, density of state (DOS), and optical properties have been studied using the Local Density Approximation (LDA) density function theory. The electronic band structure obtained shows that BiOCl crystal has a forbidden band gap of 2.45 eV indirect. Using the LDA, structural optimization for BiOCl was carried out. The outcome of BiOCl's structure optimization was contrasted with the experimental findings, and it was found to be in strong agreement with these tests. It calculates the linear photon-energy-dependent dielectric functions and some optical properties, such as the function of energy-loss, the effective number of valance electrons and the effective optical dielectric constant

KEY WORDS: BIOCL, AB-INITO, BAND STRUCTURE, OPTICAL PROPERTIES.

INTRODUCTION

Bismuth oxychloride (BiOCl), a member of compounds with the general formula A= As, Sb, Bi, B= O, S, Se and X= Cl is a wide bandgap semiconductor with a tetragonal PbFCl-type structure (space group P4/nmm: No: 129) (Keramidas et al, 1993; Peng et al, 2009; Zeng et al, 2019). This crystal has 2 BiOCl molecules in a unit cell. Therefore, this compound has a complex structure with 18 valance electrons per unit cell.

The BiOCl unit cell is shown in Figure 1 (Zhang et al, 2006) and atomic positions are given in the unit cell in Table 1 (Keramidas et al, 1993). The structure of crystals can be derived from the structure of the fluorite (CaF2). The Bi atom is paired with four O atoms in one base and four Cl atoms in another. The atom O is coordinated tetrahedrally to four atoms Bi. The Cl atom is bound in a planar square with four Bi atoms to form a pyramid, with its non-bonding electrons pointing to the other side of the square. As shown in Figure 1, through the Cl atoms along the c- axis, the (BiOCl) layers are stacked together by the nonbonding (van der Waals) interaction; the structure is therefore not heavily packed in this direction (Zhang et al, 2006; Sun et al, 2019).

Within the local density approximation (LDA) by Zhang et al, 2006, the electronic structure of BiOCl was calculated using the tight-binding linear muffin-tin orbital (TB-LMTO) method (Li et al, 2018). The calculated indirect nature is in agreement with the experimentally observed linear relationship between and (where and represent, respectively, the absorption coefficient and photon energy) although the calculated band gap is

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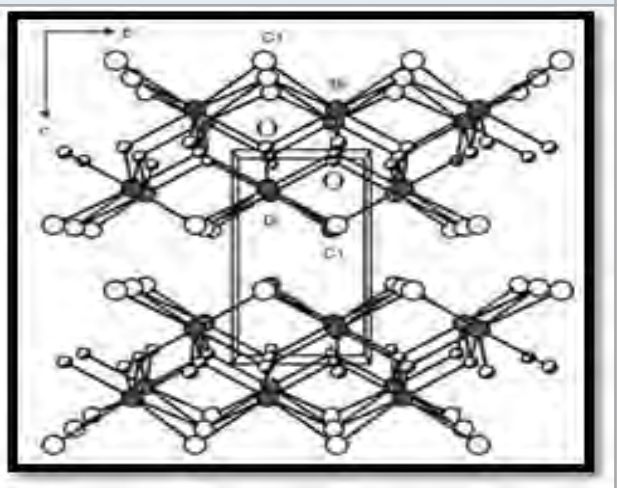
relatively narrow (Zhang et al, 2006; Li et al, 2017). BiOX's electronic band structure (X = F, Cl, Br, and I) was determined by Huang at al, 2009, using DFT method within GGA scheme. The atomic charges and bond orders were analyzed using the Mulliken population analysis (Mulliken, 1955; Srivastava et al, 2014; Sharma et al, 2015; Segall et al, 1996; Segall et al, 1196; Sun et al, 2018), describing the spatial distribution of orbital density (Huang at al, 2009) as well. No ab initio general possible measurements of BiOCl's optical properties have been documented in depth, as far as we know (Tripathi, 2019; Wang et al, 2019).

Table 1. Fractional atomic coordinates (Å) for BiOCl (Keramidas et al, 1993)

Atoms	x	Y	Z
Bi	0.25	0.25	0.1714(3)
O	0.25	0.75	0
Cl	0.25	0.25	0.6459(25)

Figure 1: The unit cell of BiOCl viewed along (100) (Zhang et al, 2006)



In the present work we have investigated the electronic band structure, total state density (DOS), structure optimization, and photon-energy-dependent optical properties of the BiOCl crystal using a pseudo potential approach based on the local density approximation (LDA) density functional theory (DFT) (Kohn et al, 1965; Liu et al, 2019).

MATERIAL AND METHODS

For this research, the SIESTA (Spanish Initiative for Electronic Simulations with Thousands of Atoms) code (Ordejon et al, 1996; Soler et al, 2002; Liao et al, 2020; Fitzpatrick et al, 2020) was used to measure BiOCl 's energy spectra and optical. In the LDA parameterized by Ceperley et al, 1980; it solves the quantum mechanical equation for the electron inside the DFT method. Separable Troullier et al, 1991; norm-conserving pseudopotential activates the interactions between the electrons and core ions. The basis set is based on the Sankey et al, 1989; finite range pseudoatomic orbital's (PAO's), expanded to include multiple-zeta decay (Heidari et al, 2020; Canpolat et al, 2019).

We have separately generated atomic pseudopotentials for Bi, O and Cl, using the atomic configurations $5s^2 5p^3$, $2s^2 2p^4$ and $3s^2 3p^5$, respectively. The cut-off radii are taken as 2.70, 1.15 and 1.65 a.u for current atomic pseudopotentials. For the Bi, O, and Cl channels s, p, d, and f respectively. SIESTA measures the potential of self-consistency in real space on a grid. In terms of an energy cut-off E_c, the fineness of this grid is calculated in analogy to the energy cut-off if the basis set contains plane waves. Here, we find an optimum value of around 300 R_y for BiOCl by using a double-zeta plus polarization (DZP) orbital's basis and the cut-off energies between 50 and 450 R_y with different base sets. 98 k-points for BiOCl were found to be sufficient for the final calculations to obtain the total energy with an accuracy of approximately 1 meV/atoms (Paudel et al, 2018).

RESULTS AND DISCUSSION

Structural Optimization: All physical characteristics contribute to total strength. For example, a crystal's equilibrium lattice constant is the lattice constant which minimizes total energy. If the total energy is measured, any physical property can be determined that is related to total energy. First, the equilibrium lattice parameter was determined by minimizing the total energy of the crystal determined for the different lattice constant values by means of Murnaghan's, 1994; state equation (EOS) as shown in Figure 2, and the result is shown in Table 2 along with the experimental and theoretical values. For tetragonal structures the lattice parameters for BiOCl are found to be a = b=3.88 which c=7.314, and are in good agreement with the experimental and theoretical values. We have used the computed lattice parameter in all of our calculations (Sun et al, 2019).

Figure 2: Energy versus volume curve of BiOCl

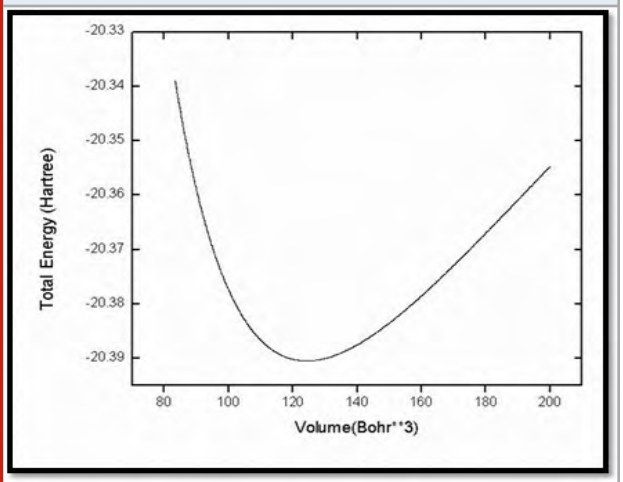
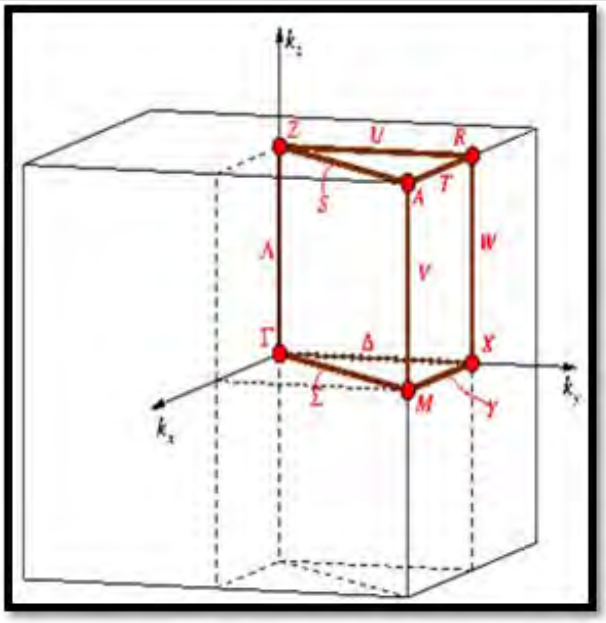


Table 2. Structure parameters of BiOCl materials

Reference	α(Å)	c (Å)	Space Group
Present	3.888	7.314	
Experimental	3.887	7.354	
Experimental	3.888	7.357	P4/nmm
Experimental	3.890	7.890	
Theory	3.824	7.243	

Electronic Band Structure: In the first Brillouin Zone (BZ) of the tetragonal system, the electronic band structures of BiOCl crystals were determined along high symmetry directions and are shown in Figure 3. The band structures were calculated along the special lines which connect the high symmetry points (1/2, 0, 0), X (1/2, 0, 0), Z (0, 0, 1/2), M (1/2, 1/2, 0), R (1/2, 0, 1/2) and A (1/2, 1/2, 1/2) in the k-space (Zhang et al, 2019).

Figure 3: First Brillouin zone for BiOCl (space group P4/nmm)



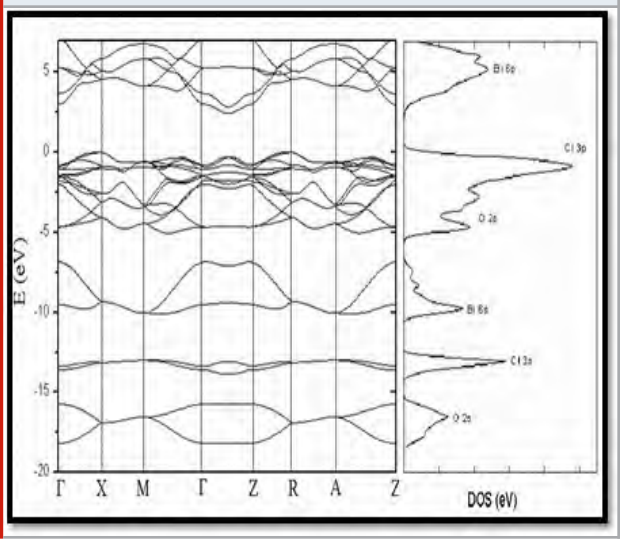
The calculation results are shown on BiOCl crystal in Figure 4. The densities of states (DOS) are shown in the rightmost panels of this Figure. Such crystals' determined band gap values are given in Table 3. In our calculations, the valance band consists of the Cl 3s and 3p states, the O atom 2s and 2p states, and the Bi atom 6s states, while the conduction band consists of the Bi atom 6p states.

Table 3. Energy band gaps for BiOCl

Referance	E_g(eV)
Present	2.45 indirect- 2.77 direct
Experimental	3.46 indirect
Theory	2.59 indirect

As can be seen in Figure 4, the top of the valance band is placed between the Z-R points near the R point and the bottom of the conductive band is located at the nearly midway point between the BZ's and Z points. Therefore the BiOCl band gap is indirect with the value 2.45 eV the lowest direct band gap value reported for BiOCl is 2.77 eV(Tripathi et al, 2017; 2016; 2015; 2016).

Figure 4: Energy band structure and DOS (density of states) for BiOCl



Ultimately, the values obtained for BiOCl in the band gap are less than those measured. The band gap values are underestimated than the experimental values for all of the crystal structures considered. Because of the use of pseudopotential method this is an expected case.

3.3. Optical Properties: It is well known that the effect of the electric field vector, E , of the incoming light is to polarize the material. At the level of linear response this polarization can be calculated using the following relation (Li et al, 2009; Zhang et al, 2019):

$$P^i(\omega) = \chi_j^{(1)}(-\omega,\omega)\, E^j(\omega) \quad (1)$$

Where $\chi_{ij}^{(1)}$ is the linear optical susceptibility tensor and it is given by (Levine et al, 1989)

$$\chi_{ij}^{(1)}(-\omega,\omega) = \frac{e^2}{\hbar\Omega}\sum_{m\,\vec{k}} f_m(\vec{k})\frac{r_m^i(\vec{k})r_m^i}{\omega_m(\vec{k})} \quad (2)$$

Where n,m denote energy bands, $f_{mn}(\vec{k}) = f_m(\vec{k}) - f_n(\vec{k})$ is the fermi occupation factor, Ω is the normalization volume. $\omega_{mn}(\vec{k}) \equiv \omega_m(\vec{k}) - \omega(\vec{k})$ are the frequency differences, $\hbar\omega_n(\vec{k})$ is the energy of band n *at* wavevector k. The $\vec{r}_{mn}$ are

are the matrix elements of the position operator and are given by

$$r^{i}_{nm}(\vec{k}) = \frac{v^{i}_{nm}(\vec{k})}{i\omega_{nm}}; \quad \omega_n \neq \omega_m \qquad \text{.....(3)}$$

$$r^{i}_{nm}(\vec{k}) = 0; \quad \omega_n = \omega_m$$

Where $v^{i}_{m}(\vec{k}) = m^{-1}p^{i}_{m}(\vec{k})$ m is the free electron mass, and $\vec{p}_{mn}$ is the momentum matrix element.

As can be seen from equation (2), the dielectric function $\varepsilon_j(\omega)=1+4\pi\chi^{(1)}_{j}(-\omega,\omega)$ and the imaginary part of $\varepsilon_j(\omega)$ $\varepsilon^{j}_{2}(\omega)$ is given by

$$\varepsilon^{ij}_{2}(w) = \frac{e^2}{\hbar\pi}\sum_{m}\int d\vec{k} f_{nm}(\vec{k})\frac{v^{i}_{m}(\vec{k})v^{j}_{m}(\vec{k})}{\omega^{2}_{m}}\delta(\omega-\omega_{m}(\vec{k})). \qquad (4)$$

The real part of $\varepsilon_j(\omega)$ $\varepsilon^{j}_{1}(\omega)$ can be obtained by using the Kramers-Kroning transformation

$$\varepsilon^{j}_{1}(\omega)-1 = \frac{2}{\pi}\wp\int_{0}^{\infty}\frac{\omega'\varepsilon^{j}_{2}(\omega')}{\omega'^{2}-\omega^{2}}d\omega'. \qquad (5)$$

Since the Kohn-Sham equations decide the properties of the ground state, there is no physical meaning to the unoccupied conduction bands as measured. If they are used in calculating optical properties for semiconductors as single-particle states, a band gap problem is included in the response calculations. In the present work, we used the 'scissors approximation' (Levine et al, 1989; Philipp et al, 1963; Zhang et al, 2019) in order to take into account self-energy results. In the present work, , the scissor shift to make the theoretical band gap match the experimental one, is 1.01 eV for BiOCl.

Expressions for the energy-loss spectrum, $L(\omega)$.

$$L_{ij}(\omega) = -\mathrm{Im}\ \varepsilon^{-1}_{j}(\omega) \qquad (6)$$

The known sum rules (Kovalev, 1965) can be used to determine certain quantitative parameters, in particular the effective number of valence electrons per unit cell N_{eff}, and the effective optical dielectric constant ε_{eff} which contributes to the optical constants of an energy crystal. One can obtain an estimate of the distribution of oscillator strengths for both intra band and inter band transitions by computing the $N_{eff}(E_0)$ defined according to

$$N_{eff}(E) = \frac{2m\varepsilon_0}{\pi\hbar^2e^2N}\int_{0}^{\infty}\varepsilon_2(E)EdE, \qquad (7)$$

Where $N\alpha$ is the density of atoms in a crystal, e and m are the charge and mass of the electron, respectively and $N_{eff}(E_0)$ is the effective number of electrons contributing to optical transitions below an energy of (E_0)

Further information on the position of the core and semi-core bands can be obtained by measuring the contribution made by the different bands to the static dielectric constant; ε_0

According to the Kramers-Kronig relations, one has

$$\varepsilon_0(E)-1 = \frac{2}{\pi}\int_{0}^{\infty}\varepsilon_2(E)E^{-1}dE \ . \qquad (8)$$

Thus, an 'effective' dielectric constant can be defined, which represents a different means of inter band transitions than that represented by the sum law, equation (8), depending on the relation

$$\varepsilon_{eff}(E)-1 = \frac{2}{\pi}\int_{0}^{E_0}\varepsilon_2(E)E^{-1}dE \ . \qquad (9)$$

The physical meaning of ε_{eff} is quite clear: ε_{eff} is the effective optical dielectric constant governed by the inter band transitions in the energy range from zero to E0, i.e. by the polarization of the electron shells.

We have chosen a photon-energy range of 0-30 eV to calculate the optical response using the calculated band structure and have seen that a photon-energy range of 0-18 eV is sufficient for most of the optical functions.

The BiOCl crystal has an optically uniaxial, hexagonal structure. For this reason the BiOCl crystal linear dielectric tensor has two independent components that are the linear dielectric tensor's diagonal elements. Figure 5 presents the measured actual parts and imaginary parts of the linear frequency-dependent dielectric function's xx- and zz- components. The function ε^{x}_{1} is equal to zero at about 6.66, 10.72, 13.90, 22.5, 22.99 and 23.23 eV (W, X, Y, Z, U and V in Figure 5), while the other function ε^{z}_{1} is equal to zero at about 7.37, 8.35, 8.49, 9.90, 12.97 and 22.28 eV (W, X, Y, Z, U and V in Figure 5). The values of the ε^{x}_{2} and ε^{z}_{2} peaks shown in Figure 5 are summarized in Table 4. This peak corresponds to the transitions from the valance to the conduction band (see Figure 5).

The calculated energy-loss functions, $L(\omega)$, are presented in Figure 6. In this Figure, L_{xx} and L_{zz} correspond to the energy-loss functions along the x- and z- directions, respectively. The function $L(\omega)$ describes the energy loss of fast electrons traversing the material. The sharp maxima in the energy-loss function are associated with

the existence of plasma oscillations (Marton, 1956). The curves of L_{xx} and L_{zz} in Figure 6 have a maximum near 24.33 and 25.23 eV, respectively and these value coincide with the V point in Figure 5.

Table 4. Comparative characteristics of linear optical functions of BiOCl crystal

	Peaks (eV)															
ε_2	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Xx	5.44	6.39	7.21	11.53	12.49	13.49	14.49	15.61	16.21	17.66	18.47	18.93	20.73	21.95	22.91	24.02
Zz	4.97	6.04	7.26	8.65	10.39	12.87	14.06	15.02	15.51	16.29	17.85	18.93	19.59	20.21	23.61	24.87

Figure 5 Energy spectra of dielectric function for BiOCl

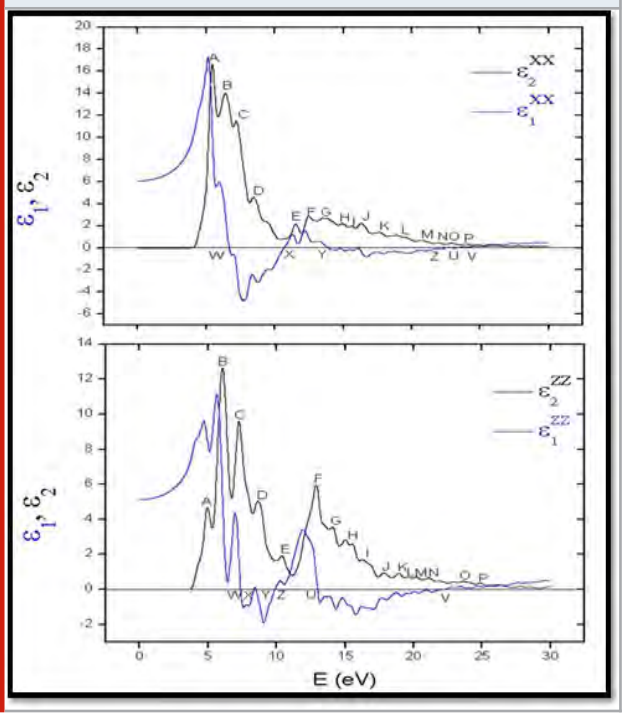
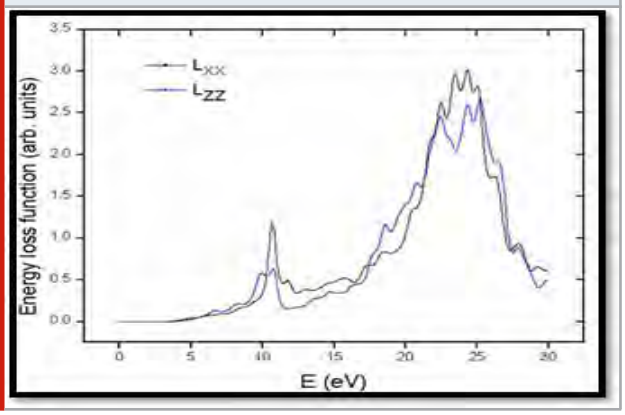
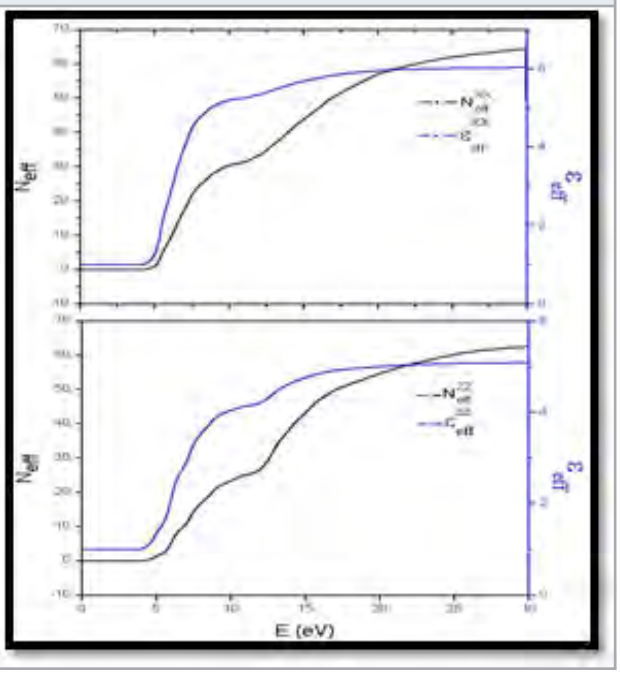


Figure 6 Energy-loss functions along the x- and z- axes for BiOCl



The calculated effective number of valence electrons N_{eff} and the effective dielectric constant (ε_{eff}) are given in Figure 7. The effective number of valence electron per unit cell N_{eff}, contributing in the inter band transitions, reaches saturation value at about 26 eV. This means that deep-lying valence orbital's do not participate in the inter band transitions (see Figure 4)

Figure 7: Energy spectra of and along the x- and z-axes



The effective optical dielectric constant ε_{eff} as shown in Figure 7, reaches a saturation value at approximately 16 eV. Dependence of the photon-energy ε_{eff} can be separated into two regions. The first is marked by a rapid rise and extends up to 10 eV. The value of ε_{eff} rises smoother and slower in the second region, and tends to saturate at 16 eV energy. This means that the greatest contribution to ε_{eff} arise from inter band transitions between 4.7 and 16 eV.

CONCLUSION

In this work we have carried out a detailed investigation of the BiOCl crystal's electronic structure and frequency-dependent linear optical properties using the density functional methods. This research had the challenge of applying the density-functional methods to a complex crystal such as the BiOCl. BiOCl crystal is seen as having the indirect forbidden gap. Our experimental results are consistent with the obtained band gap 2.45 eV values

in agreement with the previous results. The complete calculation of DOS shows that the valance band consists of 3s and 3p states of the Cl atom, 2s and 2p states of the O atom, and 6s states of the Bi atom, while the band of conduction consists of 6p states of the Bi atom. We investigated photon energy-dependent dielectric functions as well as related quantities such as power-loss ratio, the effective number of valance electrons per unit cell participating in inter band transitions and the effective optical dielectric ratio along the x- and z-axes. The results of the structural optimization carried out using the LDA are in excellent agreement with the results of the experiments.

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Effect of *Ocimum sanctum* and *Allium sativum* on Lipid Peroxidation and Antioxidant Enzymes in Alloxan Induced Diabetic Rats

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ABSTRACT

In diabetes, persistence hyperglycaemia has been reported as a cause of increased production of free radicals. Hyperglycemia induces oxidative stress and becomes the main factor for predisposing the complications in diabetes. The study is being aimed to find out the status of lipid peroxidation by measuring the levels of peroxidation end products namely,malondialdehyde (MDA) and4-hydroxynonenal (4HNE)nitrate and nitrite as well as antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) which might be helpful in risk assessment of various complications of diabetes mellitus. Treatment of the diabetic animals with *Ocimum sanctum* (Holy basil/ Tulsi) and *Allium sativum* (garlic) extract for two weeks showed protective effects evidenced by reversal of most of the parameters studied including plasma glucose level as compared to control rats.

KEY WORDS: OXIDATIVE STRESS, ANTIOXIDENTS, LIPID PEROXIDATION, OCIMUM SANCTUM AND ALLIUM SATIVUM.

INTRODUCTION

Diabetes mellitus is a serious health problem affecting millions of individuals worldwide. By the year 2025, the World Health Organization (WHO) predicts that over 300 million people worldwide will have diabetes mellitus. The reported prevalence of diabetes in adults between the ages of 20 and 79 is as follows: India8.31%, Bangladesh 9.85%, Nepal 3.03%, Sri Lanka 7.77%, and Pakistan 6.72%. Diabetes is a chronic disease, which occurs when the pancreas does not produce enough insulin, or/ and when the body cannot effectively use the insulin it produces. Diabetes mellitus is a group of metabolic diseases characterized by high blood glucose level that result from defects in insulin secretion, or action, or both (Lakhtakia et al., 2013, Gyawali et al., 2015, Animaw et al., 2017, Alotaibi et al., 2018).

Islets of Langerhans are organelles present within the pancreas and are mainly responsible for the production of insulin, glucagon, somatostatin and pancreatic polypeptide upon stimulation (Xavier et al., 2018). There is evidence that β cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acids level or a combination of both (Jezek et al., 2018). β cells are particularly sensitive to ROS due to inadequate expression of free radical quenching enzymes (Tan et al., 2018). The capability of oxidative stress to damage mitochondria and ultimately decrease in insulin secretion is therefore, obvious (Park et al., 2019).

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Oxidative stress, a potentially harmful imbalance between the levels of pro-oxidants and antioxidants (Pizzino et al., 2017). It can cause cellular injury and tissue damage by promoting several reactions e.g., lipid perxidation, DNA damage, protein glycationetc (Liguori et al., 2018). Lipid peroxides may increase the participation of advanced glycation end-products in the development of chronic vascular complications (Chen et al., 2018). The chemical modification of proteins and lipids by ROS is speculated to contribute to the pathogenesis of diabetic complications. ROS also causes base modifications and strand breaks in DNA also (Sengupta et al., 2018).

Under normal conditions, free radicals are formed in minute quantities and are rapidlyscavenged by natural cellular defense mechanisms comprisingof enzymes like superoxide dismutase (SOD), glutathioneperoxidase (GPx), glutathione reductase (GR) and catalase (CAT) (Kurutas et al., 2016). Increased production of ROS which disturbs the antioxidant defence system of the cell by increasing the functional levels of superoxide anion ($O2^{-}$.), hydroxyl radical (OH.) and hydrogen peroxide (H_2O_2) (Lu et al., 2018). There are reports that in diabetics the disturbed equilibrium between prooxidants and antioxidants alters the metabolic status of body (Sarangarajan et al., 2017).

Plants have always been the source of drugs since ancient times. Many of the currently available drugs have been derived directly or indirectaly from plants (Calixto et al., 2019). Many herbal medicines have been recommended for the treatment of diabetes (Moradi et al., 2018). The effect of *Ocimum sanctum* on the glucose, serum lipid peroxidase and antioxidant enzymes are documented (Governa et al., 2018). Antidiabetic drugs can act in different ways such as stimulation of beta cell of pancreatic islets to release insulin to resist the hormones which rise blood glucose, increase the number and sensitivity of insulin receptor, increase glycogen content, enhance the use of organ glucose in the tissue, free radicals scavenging, resist lipid peroxidation, correct the metabolic disorder of lipid and protein and promote microcirculation in the body (Lankatillake et al., 2019). The aim of the present study is to investigate the potential of *Ocimum sanctum* (holy basil or Tulsi) and *Allium sativum* (garlic) in controlling the levels of glucose, nitrate/ nitriteand antioxidant enzymes in alloxan induced diabetic rats as compared to control and diabetic rats (Jayant et al., 2016).

MATERIAL AND METHODS

Chemicals used in the present study were of highest purity/ analytical grade. Sodium chloride, potassium chloride, sodium carbonate, sodium bicarbonate, disodium hydrogen phosphate, sodium dihydrogen phosphate, triton X-100, sodium hydroxide, ethylenediaminetetraacetic acid, reduced glutathione, sodium azide, ethanol, and magnesium chloride were purchased from HIMEDIA Chemicals, India. Glutathione, Folin-Ciocalteu's reagent, glutathione reductase, nicotinamide adenine dinucleotide phosphate(reduced) were purchased from sigma Aldrich chemicals private limited, Mo, USA.

Male albino rats of Wistar strain (weight 120±20g) were used in the proposed study. Animals were obtained from the animal facilities of Defence Research and Development Establishment, Gwalior, India, and were maintained under controlled conditions of temperature (250 ± 2^{0}C), relative humidity of (50 ± 15%), and normal photoperiod (light-dark cycle of 12 h) in the animal room of our department on standard pellet diet and tap water ad *libitum*. Animals were housed throughout the experiment in polypropylene cages (with each cage housing six animals) containing paddy husk as bedding and allowed to acclimatize to the environment of animal room for seven days before the start of the experiment.

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 leaves extract of *Ocimum sanctum*, 2.5 mg/kg body weight) (Kaushal et al., 2019).
Group 3 : Treated control group (treated with extract of pods of *Allium sativum*, 0.25 mg/kg body weight) (Azantsa et al., 2018).
Group 4 : Diabetic (I.V. injection of alloxan 70 mg/kg body weight).
Group 5 : Treated diabetic group (treated with leaves extract of *Ocimum sanctum*, 2.5 mg/kg body weight).
Group 6 : Treated diabetic group (treated with extract of pods of *Allium sativum*, 0.25 mg/kg body weight).

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 (Jayant et al., 2016). The animals were checked for blood glucose level 48 h after alloxan injection, and the rats with blood sugar level above 200 mg/dl were considered as diabetic and were used for the experiment.

Ocimum sanctum (Holy basil or Tulsi) leaves were obtained from botanical garden of Jiwaji University Gwalior, cleaned and aqueous extract was prepared and 2.5 mg/kg body weight was given orally to the rats of group 2 and 5 with the help of cannula, daily, for two weeks. *Allium sativum* (Garlic)pods were purchased from the local herbal market, cleaned, and aqueous extract of *Allium sativum* pods was prepared and 0.25 mg/kg body weight was given orally to the rats of group 3 and 6 with the help of cannula, daily for two weeks.

Rats were humanely killed 24 h after the last treatment by cervical dislocation; different tissues were excised off, washed with 0.9% NaCl and used for different

estimations. Animals were handled, ethically treated and humanly killed as per the 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. Malondialdehyde (MDA) and 4-hydroxynonanal (4HNE), the two major end products of lipid peroxidation, were estimated by the method of Jacobson (1999) with minor modifications. A 10% tissue homogenate was prepared in TrisHCl buffer (20 mM, pH 7.4). Prior to homogenization, 10 µL 0.5 M

butylatedhydroxytoluene(BHT) in acetonitrile was added per 1 ml of tissue homogenate. After homogenization, the homogenate was centrifuged at 3000 g for 10 minat 4° C and clear supernatant was used for the assay. Briefly 200 µL of supernatant was transferred to 650 µL of 10.3 mM 1-methyl-2-phenylindole in acetonitrite and vortex mixed. To assay MDA + 4HNE, 150 µL of 15.4 M methanesulfonic acid (MSA) was added,vortexed and incubated at 45°C for 40 min . To assay MDA alone, 150 µL of 37% HCl was added instead of MSA, vortexed, incubated at 45°C for 60 min. After incubation, samples were kept on ice, centrifuged at 9500 g for 5 min and absorbance was measured at 586 nm. The levels of MDA and 4HNE are expressed as nmol g^{-1} tissue using extinction coefficient 1.1×105 M-1 cm^{-1}.

Nitrate and nitrite levels in the tissues were estimated as described by Miranda (2001). Tissue homogenate was prepared in 0.9% NaCl and for nitrate estimation, reaction mixture contained 100 µl supernatant, 100 µl of VCl_3 (saturated solution) and 100 µl of Griess reagent (prepared by mixing equal volumes of 2% sulfonamide in 5% HCl and 1% N[1-naphthyl] ethylenediaminedihydrochloride). Absorbance was measured at 540 nm. For nitrite estimation, 100 µl of distilled water was added instead of VCl_3. For standard curve varying concentrations of $NaNO_3$ or $NaNO_2$ ranging from 2 to 20 µmole was taken in 100 µl and added either VCl_3 (for nitrate) or distilled water (for nitrite) and 100 µl Griess reagent. Concentration of nitrate or nitrite was expressed as µmole gm-1 tissue.

Superoxide dismutase (SOD, E.C.No. 1.15.1.1): SOD activity was assayed by estimating the inhibition of auto-oxidation of epinephrine (Misra et al., 1972). The 10% tissue homogenate was prepared in 0.9% NaCl, centrifuged at 15,000 g for 15 min and the corresponding supernatant was used for enzyme assay. Reaction mixture containing 0.5 ml sodium carbonate buffer (0.3 M, pH 10.2), 0.5 ml EDTA (0.6 mM), 0.5 ml homogenate and 1.0 ml water, was incubated at room temperature for 10 min. Reaction was initiated by addition of 0.5 ml epinephrine (1.8 mM) and absorbance change per min was recorded for 5 min at 480 nm. Specific activity is expressed as % inhibition of auto-oxidation of epinephrine by the enzyme min-1 mg-1 protein.

Catalase (CAT, E.C.No. 1.11.1.6): CAT activity was estimated by the method of Aebi (1984). The 10% tissue homogenate was prepared in 1.15% KCl, centrifuged at 5000g for 10 min and the corresponding supernatant was used for CAT estimation. Reaction mixture containing 0.8 ml phosphate buffer (K2HPO4/ NaH_2PO_4, 50 mM, pH 7.0), 0.1 ml homogenate and 0.1 ml triton X-100 (0.02%) was incubated at room temperature for 10 min. Reaction was initiated by addition of 2.0 ml H_2O_2 (0.03 M prepared in potassium phosphate buffer, pH 7.0) and absorbance change per min was recorded for 5 min at 240 nm. Specific activity is expressed as µmole H_2O2 decomposed min^{-1} mg^{-1} protein.

Glutathione peroxidase (GPx, E.C.No. 1.11.1.9): GPx activity was estimated as described by Paglia (1967). The 10% homogenate was prepared in 1.15% KCl, centrifuged at 5000 g for 10 min and the corresponding supernatant was used for GPx estimation. Reaction mixture containing 0.3 ml sodium phosphate buffer (0.1 M, pH 7.4), 0.1 ml GSH (0.15 M), 0.05 ml sodium azide (2.25 M), 0.05 ml homogenate, 0.1 ml NADPH (0.84 mM) and 0.05 ml glutathione reducatse (2 U/ml) was incubated at room temperature for 10 min. Reaction was initiated by addition of 0.05 ml H2O2 (0.001 mM) and absorbance change per min was recorded for 5 min at 340 nm. Specific activity is expressed as nmole NADPH oxidized min-1 mg-1 protein.

Protein estimation: Protein in the tissue samples was precipitated by 10% TCA and the precipitate dissolved in 0.1 N NaOH for estimation. Protein was estimated by the method of Lowry (1951) using bovine serum albumin as standard.

Statistical analyses: Results are expressed as mean ± S.E. of six different sets of observation taken on different days. All the statistical analyses were performed using one-way analyses 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 AND DISCUSSION

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 aquous 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. Theresults of the present study showed that oral administration ofextract of *Ocimum sanctum* and *Allium sativum* daily for 14 days, to the diabetic rats caused 18.7%, and 15.4% decrease on 7th day and 42.8%, and 40.8% decrease in the blood glucose level on day 14th of the start of treatmet when cmpared with respective untreated diabetic rats. The results clearly showed the hypoglycemic potential of *Ocimum sanctum* and *Allium sativum* extracts.

The results of the present study that showed increased lipid peroxidation (LPO) in the tissues of alloxan induced diabetic rats. The results of the presnt study

clearly showed that alloxan adminstration in rats caused accumulation of malondialdehyde (MDA) and 4-hydroxynonanal (4HNE), the two major end products of lipid peroxidation, in the liver and the brain of rats when comapared with control. MDA and 4HNE levels were increased 28.03% and 55.6% in the liver and 51.11% and 69.8% in the brain, respectively, when compared with the control (Table 2). When the diabetic rats were given *Ocimum sanctum* treatment for two weeks, 14.8% and 27.2% decrease in MDA levels while 28.1% and 34.4% decrease in the 4HNE and *Allium sativum* treatment for two weeks, 11.4% and 23.5% decrease in MDA levels while 24.5% and 30.5% decrease in the 4HNE levels of the liver and the brain, respectively, were observed when compared with control group (Table 2). When the *Ocimum sanctum* and *Allium sativum* treated diabetic rats were compared with control group, percent increase of MDA and 4HNE levels were 9.1%, 10.1% and 13.5%, 15.5% in the liver and 11.8%, 11.3% and 17.7%, 17.9% in the brain, respectively. The results clearly showed antioxidative potential of *Ocimum sanctum* and *Allium sativum*.

Table 1. Effect of oral treatment of extracts of *Ocimum Sanctum* and *Allium sativum* on glucose level in alloxan induced diabetic rats

		Level of glucose in 14 days experimental animals		
S No.	Groups	0 day	7th day	14th day
1.	Control	104.00±1.53	105.67±1.33	106.67±1.33
2.	Control + *Ocimum sanctum*	107.33±2.19#	106.33±2.19#	104.67±1.86#
3.	Control + *Allium sativum*	107.67±2.91#	106.67±2.6#	105.67±2.6#
4.	Diabetic	423.33±7.84***	418.67±12.06***	423.33±7.84***
5.	Diabetic + *Ocimum sanctum*	422.19±12.81***	340.33±18.26***	242.33±5.04***
6.	Diabetic + *Allium sativum*	424.00±7.37***	354.00±18.77**	250.67±10.17***

Glucose concentration is expressed as mg/dl.
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 ***

Table 2. Effect of oral treatment of extracts of *Ocimum Sanctum* and *Allium sativum* for 14 days on the levels of MDA and 4HNE in the tissue of alloxan induced diabetic rats.

		MDA		4HNE	
S No.	Groups	Brain	Liver	Brain	Liver
1.	Control	27.27±0.26	61.42±0.52	16.06±0.3	25.61±0.40
2.	Control + *Ocimum sanctum*	21.82±2.33#	60.00±0.45#	11.67±0.4***	21.97±0.15**
3.	Control + *Allium sativum*	23.18±2.15#	61.06±0.66#	12.58±0.4**	23.33±0.4*
4 .	Diabetic	41.21±0.15***	78.64±0.27***	27.27±0.26***	39.85±0.4***
5.	Diabetic + *Ocimum sanctum*	30.00±0.26***	66.97±0.4***	17.88±0.4***	28.64±0.52***
6.	Diabetic + *Allium sativum*	31.51±0.15***	69.7±0.3***	18.94±0.55***	30.15±0.66***

MDA and 4HNElevels are expressed as n mole/gm.
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 ***

Administration of *Ocimum sanctum* showed antioxidative effect in the control group also by reducing the levels of MDA and 4HNE in the liver and the brain, both. When the control rats were given *Ocimum sanctum and Allium sativum* treatment for two weeks, decrease in MDA and 4HNElevels were observed when compared with untreated control group however the changes were not significant (Table 2).

Table 3. Effect of oral treatment of extracts of *Ocimum sanctum* and *Allium sativum* for 14 days on nitrate and nitrite in the tissues of in alloxan induced diabetic rats.

		Nitrate		Nitrite	
S No.	Groups	Brain	Liver	Brain	Liver
1.	Control	117.27±0.69	143.03±0.80	50.76±0.66	59.7±1.45
2.	Control + *Ocimum Sanctum*	108.03±0.99**	135.18±1.07**	45.45±2.84*	51.51±0.76**
3.	Control + *Allium sativum*	114.24±0.55*	139.21±0.12**	45.45±1.64**	52.64±0.64*
4.	Diabetic	245.91±1.31***	266.7±1.43***	86.81±1.2***	96.06±0.61***
5.	Diabetic + *Ocimum Sanctum*	152.72±0.7***	165±1.39de***	61.51±0.55***	63.48±1.18***
6.	Diabetic + *Allium sativum*	155.54±0.28***	169.09±0.52***	63.03±0.66***	65.45±1.64***

Nitrate and nitrite levels are expressed as µ mole / gm.
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 ***

Levels of nitrate and nitrite were increase in allaxan induced diabetic rat tissues. The results of the present study showed that there was significantly high concentration of nitrate and nitrite in the liver and the brain of diabetic rats. Dabetic rat tissues showed nitrate and nitrite level 109.7%, 71.02% and 86.5%, 60.9% increase in brain and liver tissues when compared with control rat tissues. Diabetic rat tissues given oral administration of *Ocimum sanctum* and *Allium sativum* extracts for 14 days,diabetic treted rats 38%, 36.7% and 29.1%, 27.4% decrease in the brain and 38.1%, 36.6% and 33.9%, 31.9% decrease in the liver of the nitrate and nitrite, respectivelywhen compered with diabetic rat tissues. Treatment of control rats with *Ocimum sanctum* and *Allium sativum* extracts for 14 days, caused about 7.8%, 2.6% and 10.9%, 10.4% decrease in the brain and 5.5%, 2.6% and 13.7%, 11.8% decrease in the liver the level of nitrate and nitrite when compared with control rat tissues (Table3).

The levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were significantly reduced in the tissues of alloxan induced diabetic rats. The hepatic activities of SOD, CAT and GPx were decreased by 50.8%, 64.7%, 46.3% and 47.1%, 70.7%, 52.0% decrease, respectively in brain of diabetic rats compared with control rats (Table 4). Oral administration of *Ocimum sanctum* and *Allium sativum* extract for 14 days showed protective effects against alloxan diabetes induced alterations in the activities of SOD, CAT and GPx. The activities of SOD, CAT and GPx were increased by 51.9%, 81.9%, 72.9% and 47.8%, 78.7%, 68.2% in the liver and 49.3%, 112.3%, 74.4% and 41.9%, 97.1%, 71.2% increased in the brain of diabetic rats given respectively *Ocimum sanctum* and Allium sativum extract for 2 weeks when compared with diabetic rat tissues while the same *Ocimum sanctum* and *Allium sativum* extracts were given to the control rats, marginal changes in the activity of SOD, CAT and GPx were observed in both the tissues which were not significant(Table 4).

Reactive oxygen species (ROS) cause lipid peroxidation and damage protein by chemical modifications through cross-linking and fragmentation (Fabrice et al., 2019). Therefore oxidative stress has been considered to contribute to the pathological processes of diabetic complications. The results of the present study revealed significant elevation in the levels of MDA and 4HNEin the tissues of diabetic rats, an indication of increased oxidative stress (Bigagli et al., 2019). Treatment of diabetic rats with *Ocimum sanctum* and *Allium sativum* extracts reduced not only the blood glucoselevel but also the MDA and 4HNE levels were also decreased in the liver and the brain of rat tissues (Joudaki et al., 2019).

In diabetes hyperglycemia has been reported to cause increased production of ROS through auto-oxidation and non enzymaticglycation (Prakash et al., 2017). Under normal physiological condition there is a critical balance in the generation of oxygen free radicals and its antioxidant defense systems used by organism to deactivate and protect themselves against free radical toxicity (He et al., 2017). The results of the present study showed thatdecrease activity of antioxidant enzymes (SOD, CAT, GPx) in the tissues of diabetic rats and increase in the tissues oxidative stress (Ighodaro et al., 2018). Diabetic rats treated with Ocimum sanctum and Allium sativum extractsincrease the activity of antioxidant enzymes such as SOD, CAT and GPx in liver and brain tissues in diabetic rats (Govindappa et al., 2015).

Diabetes mellitus increase production of nitrate and nitrite in the tissues of diabetic rats. *Ocimum sanctum* and *Allium sativum* extracts decrease activity of nitrate and nitrite in the tissues of diabetic rats.

Table 4: Effect of oral treatment of extracts of *Ocimum Sanctum* and *Allium sativum* for 14 days on the activity of superoxide dismutase, catalase and glutathione peroxidase in the tissues of in alloxan induced diabetic rats.

			SOD		CAT		GPx
S No.	Groups	Brain	Liver	Brain	Liver	Brain	Liver
1.	Control	225.31±2.20	306.32±5.14	20.31±0.35	28.41±0.88	832.74±30.58	1991.96±20.37
2.	Control + *Ocimum sanctum*	226.19±1.62#	309.25±4.82#	20.90±0.53#	28.81±1.18#	838.13±19.97#	2018.47±39.61#
3.	Control + *Allium sativum*	225.24±1.3#	306.72±5.16#	20.47±0.45#	28.57±1.17#	833.81±26.67#	1999.45±25.53#
4.	Diabetic	119.03±17.26**	150.59±10.21***	5.94±0.04***	10.02±0.20***	400.73±28.64***	1070.47±23.76***
5.	Diabetic + *Ocimum sanctum*	177.76±5.48*	228.69±9.88**	12.61±0.92**	18.23±1.62**	698.89±31.42**	1851.89±25.51***
6.	Diabetic + *Allium sativum*	168.86±6.52#	222.61±8.52**	11.71±0.61***	17.91±1.57**	686.12±39.4**	1800±33.74***

SOD, CAT and GPx activation is expressed as µ mole/ min/ mg protein.
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 ***

Ocimum sanctum and *Allium sativum* extracts showed antidiabetic potential (Suanarunsawat et al., 2016).There are about 800 plants which have been reported to show antidiabetic potential (Suryavanshi et al., 2019). A wide collection of plant derived active principles representing numerous bioactive compounds has established their role for possible use in the treatment of diabetes (Suryavanshi et al., 2019). The most common and effective antidiabetic medicinal plants of Indian origin are Babul (*Acacia arabica), bael(Aeglemarmelose), church steeples (Agrimoniaeupatoria), onion (Allium cepa), garlic (Allium sativum), ghritakumarai (Aloe vera), neem (Azadirachtaindica)* etc. All these plants are a rich source of phytochemicals having medicinal value.

Diabetes mellitus decreased the activity of antioxidant enzymes and increased serum glucose, nitrate/nitrite, MDA and 4HNE levels. Oral administration of aqueous extracts of *Ocimum sanctum* and *Allium sativum* increased antioxidant enzymes activity and decrease serum glucose, nitrate/nitrite, MDA and 4HNE in the tissues of diabetic rats. Further human studies are necessary to found the role of these herbal drugs in controlling type I diabetes and its complications (Sharifi-Rad et al., 2016).

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In vitro Evaluation of Anti-Diabetic Effect of *Trigonella foenum graecum* Leaves in Different Solvent Extracts

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ABSTRACT

Diabetes mellitus is a metabolic disease characterized by high blood glucose level resulting from defects in insulin secretion, insulin action or both. Diabetes is considered as one of the most significant diseases in developed country. There are increasing incidences of diabetes every day and this indicate the need for treatment for it. The present study aims to evaluate the anti-diabetic activity of *Trigonella foenum-graecum* leaves in different solvent extracts in in vitro. The leaves were collected, dried and subjected to ethanol and petroleum ether extraction. The extracts were than subjected for in-vitro anti-diabetic activity assays such as Alpha-amylase inhibition assays, Non-enzymatic glycosylation of haemoglobin, and Glucose uptake in yeast cell and compared with their respective standards acarbose drug, ascorbic acid and metronidazole. The obtained result signifies that higher concentration of extracts possesses high effective anti-diabetic activity. The results of the work indicate that the both extracts of plant possessed considerable in vitro antidiabetic activity by inhibition of α-amylase, ethanol extract of plant shows maximum inhibition (73.4%) of glycosylation of haemoglobin, while extracts of *T. foenum-graecum* provide uptake of glucose by yeast cells which differ with the sample and glucose concentration, maximum increase in 5mM glucose concentration. Hence, from study it is concluded that *Trigonella foenum-graecum* leaves might be considered as herbal remedies for diabetes. However, the effect need to confirm using further anti-diabetic investigation and clinical trials for its effective utilization.

KEY WORDS: ANTI-DIABETIC ACTIVITY, ETHANOL EXTRACT, PETROLEUM ETHER EXTRACT, *Trigonella foenum-graecum.*

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INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar (glucose) levels that result from defects in insulin secretion or action, or both. Hyperglycemia resulting either due to defective production or action of insulin leads to a number of complications; cardiovascular, renal, neurological, ocular etc (Gray et al., 2000). According to International Diabetes Federation it is estimated that 463 million people have diabetes in 2019. Given that half a billion people are living with diabetes and there is an urgent need for developing and implementing multi-sectoral strategies to tackle diabetes. Without urgent and sufficient actions, it is predicted that 578 million people will have diabetes in 2030 and the number will increase by 51% (700 million) in 2045 (Saeedi et al., 2019)

Medicinal plants are used by 80 % of the world population especially in developing countries to cure and improve the general health, principally due to the common belief that plant-derived drugs are without any side effects along with being economical and locally accessible (Gupta et al.,1998). There has been growing interest in the application of natural components as antidiabetic agents (Qi L et al., 2010). A wide range of products claiming to lower blood glucose levels or prevent and treat diabetes complications and comorbidities are marketed to the public, (Geil et al., 2008). Fenugreek is one of the medicinal plants specially its seeds, which is widely used in folk medicine. It has a diuretic, uterine & cardio tonic, hypotensive, hypolipidemic, hypoglycemic, antinociceptive and anti-inflammatory (Al-Khateeb et al 2012).Among the various medicinal plants documented use as a hypoglycemic agent, *Trigonella foenum-graecum* commonly known as fenugreek in English and Methi in various Indian languages is important dietary and medicinal plants (Al-Khateeb et al 2012, Nathiya et al 2014). The *Trigonella foenum-graecum* seeds solution is effective in hyperlipidemia of diabetic patients, (Geberemeskel et al., 2019).

Fenugreek seeds powder have potent hypolipidemic effects when given with atorvastatin. (Hemavardhini et al., 2018).Though several forms of treatments are available in terms of medications and injectable insulin, they are accompanied with side effects. There are many drugs available in market for the treatment of diabetes like sulfonylurea's, biguanides, and alpha glucosidase inhibitors which are more expensive and have various side effects, but natural herbal drugs have been found to have lesser side effects and also provide long term effect for therapy in treating diabetes, (Sundarrajan et al.2019).

In the perspective on the above mentioned studies, there is no evidence of *in vitro* antidiabetic activity of Trigonella foenum graecum leaves. Hence, the present study was coordinated to investigate the antidiabetic action based on the inhibitory action on alpha-amylase, glycosylation of haemoglobin and glucose utilization by glucose uptake by yeast cells method. The aim of present study is to evaluate the antidiabetic potential of different solvent extracts of *Trigonella foenum-graecum* leaves by *in vitro* antidiabetic method.

MATERIAL AND METHODS

Fresh leaves of plant were purchased from market. The leaves were washed, dried and grind into fine powder. Petroleum ether and ethanol extractions of leaves were done using Soxhlet apparatus. After all petroleum ether and ethanol were removed from respective filtrate. The extracts were stored at the refrigerator for further study. The in vitro studies were carried out according to the method of (Joshi et al., 2013) with some modifications. Alpha-Amylase inhibition assay:Alpha amylase inhibitory activity was based on the starch iodine method. In alpha amylase inhibition method 1ml substrate- potato starch (1% w/v), 1 ml of drug solution (Acarbose std drug/ extract) of five different concentration such as 50, 100, 150, 200, and 250 µg/ml, 1ml of alpha amylase enzyme (1% w/v) and 2ml of acetate buffer (0.1 M, 7.2 pH) was added. The above mixture was incubated for 1 hr. Then 0.1 ml Iodine-iodide indicator was added in the mixture. Absorbance was taken at 565 nm in UV-Visible spectroscopy. % inhibition was calculated by formula,(Gupta et al., 2012).

$$\text{Inhibition of alpha- Amylase (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

Non-enzymatic glycosylation of haemoglobin: Glucose (2%), haemoglobin (0.06%) and Gentamycin (0.02%) solutions were prepared in phosphate buffer 0.01 M, pH 7.4. 1 ml each of above solution was mixed. 1 ml of each concentration was added to above mixture. The reaction mixture was incubated in dark at room temperature for 72 hrs and then the degree of glycosylation of haemoglobin was measured colorimetrically at 520 nm. Ascorbic acid was used as a standard drug for assay and percentage inhibition was calculated using the formula

$$\%\text{inhibition} = \frac{\text{Absorbance Sample} - \text{Absorbance Control}}{\text{Absorbance Sample}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample.

Glucose uptake in Yeast cells: Yeast suspension was prepared by repeated washing (by centrifugation at 3,000×g; 5 min) in distilled water until the supernatant fluids were clear (Cirillo 1962). A 10% (v/v) suspension was prepared with the supernatant fluid. 1mL of glucose solution (5, 10 and 20 mM) was added to various concentrations of extracts (50,100,150,200 and 250 µg) and incubated for 10 min at 37°C. Reaction was started by adding 100 µl of yeast suspension, vortex and further

incubated at 37°C for 60 min .After 60 min, the reaction mixture was centrifuged (2,500×g, 5 min) and glucose was estimated in the supernatant. Metronidazole was taken as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

RESULTS AND DISCUSSION

α-Amylase inhibition assay: Ethanolic and petroleum ether extract of *Trigonella foenum graecum* leaves exhibited significant inhibition in alpha-amylase activity. From table no. 1 it is seen that as the concentration increases inhibition activity is also increases Petroleum ether extract of plant shows higher inhibition (61.4%) at 250µg/ml which considerably more than standard (61.1%) and ethanolic extract (58.8%). Inhibition of α-amylase enzyme reduced the high Postprandial (PP) blood glucose peaks in diabetes. These α- amylase inhibitors are also known as starch blockers as they prevent or slow down the absorption of starch into the body mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into other simple sugars (Banerjee et al 2017).

The amylase inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates. Acarbose is complex oligosaccharides that delay the digestion of carbohydrates. It inhibits the action of pancreatic amylase in breakdown of starch. Synthetic inhibitor causes side effect such as abdominal pain, diarrhoea and soft faeces in the colon. The reaction mechanisms involved in inhibition of α-amylase enzymes by plant protein inhibitors are not clearly understood. But there are some suggestions that the plant protein might cause conformational changes in structure (Medagama and Sinadhira 2015).

Table 1. Alpha Amylase inhibition method

Concentration in µg/ml	STD %inh	Ethanol %inh	PE %inh
50	34.8±0.7571	30.2±0.4163	31.5±0.5487
100	50.8±0.1001	48.1±0.5572	49±0.5507
150	55.5±0.5773	55.4±0.3464	55.8±0.5825
200	58.8±0.60	57.8±0.5507	59.2±0.4333
250	61.1±0.5487	58.8±0.4977	61.4±0.4333

STD-Standard, PE-Petroleum ether, % inh-Percent inhibition. Values are expressed as ±SEM.

Table 2. Alpha Amylase inhibition method

IC_{50} value	STD	Ethanol	P.E
	28.994	150.451	139.886

IC_{50} value-Inhibitory concentration at 50%

Non-enzymatic glycosylation of haemoglobin: The haemoglobin present in the red blood corpuscles has a tendency to get bound to glucose. The inhibitory activity of ethanol and petroleum ether extract of *Trigonella foenum graecum* was found and compared with the standard drug. Results showed that the ethanol extract showed higher inhibitory activity up to 73.3% (Table No. 3) which is higher when compared to petroleum ether and standard. The greater the blood-glucose concentration, the greater is the amount of glucose-bound haemoglobin which leads to the formation of reactive oxygen species (Ogundele et al., 2017). The glucose autooxidation, protein glycosylation, formation of advanced glycation end products, and polyol pathway all are involved in the generation of the oxidative stress, implicated in the origin of type II DM (Kotb and Khaldun 2015). As the concentration increases, formation of the glucose–haemoglobin complex decreases and free haemoglobin increases, this shows the inhibition of glycosylated haemoglobin.

Table 3. Non-enzymatic glycosylation of haemoglobin method

Concentration in µg/ml	STD %inh	Ethanol %inh	PE %inh
50	18±0.4910	25±0.5811	7.6±0.4582
100	28±0.6359	50±0.5773	17.2±0.3464
150	33.3±0.6350	61.2±0.2309	25±0.5573
200	36.8±0.5206	69.2±0.2728	29.4±0.36055
250	40.2±0.2309	73.3±0.3711	33.3±0.9643

STD-Standard, PE-Petroleum ether, % inh-Percent inhibition. Values are expressed as ±SEM.

Table 4. Non-enzymatic glycosylation of haemoglobin method

IC_{50} value	STD	Ethanol	P.E
	327.358	125.541	366.194

IC_{50} value-Inhibitory concentration at 50%

Table 5. Effect of Standard drug on glucose uptake by yeast cells

Concentration in µg/ml	20 mM	10mM	5mM
50	44.9±0.6064	45.2±0.2603	46.6±0.2333
100	60.2±0.1201	70.3±0.4910	72±0.1154
150	74.4±0.2886	75.1±0.2027	84.9±0.0881
200	75.4±0.4055	78.2±0.2645	87.2±0.23094
250	77.4±0.4333	81.9±0.4666	90.4±0.4255

STD-Standard, PE-Petroleum ether, % inh-Percent inhibition. Values are expressed as means ±SEM.

Table 6. Effect of Petroleum ether extract of *T.foenum-graecum* on glucose uptake by yeast cells.

Concentration in µg/ml	20 mM	10mM	5mM
50	47.2±0.5238	47.5±0.4041	48.1±0.5658
100	69.1±0.4333	70.6±0.3464	74.9±0.5487
150	72.8±0.46188	76±0.5773	78±0.72188
200	76.6±0.4333	78±0.4163	86.9±0.2403
250	78±0.5487	84±0.3179	90.5±0.5131

STD-Standard, PE-Petroleum ether, % inh-Percent inhibition. Values are expressed as means±SEM.

Table 7. Effect of Ethanol extract of *T.foenum* graecum on glucose uptake by yeast cells

Concentration in µg/ml	20 mM	10mM	5mM
50	46.2±0.5607	47.2±0.4163	48.2±0.3756
100	58±0.0333	69±0.2848	71.3±0.333
150	69.1±0.3527	71.2±0.8717	79.2±0.6936
200	73.1±0.6359	74.4±0.3055	82.9±0.5507
250	75.4±0.3055	76.6±0.3464	86.1±0.5925

STD-Standard, PE-Petroleum ether, % inh-Percent inhibition, Conc.-Concentration Values are expressed as ±SEM.

Glucose uptake by yeast cell: It is stated that the transport of glucose across yeast cell membrane occurs by facilitated diffusion down the concentration gradient. Hence glucose transport occurs only if the intracellular glucose is effectively reduced (utilized) (Vijayalakshmi. et al 2014, Shehzadi et al., 2018).The data obtained clearly suggests that the ethanol and petroleum ether extract of *Trigonella foenum graecum* leaves is capable of effectively enhancing glucose uptake which in turn suggests that it is capable of enhancing the effective glucose utilization, thereby controlling blood glucose level. The extracts of *T.foenum graecum* leaves promoted the uptake of glucose across the plasma membrane of yeast cells (Table No.6 and 7). The highest uptake of glucose was seen in 5mM glucose concentration of petroleum ether extract of *T.foenum graecum*.

Table 8. Glucose uptake in different glucose concentration

	5mM			10mM			20mM		
IC_{50} value	STD	PE	Ethanol	STD	PE	Ethanol	STD	PE	Ethanol
	22.53	17.35	14.90	26.23	18.42	12.34	47.5	14.39	52.31

IC_{50} value-Inhibitory concentration at 50%

CONCLUSION

The results indicate that the extracts of T*rigonella foenum graecum* possess antidiabetic properties. This activity may be due to the strong occurrence of phenolic compounds such as alkaloids, flavanoids, tannins, steroids and phenols. This study gives an idea that the compounds of *Trigonella foenum graecum* can be used as lead compound for designing a potent anti-diabetic drug which can be used for treatment.

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Does Spermidine Always Act as Stimulant: Kinetics for Enzyme Action and Yield Attributes as Diagnostic Criteria in four *Vigna mungo* genotypes

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ABSTRACT

An experimental approach was managed for four mash bean [*Vigna mungo* (L.) Hepper] genotypes in quest of finding the kinetics of spermidine action accompanied with findings of response variations among crop genotypes. Plant nitrate reductase activity, number of legumes plant-1, number of grains plant-1, total yield plant-1 were evaluated against spermidine concentrations of 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM. Four genotypes i.e., MASH 80, MASH 88, MASH 97 and MASH ES-1 were grown in earthen pots filled with homogenized loamy soil. Pots by number were replicated four times for each concentration of spermidine in every genotype and were arranged completely in randomized design. Plants were sprayed thrice with the said concentrations of spermidine starting from twenty days after germination with an interval of ten days each. Nitrate Reductase Activity (NRA) was measured in leave on expiry of nine days after completion of spermidine spray while yield plant-1 and its contributing factors were recorded at physiological maturity of crop. Spermidine mediated stimulation in Nitrate Reductase Activity (NRA), yield plant-1 and yield contributory factors. The most effective concentration for all the characteristics was 1.25mM to which all genotypes responded in a similar fashion. All the genotype exhibited sigmoidal expression pattern with an exception of MASH 80 for legumes development phenomenon. MASH 88 responded in the best way except for number of grain plant-1 for which MASH 97was at the most. The least responsive of the genotypes was MASH ES-1 in all the cases studied.

KEY WORDS: SPERMIDINE, KINETICS, ENZYME, YIELD, VIGNA.

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INTRODUCTION

Polyamines (PAs) are group of low molecular weight nitrogenous compounds that mediate several plant developmental events (Aguria et al.2017; Ahmad et al., 2012; Alcázar et al. 2010). Polyamines (PAs) are commonly known to have protective effects on abiotic stress (Puyang et al., 2015; Pal et al., 2015; Wang et al.2019). Their involvement in key plant processes such as membrane stabilization, protein synthesis, enzyme activation, ROS scavenging, mineral uptake, and hormonal profile regulation has been reported (Ahmad et al., 2012; Hu et al 2012; Puyang et al 2016; Li et al. 2016).

Among the common occurring Polyamines are diamine putrescine, triamine spermidine, and tetra-amine spermine, existing ubiquitously in plants and playing a crucial role in plant physiological status (Fang, 2019). These compounds mainly exist in three forms in plant cells, diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm), each of which may be present in a free, soluble conjugated or insoluble bound form. Soluble conjugated forms, such as phenolic compounds, are covalently conjugated to small molecules, whereas insoluble bound forms are covalently bound to macromolecules, such as nucleic acids and proteins (Gill and Tuteja, 2010). Due to their polycationic nature, Polyamines can interact with proteins, nucleic acids, phospholipids and cell wall components, leading to their stabilization (Ahmad et al. 2012). The differential influence of stresses on the PA metabolism has been reported (Hu et al. 2012: Puyang et al. 2016).

To adjust polyamine levels finely to the levels required by the physiological state of the cell, various organisms have evolved homeostatic mechanisms involving polyamine biosynthesis, catabolism, transport, and uptake. Among the biosynthetic enzymes, S-adenosylmethionine decarboxylase and ornithine decarboxylase (ODC) are highly regulated at the transcriptional and post-transcriptional level. In particular, both animal and plant S-adenosylmethionine decarboxylases are subject to translational negative feedback regulation (Ivanov et al., 2010; Kovacs, 2020).

The application of exogenous PAs can check the inhibition of growth, remove reactive oxygen species (ROS), increase the level of endogenous PAs, protect the activity of the enzyme system under abiotic stress (Chen et al. 2019; Nahar et al. 2016). Exogenous application of polyamines to plants helps them to grow and yield better. Spermidine application induced stimulatory effect and improved Plant height, root length, number of leaves, fresh and dry biomass, seed number and weight per plant), oil quantity, endogenous IAA content, content of chlorophyll, reducing and non-reducing sugars, total carbohydrates and total proteins (Gul et al. 2020).

Besides their protective role, polyamines also serve as signalling molecules. The exogenous application of signaling molecules is crucial in term of dose and time of application (Pal et al. 2019). In addition to exogenous application, endogenous level of polyamines is found to be closely related to regulate the plant growth regulators. Endogenous Putrescine was found to be closely related to IAA and gibberellin (GA) contents, and high levels of Putrescine and Spermidine were not conducive for the accumulation of IAA and GA (Xu, 2015).

Mash bean [*Vigna mungo* (L.) Hepper], black gram, is an important legume crop grown in Asia. It has great value as food, fodder and green manure. In addition to improving the soil fertility, it is a cheap source of protein for direct human. It has easily sufficient protein content (20.8–30.5%) and carbohydrate (56.5–63.7%) on dry weight basis (Sharma et al. 2012). Mash bean is mainly grown as a pulse crop. Sometimes it is grown as a green manure to improve soil fertility. Like other legumes, mash bean also possesses the ability for establishing a symbiotic relationship with the nitrogen fixing bacteria Mash bean can fix approximately 37–83 kg ha^{-1} of nitrogen through this symbiotic association (Mohammad et al. 2010). Keeping in view the crucial role of polyamines regarding their dose and time of application; their role in controlling the endogenous level of growth regulators, the project was designed to evaluate the dose response cures of spermidine for Mash genotypes.

MATERIAL AND METHODS

Plant growth regulator mediated regulation of plant development is dependent upon variation in cell sensitivities and response times. So in any experiment where a hormone is being used, it is important to construct a dose response curve by varying the concentration of hormone and observing the degree of response. Hence, a pot experiment was devised to find out kinetics of enzyme action and yield attributes in four mash bean [*Vigna mungo* (L.) Hepper] genotypes to evaluate the expression of various dose response curves for exogenous spermidine in term of number of legumes plant-1, Number of grains fruit-1, total yield $plant^{-1}$(g), Nitrate Reductase (EC 1.6.6.1) Activity (NRA). Seeds of four mash genotypes i.e., MASH 80, MASH 88, MASH 97 and MASH ES-1 were obtained from Pulse Section, Ayub Agricultural Research Institute (AARI), Faisalabad (Pakistan). Spermidine, N-[3-Aminopropyl]$^{-1}$, 4-butanediamine, ($C_7H_19N_3$) of Sigma Aldrich, Japan was used as plant growth regulators. The genotypes have their origin in Ayub Agricultural Research Institute (AARI), Faisalabad (Pakistan) and National Agricultural Research Centre (NARC) Islamabad (Pakistan).

Experiment was designed with complete randomization of treatments and genotypes to avoid unequal exposure of environmental factors. Each treatment was repeated ten times by pots and plants. Pots of 30 cm diameter were used. Each pot was filled with 10 kg sandy loam soils and lined with polyethylene bags ensuring seepage prevention. Sterilized seeds, similar in size and weight, of each genotype were germinated. After germination, thinning was performed to maintain one seedling in each

pot in order to avoid the imbalanced uptake of nutrients by plants. Insects and pests were control by foliar spray of Thiodon insecticides of Hoechst (Pvt) Ltd, Pakistan. Plants were irrigated with normal irrigation water. By reviewing the published data concentrations of spermidine selected for experiment were as 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM in addition to control conditions of distilled water spray (Khoshbakht et al,2018).

Solutions of spermidine in respective concentrations were prepared in estimated (pre determined by trial method) amount of water by taking the great care of their half life, temperature and other environmental hazards which cause the denaturation of PGRs solution. Plants were exposed to first spray of PGRs after twenty days of germination repeated twice after each fifteen days with a great care of avoiding falling of drops of solution from leaf surface. Tween-20 (0.1%) was used as a surfactant for foliar spray, Yousefi et al. 2019). Nitrate reductase activity of four plants of each treatment was determined on the expiry of ten days after last spermidine spray by using the method of Sym, (1984) method.using spectrophotometer (Hitachi-220).Nitrate reductase activity was estimated according to the formula:

Nitrate Reductase Activity=Graph reading × Dilution factor × O.D of sample (µmol NO_2/h/g FW)

Yield and its contributing factors were studied at the maturity of crop (80 days age). Number of legumes plant^{-1}, number of grains fruit-1and total yield plant^{-1}(g) were determined. For randomly selected four plants per treatment in each genotype. The data were analyzed for analysis of variance using COSTAT computer package (CoHort Software, Berkeley, CA). Duncan's New Multiple Range test at 5% level of probability (Duncan, 1955) was used to compare means. Differences between individual means were tested by LSD tests at 0.05% significance level where significant F values were obtained by using MSTAT-C Computer Statistical Programme (MSTAT Development Team, 1989).

RESULTS

Number of legumes plant^{-1}: According to Duncan's Multiple Range test (Table: 1), exogenous spermidine substantially altered legume development and its action was pertinent in stimulating the enhancement of legume number. This action of spermidine was not statistically justified to all levels of its application. Foliar spray of 0.75 to 1.50mM was proved to be a significantly potent factor in their effect. Generally, maximum (27.651%) elevation in legume number was conducive to the sincere thanks of 1.25mM concentration. This trend corresponded to individual genotypic response also except for MASH 80 for which this definitive relationship could not occur and maximum effect was by 1.50mM dose. From the data, it could be inferred that some of the lower spermidine concentrations, though non significantly, but impaired with the ongoing trend for spermidine action (Table 1). Among the genotypes, MASH 88 revealed maximum (13.856) and MASH ES^{-1} revealed minimum (12.023) other genotypes lying between the two.

Table 1. Number of legumes plant-1 of mash [*Vigna mungo* (L.) Hepper] exposed to three shoot system sprays of spermidine concentrations (0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM) at 20 to 40 days of age [Values represent means ± SE].Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions (upper case letters).

Spermidine (mM)	MASH 80	MASH 88 (LSD=2.726 ;n=4)	MASH 97	MASH ES-1	TREATMENTS MEANS (LSD=1.362 ;n=16)
Distilled water	23.00±1.678 [GHI]	25.500±1.568 [DEFG]	23.500±3.502 [FGHI]	22.000±2.860 [I]	23.500 d ±2.626
0.25	22.000±1.440 (-4.347) [I]	26.000±1.718 (1.960) [CDEF]	24.000±1.440 (2.127) [FGHI]	22.000±1.940 (0.000) [I]	23.500 d ±2.262 (0.000)
0.50	22.834±1.482 (-0.721) [GHI]	25.000±2.952 (-1.960) [EFGH]	24.000±1.632 (2.127) [FGHI]	22.500±1.754 (2.272) [HI]	23.582 d ±2.090 (0.348)
0.75	26.164±1.750 (13.756) [CDEF]	27.164±1.472 (6.525) [CDE]	25.000±1.382 (6.382) [EFGH]	23.000±0.862 (4.545) [GHI]	25.332 c ±2.034 (7.795)
0.100	27.000±1.274 (17.391) [CDE]	30.000±3.568 (17.647) [AB]	27.000±1.522 (14.893) [CDE]	26.164±2.134 (18.927) [CDEF]	27.540 b ±2.560 (17.191) []
1.25	30.830±1.370 (34.043) [AB]	32.164±2.134 (36.868) [A]	28.330±1.388 (20.553) [BC]	28.664±1.884 (30.290) [BC]	29.998 a ±2.244 (27.651)
1.50	32.664±1.634 (42.017) [A]	28.164±1.666 (10.447) [BCD]	26.164±1.750 (11.336) [CDEF]	24.000±1.440 (9.090) [FGHI]	27.748 b ±3.608 (18.076)
GENOTYPES MEANS →	26.356 b ±4.130	27.712 a ±3.174 (-5.144)	25.426 b ±2.410 (3.528)	24.046 c ±2.912 (8.764)	
			(LSD=1.030 ; n=28)		

Number of grains fruit^{-1}: Exogenous spermidine concentrations of 1.00 to 1.50mM, according to Duncan's Multiple Range test (Table: 2), were significant in affecting the development of grain. A definitive positive proportionate relationship occurred between grain development and spermidine concentrations in all genotypes. The greatest promise, if the term may be used, in this fashion, in enhancing grain number is of 1.25mM spermidine which raised the grain number to maximum (15.591%) extent. But MASH ES-1 showed such saturation effect by 1.00 mM spermidine. The highest level of spermidine concentration showed its effect lower than this and established a sigmoidal pattern of expression for spermidine action (Table 2). The trend of increase in grain number was, however, not reflected when 0.50mM foliar spray of spermidine was experiences on plants of MASH 80 where a slight reduction of 2.478% than control plants was observed. Among the genotypes, MASH 97 revealed maximum (8.325) and MASH ES-1 revealed minimum (7.543) other genotypes lying between the two.

Total yield plant^{-1}(g): Duncan's Multiple Range test (Table: 3), showed that yield increased under the stimulus of spermidine and generally, in all the genotypes, spermidine effect evidently could have a significant pivotal role when applied in concentration range of 0.75 to 1.50mM. Statistically no remarkable variation in yield was assessed upto 0.50mM level proving it to be a susceptable dose.

Table 2: Number of grains fruit-1 of mash [*Vigna mungo* (L.) Hepper] exposed to three shoot system sprays of spermidine concentrations (0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM) at 20 to 40 days of age [Values represent means ± SE].Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes.

Spermidine (mM)	MASH 80	MASH 88 (n=4)	MASH 97	MASH ES-1	TREATMENTS MEANS (LSD=0.427 ;n=16)
Distilled water	7.657±0.717	7.345±0.421	7.795±1.098	7.015±0.903	7.453b±0.799
0.25	7.757±0.767 (1.305)	7.277±0.434 (0.925)	8.377±0.749 (7.466)	7.267±0.416 (3.592)	7.670b±0.722 (2.911)
0.50	7.492±.345 (-2.478)	7.522±0.544 (2.409)	8.062±0.286 (3.425)	7.082±0.268 (0.955)	7.540b±0.493 (1.167)
0.75	7.805±1.103 (1.932)	7.492±0.578 (2.001)	8.252±0.576 (5.862)	7.492±0.575 (6.799)	7.760b±0.739 (4.119)
0.100	8.650±0.534 (12.968)	8.512±0.666 (15.888)	8.492±0.420 (8.941)	8.082±0.268 (15.210)	8.434a±0.493 (13.162)
1.25	8.832±0.608 (15.345)	8.760±0.555 (19.264)	8.857±0.593 (13.624)	8.012±0.301 (14.212)	8.615a±0.595 (15.591)
1.50	8.262±0.181 (7.901)	8.647±0.274 (17.726)	8.437±0.405 (8.236)	7.852±0.978 (11.931)	8.300a±0.580 (11.364)
GENOTYPES MEANS →	8.065ab±0.762	7.936b±0.767 (1.599)	8.325a±0.773 (-3.223)	7.543c±0.648 (6.472)	7.967±0.762
	(LSD=0.3239 ; n=28)				

Plant exposure to 1.25mM concentration revealed the strongest impact of spermidine on yield in all the genotypes proving it to be optimum concentration. This gradual trend of yield enhancement was not observed under the exogenous application the highest spermidine concentration (Figure 3). Some limitations in spermidine effects reside in the 0.25mM and 0.50mM dose for MASH 80 as reduction of 3.340% and 3.118% respectively from control plants were recorded. Among the genotypes, MASH 88 was the most productive (5.542) and MASH ES-1 was the least productive (4.502).Only MASH 97 differed statistically from rest of the genotypes.

Nitrate Reductase (EC 1.6.6.1) Activity (NRA): The data in Table 4, according to Duncan's Multiple Range test reveals, spermidine amplified Nitrate Reductase Activity (NRA) exponentially in a concentration dependent manner. Statistically significant impacts were laid down by concentration range from 0.75 to 1.50mM. The most promising and much more significant increase (27.609%) appeared to occur by 1.25mM concentration. This fact was solidified when individual genotypic response to same level of concentration was observed to be of maximum value. The highest concentrations could not have a pace with the dose dependent exponential amplification of Nitrate Reductase Activity (NRA) and deviated from expected linear expression model of spermidine action (Figure 4). Not obscure, but a negative, relationship was noted regarding 0.25mM and 0.50mM concentration application on plants of MASH ES-1 which yielded

a reduction of 1.844% and 1.270% respectively from control. Of the genotypes, MASH 88 revealed maximum (9.587) and MASH ES^{-1} revealed minimum (8.421) value MASH 97 was similar to MASH 88 and MASH 80 behaved like MASH ES^{-1} in response.

DISCUSSION

Experimental results revealed increase in yield and its attributes by foliar application of spermidine (Table 1-3). The increase in yield components may be ascribed to promotion of growth and developmental process of plants by exogenous spermidine. It has been reported that Polyamines play role to improve plant growth (Alsokari, 2011). Spermidine imparts maximal beneficial effects on the growth of plant (Schaller et al. 2014; Ahanger et al. 2020). Improved growth in Spermidine treated seedlings was reported earlier (Nahar et al. 2016). Increased growth following application of phytohormones results from their impact on photosynthesis. Spermidine application proved beneficial in improving photosynthesis (Ahanger et al. 2020).

Increased photosynthesis directly influences the growth and metabolite production, ultimately increasing the energy status (Galili et al. 2016). Increase in photosynthesis might be due to more chlorophyll synthesis.Spermidine application has been reported to increase the synthesis of chlorophyll intermediates and decrease chlorophyllase activity (Ahange et al. 2018; Nahar et al. 2016). The increased synthesis of photosynthetic pigments and rate of photosynthesis is directly influenced in turn by the significant impact on the uptake of key mineral ions and the regulation of stomatal characteristics.The application of Spermidine improved Nitrogen uptake and stomatal functioning. Increased Nitrogen uptake contributes to greater Rubisco generation, while stomatal functioning maintains internal CO2 concentration and water, thereby leading to temperature maintenance (Khan et al. 2016). In addition, exogenous Spermidine application reduces chlorophyll degradation and enhances the stabilization of chloroplast structures (Li et al. 2016). Hu et al. (2016) have demonstrated increased D_1 protein synthesis in chloroplasy and amelioration of the decline in photosynthesis due to the exogenous treatment of Spermidine.

Table 3. Total yield plant-1(g) of mash [*Vigna mungo* (L.) Hepper] exposed to three shoot system sprays of spermidine concentrations (0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM) at 20 to 40 days of age [Values represent means ± SE].Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes.

Spermidine (mM)	MASH 80	MASH 88	MASH 97 (n=4)	MASH ES-1	TREATMENTS MEANS (LSD=0.821 ;n=16)
Distilled water	8.980±1.122	9.530±0.588	9.410±2.328	7.844±1.316	8.940 e ±1.490
0.25	8.680±0.836 (-3.340)	9.670±0.640 (1.469)	10.294±1.194 (8.472)	8.140±0.488 (3.773)	9.196 de ±1.142 (2.863)
0.50	8.700±0.178 (-3.118)	9.550±0.648 (0.209)	9.854±0.974 (3.835)	8.130±0.558 (3.646)	9.062de ±0.914 (1.364)
0.75	10.460±2.080 (16.481)	10.380±1.206 (8.919)	10.240±0.520 **(7.903)**	**8.544±0.260 (8.924)**	**9.916 cd ±1.374 (10.805)**
0.100	**11.910±1.188 (32.628)**	**13.080±2.354 (37.250)**	**10.604±0.948 (11.738)**	**10.734±0.538 (36.792)**	11.582 b ±1.644 **(29.552)**
1.25	**13.870±0.674 (54.454)**	**14.384±1.650 (50.933)**	**12.770±0.728 (34.562)**	**11.604±0.866 (47.934)**	**13.156 a ±1.452 (47.158)**
1.50	**12.534±1.296 (39.576)**	**11.004±1.394 (15.466)**	**10.614±1.474 (11.844)**	**8.030±0.674 (2.371)**	**10.546 c ±2.012 (17.964)**
GENOTYPES MEANS →	**10.734a ±2.220**	**11.084 a ±3.700 (-3.260)**	**10.540 a ±1.522 (1.807)**	**9.004 b ±1.570 (16.117)**	**10.340±2.036**
	(LSD=0.620 ; n=28)				

Increase in yield contributing factors might be due to more flower differentiation and cotrolled flower senescence or fruit post ripening process (Gupta et al. 2019). Flower bud differentiation is triggered Polyamines (Xu, 2015). There are many reports that have shown that exogenous application Polyamines and Polyamine synthesis inhibitors affect flower bud differentiation. Exogenous Polyamines accelerate the process of flower bud differentiation and maintenance of flower bud (Xu, 2015). Polyamines were found to be more abundant in flowers than in any other organ, and the addition of exogenous Polyamines to poorly flowering plants are reported to significantly enhance flowering response (Applewhite et al. 2010).

A reduction in Polyamines content is signal for senescence (Duan et al. 2006). Exogenous Spd and Spm treatments can increase the Polyamines content in cut flowers, and delay their senescence and improve their quality (Yang and He, 2001; Cao, 2010). Delayed leaf senescence was found to be associated with a higher spermine level and reduced reactive oxygen species (ROS) level (Sobieszczuk-Nowicka, 2017). Polyamines appeared to delay senescence by inhibiting ethylene biosynthesis (Woo et al. 2013; Anwar et al. 2015).

Flower senescence may be reduced due to production of antioxidants and reduction of ROS generated during stress (Ahanger et al. 2018). The application of putrescine (Nahar et al. 2016) has been reported to reduce the accumulation of H_2O_2 and O_2. The exogenous application of Spermidine reduces chlorophyll degradation and enhances the stabilization of chloroplast structures (Li et al. 2016). Nahar et al. (2016) have also reported reduced lipoxygenase activity due to putrescine application. Lipoxygenase generates unsaturated fatty acid hydroperoxide by adding molecular oxygen to polyunsaturated fatty acids and can also produce excess acyclic or cyclic compounds due to fatty acid oxidation thereby stabilizing membrane (Porta and Rocha-Sosa, 2002). Such increased membrane stability due to the application of Spermidine may be due to the maintenance of increased antioxidant activity in them (Hu et al. 2020). Different antioxidant enzymes have specific roles and share different locations within cells (Ahmad et al.2010). Nahar et al. (2016) and Li et al. (2015) have also reported up-regulation of the antioxidant system due to the treatment of Spermidine.

Exogenous application improved the activity of nitrate reductase (Table 4). Nitrate reductase mediates the rate-limiting step in N metabolism, thereby regulating the key metabolic pathways including the amino acid and N-containing secondary metabolites (Ahanger et al. 2017). Bashri et al. (Bashri et al.2018) and Khalil et al. (Khalil et al. 2017) have also demonstrated increased nitrate reductase activity due to the application of cadaverine and Kn. Increased nitrate reductase activity results in improved N assimilation (Khalil et al. 2017), thereby influencing the protein synthesis and stress tolerance (Iqbal et al. 2015). Spermidine mediated enhancement in the nitrate reductase, and Nitrogen contents may have directly regulated the synthesis of photosynthetic enzymes and other protective compounds, (Ahanger et al. 2020).

Table 4. Nitrate Reductase (EC 1.6.6.1) Activity (NRA) of 50 days old mash [Vigna mungo (L.) Hepper] exposed to three shoot system sprays of spermidine concentrations (0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM) at 20 to 40 days of age [Values represent means ± SE].Values in parentheses represent %age increase (+)/decrease (-) over untreated of row#1 or over MASH 80 for genotypes means. Values followed by dissimilar letters, are different at P = 0.05 among means of treatments and genotypes (lower case letter) as well as among interactions

Spermidine (mM)	MASH 80	MASH 88 (LSD=0.819 ;n=4)	MASH 97	MASH ES-1	TREATMENTS MEANS (LSD= 0.410;n=16)
Distilled water	7.885±0.645 [JKL]	8.795±0.277 [EFGHI]	8.685±1.209 [FGHIJ]	7.48±0.579 [KL]	8.211e ±0.883
0.25	8.1±0.237 (2.726) [HIJKL]	8.865±0.374 (0.795) [EFGH]	9.16±0.660 (5.469) [DEF]	7.342±0.437 (-1.844) [L]	8.366 de ±0.835 (1.887)
0.50	8.072±0.274 (1.800) [HIJKL]	8.822±0.299 (0.306) [EFGH]	8.942±0.487 (2.959) [EFG]	7.385±0.087 (-1.270) [L]	8.305 de ±0.708 (1.144)
0.75	8.292±0.124 (5.161) [GHIJK]	9.22±0.564 (4.832) [DEF]	9.137±0.258 (5.204) [DEF]	8.227±1.005 (9.986) [GHIJK]	8.719 cd ±0.713 (6.186)
0.100	9.372±0.253 (18.858) [DEF]	10.547±1.176 (19.920) [AB]	9.292±0.474 (6.989) [DEF]	8.977±0.510 (20.013) [EFG]	9.547 b ±0.875 (16.270)
1.25	9.85±0.360 (24.920) [BCD]	11.347±0.692 (29.016) [A]	10.462±0.438 (20.460) [B]	10.252±0.533 (37.058) [BC]	10.478 a ±0.733 (27.609)
1.50	8.002±0.373 (1.483) [IJKL]	9.515±0.689 (8.186) [CDE]	9.317±0.746 (7.276) [DEF]	9.287±0.632 (24.157) [DEF]	9.030 c ±0.835 (9.974)
GENOTYPES MEANS →	8.510 b ±0.795	9.587 a ±1.096 (-1.056)	9.285 a ±0.796 (-13.846)	8.421 b ±1.182 (-10.260)	8.951±1.091
	(LSD=0.310 ; n=28)				

Furthermore, Spermidine Increased K content (Ahanger et al. 2020) which directly influences plant growth through its involvement in the regulation of enzyme activity and photosynthesis (Ahanger and Agarwal, 2017; Ahanger and Agarwal, 2017;Ahanger et al. 2017). The results revealed some deviations from the general exhibited trend of spermidine effects at high concentration The deviation from the augmentations for spermidine role might be ascribed to facts like free polyamines concentration varies with in a species also

(Reggiani, et al. 992): environmental factors (Kubis, 2006); Attachments of spermidine to other molecules (Gill and Tuteja, 2010)

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Detection of Leukemia and Sickle Cell Anemia Using Segmentation of Microscopic Images

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ABSTRACT

The analysis of blood cells during a microscope image can provide useful information concerning the health of the patient. Digital image processing techniques are far more efficient than manual observation to look at distinguish and diagnose blood disorders. The goal of this practice is to watch the nucleus and cytoplasm of blood cells. The project work is beneficial to detect different sorts of disease like leukemia, anemia, etc. supported the condition of the nucleus; the standard morphology of RBC undergoes specific changes as a consequence of various pathological abnormalities. One such disease is red blood cell Anemia (SCA) where the RBCs take crescentic sickle-like shape. Here during this project, correct identification of aberration in normal parameters of RBCs in an anaemic blood sample has presented using different image processing tools and techniques. Acute Myelogenous Leukemia (AML) may be a fast-maturing malignant neoplastic disease of the blood and bone substance. The demand for mechanized of leukemia detection arises since current methods require manual examination of the blood smear is that the beginning step toward diagnosis. this is often time-consuming, and also the accuracy of the tactic depends on the operator's ability. during this paper, assorted image segmentation and have extraction methods utilized for AML detection are discussed. Leukemia comes about when tons of abnormal white blood cells created by the bone substance. Hematologist makes use of the microscopic study of human ancestry, which results in needing of methods, segmentation, including microscopic colour imaging, Categorization and clustering which will allow identification of patients affected by Leukemia. Different image processing algorithms like Image Enhancement, clustering, texture analysis, Mathematical morphology; Thresholding and Labelling are implemented using MATLAB.

KEY WORDS: SICKLE CELL ANAEMIA, LEUKEMIA, AML, CLUSTERING AND FEATURES.

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INTRODUCTION

Like all other sectors of medical science, there has been a normal progression in the pathological field too. However, the current age popular haematological analyzing devices cannot identify morphological abnormalities in blood cells with 100% precision as the detection part may subject to human error (Kasmin et al., 2012). Hither comes to the helping hand of advanced engineering skill. But till today there are few fields of the biomedical field where engineering science has not shown its magic still. One of such untouched sectors of the medico technological field is the detection of Sickle Cell Anemia and Acute myelogenousleukemia using Image Processing. Using this current research work have defined a metric and implemented the algorithm to find the significant result set which successfully detects the deviation of the shape of human RBCs and WBC to diagnose the disease Sickle-cell Anemia and Acute Myelogenous Leukemia, (Talukdar et al.,2014).

White blood cells (WBCs) or leukocytes play a major part in the diagnosis of several diseases. So extracting information about them is valuable for hematologists. The cancer of the blood and bone marrow is called leukemia. The bone marrow produces a great number of abnormal white blood cells in the case of cancer of the blood. These cells are immature, and they do not function properly. Without treatment, leukemia can be a pernicious disease. Leukemia is broadly classed as 1) acute leukemia (which comes along quickly), and 2) chronic leukemia (which comes along slowly). The word "acute" in acute myelogenousleukemia denotes the rapid advancement of the disease. It is called myelogenousleukemia because it involves a group of white blood cells called the myeloid cells, which in normal develops into different types of mature blood cells.

Blood cancer or leukemia can be healed if it is discovered and treated at the earliest level. The demand for automation of leukemia detection arises since current methods involve manual examination of the blood smear is the beginning step toward diagnosis. This wastes more time, and its accuracy is much dependent on the operator's ability. Many trials have been done in past decades to construct systems that aid in acute leukemia segmentation, classification and detection. Image segmentation is a method which can be used to understand images and extract information or objects (Nazlibilek, et al. 2014 and Ghane 2017). It is the beginning step in image analysis. Feature extraction in image processing is a technique of redefining a large set of redundant data into a set of features (or feature vector) of reduced dimension. This input data is transformed into the set of features is called feature extraction (Krishnan, et al. 2014).

Sickle cell anemia is an inherited disease. They inherit two genes for sickle hemoglobin one from each parent. This leads to a propensity to the cells to assume a rigid, abnormal, sickle like shape under certain circumstances. Folks who inherit a sickle hemoglobin gene from one determine and normal genes from the opposite determine have a condition referred to as sickle cell trait; it's far different than sickle cellular anemia. Humans who have sickle cell trait don't have the disease. Humans who have sickle cell trait can pass the sickle hemoglobin gene to their kids. Anemia is a circumstance in which lineage has a lower than usual number of RBCs. This shape also can occur if red blood cells don't contain enough hemoglobin. Red blood cells are produced in the spongy marrow inside the big bones of the physical structure. Bone marrow is continually creating new red blood cells to substitute previous ones. Healthy red blood cells last about 120 days in the bloodstream and then drop dead. The abnormal sickle cells usually die in about 10 to 20 days in sickle cell anemia. The bone marrow cannot be able to reproduce new red blood cells fast enough to put back the existing ones. Thus, the decreased number of RBCs in the blood causes anemia, which has termed as Sickle Cell Anemia (Rakshit and Bhowmik, 2013). Further, the segmentation and classification overview of red blood cells has been shown in detailed by several workers, (Alzubaidi, et al 2020), (Patgiri, et al 2019) and (Raphael, et al 2019)

MATERIAL AND METHODS

To detect the abnormal blood cells and their nucleus image segmentation is needed. In the case of leukemia detection segment the WBC nucleus, it needs many feature extraction techniques. But, sickle cell anemia needs segmentation and then followed by geometrical measurement to detect the shape information. Image segmentation aims to extract important information from an input image. It has taken on a key role for the reason that efficiency of next characteristic extraction and classification relies greatly on the perfect identification of the myeloblasts. There are many algorithms for segmentation has been developed for grey-level images. Image segmentation in this system was performed for extracting the nuclei of the leukocytes using colour-based clustering. Cluster analysis is the conventional study of methods and algorithms for grouping objects according to measured or perceived intrinsic characteristics.

Cluster analysis uses class labels (i.e., K-means), which is one of the most popular unsupervised learning algorithms and is also a simple clustering algorithm; it is still widely practised. This speaks to the difficulty in designing a general-purpose clustering algorithm, and the ill-posed problem of bunching. In this report, we chose clusters corresponding to the nucleus (high intensity), desktop (high brightness level and low chroma), and other cells (e.g. Leukocyte cytoplasm and Red blood cells). Here, each pixel is assigned to one of these classes using the attributes of the cluster core.

K-Means Clustering Algorithm: The k-means algorithm requires three user fixed parameters: (1) The number of clusters k, (2) Cluster initialization, and (3) Distance metric(Nancy M. Salem 2014). A k-means clustering procedure is employed to assign every pixel to one of the clusters. Here each and every pixel is assigned to one

of these classes using the attributes of the cluster core. Every pixel of an object is classified into k clusters based on the corresponding *a and *b values in the L*a*b colour space. Thus, each picture element in the L*a*b colour space is classified into any of the k clusters by calculating the Euclidean distance between the picture element and each colour indicator. These clusters correspond to the nucleus (high intensity), desktop (high brightness level and low chroma), and other cells (e. g., erythrocytes and leukocyte cytoplasm).

Each pixel of the entire image will be labelled with a particular colour depending on the minimum distance from each index. While performing k-means segmentation of complete images, it was noticed that, in some of the segmented images. Still, the edges of the nuclei were obtained as opposed to the whole images of the nuclei. The morphological filtering is used to overcome the shortcoming of the nuclei. An image is partitioned into several areas depending on the features to be drawn out. Employing morphological filtering ensures that perceptibility and visibility of these regions improve. The next activities were done to get the desired result. Once these actions are performed, the following shape-based and textures are then extracted from these whole images they are:

1. Edge enhancement (used by the Sobel operator)
2. Canny edge detection
3. Dilation
4. Hole-filling

Feature extraction in image processing is a method of transforming large redundant data into a reduced data representation. Feature extraction is to transform the input data into the set of features. These feature extraction techniques are mainly used to detect AML from the blood images (Agaian et al., 2014) and (Sukhia et al., 2019).

Fractal Dimension: Fractals are using in medicine and science earlier for various quantitative measurements. The fractal dimension D is a statistical measure that gives an indication of how completely a fractal appears to occupy space. The most important theoretical fractal dimensions are the Renyi dimension, the HD, and the packing dimension normally, the box-counting dimension is widely practised. The procedure for Hausdorff Dimension measurement using the box-counting method is presented below as an algorithm:

- Each nucleus colour (RGB) image is converted to grey and successively to a binary image.
- The nucleus edge boundary is extracted using Canny edge detection technique.
- A grid of R squares is superimposed over the edges while counting the edge occupied squares.
- Measure 3 is continued for an increasing number of squares.

The Hausdorff Dimension HD may then be specified as follows:

$$HD = \frac{\log(R)}{\log(R(S))} \quad (1)$$

The notation of R is the number of squares in the superimposed grid, and R(s) is the number of squares or boxes (box count) which are occupied. Higher HD signifies a higher degree of roughness.

Local Binary Patterns (LBP)
LBP is a powerful feature for texture classification. This LBP feature vector is formed as follows:

- The examined window is split into cells (e.g. 16x16 pixels for each cell).
- For every pixel in cellular to evaluate the pixel cost into every one of its eight neighbours (on its right -top, right-middle, left-top, and many others.).
- Observe the pixels along a circle, i.e. either clockwise or counter clockwise.
- If the centre pixel's value is extra than the neighbour's value, write "1". Otherwise, write "0". This offers an eight-digit binary wide variety (that is usually converted to decimal for comfort).
- Compute the histogram, over the cellular, of the frequency of each "number" occurring (i.e., each mixture of which pixels are smaller and which might be greater than the centre).
- Optionally normalize the histogram.
- Concatenate histograms of all cells. This gives the window's feature vector.

Texture Features: The texture functions may be extensively categorized into spatial texture feature extraction techniques and spectral texture feature extraction methods based totally at the area from which they are extracted. Inside the first technique, texture capabilities have become through finding the local pixel systems within the authentic photo area, extracted or by means of computing the pixel data, while the second technique transforms an image into the frequency area after which calculates function from the transformed photo.

Shape Features: Shape descriptors are fixed of numbers that describe a given shape. Form function extraction techniques may be broadly categorized into two businesses, contour-based totally and place-based technologies. The first technique calculates shape capabilities most effective from the form boundary, whilst the second technique extracts capabilities from the whole location. Further, spatial courting is also taken into consideration in picture processing that tells the item place inside a photo or the relationships among objects. It includes the subsequent instances: the absolute spatial vicinity of areas and relative places of regions.

In sickle cell anemia, a normal RBC (i.e. Circular in front view and bi-concave inside view) is translated into 'sickle'-like form and plumpness of the RBC are

lost (Rakshit and Bhowmik 2013). So the confirmatory pathological diagnosis of SCA is obtained by examining the pattern of the RBC. Here a metric has been purported to identify this change in the shape of RBC in order to diagnose the disease. Essentially, this metric indicates the roundness of a confined object. It is specified as (4*pi*area) /perimeter2. So for a 2-d circle, i.e. a round object, metric value is 1for a 2-d square, it is 0.785. That is why for a normal RBC metric value is nearly one, and for sickle RBC, the value deteriorates from 1 and results in a lesser value of the metric, The overlapping of the blood cells can be easily separated using spatial colour based SC and ellipse fitting on cell contours methods (JiGe,et al. 2014).

The algorithm is described here:

- The microscopic blood smear image is fed to the program as input and is converted to a binary image.
- The input image is complemented, and for the ease of further processing, small unwanted spots are taken away from it using bwareaopen which removes all the targets in the diagram containing fewer than the number of pixels mentioned in the threshold stage.
- A number of similar components are detected using bwconncomp.
- Using region props to calculate the area on the connected objects.
- Detected connected corpuscles are displayed one by one, and then the corresponding surface area is shown in the software command window.
- Throwing out the smaller background objects, just the larger RBCs are seen and pre-processed for going through the remainder of the stairs.
- The edges of these RBCs are detected using the Sobel edge detection algorithm.
- Small holes in the objects are covered up using an infill operator to increase the precision of further calculation.
- The boundary of each of the elements is traversed and marked over the objects, and the perimeter is calculated from the obtained boundary measured.
- Metric(roundness) [i.e. (4*pi*area) / perimeter2] is calculated for each object.
- By this roundness value(metric), any deviation or change in the shape of RBC is detected, which are helpful in diagnosing some cases of anemia like sickle cell anemia.

RESULTS AND DISCUSSION

The proposed algorithm uses various methods to detect blood disorders like anemia and leukemia. The Fig 1 shows the blood cancer microscopic image it is processed by texture analysis, fuzzy clustering and GLCM techniques and finally, the detected cancer cell nucleus shown in Fig 3. The colour-based classification using k-means clustering output shown in fig 2. Table 1 indicates the various performance measures between normal and cancerous cell; these values are mainly used to identify the disease (AML). Fig 4 shows the sickle cell microscopic image the segmentation process applied to the input image. The geometric feature analysis was performed, and finally, the detected RBC cells are indicated in Fig 5. In sickle cell anemia detection based on the roundness value, it will classify as a normal cell or sickle cell if the roundness value greater than 0.75 the cells are classified as normal RBC. If the rounded value is less than 0.75, then the cells are classified as sickle cell. These features based classification algorithm is done by multilevel; it is mainly used to detect blood disorders.

Figure 1: Input Diseased Leukemia Microscopic Image

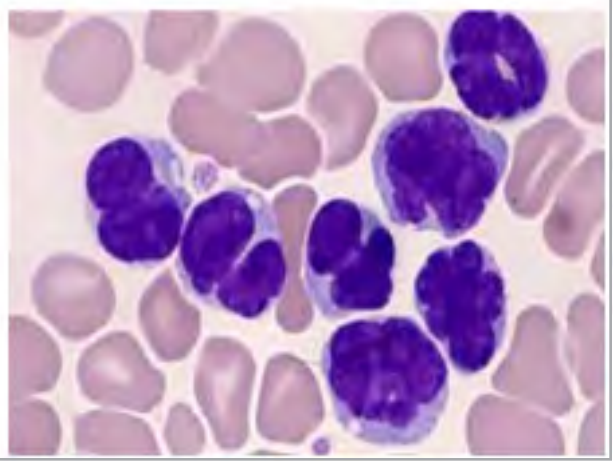

Figure 2: Detected Nucleous Using K- means Clustering

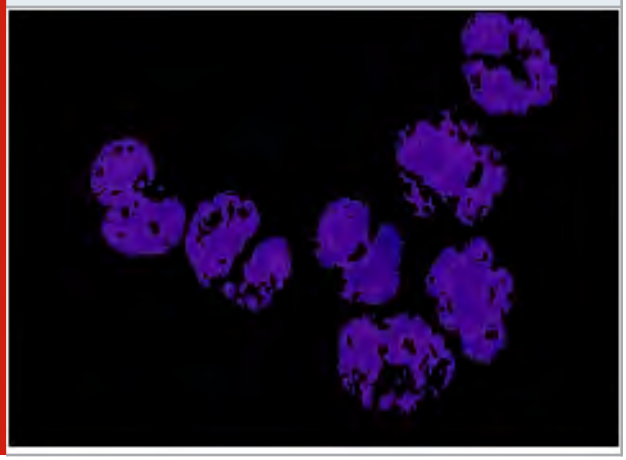

Figure 3: Cancer Detected Image

Figure 4: Sickle Cell Microscopic Image

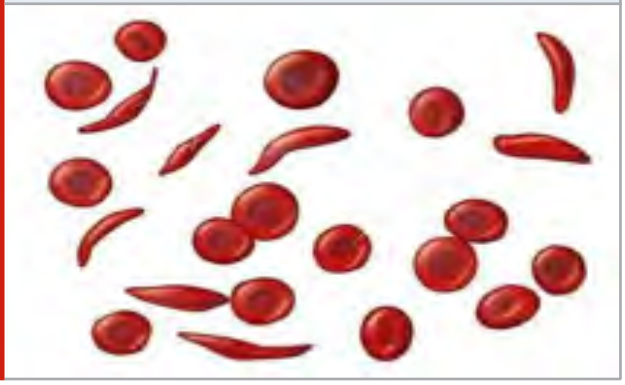

Figure 5: Detected RBC Cells

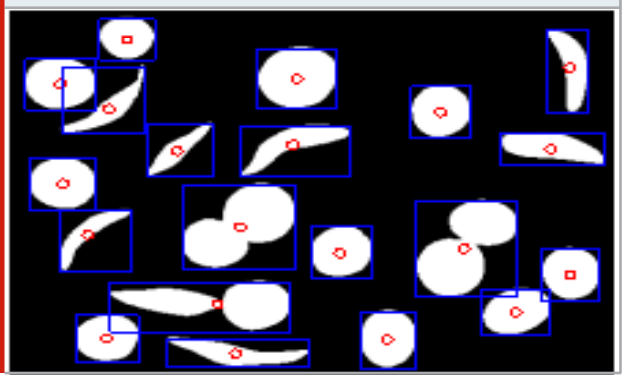

Figure 6: Sickle Cell

Figure 7: Normal Cell

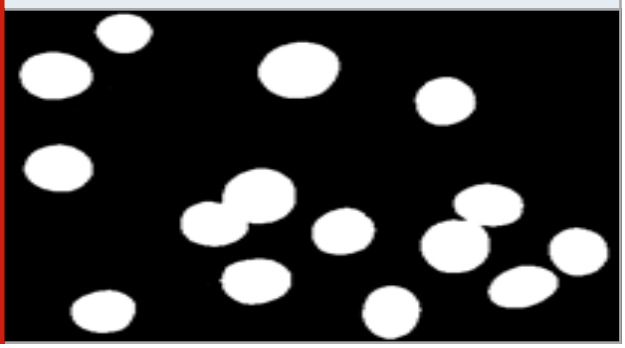

Table 1. Performance Measures of Leukemia Detection

Features	Cancerous	Normal
Mean	32.3348	38.3476
Standard deviation	47.1398	40.8327
Area	6468	1976
Perimeter	255	805.723
Elongation	1.1354	1.519
Eccentricity	0.4852	0.7543
Form factor	1.3621	0.0378
Solidity	0.5742	0.2128
Compactness	10.762	327.42

CONCLUSION

The main objective of the present work is to detect blood disorders like leukemia and anemia. The nucleus segmentation and classification is followed by feature extraction methods to detect Leukemia and anemia. Shape features of nuclei such as area, perimeter, texture feature and GLCM, etc. are considered for better accuracy of detection. The sickle cell anemia detection cell boundary segmentation and geometrical measures are mainly used. The results show that the proposed nucleus segmentation, feature extraction and statistical parameter such as mean and standard deviation based produced good segmentation performance. Besides, the fully segmented nucleus and shape of the cell can be better achieved by using MATLAB based algorithm because it is less sensitive to input image variations.

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Phytochemical Investigation and Anti Mycobacterial Activity of *Solanum pubescens*

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ABSTRACT

Tuberculosis is an infectious disease that kills approximately three million people annually worldwide. This high incidence of infection and the increased rate of multi-drug resistance and extensively drug resistance strains of the organism further complicated the problem of TB control and have called for an urgent need to develop new anti tuberculosis drugs from plants. The present study is aimed to evaluate the phytochemical investigation and anti mycobacterial activity of *Solanum pubescens*. Preliminary phytochemical analysis is done both methanolic and ethyl acetate extracts of leaves of *Solanum pubescens*. Both the extracts were subjected with estimation of total phenolic content, total flavonoid, total alkaloid and total antioxidant activity. The phenolic content, flavonoid content, alkaloid content and *invitro* anti-oxidant potential of methanolic extract was found to be 46.33±13.23, 29.66 ± 8.08, 1.46±0.26 and 48.33±12.11 respectively. The phenolic content, flavonoid content, alkaloid content and *invitro* anti-oxidant potential of ethyl acetate extract was found to be 38.33±11.23, 23.43 ±5.05, 1.24±0.16 and 43.66±9.07 respectively. Methanolic and ethyl acetate extracts were assessed for anti mycobacterial activity against mycobacterium tuberculosis using H37RV strain. Methanolic extract of leaves of *Solanum pubescens* exhibited very good anti tuberculosis activity having minimum inhibitory concentration of 6.25µg/ml. The ethyl acetate extract of *Solanum pubescens* having minimum inhibitory concentration of 10µg/ml. Furthermore work is required to be undertaken for active principle or group of compounds responsible for such activity.

KEY WORDS: ANTI-OXIDANT ACTIVITY, ANTIMYCOBACTERIAL ACTIVITY, *SOLANUM PUBESCENS*.

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INTRODUCTION

Tuberculosis (TB) continues to be one of the leading infectious causes of death in the world especially in a developing and densely populated country like India, (Rajesh Archana et al., 2017). *Mycobacterium tuberculosis* is a pathogenic organism which causes Tuberculosis (Ravindran et al., 2020). TB, an old disease that has plagued humankind for centuries, is known to be a highly infectious disease and still remains the leading killer among infectious diseases in the world (Aro et al 2019). Although anti-TB drugs have been in existence for many years, the disease continues to be prevalent. The primary reason behind this is the emergence of drug-resistant strains of Mtb. Thus, although several drugs are available, the quest for newer and more effective ones continues (Sarkar et al., 2020).

The indigenous plant resources provide medications to alleviate cough and related respiratory disorders associated with TB (Sieniawska et al., 2020). Moreover, higher plant extracts have been considered as promising sources of novel anti-TB leads (Kahaliw et al., 2017). Traditionally *Solanum pubescens* has been used in treatment of headache, menstrual pain, rheumatoid arthritis, tuberculosis, ulcers, liver disorders, diarrheal disease and cancer disorder. But there is unavailable scientific data of *Solanum pubescens* against tuberculosis. The present study is undertaken to explore the preliminary phytochemical analysis and antimycobacterial activity of *Solanum pubescens*.

MATERIAL AND METHODS

Plant collection: Fresh leaves of *Solanum pubescens,* family Solanaceae were collected from Kadiri, Ananthapuramu district, Andhrapradesh state, India.

Identification & authentication: *Solanum pubescens* was identified and authenticated by Dr. J. Raveendra Reddy, M. Pharm, Ph. D, Raghavendra Institute of Pharmaceutical Education and Research [RIPER], Ananthapuramu district, Andhrapradesh state, India.

Chemicals: The chemicals were used in this study were obtained from Hi-Media Pvt. Ltd.

Extraction procedure: The powdered plant material was weighed using analytical balance and prepared for solvent extraction. Successive extraction was done using 300g of powdered material of leaves in soxhlet apparatus. The solvents ethyl acetate (2L, 70˚C, 15-17 cycles) and methanol (2L, 70˚C, 15-17 cycles) were used (Fatemeh SR et al., 2014).

Phytochemical screening: The methanolic extract of *Solanum pubescens* (MESP) and ethyl acetate extract of *Solanum pubescens* (EAESP) were subjected to phytochemical screening (Borokini TI et al., 2012).

Estimation of total phenolic content (TPC): Folin ciocalteu's assay is used for the determination of total phenolic content of the extracts 1ml of extract or standard solution of Gallic acid at concentration of 20, 40, 60, 80, 100mg/ml is added to the 25ml of volumetric flask which is already containing 9ml of distilled deionized water. To the above solution added 1ml of Folin- ciocalteus reagent and shaken it well. Added 10 ml of 7% sodium carbonate solution after 5min to the above solution. The above solution is diluted by using 25ml of water and mixed it. Incubated the solution for 90 min at room temperature, absorbance was determined at 750 nm using UV-Visible spectrophotometer. The total phenolic content was expressed in units as milligrams of Gallic acid equivalents[GAE] per 100 grams dry mass[mg GAE/100MG] (Tab sum, SH et al., 2016).

Estimation of total flavonoid content (TFC): Colorimetric assay is used for the determination of total flavonoids content 1ml of extract and standard solutions of quercetin at concentrations of 20, 40, 60, 80, 100mg/ml was added to the 10ml volumetric flask already containing 4ml distilled water. To the above solution added 0.3 ml of 5% Sodium nitrate. Then added 0.3 ml of 10% Alcl3 after 5min and 6 min, added 2ml of 1M NaOH and total volume made up to 10 ml with distilled water. The whole solution was mix up and absorbance was measured at 510 nm in UV- Visible spectrophotometer. The data of the total flavonoid content was expressed as milligrams of quercetin equivalents [QE] per 100g dry mass [mg QE/100g] (Tabsum et al., 2016).

Estimation of total alkaloid content (TAC): TAC was also quantified by spectrophotometric method. This method is based on the reaction between alkaloid and bromocresol green [BCG]. The plant extract [1mg/ml] was dissolved in 2 N HCL and then filtered. The PH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. 1ml of this solution was transferred to a separating funnel, and then 5ml of BCG solution along with 5ml phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extract was collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm (Islam et al., 2013).

Total antioxidant activity: The antioxidant activity of the extracts was evaluated by the phosphor-molybdate method using ascorbic acid as a standard. The assay is based on the reduction of Mo [4]-Mo [5] by the extract and subsequent formation of green phosphate/ Mo[5] complex at acidic pH. An aliquot of 0.3 ml. extract was combined with 3ml of reagent solution [0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate]. The tubes containing the reaction solution were incubated at 95degree centigrade for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank using a spectrophotometer. Methanol [0.3ml] in the place of extract is used as the blank. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid (Lourenco, et al., 2007).

In vitro anti tuberculosis activity: The anti mycobacterial activity of compounds were assessed against M. tuberculosis using micro plate Alamar Blue assay [MABA]. This methodology is non toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Briefly, 200 micro liters of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 micro liters of the Middle brook 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 micro gram per ml. plates were covered and sealed with parafilm and incubated at 37 ^{0}C for5 days. After this time, 25µl of freshly prepared 1:1 mixture of almar blue reagent and 10% tween 80 was added to the plate and incubated for 24hrs. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration which prevented the colour change from blue to pink, (Lourenco et al., 2007).

RESULTS AND DISCUSSION

Tuberculosis is a chronic disease caused by *Mycobacterium tuberculosis*. Tuberculosis has been major health problem for developing countries including India. Tuberculosis is still a serious illness and a serious public health problem with medical, sociological and economic consequence that has a fatal end in more than 50% of untreated cases. The increase in multi drug resistance and extensively drug resistance strains of mycobacterium tuberculosis, there is an urgent need of finding newer anti-mycobacterial agents to combat this problem (Ngadino et al., 2018).

In the current study the focus was to assess the phytochemical screening and antimycobacterial activity of methanolic and ethyl acetate extracts of leaves of *Solanum pubescens*. The physical appearance of methanolic and ethyl acetate extracts of leaves of *Solanum pubescens* was found to be green in colour, semisolid and pleasant odour. The methanolic extract of Solanum pubescens examined for phytochemical analysis and they contained the alkaloids, flavonoids, tannins, phenolic compounds and glycosides where as ethyl acetate extract of *Solanum pubescens* consisted of alkaloids, flavonoids, tannins, phenolic compounds, steroids and saponins. Both the extracts were subjected for quantification of the total phenolic content, total flavonoid content, total alkaloid content and total antioxidant activity.

The content of the phenolic compounds in the methanolic extract of leaves of *Solanum pubescens* is expressed in gallic acid equivalents was 46.33±13.22. The content of flavonoid compounds in methanolic extract of leaves of *Solanum pubescens* is expressed in quercetin equivalents was 29.66 ± 8.087. The content of alkaloid compounds in methanolic extract of leaves of *Solanum pubescens* is expressed in atropine equivalents was 1.463±0.268. The content of total antioxidant activity of methanolic extract of leaves of *Solanum pubescens* is expressed in ascorbic acid equivalents was 48.33±12.11. The content of total phenolic compounds in ethyl acetate extract of leaves of *Solanum pubescens* is expressed in gallic acid equivalents was 38.33±11.23.

Table 1. Physical appearance of extracts of *Solanum pubescens*

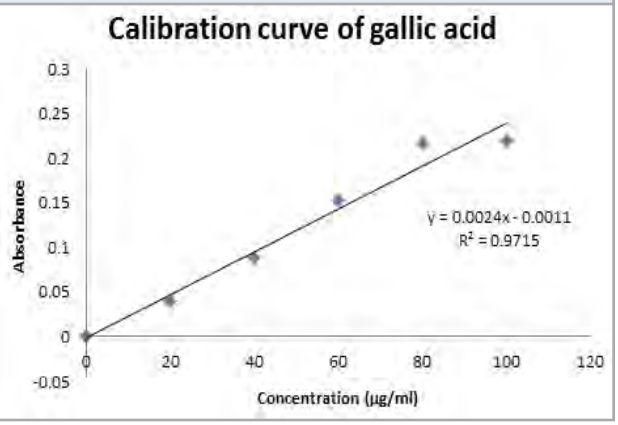
S. No.		Result	
	Physical appearance	MESP	EAESP
01	Color	Green	Green
02	Texture	Semi solid	Semi solid
03	Odour	Pleasant	Pleasant
04	% Yield	10%	8%

Table 2. Phytochemical screening of *Solanum pubescens*

S. No	Test	MESP	EAESP
01	Alkaloids	+	+
02	Flavonoids	+	+
03	Phenolic compounds	+	+
04	Tannins'	+	+
05	Steroids	+	+
06	Saponins	+	-
07	Glycosides	+	-

+ indicates present, - indicates absent

Figure 1: Estimation of total phenolic content



The content of flavonoid compounds in ethyl acetate extract of leaves of *Solanum pubescens* is expressed in quercetin equivalents was 23.43±5.057. The content of alkaloid compounds in ethyl acetate extract of leaves of *Solanum pubescens* is expressed in atropine equivalents was 1.246±0.168. The content of total antioxidant activity of ethyl acetate extract of leaves of *Solanum pubescens* is expressed in ascorbic acid equivalents was 43.66±9.0.

Figure 2: Estimation of total flavonoid content

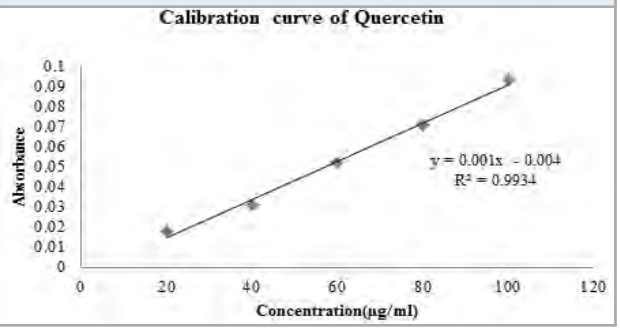


Figure 3: Estimation of total alkaloid content

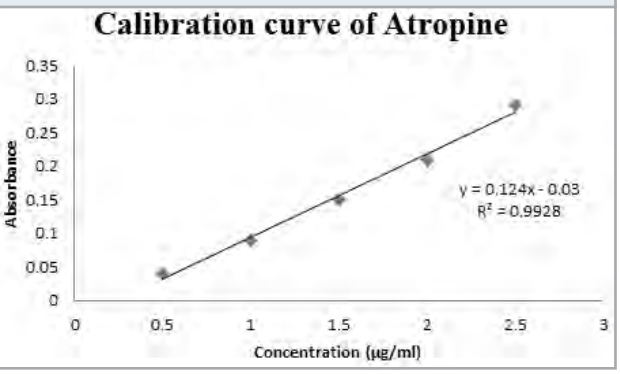


Figure 4: Total anti - oxidant activity

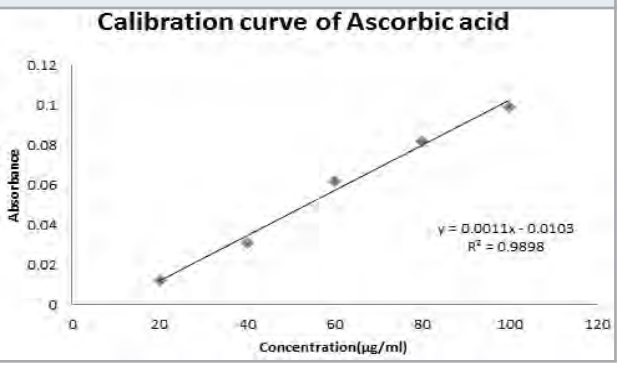


Table 3. Total TPC, TFC, TAC contents of methanolic extract of *Solanum pubescens*

Name of the plant	Solanum pubescens
Family	Solanaceae
Parts used	Leaves
Extract investigated	Methanolic
TPC (mg of GAE/gm)	46.33±13.23
TFC(mg of QE/gm)	29.66±8.087
TAC(mg of /gm)	1.463±0.268
Total antioxidant activity	48.33±12.11

Each value represents the mean ± S.E.M.(n=5).GAE=Gallic acid equivalents, QE= Quercetin equivalents.

Table 4. Total TPC, TFC, TAC contents of ethyl acetate extract of *Solanum pubescens*

Name of the plant	*Solanum pubescens*
Family	Solanaceae
Parts used	Leaves
Extract investigated	Ethyl acetate extract
TPC (mg of GAE/gm)	38.33±11.23
TFC(mg of QE/gm)	23.43±5.057
TAC(mg of /gm)	1.246±0.168
Total antioxidant activity	43.66±9.07

Each value represents the mean± S.E.M.(n=5) GAE= Gallic acid equivalents, QE= Quercetin equivalents

Table 5. Anti TB activity of methanolic extract by using MABA

Samples(µg/ml)	*Mycobacterium tuberculosis*
0.8	R
1.6	R
3.12	R
6.25	S
12.5	S
25	S
50	S
100	S

Table 6. Anti TB activity of ethyl acetate extract by using MABA

Samples(µg/ml)	*Mycobacterium tuberculosis*
1.2	R
2.5	R
5	R
10	S
20	S
40	S
80	S
100	S

The anti-mycobacterial activity of methanolic and ethyl acetate extract of leaves of *Solanum pubescens* is evaluated by micro plate alamar blue assay using H37RV, a standard strain of *Mycobacterium tuberculosis*. The plant extracts showed anti mycobacterial activity to different extents but methanolic extract of leaves of Solanum pubescens exhibited good anti tuberculosis activity having minimum inhibitory concentration of 6.25µg/ml. The ethyl acetate extract of *Solanum pubescens* having minimum inhibitory concentration of 10µg/ml (Showed in table 6 and 7). Furthermore work is

required to be undertaken for active principle or group of compounds responsible for such activity.

CONCLUSION

The methanolic extract of *Solanum pubescens* was shown to be very active as is quite evident from its MIC of 6.25µg/ml. The actual compound responsible for activity needs to be identified.

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Conflicts of Interest: The authors declare no conflict of interest

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Toxicological Communication

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Acute Toxicity Impact of Sodium Arsenite on Behavioural Changes and Histopathology of Kidney and Intestine of the Freshwater Fish, *Channa punctatus* and its Revival With Aqueous Garlic Extract

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ABSTRACT

Toxic metals get accumulated in our environment due to anthropogenic activities. Arsenic, a metalloid, disturbs haematological and biochemical factors of an organism as well as depletes cells major antioxidants and enzymes. The present work was designed to observe behavioural changes, histopathological and ultrastructural changes of kidney and intestine of *Channa punctatus* exposed to sub-lethal concentration (10% and 50% of LC50) sodium arsenite and its revival with aqueous garlic extract (AGE). The traditional LC50 test is used to measure the potential risk of sodium arsenite and the static renewal technique has been used to observe the morphological and behavioural changes of *Channa punctatus*. Histopathological lesions were examined and photographed with the help of Bright Field Microscope. Scanning Electron Microscope was used to observe the ultrastructural changes in the tissues of *Channa punctatus*. Histopathological and ultrastructural studies of kidney and intestine tissues reveal various alterations in the tissues. Hypertrophy of the epithelial cells of renal tubules along with reduction in the size of the tubular lumens was seen in the kidney tissue of the fishes with acute exposure. Hypertrophied nuclei, dilation, oedema were seen in the renal tubules. Vacuolisation due to disintegration of cytoplasm was quite evident. The revival group with aqueous garlic extract showed a lesser extent of vacuolisation and comparatively low enlargement of renal tubules. The intestine of the arsenic treated group revealed severe degenerative changes in the intestinal mucosa, elongated lumen in villi, fusion of villi and atropy in the muscularis. The cytoplasm demonstrated vacuolisation, apoptotic, and necrotic cells. The degenerative changes were found to be less severe in the arsenic + AGE treated group than the group treated with only arsenic. Hence the protective role of garlic can be established from the normalization of the behavioural changes and restoration of histological and surface ultrastructural damages in arsenic induced *Channa punctatus*.

KEY WORDS: KIDNEY, INTESTINE, ARSENIC, TOXICITY, GARLIC, REVIVAL

ARTICLE INFORMATION

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INTRODUCTION

Arsenic, a metalloid, is an important environmental toxicant. Arsenic exists in aquatic environment either in arsenite (As3+) or arsenate (As5+) form that interconverts through redox and methylation reactions (Ghosh et al., 2006). However, the arsenite form has been found to be more toxic both in *in vivo* and *in vitro* experiments than the arsenate form (Cervantes et al., 1994). Fishes are continuously exposed to arsenic through gills and intake of arsenic contaminated food. In fish, arsenic varies in their toxicity by remaining in two oxidation states, methylated species, arseno-sugars and arseno lipids (Bears et al., 2006). The pathophysiology of arsenic in fish tissue takes place due to the combination of these forms (Wrobel et al., 2002).

Fish population is an important component of the food chain. Hence, any effect of such pollution would have adverse influence on the nutritive value of fish and on man through their ingestion. Some amount of histopathological works are available on the effects of different pollutants on fish kidneys (Gupta and Srivastava 2006) but much is not known about the effect of arsenic on the histopathology of exposed fishes.It has been found from previous literatures that acute toxicity of arsenic compounds varies from one species to another species and even in strains of the same species. It was also seen that toxicity of arsenic compounds depends on species, sex, age, dose, exposure period, nature, concentration and organic and inorganic form of the arsenic (Luh et al., 1973). In aquatic toxicology among different types of toxicity tests the traditional LC50 test or the acute toxicity test is often used to measure the potential risk of a particular chemical (Roy et al., 2006; Samanta et. al., 2020).

Garlic (*Allium sativum* L.) is one of the most important vegetables and one of the most ancient medicinal plants which originated from Central Asia over 6000 years ago. The products derived from garlic such as aged garlic extract (AGE) is found to have a higher antioxidant activity due to the presence of stable and highly bio available water soluble organosulfur compound like S-allyl cysteine sulphoxide (SAC) and S-allyl mercapto cysteine (SAMC), with highly potent antioxidant activity (Imai et al., 1994). The protective or ameliorative effect of dietary garlic and its oil was found on common carp (*Cyprinus carpio*) (Yousefi et. al., 2020), growing rabbits (Attia et. al., 2020) and Rohu fish (*Labeo rohita*) (Khan et. al., 2020) against the toxicity of ambient ammonia, lead and silver nanoparticles respectively. Therefore, the present work was mainly aimed to study the behavioural changes as well as histopathological and ultrastructural studies of kidney and intestine of *Channa punctatus* exposed to sodium arsenite and its revival with aqueous garlic extract (AGE).

MATERIAL AND METHODS

Fish: Healthy and disease free *Channa punctatus* were collected from local markets in Guwahati. Their weight was approximately in between 25-45 gm and average length was 14 cm. Fishes were brought to ambient laboratory condition.

Acclimatization of the Fishes: Fishes were disinfected with a dip of 2% potassium permanganate (KMnO4) solution and were acclimatised in aquaria for two weeks before initiation of experiment. The water provided in the aquaria was from the tap in the laboratory and was changed on the following day. The fishes were fed everyday with fish food available in the market during acclimatization period. Proper aeration was done in all the processes. Following the standard procedure given by APHA the physicochemical parameter of the test water such as dissolved oxygen, carbon dioxide concentration, total ammonia, temperature, pH, and hardness were monitored.

Preparation of Stock Solution of Arsenic and its Treatment: Sodium Arsenite (NaAsO2), molecular weight- 129.91 Merck, India (Ltd.) was procured for performing the experiment. A stock solution was prepared by adding 5 mg of sodium arsenite to 100 ml of distilled water. The test concentration was prepared by diluting the stock solution with appropriate amount of distilled water. Physicochemical parameters of the water used in test solution were maintained according to the standard procedures given by APHA, 2005. The control group of fishes were kept in similar conditions without adding sodium arsenite.

Preparation of Garlic extract: The outer layer of garlic (*Allium sativum* L.) cloves were removed, and crushed mechanically in a mortar-pestle with 100 ml of autoclaved distilled water for 1 gm of garlic. The homogenate was shaken for 20 minutes, filtered successively through gauze and 0.22 micron membrane filter to obtain the aqueous garlic extract (AGE) (Chowdhury et al., 2008). 10 ml of AGE was added in each plastic pool during the experimental procedure.

Determination of 96 h- LC 50 for Arsenic: Fishes were transferred to different plastic buckets and exposed to five ascending concentrations of sodium arsenite, such as 5, 15, 25, 35 and 45 ppm (parts per million). In all cases, control groups of fishes were maintained. Each experimental trial was carried out for a period of 96 hours. The mortality rate of the fish was recorded at logarithmic time intervals that is, after 6, 12, 24, 48, 72 and 96 hours of exposure. The LC50 was determined statistically to different concentration of sodium arsenite in water which killed 50 percent of the test species during a specific time interval and under similar experimental conditions (Sparaque 1973). The test media was renewed daily during the experimental period. The data obtained in course of the investigation were analysed statistically to see whether there is any influence of different treatment concentrations on the mortality of the fish.

Experimental Design: To study the histopathological and ultrastructural changes of different tissues of *Channa punctatus* two experiments had been designed. In the

first experiment fishes were exposed to sub lethal concentration i.e. 12ppm (approximately 50% of LC50 value) of sodium arsenite along with a control group for 96 hours. In the second experiment fishes were exposed to sub lethal concentration i.e. 2.5ppm (10% of LC50 value) of sodium arsenite along with a control group for 15 days. Six fishes were used for each set and two sets of experiments were carried out for 96 hours and four sets of experiments were carried out for 15 days. In the 96 hours experiment first set was taken as control without any dose and the second set was given the sub-lethal dose (12 ppm) of sodium arsenite. In the 15 days experiment, first set was taken as control without any dose and the second set was given the sub-lethal dose (2.5 ppm) of sodium arsenite. Another set of fishes received the same dose of sodium arsenite (2.5 ppm) mixed with 10 ml of Aqueous Garlic Extract (AGE). The water, arsenic and garlic treatments of each plastic pool were changed after 48 hour. An experiment was also performed by treating a group of fish only with aqueous garlic extract for 15 days.

Morphological and Behavioural Studies: Morphology and the behaviour of the fishes were observed thoroughly in all the experiments. A control experiment was run parallel by taking same number of fishes and same quantity of water for comparison. The fishes in each group were examined within 96 hours of experimental duration for any morphological and behavioural changes. Their behaviours were also recorded in control and garlic treated groups. In this study behaviours of the fishes such as hyper excitability, fin movements, yawn, fin flickering, S jerk, nudge, nip, cough, opercular movements, imbalanced swimming were considered as observational parameter for all the experiments. Together with the behavioural changes the alteration observed in the skin of the fishes were recorded.

Histological studies: Fishes from control and treated group were randomly selected and the kidney and intestine tissues were isolated for histopathological examinations. Physiological saline solution (0.75% NaCl) was used to rinse and clean the tissue. They were fixed in aqueous Bouin's solution for 24 hours, processed through graded series of alcohols, cleared in xylene and embedded in paraffin wax. Sections were cut at 4 micron thickness and stained with Hematoxylin and eosin stain. Histopathological lesions were examined and photographed with the help of computer attached Bright Field Microscope (LeicaDM3000) (Magnification x400 and x100).

Scanning Electron Microscopic Analysis: Kidney and intestine tissues of control and treated groups were rapidly removed and processed routinely for scanning electron microscopic studies. The tissues were cut into small pieces of 1 mm thickness and fixed in 2.5 % glutaraldehyde prepared in cacodylate (sodium phosphate) buffer adjusted to pH 7.4 for 24 hours and afterward washed in phosphate buffer for 15 min. After dehydration in ascending series of acetone, samples were immersed in Tetra Methyl Silane for 10 minutes at 4 degree centrigrate. Then they were brought to room temperature to dry. The specimens were mounted on Aluminium Stubs coated with gold and observed through scanning electron microscope in Sophisticated Analytical Instrument Facility (SAIF), North-Eastern Hill University (NEHU), Shillong – 793022.

RESULTS AND DISCUSSION

The observed physico-chemical parameters of the tap water used in the experiment were temperature, pH, dissolved oxygen, dissolved carbon dioxide, total hardness and ammonia. These parameters were monitored throughout the acclimatization period and the trial periods in which the fishes were exposed to sodium arsenite and garlic. The observed physico-chemical parameters of the test water are listed in Table 1. The parameters of the test water were measured according to the experimental procedure described by APHA (1998) and according to their recommendations the fluctuation in temperature should not exceed 4°C and the oxygen content must not fall below 4 mg/L for the warm water fish.

Figure 1: Relationship between concentration of sodium arsenite and probit mortality of *Channa punctatus* after 96 hours.

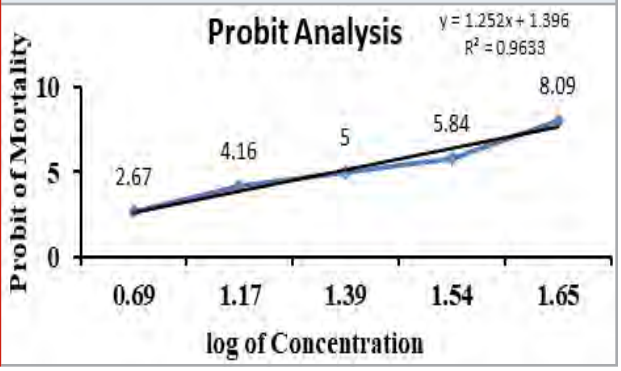
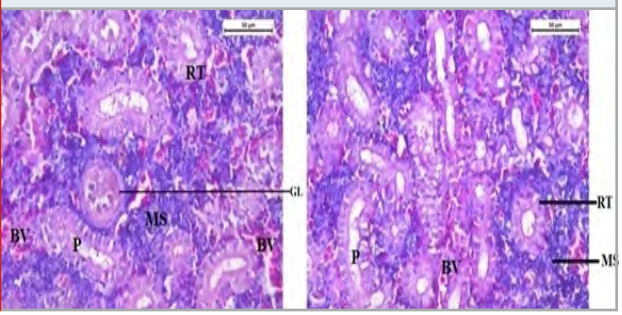


Figure 2: Histology of kidney tissue in the control group of *Channa punctatus*. (GL- Glomerulus, RT- Renal Tubule, MS- Mesenchyma, P-Proximal Tubule, BV- Blood Vessels)



In the present study, it was observed that 45 ppm sodium arsenite in water induced death of all the exposed fishes within 96 hours. The 96 hours LC50 of sodium arsenite for *Channa punctatus* was found to be 25 ppm and its probit analysis graph can be seen in the Figure 1. Fishes treated with a concentration of 5, 10 and 12 ppm survived for more than 90 days with zero mortality

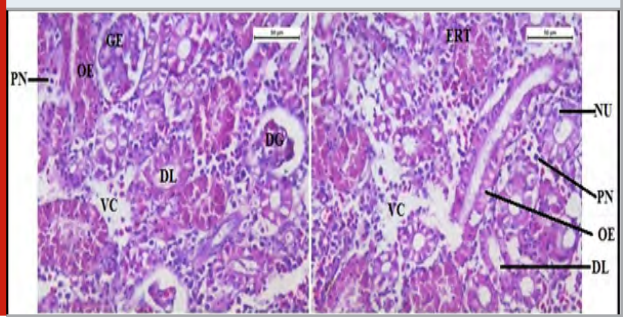
rates. In the present study it was observed that (Table 2) during the initial period of sodium arsenite exposure fishes at all the tested concentrations became restless, moved faster, showed rapid opercular movements and hyper excitability and had the tendency of jumping out from the arsenic contaminated water. Frequency of erratic swimming, jumping and gulping of air at the water surface was found to be increased during the initial period (30 hours) of exposure in both the two test concentrations but during the later period of exposure, these activities reduced.

This might be due to their adaptation in the arsenic contaminated environment or because of reduction of their enzymatic activity. It was seen that, the changes of above activities depended on the concentration of sodium arsenite. When the fish were exposed to median lethal concentration (25 ppm) the observed changes in the behavioural activities were relatively increased initially (of initial 10 hours period of exposure time) and then reduced afterwards. It was also observed that for median lethal arsenic concentration fish were seen with increased body depigmentation along with lot of mucus all over the body. Towards the end of exposure period the fish struggled for breathing and had lost their swimming ability. The fish gradually lost their sense of equilibrium and became lethargic. These behavioural changes were more prominent for higher concentration of sodium arsenite than in the lower concentrations.

When the fishes were exposed to Garlic treated water for the first 30 hours the fishes showed a little of excitability, opercular movement and fin flickering but they did not show any imbalanced swimming, depigmentation and mucus secretion during entire 96 hours exposure period. Another group of fishes first exposed to sublethal concentration (2.5 ppm) of sodium arsenite for 96 hours and then they were transferred to only AGE treated water for 96 hours. In this case the fishes showed a sign of relaxation and found to have less excitability, opercular movements, fin flickering, and depigmentation. It was observed that the test fishes showed almost similar behavior as of the fishes of control group towards the end of 96 hours of observation. At the end of the exposure period of median lethal concentration (25 ppm) of arsenic almost fifty percent of the fishes were found dead, scattered at the bottom of the tank with their mouth open. In the other case fish remained alive and relatively active throughout the experimental period.

The histology of the kidney tissue of the control group of *Channa punctatus*, is shown in the Figure 2. The kidney tissue of *Channa punctatus* revealed normal arrangement of the renal tubules and Bowman's capsules with glomeruli distributed in the interstitium of haematopoietic tissue. Regular arrangement of renal tubules, proximal tubule and blood vessels were clearly visible. Histopathology of the kidney tissue exposed to sodium arsenite for 15 days revealed hypertrophy of the epithelial cells of renal tubules along with reduction in the size of the tubular lumens was seen in the acute exposure (Figure 3). Hypertrophied nuclei (NU), dilation (DG), oedema (OE) were prominent in the renal tubules. Vacuolisation (VC) due to disintegration of cytoplasm was quite evident. Pyknotic nuclei (PN) could be observed in mesenchymal tissue. Vacuolization (VC) and disorganized blood capillaries could be seen in Glomeruli.

Figure 3: Histology of kidney tissue in the group of *Channa punctatus* treated with sodium arsenite for 15 days. (VC- Vaculisation, NU-Hypertrophied Nuclei, DS- Dequamation of Epithelial Lining, ERT- Enlargment of Renal Tubules, PN- Pyknotic Nuclei, DL-Dilation of Renal Tubules, OE- Qedema od Renal Tubules, DG-Dilation of Glomerulus, GE- Glomerular Expansion)



Histology of the kidney tissue of the fishes treated with only garlic for 15 days (Figure 4) represented normal structure of the kidney tissue similar to the structure of the control fishes. Histology of kidney tissue exposed simultaneously to sodium arsenite and AGE for 15 days (Figure 5) revealed that the histoarchitechture of proximal tubule, glomeruli and mesenchyma remained unaffected whereas few vacuolisation and comparatively low enlargement of renal tubules could be seen (Figure 5). Ruptures of few blood vessels were also prominent. A comparison of the histological changes of sodium arsenite treated kidney tissue with sodium arsenite + AGE treated group revealed lesser damage in the histoarchitechture of the kidney tissue. This is probably due to the protective effect of garlic which may prevent the accumulation of arsenic in the kidney tissue.

Figure 4: Histology of kidney tissue in the group of *Channa punctatus* treated with only garlic for 15 days (RT- Renal Tubule, P- Proximal Tubule, BV-Blood vessels, MS-Mesenchyma)

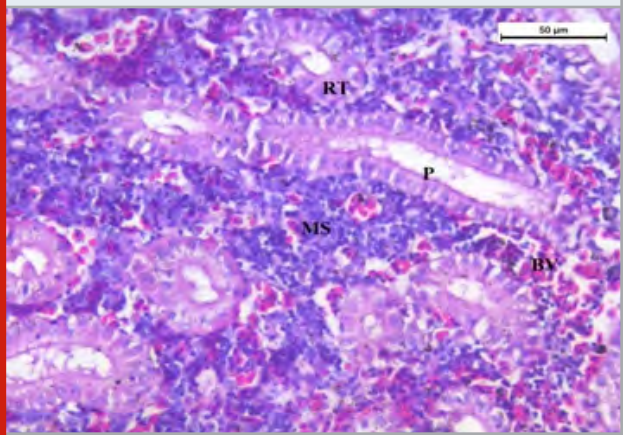
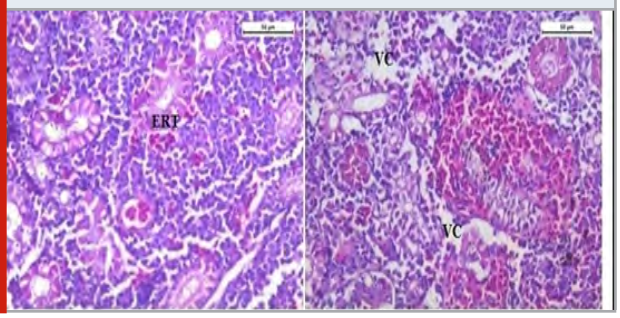


Figure 5: Histology of kidney tissue in the group of *Channa punctatus* simultaneously treated with sodium arsenite and aqueous garlic extract (AGE).



Ultrastructure of the kidney tissue of control group of *Channa punctatus* for 96 hours and 15 days experiments showed normal surface structure and regular arrangement of different components of the tissue (Figure 6). SEM images of the kidney tissue exposed to sodium arsenite for 96 hours as well as for 15 days (Figure 7) revealed many outgrowths in the surface of the tissue exposed to sodium arsenite in both 96 hours and 15 days of treatment. Although, it could be seen that the extent of the growth of the tumour like structures were more in the 15 days exposure as compared to 96 hours exposure. It was also observed that when the *Channa punctatus* were treated with only garlic extract the ultrastructure of the kidney tissue appeared to be similar to that of control group (Figure 8). SEM images of the kidney tissue exposed simultaneously to sodium arsenite and AGE for 15 days duration (Figure 9) showed significant recovery in the surface ultrastructure as compared to the deformation observed in the kidney tissue exposed only to sodium arsenite (Figure 7).

Figure 6: SEM images of kidney tissue in the control group of *Channa punctatus*. (A- 96 hours observation, B- 15 days observation)

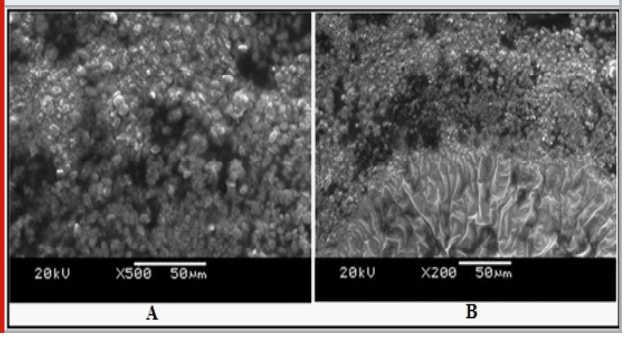


Figure 7: SEM images of kidney tissue in the treated group of *Channa punctatus*. (A- 96 hours treatment, B-15 days treatment, O-Outgrowth or tumour like structure)

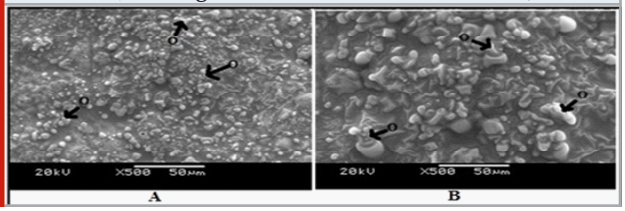


Figure 8: SEM images of kidney tissue in the group of *Channa punctatus* which was treated with only garlic for 15 days.

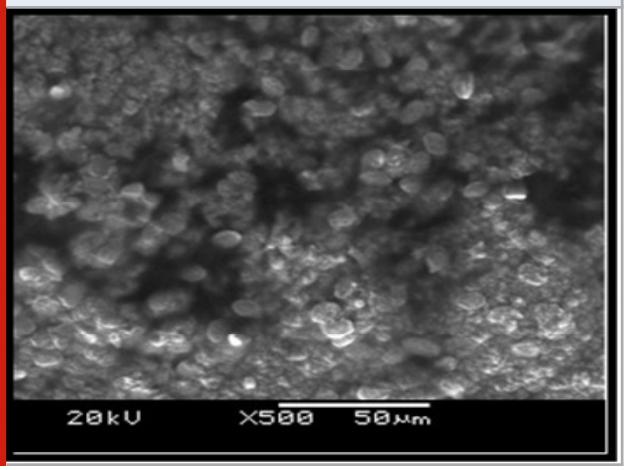
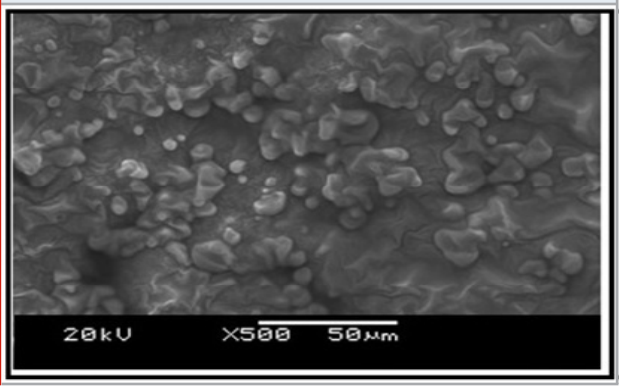


Figure 9: SEM image of kidney tissue in the group of *Channa punctatus* exposed with sodium arsenite and aqueous garlic extract simultaneously



Histologically, the intestinal wall of *Channa punctatus* is comprised of mucosa, submucosa, muscularis and serosa. The mucosal layer extends into prominent slender folds called villi which has intestinal glands. Figure 10 shows the normal histology of intestine in the control group of *Channa punctatus*. The intestinal histology of the treated groups showed severe degenerative changes in the intestinal mucosa, elongated lumen in villi, fusion of villi and atropy in the muscularis. On days upwards of the exposure, the cytoplasm demonstrated vacuolization, apoptotic, and necrotic cells in greater numbers in 15 days arsenic treated fishes (Figure 11).

Figure 10 : Optical Micrograph of the intestine in the control group of *Channa punctatus*. (SR-Serosa, MM-Muscularis Mucosa, MU-Mucosa, VI-Villi)

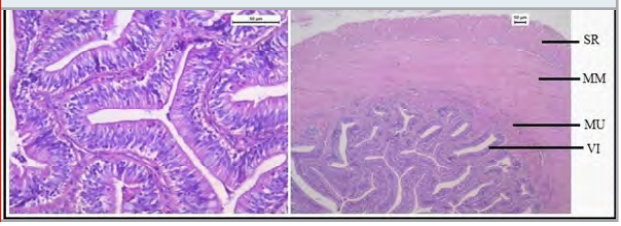


Figure 11: Optical Micrograph of the intestine tissue of *Channa punctatus* treated with arsenic. (EL- Elonggated Lumen in Villi, VC – Vacoulisation, N- Necrosis)

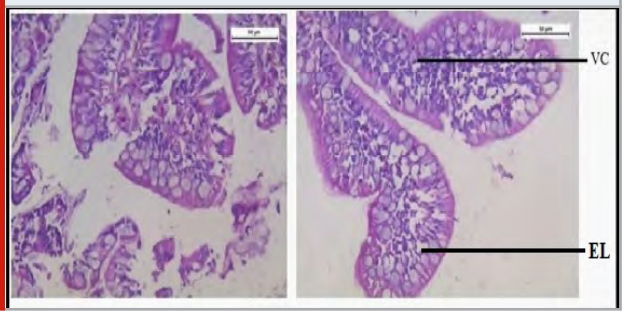


Figure 12 shows normal histology of the intestine in the group of fishes treated with only garlic extract. The histological study of intestine in the arsenic and AGE treated group revealed vacuolisation, partially damaged mucosa and distorted villi. However the damages were partially recovered (Figure 13).SEM photograph in the control group revealed normal ultrastructure of the intestine tissue (Figure 14). However in the treated group surface wrinkling and bulbous epithelium were observed (Figure 15). Intestinal mucosal folds were found to be damaged and epithelial cells were distorted. The degenerative changes were found to be less severe in the arsenic + AGE treated group than the group treated with only arsenic (Figure 17). The SEM images of intestine tissue treated with only garlic which was in accordance to the structure of the control group (Figure 16).

Figure 12: Optical micrograph of intestine tissue of *Channa punctatus* treated with only garlic extract

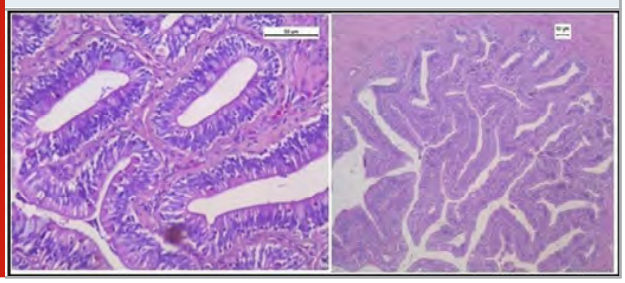


Figure 13: Optical micrograph of intestine tissue of *Channa Punctatus* treated with arsenic and garlic extract simultaneously for 15 days duration (DV- Distorted Villi).

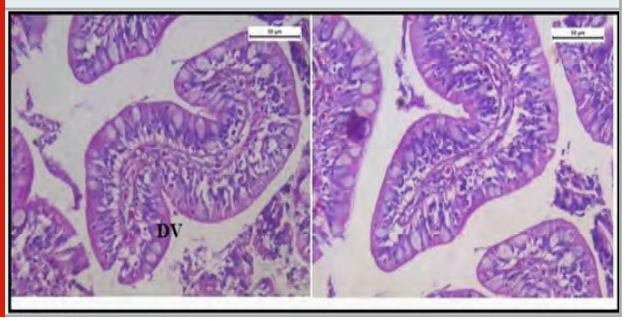


Figure 14: SEM image of intestine tissue in the control group of *Channa punctatus*

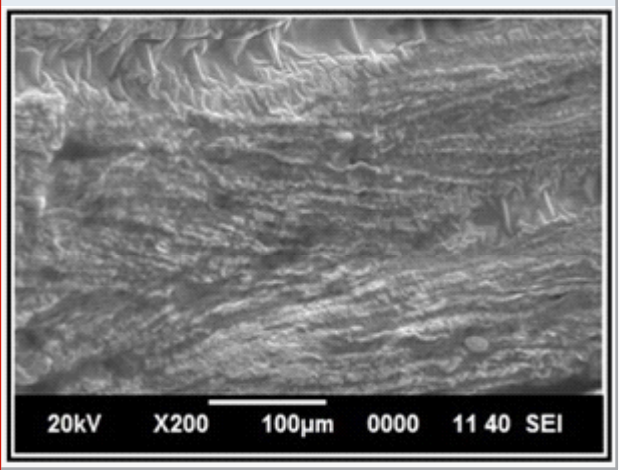


Figure 15: SEM images of intestine tissue of *Channa punctatus* treated with arsenic. (A- 96 hours treatment, B1 & B2 - 15 days treatment, BE- Bulbous Epithelium, SW- Surface Wrinkling).

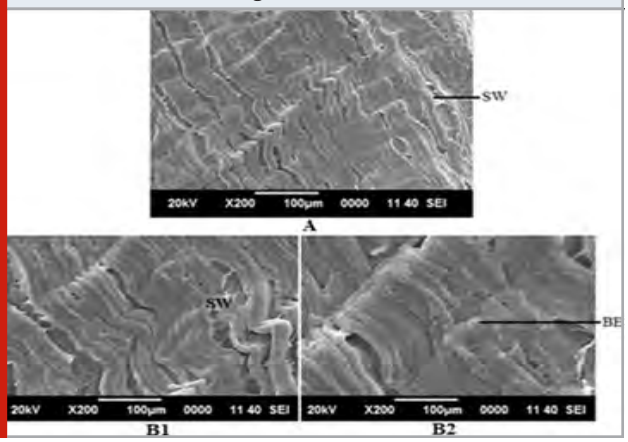


Figure 16 : SEM image of intestine in *Channa punctatus* treated with only Aqueous Garlic Extract (AGE).

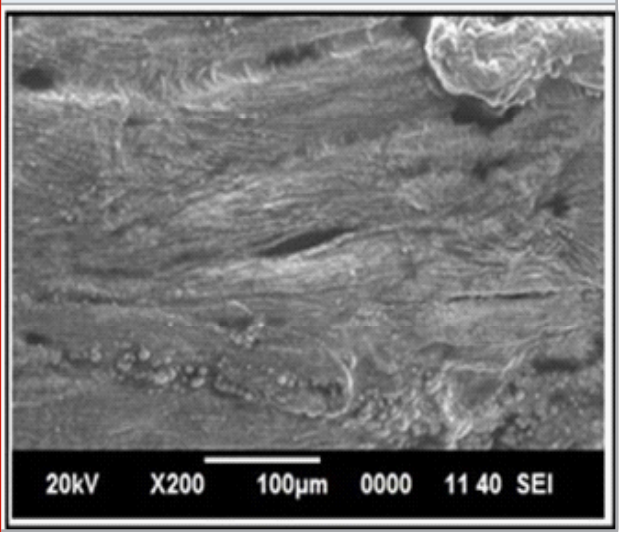
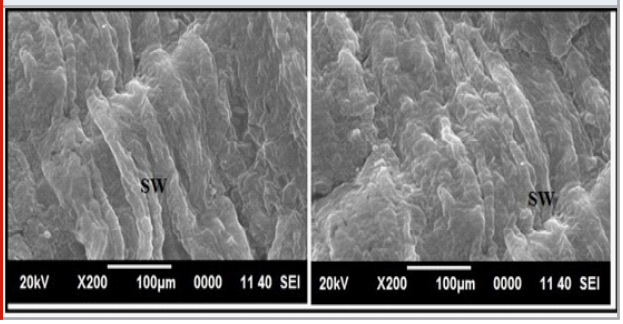


Figure 17: SEM images of intestine tissue in the group of *Channa punctatus* treated with arsenic and AGE for 15 days duration (SW-Surface Wrinkling).



This study was aimed to analyse behavioural and histological alterations on acute exposure of sodium arsenite on *Channa punctatus* and to study the ameliorative effect of garlic against arsenic toxicity. An excellent tool to assess the well-being of a particular organism has been the behaviour. It was observed that the different concentration of sodium arsenite initiated various pattern of behavioural changes in the test fish. The cause of such abnormal behaviour and altered movements might have been due to some enzymatic and ionic imbalances in blood and tissues (Larsson et al., 1981). It was also noted that, the changes of above activities depended on the concentration of sodium arsenite. The group of fishes exposed to sublethal concentration (2.5 ppm) of sodium arsenite for 96 hours was transferred to only AGE treated water for 96 hours.

In this case the fishes showed a sign of relaxation and were found to have less excitability, opercular movements, fin flickering, and depigmentation. Many researchers observed abnormal behaviours in different fishes treated with various heavy metals (Santha et al., 2000; Karuppasamy 2001; Subathra and Karuppasamy 2003; Sivakumar et al., 2006). The stressful toxic environment along with sensory stimulus might have increased the opercular movement for proper ventilation of gills to cope up with hypoxia (Lata et al., 2001). The neurotoxic effects and the disturbances to receptor system of the body might be the cause of abnormal behaviours in the fishes (Bhavani and Karuppasamy 2014).

Table 1. Physico-chemical parameters of the test medium.

Physico-chemical parameter	Value	Mean
Temperature (°C)	25-27	25.8 ± 0.4
PH	7.1-7.4	7.14 ± 0.18
Dissolved oxygen (mg/L)	6.5-7.3	6.96 ± 0.12
Dissolved Carbon dioxide (mg/L)	10.3-10.9	10.56 ± 0.18
Total Hardness (mg/L)	189-197	193.2 ± 0.68
Ammonia (mg/L)	0.15-0.18	0.164 ± 0.02

Table 2. Effect of Sodium arsenite on the behavioural activity of *Channa Punctatus* during 96 hours exposure to the sub-lethal and medium lethal concentration of Sodium arsenite and AGE (Aqueous Garlic Extract)

Nature of Behaviour	Control Group	Arsenic exposure concentrations		AGE treated group	Post arsenic-AGE treated group
		2.5 ppm	25 ppm		
Opercular movement	+	++	+++	+	+
Hyperexcitability	_	++	+++	+	_
Fin movement	+	++	+++	+	+
Imbalance swimming	_	+	+++	_	_
Mucus secretion	_	+	+++	_	_
Depigmentation	_	+	++	_	_
Mortality	_	_	+	_	_

- = Normal effect; + = mild effect; ++ = Moderate effect; +++ = Strong effect

Behavioural responses, abnormal swimming, restlessness, fin flickering, hyper excitability, jumping out and to and fro movement of the fish observed in the present study might be the avoidance responses of the fishes towards the toxicant. The avoidance response may be because of changes in sensitivity of chemoreceptors. Similar observations were reported by researchers like Svecevieus (2001) and Agarwal (1991). Lethargy observed in this study towards the end of exposure period might be because of loss of energy resulted due to erratic swimming, jumping and restlessness. The impairment of nervous system might cause the lateral swimming and loss of equilibrium in the fishes (Sinha and Kumar, 1992).

The histology of the kidney tissue exposed to sodium arsenite represented expansion of renal tubules and

distinct separation of their epithelial lining from the tubular cells. Most of the renal tubules were observed to lose their cellular integrity. Hypertrophied nuclei (NU), Dilation (DG), oedema (OE) were prominent in the renal tubules. Vacuolisation (VC) due to disintegration of cytoplasm was quite evident. Pyknotic nuclei (PN) could be observed in mesenchymal tissue. Vacuolization (VC) and disorganized blood capillaries could be seen in Glomeruli. Similar histological changes in the kidney of *Channa punctatus* (Bloch) due to the effects of sub-lethal concentrations of zinc were reported earlier (Gupta and Srivastava 2006). Pathological changes in the kidney of different fishes exposed to various other toxicant have earlier been reported by many researchers (Hadi and Alwan 2012; Anitha Kumari and Sree Ram Kumar 1997; Prashanth 2011; Banerjee and Bhatacharya 1994., Wang et. al., 2020).

The degenerative damages takes place due to the presence of high concentration of heavy metals in the kidney tissue (Anitha Kumari and Sree Ram Kumar 1997; Venkataramana and Radhakrishnaiah 1987).In the present study it has been observed that the extent of damages in the kidney tissue reduced to some extent in the fishes treated simultaneously with sodium arsenite and the Aqueous Garlic Extract (AGE) for 15 days duration. Histology of the kidney tissue of the fishes treated with only garlic for 15 days represented normal structure of the kidney tissue similar to the structure of the control fishes. The histopathology of the kidney of the group of fishes treated with sodium arsenite and AGE for 15 days showed that the histo-architecture of proximal tubule, glomeruli and mesenchyme remained unaffected. The histological alterations observed were vacuolisation, enlargement of renal tubules and ruptures in few blood vessels. A comparison of the histological changes of sodium arsenite treated kidney tissue with the revival group showed that there was a lesser extent of damage in the micro structure of the kidney tissue. This is probably due to the protective effect of garlic which might have prevented the accumulation of arsenic in the kidney tissue.

Ultrastructural changes of the kidney tissue exposed to sodium arsenite showed many tumour like outgrowths in the surface of the tissue. It was also observed that when the *Channa punctatus* were treated with only garlic extract the SEM images of the kidney tissue appeared to be similar to that of the control group. Massar et al., (2014) observed similar abnormal outgrowths at the surface of the kidney tissue of Common Carp (*Cyprinus carpio* L.) inhabiting a polluted reservoir. Kidney tissue of different fishes exposed to Glyphosate-based Herbicide, Excel Mera 71, Cadmium Nanoparticles and the insecticide monocrotophos showed abnormalities in its ultrastucture (Samanta et al., 2018; Sangeetha et al., 2017; Singla 2015). Recently it has been observed that exposure of toxicant like copper significantly disorganized the morphology of kidney in rat (Wan et. al., 2020).

Ultrastructural changes of the kidney tissue exposed simultaneously to sodium arsenite and AGE showed a lesser extent of deformation as compared to the deformation observed in the kidney tissue exposed only to sodium arsenite. The extent of the growth of the tumour like structure was found to be more in the arsenic treated fishes than the ones treated with arsenic +AGE. These results indicate that there is a protective role of AGE that prevented the adverse effects of sodium arsenite on kidney tissue.The histology of the intestinal tissue of arsenic treated group of *Channa punctatus* showed severe degenerative changes in the intestinal mucosa, elongated lumen in villi, fusion of villi and atropy in the muscularis. The cytoplasm demonstrated vacuolization, apoptotic, and necrotic cells in greater numbers. The damages in the intestinal villi might be due to the defensive mechanism of fish against arsenic toxicity. Lifting of columnar epithelium of villi could be a defence mechanism of the fish against the toxic environment.In the arsenic + AGE treated group partially damaged mucosa, vacuolisation and epithelial lifting of intestinal villi were observed. The damages were less severe than the arsenic treated group. Normal histology of the intestine in the group of fishes treated with only garlic extract was seen.

The present findings are in accordance with the findings of earlier researchers. Similar histopathological changes in the intestine of arsenic treated *Channa punctatus* was reported by Hossain (2012). Dilation of vascular elements, oedema, and increased width of the sub mucosa, lumen and serosa were noted. The effect of different toxicants on the intestine of Tilapia fish were observed by Shah et al., (2009). The findings of Kaoud et al., (2011) revealed that the intestine tissue of *Oreochromis niloticus* when exposed to cadmium showed necrosis in mucosa and sub mucosal hemorrhage. The intestinal histology of chicken was also found to be altered by subchronic exposure of mercuric chloride (Zhou et. al., 2020).

The surface ultrastructural changes of the intestine of the arsenic treated group showed surface wrinkling and bulbous epithelium. Intestinal mucosal folds were found to be damaged and epithelial cells were distorted. The degenerative changes were found to be less severe in the arsenic + AGE treated group than the group treated with only arsenic. The SEM image of intestine tissue treated with only garlic was in accordance to the structure of the control group. Samanta et al., (2018) revealed that *A. testudineus* after glyphosate based herbicide exposure in laboratory condition under scanning electron microscopic showed severe damages in columnar epithelial cells and excess mucus secretion in the mucosal folds. Ghosh et al., (2001) observed similar changes in intestine tissue through SEM investigation in arsenic treated *Notopterus notopterus*. Histopathological Examination and Transmission Electron Microscopy mercury-exposed mice showed intestinal injury (Zhao et. al., 2020).

CONCLUSION

The ameliorative effect of garlic against arsenic toxicity was shown in the normalisation of behavioural changes and histopathological damages in arsenic induced *Channa punctatus*. From this study it can be concluded that the sensitivity of the fish *Channa punctatus* to sodium arsenite is very high and it can be regarded as a toxicant. It can also be concluded that garlic as a natural anti-oxidant do not cause any type of excitability or irritant nature in *Channa punctatus*. Hence it has been used as a treatment for revival against arsenic toxicity. The protective effect of garlic was shown in the normalisation of lesions in the histopathological and surface ultrastructural studies of arsenic induced tissues of *Channa punctatus*. Hence from the present investigation it can be concluded that garlic extract could be an effective natural agent to reduce the cellular damages in the arsenic exposed tissues and it definitely have firm role in the recovery of fish tissues after exposure to sodium arsenite.

Authors' contributions: Titikksha Das contributed to the study design, performed the experimental work, analyzed the results and wrote the paper; Mamata Goswami contributed to the study design and analyzed the results.

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Social Anxiety Disorder Among Adolescents in Relation to Peer Pressure and Family Environment

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ABSTRACT

Adolescence is a crucial developmental stage of transition where they are more influenced by outside factors such as parents, peers, family, school and society at large. This influence can be both positive and negative. The positive influence could increase self-confidence, good relations with peer, family and society, academic achievements whereas negative impact could lead to low self-esteem, inability to deal with social situations etc. leading to great stress and social anxiety among adolescents. Social anxiety disorder (or social phobia) is a mental disorder with hampered ability of social interaction which causes impairment in academic and social functioning and can seriously limit adolescent's self-beliefs and his ability to succeed. The present study is an attempt to investigate the role and impact of peer pressure and family environment on social anxiety disorder among adolescents. For this purpose, 520 adolescents studying in government and self-financed schools in the state of Punjab were selected randomly using multi-stage sampling technique. The tools used for this purpose were Social Anxiety Disorder scale (2018) by Nagpal, Peer Pressure Scale (2010) by Singh and Saini, Family Environment Scale (2012) by Bhatia and Chadha. The study is descriptive in nature. The major findings of the study revealed that (i) a significant positive relationship exists between social anxiety disorder and peer pressure among adolescents; (ii) a significant negative relationship exists between social anxiety disorder and family environment of adolescents; the conjoint effect of peer pressure and family environment on social anxiety disorder among adolescents is higher than their individual effects. The study will prove to be an eye opener for society who always judge adolescents as ruthless, insensitive, irresponsible and incapable to handle social pressures. It will also help the parents to redefine their role in relation to their children especially when they are at adolescent stage and also take the friends of their wards with positive but cautious note without undermining the positive influence of peers.

KEY WORDS: SOCIAL ANXIETY DISORDER, PEER PRESSURE, FAMILY ENVIRONMENT.

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INTRODUCTION

Adolescence is a beautiful phase of life when dreams are built and the child turns into energetic and confident individual. They are considered to be as corner stone of a family, society and country. It is a transitional stage of physical and mental human development that occurs between childhood and adulthood. This stage involves biological, social and psychological changes, though the biological ones are the easiest to measure objectively, but as they grow and develop, they are influence by outside factors such as: parents, peers, community, culture, religion, school, world events and the media (Spano, 2004). These factors play an important role in the all round development of adolescents. It has been seen that they suffer from mental, emotional and behaviour problems which are very painful and costly to both themselves and their family members. These problems not only disrupt a child's ability to function socially, academically and emotionally but also affect the person as well as his or her family, school, community and the larger society.

This has resulted poor performance in the studies, due to the failures, students' feel stress, tension and anxiety. Empirical data reveals that anxiety is characterized by feelings of distress and tension about real or anticipated threats that may manifest in cognitive, behavioural or physiological patterns (Huberty, 1997) and particularly social anxiety or phobia which has been ranked by Alonso et al. as one of the ten leading chronic disorders – mental or physical due to its effects on objective outcomes, such as the reduced quality of life in relation to health while Bruce and Saeed (1999) has ranked it as the third most common psychiatric disorder. Results of the various studies found that it is a common disorder with a prevalence of 12.1 % among the people (Kessler et al., 2005).

Social anxiety disorder has both lifetime and 12 month prevalence rates of 12.1% and 7.1% respectively, (Ruscio et al., 2008).Social phobia is highly prevalent among the girls and it has no significant effect on the students demographically (Rizwan, Inam and Abiodullah, 2015). Social phobia is the most chronic type of anxiety disorder and it effects occupational, educational and social affairs of the individual. It is also known for its association with substance use disorder and depression among school adolescents (Mekuria et al., 2017). Students with social anxiety disorder are more likely to experience depressive symptoms and have poor quality of life and vice versa. Females are mor likely to experience Social anxiety disorder (Ratnani et al., 2020).

It is "uncomfortable and unwanted" psychological problem (Ahmed, 2009). It is due to this disorder adolescents feel fear of meeting new people or embarrassing oneself in social situations.Pagano, Wang, Rowles, Lee and Johnson (2015)studied the influence of social anxiety disorder on clinical severity at intake, peer- helping during treatment and outcomes in 195 adolescents (14-18 years) and reported that 42% of adolescents have shown persistent fear of being humiliated or scrutinized in social situations, and 15 % met current diagnostic criteria for social anxiety disorder. The influence of social anxiety disorder on peer- helping and outcomes was examined by hierarchical linear regression and event history methods.

Pickering et al., (2019) have studied the role of peers in the development of social anxiety in adolescent girls: a systematic review and results shows that while low peer acceptance was significantly associated with increased social anxiety for boys and girls, negative friendship experiences, limited close relationships, and relational victimisation were highlighted as risk factors specific to girls. The review enabled the researchers for the development of prevention and intervention methods to support girls at increased risk and that focus on improving the quality of peer relationships. At adolescence stage of development, susceptibility to peer pressure is particularly high. The fear of isolation and the fear of ridicule may play a role in this. Adolescence is the time when person is most susceptible to peer pressure because peer becomes an important influence on behaviour during adolescence and peer pressure has been called a hallmark of adolescent experience. Peer conformity in young people is most pronounced with respect to style, taste, appearance, ideology and values (Campbell, 2018).

Adolescents are under constant pressure- pressure to perform academically, to become their own person independent of their parents and to deal with the hormonal and physical changes that are happening to their bodies. They are also under constant scrutiny from their classmates, and are often subject to mounting pressure to fit in or do things that earn them approval from their peers. This constant pressure to fit in and to gain approval can be overwhelming for many youngsters, ultimately leading to social anxiety disorder. Peer pressure is not always negative. We are all influenced by our peers/friends at any age, (Yael, 2015).

Family is a primary socialization context and is considered to be a strong factor influencing child development. A family is a source of stability, security and happiness. It can nurture love and togetherness but in the absence of these factors it can become a source of stress and turmoil. A conflictual family environment is associated with adolescent's insecurity and psychological distress, as well as aggressive behaviour and conduct disorder. After informal learning in home child goes to school for formal learning and school is another important factor influencing adolescent's development. A positive, supportive environment can help children feel safe. Adolescents who feel that there are people who care about them at school and feel connected to the school are more likely to be academically motivate and less likely to engage in a variety of negative behaviours including drug use, violence and sexual activity but on the other side, if the school environment is bad it may have drastic effects on their development which further leads to stress and anxiety among adolescents (Nagpal, 2018).

Xu et al., (2017) studied on the relationship between parenting styles and adolescents' social anxiety in migrant families. The data was collected from 1345 adolescents in migrant families from four non-government funded junior middle schools in Guangdong, China. Scales used for measuring parenting style was short form of the EgnaMinnenBetraffandeUppfostran and social anxiety measured by social anxiety subscale of self-consciousness scale. The result shows that mother's overprotection is associated with adolescents' social anxiety in migrant families and furthermore, in migrant families, parental emotional warmth could decrease adolescent's social anxiety, whereas maternal overprotection could increase it.

Tahirovic and Demir (2018) conducted a study on influence of parental divorce on anxiety level of adolescents and found that adolescents from divorced families shows higher level of social anxiety than adolescents from intact families and furthermore adolescents divorced families and intact families do not vary in their anxiety level in relation to gender. The sample for the study consists of 162 adolescents from five different high schools from Istanbul, Turkey and for assessment of anxiety level of participants Beck Anxiety Inventory was used.

Recently, Gomez-Ortiz et al., (2019) investigated on parenting practices and adolescent social anxiety: a direct or indirect relationship? The representative sample consisted of 2060 Andalusian students (47.7% girls. Mage = 14.37) assessed by various self reports and found a direct relationship between maternal and paternal educational practices and social anxiety and indirect relationship between parental practices and social anxiety, mediated by negative self-esteem and emotional suppression.

Social Anxiety Disorder: Social anxiety disorder is explained by extreme and consistent fear of meeting new people or embarrassing oneself in social situation. DSM-V defines social phobia as marked or intense fear or anxiety of social situations in which the individual may be scrutinized by others and this situation interferes significantly with routines, occupational (academic) functioning, social activities and relationships. Coleman (2007) defined social anxiety disorder as an anxiety disorder characterized by a phobia of scrutiny by others or of being the focus of attention in social situations involving strangers. Exposure to such social situation either generates anxiety about behaving in an embarrassing or humiliating way, or triggers a panic attack, but the reaction is recognized by the afflicted person (an adolescent or adult) as excessive or irrational, and the avoidance behaviour or anxious anticipation interferes significantly with everyday life, occupational or academic performance, or social relationships.

According to Goldberg (2014) social anxiety disorder, also called social phobia is an anxiety disorder in which a person has an excessive and unreasonable fear of social situations. Anxiety (intense nervousness) and self-consciousness arise from a fear of being closely watched, judged, and criticized by others. Nagpal (2018) defined social anxiety disorder is a persistent and significant fear of being scrutinized or humiliated by others in social situations wherein the person may have to perform or meet a lot of unfamiliar persons. When these fears become excessive they impede the person's functioning and routine. Furmark (2020) explained social anxiety disorder seems to be a problem that is strongly intertwined with personality, but at the same time it shows great variation. Peer relationships, school functioning and family functioning may all suffer as a result of child's social anxiety disorder.

Peer Pressure: Peer groups play an important role in young people's lives particularly during adolescence. Peer Pressure refers to the influence that these groups can have on how an individual thinks and acts. Peer pressure is the direct influence on people by peers, or the effect on an individual who gets encouraged to follow their peers by changing their attitude, behaviours. Santor, Messervey & Kusumakar (2000) defined peer pressure as the insistence and encouragement of the same age group individuals to make or force the individual to do something. Elliot &tMcGregor (2001) explained peer pressure as the influence exerted by a peer group in encouraging a person to change his/her attitude, values, behaviour in order to confirm the group norms. Sharry (2004) stated peer pressure as pressure coevals is associated with wrong decisions, rebellion and humour fluctuations, which will lead to a poor academic performance, because of the fact that adolescents are not cooperative in the process of learning outcomes and as a result their school results fall weakly, Dumas et al (2012).

The pressure among peer group among its member may engage to do undesired things or negative behaviour with the presence of a particular peer group leader who engage its member to do deviant acts or promote undesirable things to the group.Gulati (2017) stated peer pressure towards persons behaviour is said to be a social phenomenon where the members of a particular society or may not be influence negatively but majority are affected by the undesirable behaviour of those people who resist what others do. Moldes et al., (2019) stated peer pressure is often seen during the adolescence stage of a teenagers because they often seek comfort among their peers and intend to do what their peers does without knowing if it is good or bad for them.

Family Environment: Family is the most important environmental cluster of variables which influence the development of a child. Family environment is the first social setting in which an infant takes birth and start learning, responding and acting. Pfeiffer and Aylward (1990) was of the view that family climate affects the child's cognitive and social development, which in turn influences his/her self-confidence, self reliance, assertiveness, personality characteristics coping skills, academic motivation and success. Ranhotra (1996) defined family environment as the complex of social and cultural conditions, the combinations of external or

extrinsic physical conditions that affect and influence the growth and development of the member of family, the most intrinsic fundamental social group which includes parents and their children. Kayhan, Yazicioglu (2007) Family is a social structure making a person happy to be a member of it; it is a place where a person feels protected in every aspect of life. Basic needs in life are fulfilled by family. The first interaction is experienced within the family. Basic behaviours and habits related to life are acquired by an interaction in the family.Meadows (2010) defined family is the first place of learning which is very important for the child's social and emotional development. Kamble (2014) mentioned that family is major socialization agency and has great influence and bearing on the behaviour of children.

Recently, Lakhotia and Dubey (2020) explained a family consists of people who look after us, play an essential role in upbringing us and teach us the lesson in life. Family environment is considered as a system where the behaviour and relationship among all family members is interdependent. A stimulating physical environment, encouragement of achievement and affection are repeatedly linked to better performance of children. Every individual bears an impact ofthe environment in which she is brought up. Family is almost the exclusive environmental factor, which influences the first few primitive years of life.

MATERIAL AND METHODS

Emergence of the study: Adolescents' relationships with friends and peers play a critical role in the development of social skills and feelings of personal competence that are extremely important for adult functioning. It has been seen that peer relationships appear to be instrumental in facilitating adolescents' sense of personal identity and increasing their independence from family influence (Dusek, 1991, Ingersoll, 1989). Consequently, this area has become a great concern to parents, teachers, examination bodies, counsellors and psychologists. In this connection, social anxiety may be an important factor to examine among adolescents. Although social anxiety has long been recognized as an essential factor for understanding adults' interpersonal behaviour and psychological functioning (Leary, 1983), the study of social anxiety among adolescents has been an emerging phenomenon. Thus, the present study is an attempt to investigate the relationship between social anxiety disorder and peer pressure. Among from peer pressure, family environment is another important factor in influencing child's development. A family is a source of stability, security and happiness. It can nurture love and togetherness but in the absence of these factors it can become a source of stress and turmoil.

An ideal home environment is one where there is proper reward to strengthen the desired behaviour, a keen interest in and love for the child provision of opportunities to express its views freely, where parents put less restrictions to discipline the child, not preventing the child from acting independently, not continuing infantile care and optimum use of physical and affective punishment where children are not compelled to act according to parental desires and expectations (Bandhana & Sharma, 2012). The environment can be strong source of support for developing adolescents, providing close relationship, strong parenting skills and good communication and modelling positive behaviour. However, a review of related literature reveals that the social anxiety disorder has not received attention in the study of peer pressure in the realm of family environment. Definitely, the scarcity of research in this area especially in India provides a convincing rationale to undertake further investigation into examining the relationship between social anxiety disorder, peer pressure and family environment. Therefore, investigator made an attempt to study social anxiety disorder in relation to peer pressure and family environment among adolescents.

Operational definitions of the terms used: Social anxiety disorder:Social anxiety disorder is a persistent and significant fear of being scrutinized or humiliated by others in social situations wherein the person may have to perform or meet lot of unfamiliar persons. When these fears become excessive they impede the person's functioning and routine (Nagpal, 2018)

Peer Pressure: Peer pressure refers to as any attempt by one or more group peers to compel an individual to follow in the decisions or behaviours favored by the pressuring individual or group.

Family environment: It is the general environment of the family consisting of eight dimensions namely cohesion, expressiveness, conflict, and acceptance and caring, independence, active-recreational orientation, organization and control (Bhatia & Chadha, 2012).

Objectives of study: 1.To investigate the significance of relationship between social anxiety disorder and peer pressure among adolescents. 2. To study the significance of relationship between social anxiety disorder and family environment among adolescents. 3. To study the conjoint effect of peer pressure and family environment on social anxiety disorder among adolescents.

Hypotheses of the study: 1. There is a significant relationship between social anxiety disorder and peer pressure among adolescents.2. There is a significant relationship between social anxiety disorder and family environment among adolescents 3. The conjoint effect of peer pressure and family environment on social anxiety disorder among adolescents is higher than their individual effects. The Present study was a descriptive survey method conducted on 520 adolescents studying in government and self-financed schools in the state of Punjab. The sample was drawn from ten randomly selected districts of Punjab. Multistage randomization was followed at the district, school and adolescent level. Data collection instruments are Social anxiety disorder scale (2018) by Nagpal, Peer Pressure Scale (2010) by Singh and Saini, Family Environment Scale by Bhatia and Chadha (2012).

RESULTS AND DISCUSSION

In order to test the first hypothesis stating'there is a significant relationship between social anxiety disorder and peer pressure among adolescents',coefficient of correlation was calculated using product moment method between the scores of social anxiety disorder and peer pressure among adolescents. The value of correlation is given in Table 1

Table 1. Showing Coefficient of Correlation between Social Anxiety Disorder and Peer Pressure among adolescents

Category	Variables	N	r	Inference
Adolescents	Social anxiety disorder and peer pressure	520	0.45	Significant at 0.01 level
	Social anxiety disorder and family environment		-0.46	Significant at 0.01 level

The coefficient of correlation between social anxiety disorder and peer pressure among adolescents as depicted in Table 1 is 0.45 which is significant at 0.01 level of significance indicating that there is significant and positive relationship between social anxiety disorder and peer pressure. It implies that more the peer pressure on the adolescents more is the social anxiety disorder in them. Results of the present study are consistent with earlier researches (Pickering, Hadwin,Kovshoff, 2019) which also results low peer acceptance was significantly associated with increased social anxiety for boys and girls, negative friendship experiences, limited close relationships and relational victimization were highlighted as risk factors specific to girls and (La Greca and Lopez , 1998) resulted adolescents with higher levels of social anxiety reported poorer social functioning(less support from classmates, less social acceptance and less intimacy).

The findings of present study is supported by Tilltors et al. (2012) which resulted that social anxiety was negatively and significantly correlated with peer relationship support at baseline. Cavanaugh and Buehler (2016) found the association between cumulative social support from parents, teachers and peers was associated with decreased anxiety in early adolescence, with this association being stronger for boys than girls. A recent study Moldes et al. (2019) also explored the correlation between the perceived level of peer pressure in terms of curiosity, social belongingness, education and cultural-parenting orientation of parents. In general, adolescents are expected to face the effects of peer pressure optimistically to cope up the negative influence of peer pressure. Effects of student peer pressure are based on their approach towards their peers. Hence the above stated objective that there exists a significantrelationship between social anxiety disorder and peer pressure among adolescents' accepted.

Table 2. Showing the conjoint effect of peer pressure and family environment on social anxiety disorder among adolescents (N=520)

Variable	R	R2	% Variance	F	Inference	Step-up Regression Equation
YX1	0.448	0.201	20.1	130.15	Sig at 0.01 level	Y=69.05+0.90X1
YX2	0.458	0.210	21.0	137.84	Sig at 0.01 level	Y=213.88-0.33X2
YX1X2	0.584	0.341	34.1	133.72	Sig at 0.01 level	Y=141.40+0.75X1 -0.27X2

Here, Y stands for Social anxiety disorder, X1 - Peer pressure and X2 - Family environment

Further, the results presented in Table 1 show that value of coefficient of correlation between social anxiety disorder and family environment is -0.46 which is significant at 0.01 level of confidence indicating that there is significant and negative relationship between social anxiety disorder and family environment among adolescents, which show that adolescents who are more under parental control and overprotection higher the social anxiety disorder in adolescents.The result of this study also shows that parents of children with high anxiety are likely to be more protective and less accepting than those of low anxious children which is consistent with previous study (Bögel et al.; Fiesta and Ginsberg, 2011). This result of present study is in line with the studies which explore parents' overprotective attitudes, especially mother's attitude, can increase children's social anxiety (Verhoeven et. al. 2012).Results of the present study also support (Tahirvoic and Demir, 2018) adolescents from divorced families shows higher level of social anxiety than adolescents divorced families and intact furthermore adolescents divorced families and intact families do not vary in their anxiety level in relation to gender. Ortiz et al. (2019) also explored parental education practices seem to act as a family asset which either hinders or promotes the development of self-esteem, basic attitudes ad by doing this , either prevents or encourages the emergence of problems such as social anxiety disorder.

Therefore, on the basis of above result, aforesaid Hypothesis 'there exists a significant relationship between Social anxiety disorder and family environment among adolescents' stands accepted. The findings of the present study extend the association of social anxiety in adolescents by testing the relative importance of peer factors and parental. Previous researches has shown that both parental and peer factors are strong predictors of social anxiety in adolescents, however there is a lack of research exploring the association of these factors to adolescents in India. Further research on risk factors for social anxiety disorder with a larger sample of adolescents of India be conducted to broaden and strengthen the findings of this study.

Regression results: Table 2 showing the conjoint effect of peer pressure and family environment on social anxiety disorder among adolescents (N=520)

To verify hypothesis 3 i.e. 'The conjoint effect of peer pressure and family environment on Social anxiety disorder among adolescents is higher than their individual effects'

To test this hypothesis, the step-up regression technique was employed. The effect of peer pressure on Social anxiety disorder among adolescents was found significant at .01 level (F (1, 518) =130.15). The computed value of R2 of peer pressure and Social anxiety disorder among adolescents (YX1) is 0.201 which indicates that the contribution of peer pressure on Social anxiety disorder among adolescents is 20.1%. The Social anxiety disorder among adolescents can be predicted with the equation Social anxiety disorder=69.05+0.90 x Peer Pressure

i.e. for every unit of increase in peer pressure, Social anxiety disorder among adolescents increase .90

The effect of family environment on Social anxiety disorder among adolescents was found significant at .01 level (F (1, 518) =133.84). The computed value of R2 of family environment and Social anxiety disorder among adolescents (YX1) is 0.210 which indicates that the contribution of peer pressure on Social anxiety disorder among adolescents is 21%. The Social anxiety disorder among adolescents can be predicted with the equation Social anxiety disorder= 213.88-0.33 x Family Environment.

i.e. for every unit of increase in family environment, Social anxiety disorder among adolescents decrease .33

The conjoint effect of both peer pressure and family environment on Social anxiety disorder among adolescents was found significant at 0.01 level of significance (F(2,517)=133.72). The computed value of R2 of Social anxiety disorder with peer pressure and family environment (Y1X1X2) is 0.341 which indicates the contribution of peer pressure and family environment on Social anxiety disorderamong adolescents is 34.1%. As %age variance(=34.1) of variables of peer pressure and family environment conjointly on Social anxiety disorderamong adolescentsshows increase in its value from peer pressure (%age variance=20.1) and family environment (%age variance=21), it indicates that the conjoint effect of peer pressure and family environmenton Social anxiety disorderamong adolescents is higher than that of peer pressure and family environment separately.

Social Anxiety Disorder can be predicted with the equation

Social Anxiety Disorder =12.49+0.10 x Peer pressure +0.19 x family environment

Hence hypothesis stating that 'The conjoint effect of peer pressure and family environment on social anxiety disorder among adolescents is higher than their individual effects' stands accepted.The revelation clearly indicates that when family and friends together, the influence is more pertinent and hence instead of always doubting and blaming friends of own wards for all odds in their behaviour and personality, parents should develop a good affinity with them, of course with a watchful eye.

CONCLUSION

There exists a significant relationship between social anxiety disorder and peer pressure among adolescents. There exists a significant between social anxiety disorder and family environment among adolescents.The conjoint effect of peer pressure and family environment on Social anxiety disorder among adolescents is higher than their individual effects. Educational Implications :The study is beneficial for planners and educational authorities to consider the relationship between social anxiety disorder, peer pressure and family environment and should try to provide suitable environment in educationally institutions so that all round development of student takes place.

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Assessment of Butterfly Diversity of a Tropical Forest Division of Maharashtra, India

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ABSTRACT

A study was conducted to estimate the butterfly diversity in the Pandharkawada Forest Division of Maharashtra, India. The study revealed presence of 103 species of butterflies belong to 5 family dominated by family Nymphalidae (34.95 %), Lycaenidae (27.18 %) followed by Pieridae (18.45 %), Hesperiidae (12.62 %) and Papilionidae (6.80 %). On the basis of Occurrence of species in study area 28.155 % species was categorized as abundant species whereas 36.89 % species was common, 11.65 % species was frequent, 18.44 % was occasional, and 4.85 % species was rare. On the basis of level of protection provided by Indian Wildlife Protection Act, 1972, 16 species recorded from study area belong to different Schedules of this act of which 3 species are in schedule 1. It appears that the butterfly abundance increased from monsoon to winter while decreased in the summer and pre-monsoon possibly due to the unavailability of the nectar and changes in temperature and humidity of the habitats concerned. The results of the study prove that the Pandharkawada forest division, Maharashtra has a healthy environmental setup that accommodates rich butterfly diversity.

KEY WORDS: ABUNDANCE, BUTTERFLY, DIVERSITY, OCCURRENCE, PANDHARKAWADA.

INTRODUCTION

Pandharkawada forest division is situated in Yavatmal District of Indian state of Maharashtra. This area lies at south eastern part of the district, located between the geographical confines of east longitude 78°14' and 79°13' East and 19°45' and 20°20' North, spread over the area of 655.336 sq. km. The climatic condition of this area is characterized by a hot summer, well-distributed rainfall during the south-west monsoon season and generally dry weather during rest of the year. The cold season is from December to February (Yavatmal Gazetteer 2019). Area constitutes honey comb pattern with compact patches of vegetation, meadows, open-scrubs, waterbodies and seasonal wetlands intersperse with agriculture. These varied ecosystems show great utility for conservation of biological diversity. Varied habitats and seasonal variation in floral composition of this dry deciduous forest attract verities of insect species. The diversity of insects plays an important role in the terrestrial and aquatic ecosystems by providing ecosystem services such as pollination, pest control, nutrient decomposition, and maintenance of ecosystem (Koh and Sodhi 2004; Losey and Vaughan 2006). Among insects, butterflies are the most attractive elements of the universe. They perform prominent roles in pollination (Tiple et al., 2006; Tiple 2018).

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Adult butterflies are dependent on nectar and pollen as their food while the caterpillars are dependent on specific host plants for foliage (Nimbalkar et al., 2011), this facilitate pollination. Butterflies are considered as the best indicators of the health of any kind of ecosystem. They bear a history of long-term co-evolution with plants. (Thomas 2005; Bonebrake et al., 2010). Butterflies are therefore treated as an important model group to study ecology of any landscape and its conservation status (Watt and Boggs 2003; Ehrlich and Hanski 2004; Mukherjee et al., 2015). Temperature and relative humidity are the important factors in distribution and assemblage of Butterfly species (Gupta et al., 2019)

Many butterfly species are vulnerable due to the habitat loss caused by modern agricultural practices and urbanisation, other major negative impacts are due to the widely increasing global environmental change. In the view of this changing scenario to ensure essential ecosystem services rendered by butterflies, it is essential to document these ecologically important vividly hued winged beauties. This study is design to estimation butterfly diversity in the Pandharkawada forest division, Maharashtra, India. This will work as biological instrument in devising sustainable conservation strategies for these beautiful creatures and to understand their role in maintaining ecological dynamics of this landscape.

MATERIAL AND METHODS

Study Area: Study was conducted at Shibla Forest (canopy covered with associated grassy belts), Gopalpur Nursery (Forest Nursery), Shindola Forest (Scrub with Sandy Soil), Nilgiri Ban (Eco-Park), Saykheda (Water Reservoir and Seasonal Wetland). Ecological conditions are as every study sight is different than other.

Survey method: The butterflies were observed and photographed in the sampling sites for a period of 1 year between January 2019 and December 2019. During the survey, an efficient protocol was adopted. The survey was made using a "Pollard Walk" method (Pollard 1977; Pollard and Yates 1993) with necessary modifications. Study area was visited twice a month/Study site from morning 8 AM to afternoon 11 AM during good weather periods.

Species identification: After detection, a specimen was photographed (Nikon D7100; Nikon Inc., Tokyo, Japan) and identified with the help of visible structural features. For identification and comparative studies of observed specimens, keys and methods suggested by Evans (1932), Wynter-Blyth (1957), Haribal (1992), Kunte (2000) and Kehimkar (2008) were adopted.

Data analysis: Species occurrence analysis was carried out by Microsoft excel program with using the following formulas. Relative Dominance (RD) of species was calculated as [RD=Ni × 100/Nt] where, Ni is number of individuals of species and Nt is total number of individuals all species (Basavarajappa 2006; Joshi 2014). Relative Occurrence (RO) of family was calculated as [RO= Ns × 100/Nt] where, Ns is number of species of each family and Nt is total number of all species (Basavarajappa 2006; Joshi 2014). Mean percent occurrence (M%) for month was calculated as [M% = Nm × 100 /Nt] where, Nm is number of individuals in each month and Nt is total number of individuals during complete study tenure (Basavarajappa 2006; Joshi and Tantarpale 2016). The mean values of the pooled species occurrence data were used to calculate the monthly diversity and to categorize the local status of species.

The diversity assessment enabled highlighting the observed species richness pattern of the butterfly species. The diversity indices were quantified with the help of PAST Version 1.60 software (Palaeontological Asso., Norway; Hammer et al., 2001). The species diversity was calculated using Shannon diversity index that calculated as $[H' = -\sum_{i=1}^{R} Pi \log Pi]$, where Pi is proportion of the first species which is given by Pi= ni/N (Magurran 1988); species richness was obtained by using Margalef equation [R= (S-1)/ log N], Where, R is Index of species richness, S is Total number of species and N is Total No. of individuals (Magurran, 1988); while Species equitability was determined by equation of Pielou [J= N1/N0] where N1 is Number of abundant species in the sample and N0 is Number of species in the sample (Hammer et al., 2001). The similarity association matrix upon which the cluster based was computed using the nearest neighbour pair linkage algorithm of Euclidean distance index for presence and absence data (Hammer et al., 2001). The differences between the diversity and evenness indices of with species occurrence among different study months were statistically analysed by using Analysis of variance (ANOVA). The statistical analyses were performed following Zar (1999) using the SPSS version 10 (SPSS Inc., Chicago, Il, USA; Kinnear and Gray 2000).

RESULTS AND DISCUSSION

During this study, 103 butterfly species under five families were recorded in study area (Table 1). Based on value of butterfly relative dominance in study area, 28.155 % species was categorized as abundant species whereas 36.89 % species was common, 11.65 % species was frequent, 18.44 % was occasional, and 4.85 % species was rare (Figure 1). The maximum number of butterfly species were recorded under family Nymphalidae (34.95 %), Lycaenidae (27.18 %) followed by Pieridae (18.45 %), Hesperiidae (12.62 %) and Papilionidae (6.80 %) (Figure 2).

A dendrogram developed by Euclidean distance cluster analysis was observed to be multifaceted and showed variation in the level of similarity in the number of butterfly species in 12 months. The months with the minimum to moderate number of species belong to one cluster, whereas the rest of the months with moderate to maximum number of species formed another cluster (Figure 3). It appears that the butterfly abundance increased from monsoon to winter while decreased

in the summer and pre-monsoon possibly due to the unavailability of nectar and the changes in temperature and humidity of the habitats concerned

Mean percent abundance of butterflies was significantly different ($F = 145.5$, $df = 11$, $p < 0.05$); Shannon diversity values of butterflies were significantly different ($F = 189.2$, $df = 11$, $p < 0.05$); species evenness among different months was significantly different ($F = 196.3$, $df = 11$, $p < 0.05$) while species richness among the study months was significantly different ($F = 188.3$, $df = 11$, $p < 0.05$). A trend in mean % abundance, Shannon diversity, species richness and species equitability showed the contradictory patterns (Figure 4).

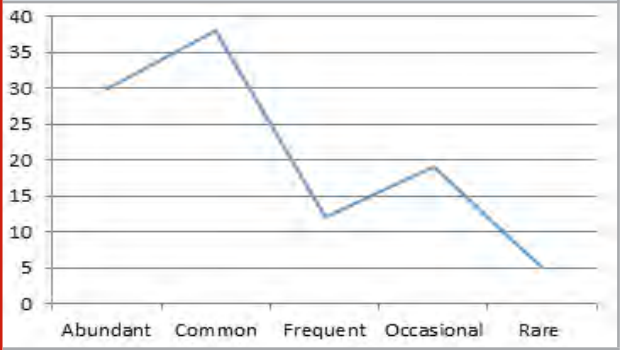
Table 1. Diversity of Butterflies during January 2019 to December 2019 in the Pandharkawada Forest Division, Maharashtra, India

Common Name	Scientific Name	Relative Dominance	Local Status	IUCN Status	WPA Status
Family: Papilionidae					
Tailed Jay	*Graphium agamemnon* (Linnaeus, 1758)	1.163	Common	NE	
Common Jay	*Graphium doson* (Felder and Felder, 1864)	1.098	Common	NE	
Common rose	*Pachliopta aristolochiae* (Fabricius, 1775)	0.994	Common	LC	
Crimson rose	*Pachliopta hector* (Linnaeus, 1758)	0.941	Common	NE	Sch.I
Lime Butterfly	*Papilio demoleus* (Linnaeus, 1758)	1.321	Abundant	NE	
Common Mormon	*Papilio polytes* (Linnaeus, 1758)	1.237	Abundant	NE	
Spot Swordtail	*Graphium nomius* (Esper, 1793)	0.599	Occasional	NE	
Family: Pieridae					
Common Albatross	*Appias albino* (Fabricius, 1775)	1.039	Common	NE	Sch.II
Indian Pioneer	*Belenois aurota* (Fabricius, 1793)	1.407	Abundant	NE	
Common Emigrant	*Catopsilia pomona* (Fabricius, 1775)	1.220	Abundant	NE	
Mottled Emigrant	*Catopsilia pyranthe* (Linnaeus, 1758)	0.920	Common	NE	
Common Gull	*Cepora nerissa* (Fabricius, 1775)	1.368	Abundant	NE	Sch.II
Small salmon Arab	*Colotis amata* (Butler, 1870)	0.604	Occasional	NE	
Large Salmon Arab	*Colotis fausta* (Olivier, 1804)	0.531	Occasional	NE	
Crimson Tip	*Colotis danae* (Fabricius, 1775)	0.578	Occasional	NE	
Small Orange Tip	*Colotis etrida* (Boisduval, 1836)	1.051	Common	NE	
White Orange Tip	*Ixias Marianne* (Cramer, 1775)	1.024	Common	NE	
Yellow Orange Tip	*Ixias pyrene* (Linnaeus, 1764)	0.712	Occasional	NE	
Common Jezebel	*Delias eucharis* (Drury, 1773)	1.114	Common	NE	
One Spot Grass Yellow	*Eurema andersoni* (Moore, 1865)	1.148	Common	LC	
Three Spot Grass Yellow	*Eurema blanda* (Boisduval, 1836)	1.003	Common	NE	
Small Grass Yellow	*Eurema brigitta* (Stoll, 1780)	1.131	Common	LC	
Common Grass Yellow	*Eurema hecabe* (Linnaeus, 1758)	1.294	Abundant	NE	
Spotless Grass Yellow	*Eurema laeta* (Boisduval, 1836)	1.359	Abundant	NE	
Psyche	*Leptosia nina* (Fabricius, 1793)	0.703	Occasional	NE	
Common Wanderer	*Pareronia valeria* (Cramer, 1776)	1.116	Common	NE	
Family: Nymphalidae					
Tawny Castor	*Acraea violae* (Fabricius, 1775)	0.976	Common	NE	
Angled Castor	*Ariadne ariadne* (Linnaeus, 1763)	1.157	Common	NE	
Common Castor	*Ariadne merione* (Cramer, 1779)	1.077	Common	NE	
Common Sergeant	*Athyma perius* (Linnaeus, 1763)	0.502	Occasional	NE	
Plain Tiger	*Danaus chrysippus* (Linnaeus,1758)	1.389	Abundant	NE	
Striped Tiger	*Danaus genutia* (Cramer, 1779)	1.270	Abundant	NE	
Common Crow	*Euploea core* (Cramer, 1780)	1.439	Abundant	LC	
Double Branded crow	*Euploea Sylvester* (Fabricius, 1793)	0.490	Occasional	NE	
Baronet	*Euthalia nais* (Cramer, 1779)	0.935	Common	NE	
Common Baron	*Euthalia aconthea* (Cramer, 1777)	0.304	Rare	NE	
Great Eggfly	*Hypolimnas bolina* (Linnaeus, 1758)	1.065	Common	NE	
Danaid Eggfly	*Hypolimnas misippus* (Linnaeus, 1764)	0.959	Common	NE	Sch.II
Peacock Pansy	*Junonia almana* (Linnaeus, 1758)	1.312	Abundant	LC	

Grey Pansy	*Junonia atlites* (Linnaeus, 1763)	1.056	Common	NE	
Yellow Pansy	*Junonia hierta* (Fabricius, 1775)	1.110	Common	LC	
Chocolate Pansy	*Junonia iphita* (Cramer, 1779)	0.970	Common	NE	
Lemon Pansy	*Junonia lemonias* (Linnaeus, 1758)	1.184	Abundant	NE	
Blue Pansy	*Junonia orithya* (Linnaeus, 1764)	1.418	Abundant	NE	
Common Evening Brown	*Melanitis leda* (Linnaeus, 1758)	1.249	Abundant	NE	
Dark Evening Brown	*Melanitis phedima* (Cramer, 1780)	0.724	Occasional	NE	
Common Bush Brown	*Mycalesis perseus* (Fabricius, 1775)	0.947	Common	NE	
Dark Brand Bush Brown	*Mycalesis mineus* (Linnaeus, 1758)	0.788	Frequent	NE	
Common Sailer	*Neptis hylas* (Linnaeus, 1764)	0.929	Common	NE	
Common Leopard	*Phalanta phalantha* (Drury, 1773)	1.032	Common	LC	
Blue Tiger	*Tirumala limniace* (Cramer, 1775)	1.213	Abundant	NE	
Commander	*Moduza procris* (Cramer, 1777)	1.140	Common	NE	
Painted Lady	*Synthia cardui* (Linnaeus, 1764)	0.911	Common	NE	
Joker	*Byblia ilithyia* (Drury, 1773)	0.902	Common	NE	
Common Three Ring	*Ypthima asterope* (Klug, 1832)	1.143	Common	NE	
Large Three Ring	*Ypthima nareda* (Kirby, 1871)	0.831	Frequent	LC	
Common Four Ring	*Ypthima huebneri* (Kirby, 1871)	0.782	Frequent	LC	
Common Five Ring	*Ypthima baldus* (Fabricius, 1793)	0.791	Frequent	NE	
Anomalous Nawab	*Polyura agrarian* (Linnaeus, 1764)	0.674	Occasional	NE	
Common Nawab	*Polyura athamas* (Drury, 1773)	0.481	Occasional	NE	Sch.II
Black Rajah	*Charaxes solon* (Fabricius, 1793)	0.546	Occasional	NE	Sch.II
Towny Rajah	*Charaxes bernardus* (Fabricius, 1793)	0.680	Occasional	NE	Sch.II
Family: Lycaenidae					
Pointed Ciliate Blue	*Anthene lycaenina* (C. Felder, 1868)	0.758	Frequent	NE	Sch.II
Large Oak Blue	*Arphopala amantes* (Hewitson, 1862)	0.368	Rare	NE	
Dull Babool Blue	*Azanus uranus* (Butler, 1886)	0.795	Frequent	NE	
Bright Babool Blue	*Azanus ubaldus* (Stoll, 1782)	1.023	Common	NE	
Lime Blue	*Chilades lajus* (Stoll, 1780)	1.430	Abundant	NE	
Gram Blue	*Euchrysops cnejus* (Fabricius, 1798)	1.199	Abundant	NE	Sch.II
Pea Blue	*Lampides boeticus* (Linnaeus, 1767)	1.229	Abundant	NE	Sch.II
Zebra Blue	*Leptotes plinius* (Fabricius, 1793)	1.377	Abundant	NE	
Dingy Line Blue	*Petrelaea dana* (de Niceville, 1884)	1.033	Common	NE	
Tailless Line Blue	*Prosotas dubiosa* (Semper, 1879)	1.018	Common	NE	Sch.II
Common Line Blue	*Prosotas nora* (Felder, 1860)	1.125	Common	NE	
Guava Blue	*Virachola isocrates* (fabricius, 1793)	0.659	Occasional	NE	Sch.I
Dark Grass Blue	*Zizeeria karsandra* (Moore, 1865)	1.258	Abundant	NE	
Lesser Grass Blue	*Zizina otis* (Fabricius, 1787)	1.181	Abundant	NE	
Tiny Grass Blue	*Zizula hylax* (Fabricius, 1775)	1.318	Abundant	NE	
Plum Judy	*Abisara echerius* (Moore, 1901)	0.688	Occasional	NE	
Common Pierrot	*Castalius rosimon* (Fabricius, 1775)	0.786	Frequent	NE	Sch.I
Forget-Me-Not	*Catochrysops strabo* (Fabricius, 1793)	1.282	Abundant	NE	
Plains Cupid	*Luthrodes pandava* (Horsfield, 1829)	0.864	Frequent	NE	
Indian cupid	*Cupido lacturnus* (Godart, 1824)	0.985	Common	NE	
Grass Jewel	*Freyeria trochylus* (Freyer, 1845)	1.344	Abundant	NE	
Common Cerulean	*Jamides celeno* (Cramer, 1775)	1.359	Abundant	NE	
Indian Red Flash	*Rapala airbus* (Fabricius, 1787)	0.305	Rare	NE	
Slate Flash	*Rapala manea* (Hewitson, 1863)	0.229	Rare	NE	
Common Silverline	*Spindasis vulcanus* (Fabricius, 1775)	0.755	Frequent	NE	
Common Shot Silverline	*Spindasis ictis* (Hewitson, 1865)	0.567	Occasional	NE	
Rounded Pierrot	*Tarucus extricates* (Kollar, 1848)	1.175	Abundant	NE	
Peacock Royal	*Tajuria cippus* (Fabricius, 1775)	0.163	Rare	NE	Sch.II
Family: Hespiridae					
Brown awl	*Badamia exclamationis* (Fabricius, 1775)	1.338	Abundant	LC	
Plain Banded Awl	*Hasora vita* (Cramer, 1780)	0.792	Frequent	NE	SchIV

Bevan's Swift	*Borbo bevani* (Moore, 1878)	0.534	Occasional	NE
Rice swift	*Borbo cinnara* (Wallace, 1866)	1.427	Abundant	NE
Blank Swift	*Caltoris kumara* (Moore, 1878)	0.543	Occasional	NE
Small branded swift	*Pelopidas mathias* (Fabricius,1798)	1.226	Abundant	NE
Conjoined Swift	*Pelopidas conjuncta* (Moore, 1878)	0.878	Common	NE
Paintbrush Swift	*Baoris farri* (Moore, 1878)	0.810	Frequent	NE SchIV
Common Straight Swift	*Parnara guttatus* (Bremer and Gray, 1853)	1.104	Common	LC
Indian Palm bob	*Suastus gremius* (Fabricius, 1798)	0.896	Common	NE
Dark Palm-Dart	*Telicota ancilla* (Moore, 1878)	1.045	Common	NE
Indian skipper	*Spialia galba* (Fabricius, 1793)	0.751	Frequent	LC
Grass Demon	*Udaspes folus* (Cramer, 1775)	0.635	Occasional	NE

Figure 1: Relative occurrence of butterfly Species in Pandharkawada forest division



Abundant- 29 (28.155%), Common-38 (36.89%), Frequent-12 (11.65%), Occasional-19 (18.44%), Rare- 05 (4.85%)

Figure 2: Relative dominance of butterfly families in the Pandharkawada forest division, Maharashtra, India

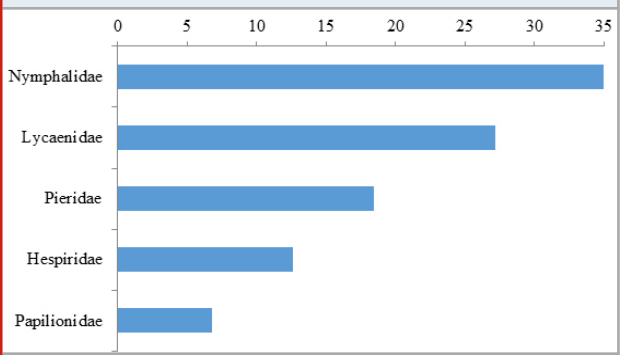


Figure 3: Dendrogram showing similarity in number of butterfly species composition among the studied month during January 2019 to December 2019

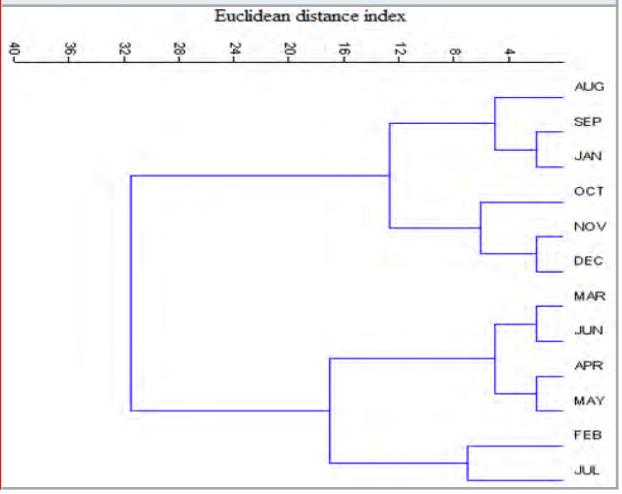
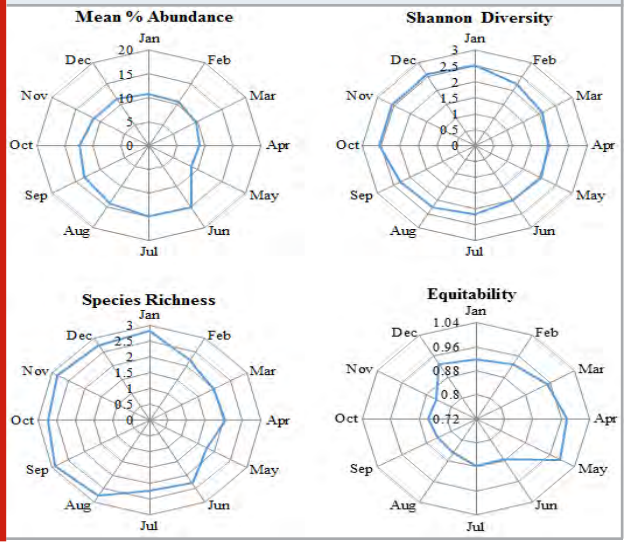


Figure 4: The values of the diversity indices in different months observed through the random sampling of butterflies in the Pandharkawada forest division, Maharashtra, India



The butterflies are the ecologically important creature that serves as indicators of environmental conditions (Stefanescu et al., 2004). Observations on the butterfly

diversity provide the information about variations in the species richness and the abundance in relation with the vegetation along the landscape and the species interactions (Öckinger and Smith 2006; Öckinger et al 2006; Mutmainnah and Santosa 2019). In this context, the diversity of Butterflies in the Pandharkawada forest division, Maharashtra, India was studied during January 2019 to December 2019. The study area was dominated by the dense vegetation with variety of plant species that host the butterfly populations. The earlier studies showed that heterogeneity of the habitats in terms of the available plant species supports the rich butterfly diversity (Kuussaari et al 2007; Mukherjee et al., 2015).

Studies on the butterfly diversity in the forest landscape contrast to the urban and suburban regions show that the richness increased with the availability of the green space and the heterogeneity of the habitats in terms of the available plant species (Öckinger et al., 2009; Mukherjee et al., 2015). Consistent with these studies the present observation records a total of 103 species belonging to five families from study area.The maximum number of butterfly species was recorded under family Nymphalidae, Lycaenidae followed by Pieridae, Hesperiidae and Papilionidae. Among these 103 species Based on value of butterfly relative dominance in study area, 28.155 % species was categorized as abundant species whereas 36.89 % species was common, 11.65 % species was frequent, 18.44 % was occasional, and 4.85 % species was rare. The rare species recorded are *Rapala airbus Rapala manea Tajuria cippus Euthalia aconthea Arphopala amantes*.

Out of these 103 butterfly species, 16 species specified under Indian Wildlife (Protection) Act, 1972 were encountered in good numbers. The butterflies *Pachliopta hector Castalius rosimon* and *Virachola isocrates* are placed in Schedule I Part IV, the species *Appias albino, Cepora nerissa, Hypolimnas misippus, Polyura athamas, Charaxes bernardus, Anthene lycaenina, Charaxes solon, Euchrysops cnejus, Lampides boeticus. Prosotas dubiosa* and *Tajuria cippus* are protected under Schedule II Part II, while *Hasora* vita and *Baoris farri* are categorized as Schedule IV. It is established that the butterfly abundance increased in monsoon as population is at its peak in June and July. It decreased in the summer and pre-monsoon possibly due to the unavailability of nectar and the changes in temperature and humidity of the habitats concerned, as temperature and relative humidity are the important factors in distribution and assemblage of butterfly species (Gupta et al., 2019)

Observations on the monthly variations of butterfly species encounter indicates peak from August to November and December while low from January to May. The present observations remain consistent with the records and views of the butterfly species in different parts of the world (Wilson et al 2004; Tiple et al., 2006; Sodhi et al., 2010; Tiple 2018). The number of species observed in the present study remained similar to the observations on the species in different parts of India bearing similar landscape patterns (Roy et al 2012; Harsh 2014; Saikia 2014; Mukherjee et al., 2015). As revealed through the present study, 103 butterfly species are available in different numbers across the study area. Dominance of the butterflies of the family Nymphalidae is similar to that observed in other parts of the world (Mutmainnah and Santosa 2019).

In parity with the species diversity observed in Pandharkawada Forest Division, Maharashtra, India, it may be assumed that the butterflies play diverse functional roles for the sustenance of the ecosystems. The richness in species composition in study area was also prominent in present investigation. The availability of the vegetation, seasonal wetlands and allied factors render stability to the butterfly population and assemblages in the landscapes, these are possibly important contributors to the observed variations in the butterfly species recorded in the present study. The observations on the diversity of the butterflies in the study area suggested that the intensive conservation management is required to ensure sustenance of ecosystem services derived from the butterflies. The present diversity study was confined to a limited area and selected habitats. There is, in the future, a chance of more species being reported because of few pockets and habitats in the studied area requiring more extensive exploration.

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Probiotic Composition Influences the Major Rumen Microbial Shifts and Fermentation Profile as Evaluated Using Quantitative PCR and In Vitro RUSITEC System

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ABSTRACT

Complex microbial interactions govern rumen functionality. We report use of quantitative PCR (qPCR) and rumen simulation technique to study the effect of probiotics, PF 1 and PF 2 on *Prevotella ruminicola, Fibrobacter succinogenes, Ruminococcus flavefaciens, Selenomonas ruminantium*, and *Streptococcus bovis* followed by volatile fatty acid and fiber reduction. Probiotic PF 2 up-regulated *R. flavefaciens* by 60.54%, down-regulated *F. succinogenes*, and doubled the concentration of propionic acid from 111 ± 16 mg/L to 251 ± 12.58 mg/L ($P<0.05$). PF 2 dosage improved the fiber digestibility; with fiber reduction from 37.42 ± 0.5 to 6 ± 0.05% w/w ($P<0.05$). Principal component analysis revealed a direct relationship between probiotic, days, and number of copies of targeted microbes ($P<0.05$). It demonstrated a positive correlation between *P. ruminicola* and *S. bovis* with butyric and propionic acids. In contrast, a strong negative correlation seen established between butyric and propionic acids with *F. succinogenes* and fiber content. We report that, rumen microbial shifts possess unique pattern upon probiotic interventions, revealing distinct fiber digestibility. To our knowledge; this is the first report of rumen bacterial quantification upon probiotic intervention using qPCR.

KEY WORDS: *IN VITRO* STUDIES, PROBIOTICS, PRINCIPAL COMPONENT ANALYSIS, QUANTITATIVE PCR, RUMEN, RUSITEC.

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INTRODUCTION

The ruminal microbial community is diverse. The ruminants possess a unique digestive system, attributed towards fibrous feed fermentation, prior to the classical enzymatic phase. The reticulorumen hosts a highly specific anaerobic microbiome, that performs fiber degradation and is critically influenced by microbial and biochemical characteristics of the rumen. Phyla *Bacteroidetes* and *Firmicutes* dominate among bacterial, archaeal, fungal, and protozoal species *Fibrobacter succinogenes, Ruminococcus flavefaciens, Ruminococcus albus, Butyrivibrio fibrisolvens, Prevotella ruminicola, Streptococcus bovis*, and *Eubacterium ruminantium* display major fibrolysis functions. Where, non-fibrolytic *Selenomonas ruminantium* has association with fibrolytic bacteria. The rumen microbiome complexity uplifts their sensitivity to environmental factors (Ghorbani et al., 2002, Weimer 2015).

In this context, an intensive farming practice demonstrates usage of high fermentable carbohydrates, aiming to enhance the animal performance. It causes disturbance of the rumen microbial balance that eventually leads to severe metabolic disorders, and impairs animal health and productivity (Chaucheyras-Durand, 2012). Therefore, there has been an increased interest towards animal probiotic application since few decades that has resulted into improved pH regulation, prohibition of pathogenic bacteria, with maintenance of animal health and productivity (Beauchemin, 2006). Besides having probiotic assisted benefits to the ruminants, decipheration of critical changes in major rumen bacteria is being considered as equally significant, although previous researches focused understanding the dietery intervention effect on the rumen microbiome (Huws et al., 2018; Abbasi et al., 2019).

It created an urge to understand the complex networking of the rumen microbiome. Despite several attempts to isolate the rumen bacterial strains over the past 50 years, the majority of rumen bacteria are yet to be identified and characterized. Recent advances in molecular techniques have reported a predominance of uncultured bacteria in the rumen (Neves et al., 2017). Advanced molecular techniques explored rumen bacteria, wherein; determination of bacterial abundance targeting the inter-species specificity is preferred (Aphale and Kulkarni, 2018). While, quantitative PCR (qPCR) is of choice for rumen microbial quantification (Fremah et al., 2018).

For these microbial ecology studies, rumen is an ideal yet complex environment. However, native complexity of rumen limits the establishment of external strains. Also, monitoring an intervening effect in the animal systems is cumbersome and time consuming. Thus, rumen simulation technique (RUSITEC) is most suitable for preliminary evaluation purpose. It allows studying multiple treatments in short time. Duarte et al., (2017) and Abbassi et al., (2018) used a RUSITEC system to understand the effect of forage and low protein diet on rumen microbiome. Wetzels et al., (2018) used a RUSITEC system for studying an effect of *Clostridium perfringens*.

The present research aims to quantify *P. ruminicola, F. succinogenes, R. flavefaciens, S. ruminantium*, and *S. bovis* upon probiotic interventions using qPCR and a RUSITEC system. The present study analyses volatile fatty acids (VFA), pH, and fiber profile among control and test samples; the principal component analysis (PCA) of which collectively inferred about probiotic assisted microbial changes and its relationship with fiber digestibility.

MATERIAL AND METHODS

PF 1 and PF 2 probiotic strains: The in-house probiotic formulations, PF 1, contained *Bacillus subtilis* (MTCC 2414), *Bacillus amyloliquifaciens* (MTCC 10456) and *Propionibacterium freudenreichii* (NCIM 2111), whereas, PF 2 contained *Bacillus licheniformis* (NCIM 2051).

Probiotic formulation: Glycerol stock of *B. subtilis, B. amyloliquifaciens*, and *B. licheniformis* was inoculated into 100 mL nutrient broth (HiMedia). Whereas, *P. freudenreichii* was inoculated into 100 mL deMan, Rogosa and Sharpe (MRS) broth (HiMedia) respectively. It was incubated at 37°C for 24–48 h. The cell broth was centrifuged at 4000 × g for 30 min and pellet was mixed with 1% pre sterilized maltodextrin and 0.5% carboxymethyl cellulose (1:1, v/v). The resulting lyo-slurry was lyophilized using Heto PowerDry LL3000 Freeze Drier, at –55°C for 36–48 h and total viable count was estimated, expressed as colony forming units (CFU)/g.

RUSITEC experiment: The RUSITEC has four cylindrical chambers treated as independent fermentation vats, designated A–D in the present study. The experimentation involved 'control' which had not been exposed to probiotic intervention. To begin with, sheep rumen digesta was obtained from slaughterhouse under controlled environmental conditions. It was diluted to four liter using artificial saliva ($NaHCO_3$, 9.80 g/L; Na_2HPO_4, 4.97 g/L; KCl, 0.57 g/L; NaCl, 0.47 g/L; MgCl2, 0.123 g/L; $CaCl_2$, 0.04 g/L) and was heated to 39 ± 2°C before use. The experiment was initiated with inoculation of 800 mL diluted rumen content in each of the chambers of one liter capacity. Crude rumen solids (80 g) were suspended in each chamber, packed in a nylon bag of 100 µm pore size. Chamber A was designated as the control whereas B–D chambers were challenged with probiotics. The assembly was fitted into the water bath maintained at 38 ± 2°C. The rumen content inside the chambers was stirred at 20 rpm. Artificial saliva was infused at a rate of 0.20 mL/min. Saliva feeding was commenced after 6 h of stabilization.

After 24 h, nylon bags were removed. Mixed cattle feed was provided for chambers A–D, every 24 h, at the dosage of 1 g/d. Chambers B–D were supplemented with 0.1 g PF 1 and PF 2 in separate experiments with

independent controls. Fermentation gas was collected in gas bags. Three experimental replications were performed to determine the results of probiotic interventions in vitro.

Community DNA extraction: The fermented RUSITEC fluid of d3 and d7, from A–D chambers was gauze filtered and centrifuged at 4200 × g for 30 min. The pellet (200 mg) was subjected to community DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen, 51504). The quality of DNA was determined using 0.8% (w/v) agarose gel electrophoresis and absorbance ratios at A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} using NanoDrop spectrophotometer.

Standard PCR: Primer specificity of the target genes was validated through standard PCR using Bio-Rad thermal cycler. The 20 µL reaction had 0.2 µL Q5 Hot Start High-Fidelity DNA Polymerase system (M0493, New England Biolabs), 0.2 mM of each dNTPs (N0446S, New England Biolabs), 100 ng DNA template, and 10 nM of forward/reverse primer each (Table 1). The PCR program used was: 98°C for 10 min; 35 cycles of 98°C for 10 s; optimized annealing temperature (Table 1) for 30 s; 72°C for 1 min; and a final extension step of 72°C for 10 min. Amplification was determined by 2% (w/v) agarose gel electrophoresis and SYBR Safe DNA gel stain (Invitrogen) where purity was visualized as a single band with absence of primer dimer and non-specific products.

Table 1. Species-Specific 16S rRNA Gene Primers used for the Quantification of Ruminal Bacteria using Real-Time PCR Assay

Targeted rumen bacteria	Primer	5'-3' sequence	T_a (°C)*	Amplicon size (bp)	Reference
Prevotella ruminicola	PR-F	GGTTATCTTGAGTGAGTT	50	485	(Singh et al., 2014)
Fibrobacter succinogenes	PR-R FS-F	CTGATGGCAACTAAAGAA GTTCGGAATTACTGGGCGTAAA	60	121	(Denman et al., 2006)
Ruminococcus flavefaciens	FS-R RF-F	CGCCTGCCCCTGAACTATC CGAACGGAGATAATTTGAGTTTACTTAGG	50	132	(Denman et al., 2006)
Selenomonas ruminantium	RF-R Sel-Mit-F	CGGTCTCTGTATGTTATGAGGTATTACC TGCTAATACCGAATGTTG	57	513	(Singh et al.,2014)
Streptococcus bovis	Sel-Mit-R SB-F SB-R	TCCTGCACTCAAGAAAGA ATGTTAGATGCTTGAAAGGAGCAA CGCCTTGGTGAGCCGTTA	60	90	(Klieve et al.,2003)

* T_a provided in above table is optimized annealing temperature used in quantitative PCR studies

Preparation of 16S rRNA gene standards: Absolute quantification involved standard curves prepared with gel extracted amplicons of the 16S rRNA gene amplified from *P. ruminicola, F. succinogenes, R. flavefaciens, S. ruminantium,* and *S. bovis* using specific primers (Table 1). Standard PCR was performed using Bio-Rad thermal cycler. The 20 µL reaction consisted of 0.2 µL Q5 Hot Start High-Fidelity DNA Polymerase system (M0493, New England Biolabs), 0.2 mM of each dNTPs (N0446S, New England Biolabs), 100 ng DNA templates, 10 nM of forward and reverse primer each. The PCR program used was: 98°C for 10 min; 35 cycles of 98°C for 10 s; optimized annealing temperature (Table 1) for 30 s; 72°C for 1 min; and a final extension step of 72°C for 10 min. The amplification was determined by 2% (w/v) agarose gel electrophoresis. The elution of DNA fragments was performed using Macherey Nagel NucleoSpin Gel and PCR Clean-up Kit (740609.50 MN). The concentration of gel extracted products was determined at A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} using NanoDrop spectrophotometer.

Real-time PCR assay conditions: Quantitative PCR was performed using CFX 96 Bio-Rad real-time PCR system. Indicator rumen bacteria were quantified by targeting 16S rRNA gene. The target gene was amplified from community DNA using primer sets (Table 1). The assay involved optimization of primer and template DNA concentration, reaction volume and reaction conditions. A 10 µL reaction in 96-well plate was run in triplicates (Bio-Rad). It consisted of 5 µL 2× QuantiTect SYBR Green RT-PCR Master Mix (QuantiTect SYBR Green RT-PCR Kit, Qiagen, 204243), forward and reverse primers (0.5 µM for *P. ruminicola*, and *S. ruminantium,* 0.3 µM for *S. bovis, R. flavefaciens*, and *F. succinogenes*) and DNA template (50 ng for *P. ruminicola, S. ruminantium, S. bovis*; 60 ng for *R. flavefaciens*; 100 ng for *F. succinogenes)* respectively. The PCR program used was: 95°C for 15 min; 40 cycles of 94°C for 15 s; optimized annealing

temperature (Table 1) for 30 s and elongation at 72°C for 1.5 min for *P. ruminicola, S. ruminantium* followed by 72°C for 30 s for *R. flavefaciens, F. succinogenes*, and *S. bovis* respectively. Amplicon specificity was determined using melt curve analysis of qPCR end products by increasing the temperature at a rate of 0.5°C every 0.05 s from 60°C–95°C.

Real-time PCR: specificity and sensitivity: Real time PCR amplicons were confirmed by 2% (w/v) agarose gel electrophoresis. To minimize variations, and screen for impurities or dimers; triplicates of each template DNA and a negative control were loaded on each plate. Sensitivity of the qPCR was determined using serially diluted gel eluted products of 16S rRNA gene corresponding to targeted rumen bacteria. The dynamic range consisted of 10 fold dilutions from 101 to 10^{10}.

The correlation between the 16S rRNA gene dilutions and threshold cycle (Ct) values in qPCR were analyzed by plotting a standard curve. Logarithms of the DNA concentrations were plotted against the Ct value with linear correlation coefficient (R^2), slope, and reaction efficiency. Only assays that fell in the range of 90–110% efficiency, clear melt curves with slope of –3.3 to –3.5 and R2 ≥ 0.99 were considered. The mean, standard deviation (SD), and coefficient of variation (CV) were calculated separately for each 16S rRNA gene dilution in order to determine the intra-assay variation. The results for counting of each species were expressed as the number of copies/g of rumen content using the formula available in (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr).

Volatile fatty acid, pH, and fiber digestion profiling: The effluent collected per day during fermentation (60 mL) was analyzed for VFA (acetic, propionic, butyric, iso-butyric, iso-valeric, and valeric acids, mmol/L) and pH. The fiber digestibility calculation was based on d0 and d7 residual fiber analysis (Neubert et al., 1940).

Statistical analysis: Multivariate one-way analysis of variance model plotting response of VFA against probiotic intervention was used to determine significant differences between acetic, propionic, and butyric acids profile among three independent RUSITEC replication experiments. Differences between means were considered significant at a P value of 0.05. Significant differences between fiber digestion of control, PF 1, and PF 2 were analyzed using one–way analysis of variance at CI of 95%. Statistical analysis was performed using Minitab 17.1.0 (Minitab Inc.).

Bioinformatics analysis: Bioinformatics analysis was performed using R packages (version 3.1.4). Principal component analysis was performed for targeted rumen bacteria using "devtools" and "factoextra" packages available in R plots package list with number of copies dataset generated upon probiotic interventions across different time period. In addition, PCA analysis was conducted to analyze correlation between number of copies, VFA, and fiber profiling dataset of day 7 after probiotic interventions.

RESULTS AND DISCUSSION

The microbial community inhabiting the rumen is extremely diverse. The microbes live in a symbiotic relationship and functionally interact with the host, playing an imperative role in maintaining a stable intra-ruminal environment and bacterial ecosystem. Rumen fibrinolysis though involves protozoa, archaea, and fungi; it's mainly driven by three predominant bacterial species including *F. succinogenes, R. albus,* and *R. flavefaciens* (Li et al., 2017). In conjunction, non-fibrolytic *P. ruminicola* has been reported to synergize with fibrolytic bacteria through interspecies hydrogen transfer and removal or exchange of metabolites. S. ruminantium one of the major non-fibrolytic bacteria also reported for synergism with *R. flavefaciens* and *F. succinogenes* towards propionic acid driven fermentation (Sawanon et al., 2017). This synergism triggers fiber fermentation in the rumen for efficient digestion in the ruminant animals.

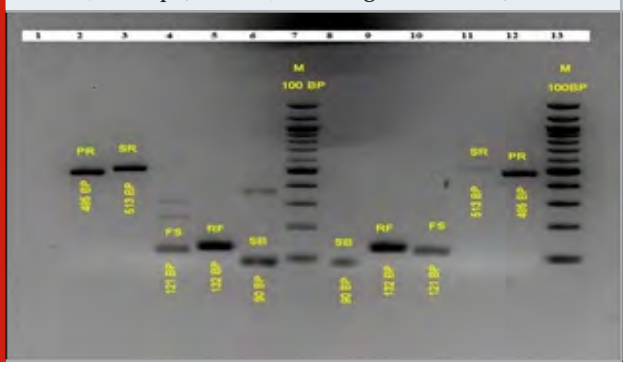
These changes, the functionalities, and mechanism of action of rumen microorganisms are recently being addressed using molecular biology tools and techniques. Qualitative investigation into the presence or absence rumen bacteria has been reported earlier (Aphale and Kulkarni, 2018). In this line, qPCR serves as one of the established platform for direct quantitation of microorganisms. Tajima et al., (2001) reported qualitative and quantitative shifts of ruminal *P. ruminicola, Prevotella bryantii, Prevotella albenis, F. succinogenes, Ruminococcus amylophilus, S.ruminantium-Mitsuokella multiacida, Treponema bryantii, S. bovis*, *Eubacterium ruminantium*, *Anaerovibrio lipolytica*, *Succinivibrio dextrinosolvens,* and *R. flavefaciens* due to diet dependent changes. Lettat et al., (2012) reported the effect of *Propionibacterium* and *Lactobacillus* based probiotics on sheep acidosis where, qPCR based quantitation of *F. succinogenes, Ruminococcus albus, R. flavefaciens, genus Prevotella,* and *Streptococcus* was performed. Schofield et al., (2018) reported the beneficial effects of *B. amyloliquifaciens* H57 probiotic mixture using qPCR.

Bacteria-based probiotics are comprised of a variable number of species and strains of beneficial bacteria known to have positive implications on animal health and performance. Bacterial probiotics have been reported to improve the rumen-predominant microorganisms (Chiquette et al., 2012). Probiotics also reduce the risk of rumen acidosis in the dairy cows receiving a combination of *Lactobacillus* and *Enterococcus* mixture. Qadis et al., (2014) reported that, lactic-acid bacteria based probiotics promote the rumen microbiome stability that in turn improves the dry matter intake, weight gain, and health of the animal (Qadis et al., 2014). In the current study, we have targeted the qPCR based quantification of *P. ruminicola, F. succinogenes, R. flavefaciens, S. ruminantium,* and *S. bovis* upon PF 1 and PF 2 probiotic dosage, using in vitro RUSITEC system. Duarte et al., (2017) and Guyader et al., (2017) have reported the

dietery intervention studies using a RUSITEC system. However, the probiotic intervention experiments have not been reported earlier using in vitro RUSITEC system. The probiotic formulations developed in-house had a bacterial composition of *B. subtilis, B. amyloliquifaciens, P. freudenreichii* (PF 1) and *B. licheniformis* (PF 2), with CFU/g of: 7×10^6 CFU/g, 5×10^6 CFU/g, 4.7×10^6 CFU/g, and 6.2×10^7 CFU/g, respectively.

For the purpose of current qPCR study, we chose PCR products of 16S rRNA gene as indicated in Table 1. While the design algorithm for qPCR utilizes many criteria, amplicon size between 50–210 bp is one of the pivotal prerequisite. Also, community DNA extraction targeted from control, PF 1, and PF 2 samples yielded 50 ng/µL, 60 ng/µL, and 75 ng/µL community DNA with absorbance ratios of 1.8 ± 0.01 (A_{260nm}/A_{280nm}) and 2.3 ± 0.05 (A_{260nm}/A_{230nm}) respectively. We report that, the primers used in this study lie between 90–132 bp thus fitting into the key criteria. This was reiterated through standard PCR results (Figure 1), which confirmed the presence of targeted rumen bacteria with amplicons in desired size range (Table 1). The successful detection of targeted microbes reveals their key functionalities within the rumen, associated with fibrolysis (Sawanon et al., 2017).

Figure 1: Standard PCR of Targeted Rumen Bacteria upon PF 1 and PF 2 Intervention. The PCR Amplicons Constitute Interventions by PF 1, Lane: 1 blank, Lane 2: *Prevotella ruminicola*, Lane 3: *Selenomonas ruminantium*, Lane 4: *Fibrobacter succinogenes*, Lane 5: *Ruminococcus flavefaciens*, Lane 6: *Streptococcus bovis*, Lane 7: Ladder, 100 bp (N3231S, New England Biolabs) and PF 2, Lane 8: *S. bovis*, Lane 9: *R. flavefaciens*, Lane 10: *F. succinogenes*, Lane 11: *S. ruminantium*, Lane 12: *P. ruminicola*, Lane 13: Ladder, 100 bp (N3231S, New England Biolabs)



Standard PCR of d7 fermented RUSITEC samples revealed the presence of P. ruminicola (485 bp), *S. ruminantium* (513 bp), *F. succinogenes* (121 bp), *R. flavefaciens* (132 bp), and *S. bovis* (90 bp) as represented in Figure 1. Real-time PCR performed to quantitatively determine population size of targeted rumen bacteria was analyzed using standard curve method (Brankatschk et al., 2012). The parameters included slope, intercept, and reaction efficiency (E %). Its details along with melting temperature (Tm, °C) and intra-assay variation (mean SD and mean CV) are presented in Table 2.

Specificity and sensitivity of entire qPCR analyses displayed a slope in the range of – 3.2 to – 3.5 having reaction efficiency between 93.07 to 103% respectively. Single sharp peak observed in the melt curve analysis indicated no primer-dimer formation or non-specific amplification, which was also confirmed by 2% (w/v) agarose gel electrophoresis. Linear correlation coefficient (R^2) was observed to be ≥ 0.99, stated the qPCR precision. The generated standard curve covered a linear range of three to eight orders of magnitude for each selected bacteria and showed linearity over the entire range of quantification, which is in alignment with minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009).

Probiotic PF 1 decreased the proteolytic, cellulolytic, and amylolytic bacterium, *P. ruminicola* population by 19.93%, whereas the same was increased by 27.69% by PF 2 dosage, after a drastic decrease on d3 unlike the case of PF 1 where the decrease persists. This signifies the ability of the population of this microbe to demonstrate dramatic shift. Since this microbe serves three key functions, a much higher (doubling in numbers) demonstrated by PF 2 from d7 onwards indicates the reinstatement of functionality associated with it. *B. licheniformis* as a compositional element of PF 2; has been reported for higher milk yield, protein, ruminal digestibility, and total VFA concentration in ruminants (Qiao et al., 2010).

One of the major fibrolytic bacteria; *P. ruminicola* may be a part of this induction. *B. licheniformis* supplementation leads to higher VFA and acetic acid concentrations; probably because it stimulates increase in population of cellulose digesting bacteria and thereby high fiber degradation (Ghorbani et al., 2002). Also, population of *P. ruminicola* was stable on d3 with either of PF 1 and PF 2 intervention with no log reduction as compared to control. This supports the fact about establishment of rumen consortia in distinguishing and complex manner where each bacterium behaves differently.

In addition, the similar response pattern was noted for *S. ruminantium* where; 5.70% reduction observed after PF 1 dosage, had 27.18% increase after PF 2 intervention. The probiotic PF 1 and PF 2 led to rise in S. bovis population by 22.68% and 3.20% respectively. *S. ruminantium* is a common Gram-negative rumen bacterium that can account for up to 51% of the total viable bacterial counts within the rumen (Abdelmegeid et al., 2018). It utilizes the succinate-propionic acid pathway to transform lactic acid into propionic acid, generating the milk (Wang et al., 2017). A previous research by Henderson et al., (2013) report that, lactate produced by *S. bovis* and *Lactobacillus spp*. serves as the major fermentable substrate for *S. ruminantium* and *Megasphaera elsdenii*. Lactate metabolism further generates acetic acid, propionic acid, and butyric acid (Gonzalez-Garcia et al., 2017). Induction of the propionic acid pathway in the present study indicates that, the population shifts towards a buffered stable rumen of milch cattle where lactic acidosis is avoided.

Result indicates that, this intervention would lead to improved milk yield in the field conditions (Mendelez et al., 2018).

Propionic acid is the most important substrate for gluconeogenesis and its concentration in the rumen is dependent on the number and type of propionic acid-generating bacteria present, particularly *M. elsdenii* and *S. ruminantium* (Wang et al., 2012). In this context, significantly higher d7 population of *S. ruminantium* after PF 1 and PF 2 dosage suggests possible population induction of *S. ruminantium* by *S. bovis*. In contrast, there was a reduction in *F. succinogenes* population by 26.82% and 27.31% after PF 1 and PF 2 administration. Moreover, PF 1 ad PF 2 intervention led to 45.23% and 60.54% rise in *R.flavefaciens* population. Phylum Bacteroidetes abundances were strongly and positively correlated with propionic acid levels. Similarly, a positive correlation was observed between *Firmicutes* and butyric acid levels (Moran and Shanahan 2014). Figure 3a, 3b, and 3c represents VFA, fiber digestion, and pH profiling evaluation.

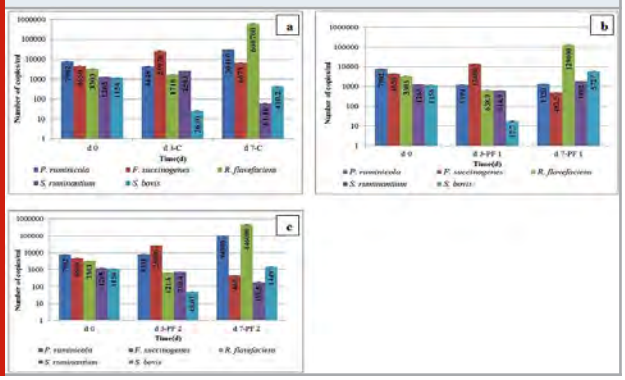
Table 2. Assay Performances of Day 3 and Day 7 with Five Targeted Rumen Bacteria under Control and Test Conditions

	Time (d)	*Prevotella ruminicola*			*Fibrobacter succinogenes*			*Ruminococcus flavefaciens*			*Selenomonas ruminantium*			*Streptococcus bovis*		
		*C	†PF 1	‡PF 2	C	PF 1	PF 2	C	PF 1	PF 2	C	PF 1	PF 2	C	PF 1	PF 2
Tm (°C)	§d0	84.0			82.5			78.5			84.5			78.5		
	§d3															
	§d7															
Inter-cept	d0	2.25			1.22			1.92			3.98			1.10		
	d3	2.25			1.22			1.92			3.98			1.10		
	d7	2.25			1.52			4.75			6.21			1.86		
Slope	d0	−3.26			−3.29			−3.36			−3.50			−3.25		
	d3	−3.26			−3.29			−3.36			−3.50			−3.25		
	d7	−3.50			−3.35			−3.46			−3.30			−3.36		
E (%)	d0	102.43			101.22			94.36			92.74			103.00		
	d3	102.43			101.22			94.36			92.74			103.00		
	d7	93.07			98.58			98.51			100.92			98.19		
¶Mean SD	d0	0.11			0.005			0.04			0.07			0.11		
	d3	0.13	0.02	0.04	0.005	0.04	0.10	0.11	0.14	0.08	0.18	0.08	0.005	0.02	0.05	0.15
	d7	0.09	0.05	0.03	0.18	0.09	0.10	0.13	0.17	0.12	0.13	0.06	0.08	0.07	0.03	0.06
**Mean CV	d0	0.58			0.02			0.17			0.27			0.46		
	d3	0.63	0.11	0.20	0.02	0.19	0.54	0.47	0.54	0.31	0.73	0.31	0.01	0.06	0.17	0.53
	d7	0.40	0.20	0.20	0.86	0.37	0.40	0.70	0.80	0.60	0.40	0.20	0.20	0.28	0.16	0.26

*C: Control, †PF 1: Probiotic 1 formulation, ‡PF 2: Probiotic 2 formulation, §d0: Day 0, §d3: Day 3 and §d7: Day 7, ¶Mean SD: Mean Standard deviation, **Mean CV: Mean coefficient of variation. Mean SD and Mean CV were calculated for intra-assay variations of quantitative PCR

Among all VFA analyzed, there was a reduction in the acetic acid concentration with PF 1 (1743 ± 52 mg/L) and PF 2 (990 ± 45.82 mg/L) interventions in comparison with control (1943 ± 140.32 mg/L). A linear rise in the propionic acid and butyric acid concentrations was observed with PF 1 (180 ± 21 mg/L, 158 ± 12.58 mg/L) and PF 2 (251 ± 12.58 mg/L, 203 ± 15.27 mg/L) dosage, in comparison with control (111 ± 27.53 mg/L, 106 ± 16 mg/L). We observed a significant difference ($P < 0.05$) between acetic acid, butyric acid, propionic acid concentration, and type of probiotic intervention applied. PF 1 dosage led to increase in the iso-butyric acid concentration from 30.93 ± 2.72 mg/L to 43.24 ± 1.77 mg/L, whereas PF 2 intervention increased the same to 54.50 ± 1.34 mg/L respectively. A similar trend was observed for iso-valeric acid and valeric acid respectively. In detail, 58.48 ± 3.15 mg/L of iso-valeric acid under control condition was increased to 76.82 ± 5.84 mg/L upon PF 1 dosage and 112.94 ± 9.06 mg/L upon PF 2 administration.

Figure 2 Copy Number Estimation of Targeted Rumen Bacteria using Quantitative PCR. Figure depicts Population Comparison of (a) day 0 vs. Day 3 and Day 7 under Control Conditions (b) Day 0 vs. Day 3 and Day 7 upon PF 1 Intervention (c) Day 0 vs. Day 3 and Day 7 upon PF 2 Intervention



Whereas, 37.19 ± 2.39 mg/L of valeric acid was shifted to 41.61 ± 1.43 mg/L upon PF 1 intervention, and 80.59 ± 5.42 mg/L with PF 2 dosage respectively. In support with this; highest production of propionic acid, butyric acid, iso-butyric acid, iso-valeric acid, and valeric acid was observed after PF 2 dosage in the current study. Therefore, the VFA profile indicates that; microbial shifts described in the present research has correlations with the proportions of the VFA produced. Here, *S. ruminantium* might induce fibrolytic bacteria towards establishment of synergistic relationship through an interaction termed 'cross-feeding 'or non-fibrolytic *S. ruminantium* may possibly involve in the rumen fiber digestion (Sawanon et al., 2017).

In addition, the administration of lactic acid bacteria (LAB) based probiotics is thought to help the rumen microflora for adaptation towards the presence of lactic acid, thereby prevents lactate accumulation in rumen (Qadis et al., 2014). In accordance with this hypothesis, the current research observed an up-regulation of *S. bovis* and *S. ruminantium* following PF 1 and PF 2 administrations and thereby indirectly decreased lactate accumulation.There was a fiber reduction observed upon PF 1 and PF 2 dosage, from 37.42 ± 0.5% w/w to 7.8 ± 0.26% w/w and 6 ± 0.05 % w/w respectively. We also observed a significant difference ($P < 0.05$) between type of probiotic and efficiency of feed digestion. There was no major change in the pH as observed during the course of fermentation from d1 to d7 with PF 1 and PF 2 interventions as compared to control. In addition; the PCA of targeted microbial copies across different time period and probiotic interventions is represented in Figure 4a. Figure 4b depicts an interrelationship between the targeted microbial copies, VFA, and fiber.

Figure 3: Fermentation Profile of in vitro RUSITEC Studies; (a) Volatile Fatty Acid Profile of Control, PF 1, and PF 2 Samples from Day 0 to Day 7 (b) Fiber Digestion Profile of Day 0 vs. Day 7 after PF 1 and PF 2 Intervention (c) pH Profile of Control vs. PF 1 and PF 2 Interventions from Day 1 to Day 7

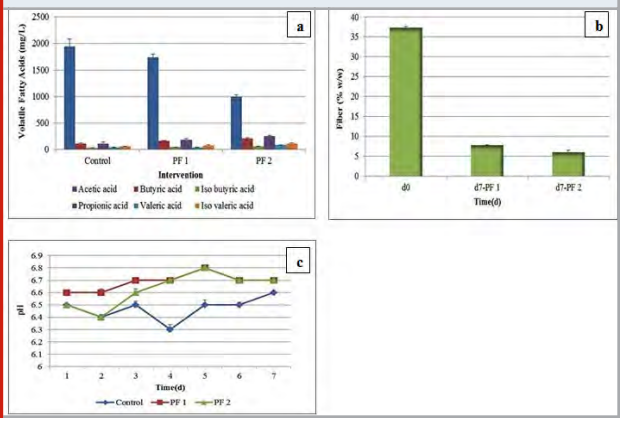


Figure 4 (a): Correlation Analysis of Targeted Rumen Bacteria upon Probiotic Interventions and Different Time Periods. Principal Component Analysis (PCA) of the Targeted Rumen Bacteria from in vitro RUSITEC Studies (b) PCA of the Targeted Rumen Bacteria, Volatile Fatty Acid Profile and Fiber Analysis of Day 7

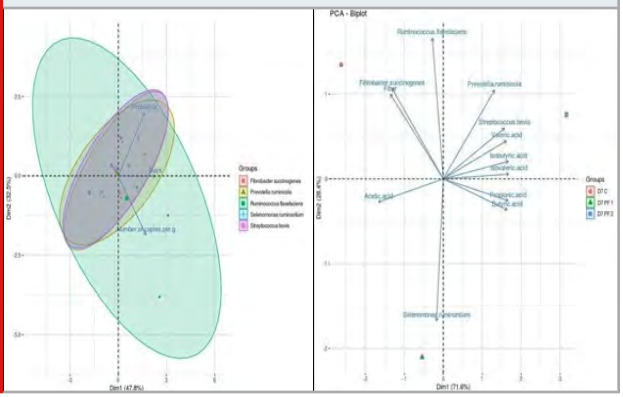


Figure 4a demonstrated total 80.5% variance, explained by PC1 and PC2 axis in the dataset. The analysis explained that, the experimental parameters; probiotic, days, and number of copies are directly related to each

other (P<0.05). The eclipse among targeted microbes revealed highest variation in *R. flavefaciens* across the experimental period and the treatments evaluated. Population of *R. flavefaciens* was seen up-regulated upon PF 1 and PF 2 dosage, by 45.23% and 60.54% respectively, a highest increase noted among all targeted bacteria. It remarks slow establishment of *R. flavefaciens* in the rumen. PCA analysis also illustrated highest variation of *R. flavefaciens* which was noteworthy. Sawanon and Kobayashi (2006) reported an improved fiber digestion with co-culture of *S. ruminantium* and *R. flavefaciens*, where succinic acid from *R. flavefaciens* is converted into propionic acid, resulting into improved rumen functions. A similar relationship was under supposition for the combination of *S. ruminantium* and *F. succinogenes*.

Evaluation of this synergy can be a key towards high fiber degradation, as *F. succinogenes* is considered to be the most important fibrolytic species. Principal component analysis (Figure 4b) revealed total 100% variance, explained by both the axis in the dataset. The significance level (P<0.05) was used in the present analysis. The results showed that; *R. flavefaciens* and *F. succinogenes* had strong positive correlation with fiber content of day 7 under control condition. PF 2 intervention revealed strong positive correlation of *P. ruminicola* and *S. bovis* with valeric, iso-valeric, and iso-butyric acids respectively. Here, *P. ruminicola* and *S. bovis* also had positive correlation with butyric and propionic acids.

The present study demonstrating population rise of P. ruminicola and *S. bovis* after PF 2 intervention have also revealed positive effect on butyric (106±16 mg/L to 203±15.27 mg/L) and propionic acid (111± 27.53 mg/L to 251±12.58 mg/L) levels. Whereas, butyric and propionic acids had strong negative correlation with *F. succinogenes* and fiber content. It states that, PF 1 and PF 2 mediated reduction of *F. succinogenes* is associated with rise in propionic and butyric acid levels; resulting into efficient fiber digestion. It's also predicted that, probiotic assisted seven days effect may have reduced the *F. succinogenes* population, because antagonistic effects of probiotic bacterial strains onto rumen inherent flora have been reported by Chiquette et al., (2012). PF 1 intervention demonstrated strong negative correlation of acetic acid with *P. ruminicola*, *S. bovis*, valeric, iso-valeric, and iso-butyric acids respectively. The current study depicting, PF 1 mediated acetic acid reduction, is attributed to rise of *S. bovis* population. Combined together, it has resulted into increase in other VFA, conferring high fiber digestion.

Probiotics have been reported to improve anaerobiosis, stabilize pH, and supply nutrients to ruminal microbes in their microenvironment (Khattab et al., 2017). The current research of correlation analysis of total VFA, nutrient digestibility, pH, and qPCR of targeted rumen bacteria suggests that; bacterial probiotics influence specific VFA production in the rumen with selective and unique pattern of up or down-regulation of rumen microorganisms; buffering the rumen environment and overall rumen functions (Figure 3).

Earlier research reports the quantitative PCR shifts of the rumen bacteria, upon dietery or probiotic intervention using *in vivo* animal experiments. Pinloche et al., (2017) and Qadis et al., (2014) studied the effect of probiotic yeast and LAB bacteria on the rumen microbiome, VFA, and pH of cattle rumen and Holstein calves. To the best of our knowledge, this is the first report wherein, qPCR based profiling has been performed in a RUSITEC system to determine the rumen microbial shifts upon probiotic intervention. The current findings have added new dimension to the community analysis with distinguishing mark over specific rumen bacteria; revealing the effect of their critical shifts on entire rumen environment and ultimately over fiber digestion, VFA profile, pH etc. It has established broad insights about rumen population dynamics due to probiotics dosage. It has also been demonstrated that, high levels of fluctuations occur within initial seven days of rumen intervention. At the end of d7, symbiotic relationship established between rumen microorganisms leading to changed functionality and an outcome.

CONCLUSION

To conclude, the current findings add new dimension to the rumen microbial shifts upon probiotic intervention and reveal the effect on fiber digestion, VFA, and pH. The study depicts that; symbiotic relationship after d7 leads to distinct functionality. The study emphasizes differential modulation of rumen bacteria by combination of probiotic microbes, where *B. licheniformis* was major determinant of microbial shifts. The direct relation between probiotic, days, and number of copies demonstrated through PCA, highlighted highest variation in *R. flavefaciens*. The strong correlation between *P. ruminicola* and *S. bovis* towards increase in butyric, propionic, and other minor VFA was noteworthy. Also, antagonism between probiotic strains and *F. succinogenes*, as well as its strong negative correlation with butyric and propionic acids was deciphered. The gradual establishment of *R. flavefaciens* upon probiotic influence was noted. Therefore, the study explains how rumen bacteria respond to probiotic intervention, setting up a guideline for the product development.

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Response of Differential Application Frequency of Poultry Litter on Mineralization Potential in Fish Culture Tanks with Special Reference to the Abundance of Ammonia Oxidizing Bacteria and Fish Growth

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ABSTRACT

The primary concern of the fish culture is nutrient budget of the particular ecosystem. Nitrogen is the chief macronutrient of aquatic system and biological nitrogen transformation in the aquaculture pond is dominated by ammonifying, nitrifying and denitrifying bacteria. The first step of nitrification is mediated by Ammonia Oxidizing Bacteria (AOB) and the functional response of AOB is influenced by substrate availability. Livestock manures are enriched with essential nutrient elements having pronounced effect in increasing organic contact of the soil sediment in aquaculture pond. So, input of organic manure such as poultry litter provides major source of macronutrients and also produces more substrate for AOB by enhanced degradation of organic matter. Although excess input of organic matter may produce deleterious effect due to accumulation of toxic nitrogen species such as ammonia and nitrite. Greater abundance of AOB is beneficial for subsequent transformation of ammonia into valuable by-products. So, the present study was conducted to establish a cost effective scientific protocol for administration of poultry litter in fish pond with special reference to the abundance of AOB. Weekly application of poultry litter at a dose of 50 Kg ha^{-1} day^{-1} with bimonthly lime application at a dose of 37.5 Kg ha^{-1} $month^{-1}$ was found to be beneficial for fish ponds. The study also explained the impact of the abundance of AOB on absolute growth rate of fish.

KEY WORDS: AMMONIA OXIDIZING BACTERIA, ABSOLUTE GROWTH RATE, POULTRY LITTER AND FISH GROWTH.

ARTICLE INFORMATION

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INTRODUCTION

Nitrogen is the prime regulating nutrient of aquaculture pond which greatly influences the productivity of aquatic ecosystem. Availability of nitrogen in aquatic system depends upon the nitrogen mineralization. Nitrification is the crucial step of nitrogen mineralization for nitrogen cycling in aquatic environment (Lu et al., 2015). Bacterial dynamics regulate the chemical transformation of organic nitrogen into simpler one through ammonification, nitrification and denitrification. In the first step of nitrification, ammonia-oxidizing bacteria (AOB) plays the pivotal role for oxidation of ammonia to nitrite which is subsequently, oxidized to nitrate by nitrite-oxidising bacteria (NOB) (Kim et al., 2005; Abeliovich, 2006; Kumari et al., 2011; Daims et al., 2016, Ghosh et al 2019).

Ammonia accumulation beyond the threshold level is detrimental to aquatic life depending on the pH of the water body (Hargreaves, 1998). In the aquatic environment, ammonia can be utilized by phytoplankton directly or it can be oxidized by ammonia-oxidizing microorganisms (Ebeling et al., 2006) to be transformed into valuable by-product. Very low proportion of nitrifiers (AOB and NOB) leads to nitrogen toxicity in the system (Jiao et al., 2009). Aquaculture ponds in which inorganic fertilisers are applied for rapid solubilisation have inadequacy in essential nutrients and may exerts untoward effects on soil structure, composition, micro-flora, macro-flora and many other characteristics of the pond ecosystems (Jana et al., 2001b).

However, organic manures are rich in both macro and micro nutrients that enrich the organic matter content of pond soil (Jana et al., 2001a) and incorporation of animal wastes as a source of organic matters in the aquatic system increase trophic status of ponds there by enhance fish production (Orhibhabor and Ansa, 2006). A minor portion of livestock manure applied to the aquaculture pond can be directly consumed by fish, and the remaining portion release nutrients to enhance the growth of photosynthetic organisms (Little and Edwards, 1999) as the excreta is enriched with nutrient of recovered 72-79% of Nitrogen (N), 82-92% of Potassium (K) and 61-87% of Phosphorus (P) in feed given to animals (Singh et al., 2014 Ghosh et al 2019).

The litter is very useful fertilizer for the fish farming ponds as it is loaded with crucial nutrients such as nitrogen, phosphorus, potassium (Gupta et al., 2012) and different trace elements, such as Cu, Zn and As (Bolan, 2010). Digestive tracts of poultry birds are short and therefore 80% of chicken manure may remain as undigested feed-stuff (Chen, 1981) which may contain about 20-30% total protein (Pudadera et al., 1986). Intensification of the macronutrients may lead to stressed condition to aquatic lives with deterioration of water and sediment qualities. Hence, organic manure should be applied in fish ponds at proper doses to promote fish production (Jhingran, 1995) because application of excess nitrogen in the form of fertilizer may have adverse effects on water quality by the accumulation of toxic nitrogenous compounds such as ammonia and nitrite. That can be controlled by maintaining the optimal environment for the proliferation of nitrifying bacteria which will cause removal of the ammonium nitrogen (Shan and Obbard, 2003). Although fish cultures under the practice of integrated poultry-fish farming system are recommended to be safe for human consumption (Oribhabor and Ansa, 2006). Poultry litter input at a dose of 50 kg ha^{-1} day^{-1} keeps the system conducive for fish farming (Tamuli, 2006). Magnitude of the abundance of bacteria highly depends on the time taken for mineralization of the applied organic matter. Taking this into consideration, the present study was carried out to find out a rational and scientific protocol for application of poultry litter in fish ponds.

The present investigation was carried out on a freshwater minor carp, bata (*Labeo bata*). It was selected for the present study because of its wide acceptability in India due to its good taste, omnivorous feeding habit, very well growth rate in shallow waters (Ghosh et al., 2019) and availability of artificially propagated seed. The experiment was conducted to ascertain the optimal mode of application of poultry litter (daily, weekly and monthly) with a dose of 50 kg ha^{-1} day^{-1} to obtain maximum abundance of AOB thereby enhancing the nitrogen cycling and growth rate of *Labeo bata* for sustained biological productivity of fish ponds.

MATERIAL AND METHODS

A120-day experiment was conducted with 3 different application frequencies (monthly, weekly and daily) in twelve tanks to find out the most effective mode of application of poultry litter with special reference to the AOB abundance during January 02 – April 30, 2017 in Gayeshpur, Nadia West Bengal (22˚58'7.788' N, 88˚29'44.556"E). Twelve circular tanks with water holding capacity of 300L were selected for the experiment (three replication for each) as control (C) with no organic input, treatment 1 (T_1) with monthly input of poultry litter, treatment 2 (T_2) with weekly input of poultry litter and treatment 3 (T_3) with daily input of poultry litter. Each of the tanks were provided with virgin alluvial soil base of 15 cm and filled with ground water, 40 days prior to initiation of the experiment. After initial liming to maintain the water pH, poultry litter was added to each tank for manuring and 15 well acclimatized fish fingerlings (*Labeo bata*) were stocked in each tank. Previously selected dose of poultry litter (50 Kg ha^{-1} day^{-1}) through a pilot study was preferred as the optimal dose of application for the present experiment with bimonthly lime application of 37.5 Kg ha^{-1} $month^{-1}$ (Table:1).

On day 0 of the experiment, treatment specific poultry litter was applied for the 1st time and samples were collected after manuring. Then the treatment specific poultry litter application frequency (monthly, weekly and daily) was followed all throughout. Samples were collected prior to poultry litter application on the day of sampling during remaining period of the experiment.

The samples were collected at fixed hour (8.00-9.00a.m) of the day during the study period from 3 places of the tank to create a homogeneous mixture of sample of water as well as of sediment.

For microbial study aliquots were prepared with serial dilution of 10^{-2}-10^{-4} for sediment samples. For sediment sample a homogeneous sediment suspension was prepared by mixing 1 g of the wet soil with 99 mL sterile distilled water. Then the aliquot dilutions were subjected to pour plate technique for microbial analysis. Selective medium was prepared for the isolation of AOB from sediment samples following standard methodology (Drews, 1974). Composition of the medium was as follows (g L^{-1}): ammonium sulphate, 1.0; potassium hydrogen phosphate (K_2HPO_4), 0.5; sodium chloride, 2.0; magnesium sulphate, 0.2; ferrous sulphate, 0.05; calcium carbonate, 6.0; cresol red (0.5%), 0.1; agar, 15 and a volume of 1L medium was prepared with adding distilled water. Mostly abundant bacterial species were subjected to SEM (Scanning Electron Microscopy) analysis to produce a magnified image of those particular organisms for further study (Photograph: 1 & 2). Physico-chemical parameters (viz. pH, alkalinity, free CO_2, NH_4-N, NO_2-N, NO_3-N and PO_4-P) of the surface water samples were monitored following standard methods (APHA, 1995).

Dissolved oxygen (DO) and chemical oxygen demand (COD) were determined by standard methodology of Wetgel & Likens (1991) and Golterman et al. (1978), respectively. Primary productivity was monitored following dark and light bottle method (Vollenweider, 1974). All the fish from each tank were harvested at the end of 120 days and length and weight of the 10 fish from each treatment were recorded. Absolute growth rate and specific growth rate of *Labeo bata* were measured using the formula of: $\Delta W = (W_t - W_i)/ t$ and $SGR = (\log W_t - \log W_i) \times 100/t$ respectively, where W_i is the initial weight, W_t is the final weight and t is days on trial (Lugert et al., 2014). Observed data were statistically evaluated (SPSS, 20). To determine the treatment difference minutely, one way analysis of variance (ANOVA) was performed among treatments and days of sampling. The level of significance was accepted at $p<0.05$, $p<0.01$ and $p<0.001$.

Table1. Experimental protocol

	a. Treatment variation				
Input	**Experimental input**	**Control (C)**	**Treatment 1 (T_1)**	**Treatment 2 (T_2)**	**Treatment 3 (T_3)**
Poultry litter	Frequency of application	0	Monthly	Weekly	Daily
	Dose of application	0	45g	11.25g	1.5g
	b. Common input				
Input	**Common input**	**Control (C)**	**Treatment 1 (T_1)**	**Treatment 2 (T_2)**	**Treatment3 (T_3)**
Lime	Frequency of application	Bimonthly after sampling in all the experimental tanks			
	Dose of application	37.5 Kg $ha^{-1}month^{-1}$ in all the experimental tanks			

RESULTS AND DISCUSSION

Interactions between water quality parameters and AOB and that in turn with the biological productivity of the aquatic system were established by the experiment. In the present investigation water temperature and dissolved oxygen (DO) were higher in control tanks than the treatment tanks. On the other hand free CO_2, carbonate alkalinity, bicarbonate alkalinity and chemical oxygen demand in all the treatments were greater than control (Table:2). Ammonia and nitrite accumulation beyond the assimilatory capacity of the particular system (Paul et al., 2020) is detrimental for the system where as nitrate is the mostly preferred form. Ammonia can be utilized by phytoplankton as the preferred N substrate, only when it is present within the desirable limit (Hargreaves, 1998). The unionized form of ammonia (NH_3) is much more toxic than ionized ammonia ($NH4^+$) because of its capability to diffuse across cell membranes (Fernandes et al., 2010).

Transition between the two forms of ammonia greatly depends on pH, at pH 7.3 about 1% get unionized; at pH 8.3 about 10% of ammonia get unionized and at pH 9.3 about 50% of ammonia get unionized (Hargreaves, 1998). In the present experiment pH of all the tanks ranged between7.59-8.54, thereby maintaining ammonia concentration supportive for fish growth. Both carbonate (CO_3^{2-}) and bicarbonate ($HCO3^-$) alkalinity ware observed in the treatment tanks whereas in case of control carbonate alkalinity was absent throughout the experiment. The net primary production in all treatments gradually increased with time in response to poultry litter load and was highest in T_3 followed by T_2, T_1 and control.

Table 2. Mean±SE values of water quality parameters excepting pH (range values for pH) of control and treatments tanks throughout the period of investigation

Parameters	Control	Treatment 1	Treatment 2	Treatment 3
Temperature (°C)	22.84±5.35	21.69±6.17	22.14±6.26	21.73±6.37
pH of water	7.63-8.14	7.73-8.42	7.59-8.51	7.65-8.54
Free CO_2 (mgL^{-1})	16.56±6.99	28.74±4.78	29.78±5.26	30.81±5.89
Carbonate Alkalinity (mgL^{-1})	0	12.33±2.13	18.33±4.81	15.5±4.84
Bicarbonate Alkalinity (mgL^{-1})	242.21±14.83	273.38±21.02	279.74±32.94	280.82±34.75
*DO(mgL^{-1})	10.67±0.68	10.08±0.71	10.11±0.91	10.13±1.09
*COD(mgL^{-1})	31.21±4.50	45.12±9.94	47.74±14.73	47.92±16.08
Ammonium- Nitrogen (mgL^{-1})	0.203±0.0390	0.2707±0.0726	0.2741±0.0889	0.2711±0.0976
Nitrite- Nitrogen (mgL^{-1})	0.011±0.0074	0.0194±0.0104	0.0193±0.0123	0.0194±0.0128
Nitrate- Nitrogen (mgL^{-1})	0.344±0.0187	0.4339±0.0596	0.4431±0.0729	0.4483±0.0741
Ortho- phosphate (mgL^{-1})	0.038±0.0109	0.0594±0.022	0.0603±0.0276	0.0597±0.0293
*NPP(mgC/m3/hr)	201.25±12.65	285.12±30.28	300.25±27.28	298.56 ±35.25

*DO=Dissolved Oxygen, COD=Chemical Oxygen Demand, NPP= Net primary productivity

Abundance of Ammonia Oxidizing Bacteria (AOB): Abundance of AOB in different treatments varied remarkably (Table: 3) with differential application frequencies of poultry litter @ 18,250 Kg ha^{-1} yr^{-1} (monthly, weekly, daily), throughout the 120 days period of experiment which were initially indifferent ($P>0.05$, ANOVA). From 4th to 120th day the AOB counts ranged between 9×10^2 cfu g^{-1} to 42×10^2 cfu g^{-1}, 24×10^2 cfu g^{-1} to 103×10^2 cfu g^{-1}, 18×10^2 cfu g^{-1} to 138×10^2 cfu g^{-1} and 17×10^2 cfu g^{-1} to144×10^2 cfu g^{-1} in control (C), treatment-1 (T_1), treatment-2 (T_2) and treatment-3 (T3) respectively. The average count was maximum ($68.79\times10^2 \pm 40.63\times10^2$ cfu g^{-1}) in T_3, followed by T_2 ($67.12\times10^2\pm 38.78\times10^2$ cfu g^{-1}), T_1 ($54.51\times10^2\pm 21.40\times10^2$ cfu g^{-1}) and lowest in control ($20.36\times10^2\pm 9.22\times10^2$ cfu g^{-1}). Abundance of AOB within the range 780-2325 cells g^{-1} in sediment was reported by Jana and Roy (1985).

Each AOB contains two or three *amoA* gene copies which code for the enzyme ammonia monooxygenase. Ammonia monooxygenase catalyses the oxygenation of ammonia to hydroxylamine. Quantitative analyses for AOB by RtPCR reported that the concentrations of the AOB *amoA* gene in the freshwater aquaculture pond sediments ranged from $4.05\pm3.83\times10^4$ to $3.11\pm1.65\times10^5$ copy g^{-1} in China (Lu et al., 2015). In a constructed wetland *amoA* gene copy numbers of AOB in water samples during summer observed within the range of $5.3\pm0.6\times10^4$ copy mL^{-1} to $8.1\pm0.5\times10^6$ copy mL^{-1} (Sims et al., 2012). Although the systems as well as methodology ware different but all the study concluded more or less similar range of AOB population.

Variability of AOB counts in all the treatments and control group were changed remarkably as time progressed. Temporal variation of AOB count was highest (8.01 folds) in T_3, followed by T_2 (7.69 folds), T_1 (5.55 folds) and lowest (2.53 folds) in C during the period of investigation. There was continuous increase in the population of AOB in T_1, T_2 and T_3 from the initial day while lowest count of AOB was observed on 21st day of experiment in C. On the other hand maximum count was observed on 75th day of experiment in T_1, T_2, T_3 and on 60th day of experiment in C.

The experiment clearly revealed that counts of AOB were increased with time due to gradual increment of organic inputs (poultry litter) in all the treatments whereas in control it was decreased until 21st day of the experiment and increased thereafter. In T_1, administration of the monthly dose in a single application contributed significantly higher concentration of AOB until 15th day and then gradually declined up to 30th day and again increased subsequently as time progressed with the increment of organic input. On the other hand as split doses were applied in T_2 and T_3, they received continuous supply of organic matter and the abundance of AOB was increased with time throughout the period of experiment. While after 90th day, as the temperature raised beyond 30°C that had a negative impact on the abundance of AOB and as a result the bacterial count declined up to 120th day in all the treatment tanks and also in control.

On 4th day there were significant differences between the C & T_1 ($P<0.01$, ANOVA), C & T_2 ($P<0.05$, ANOVA) and C & T_3 ($P<0.05$, ANOVA). During the first few days population of AOB in T_2 and T_3 were much lower than T_1 and the degree of differences were maximum on 11th and 15th days ($P<0.01$, ANOVA) while T_2 and T_3 were mostly alike ($P>0.05$, ANOVA). On 21st day for the 1st time after beginning, there were no significant ($P>0.05$, ANOVA) differences between T_1, T_2 and T_3. On 30th day, the abundance of AOB was changed as lime was applied on day 11 which caused decrease of AOB in T_1 until the 2nd monthly application of poultry litter. On the other hand T_2 and T_3 received continuous supply of organic manure at weekly and daily intervals, respectively.

That is why the abundance of AOB in T_2 and T_3 exceeded the count of T_1 on day 30. After 60th day even after manure application T1 failed to reach the AOB abundance of T_2 and T_3 and the magnitude of difference increased significantly (P<0.01, ANOVA) up to 120th day of experiment. AOB population of C was much lower than T_1, T_2 and T_3 and the degree of difference (P<0.001, ANOVA) was increased day by day. The fact implies the impact of the application of poultry litter and the frequency of application in T2 and T3 were found to be beneficial over T1 (P<0.05, ANOVA). This suggests that weekly dose of the poultry litter application (T2) is indifferent from AOB abundance of daily dose (T3) in fish tanks but weekly application is beneficial and viable as it requires less labour and time.

Figure 1: Scanning electron microscopy of isolated AOB

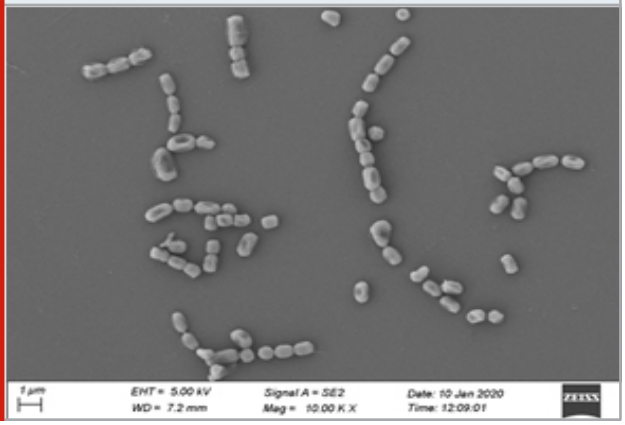
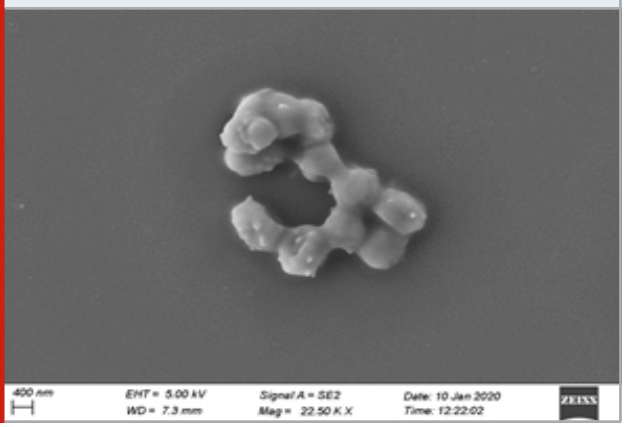


Figure 2: Scanning electron microscopy of isolated AOB



Interaction between water quality and AOB: As ammonia oxidizers obtain energy by oxidation of ammonia so ammonia concentration is likely to be correlated with AOB abundance (Jana and Roy, 1985). In the present study distribution pattern of AOB against treatment specific ammonia concentration was checked properly (Figures: 1, 2, 3, 4). In the present study, all the species of nitrogen (Ammonium-N, Nitrate-N, Nitrite-N) were positively correlated (p<0.05) with AOB abundance. Highest concentrations of ammonium-N and nitrate-N were observed in T_3 followed by T_2, T_1 and control whereas highest concentration of nitrite-N was seen in T_2 followed by T_3, T_1 and control. Concentrations of the various species of nitrogen were more or less similar in T_2 and T_3 which corroborated the similar findings regarding the abundance of AOB.

Probable reason of such distribution pattern of nitrogen is the application difference of poultry litter among the systems. Although doses were same for T_1, T_2 and T_3 but split dose of application resulted in continuous supply of the substrate in the system where as in case of monthly application substrate level started to fall after 15 to 21 day. The control system received no organic input and the only source of nitrogen was fish excreta and dead parts of plankton. So, ammonium-N, nitrate-N and nitrite-N were much less in control tanks than the treatments. In addition to that as the control systems did not have any source of phosphate so ortho-phosphate ranges of the control tanks were negligible throughout the experiment.

Table 3. Mean±SE values of AOB abundance in control and treatment tanks in different days of sampling during the period of investigation

Ammonia oxidizing bacteria(cfu×10^2 g^{-1})				
Days	Control	Treatment 1	Treatment 2	Treatment 3
0	15.67±2.05	16.67±1.25	17.33±1.25	17±0.82
4	13.00±1.63	26.67±2.05	19.67±1.25	18.67±1.25
7	13.33±2.49	34.33±2.49	24.33±2.49	23±1.63
11	12.67±1.25	43.00±3.27	28.67±1.70	26.67±2.05
15	10.67±1.25	63.33±4.9	36.67±2.05	38.33±2.62
21	10.00±0.82	52.00±3.74	45.00±1.63	48.00±2.16
30	22.67±3.86	48.33±2.49	86.67±7.76	90.33±5.73
45	35.00±2.45	78.00±5.10	97.67±5.44	110.33±2.87
60	39.67±1.70	73.67±4.99	117.33±5.31	120.33±4.99
75	28.00±2.45	92.67±8.58	133.33±4.64	136.33±5.44
90	22.67±1.25	70.67±3.68	101.33±7.36	102.00±5.35
105	21.00±2.94	64.00±6.16	84.00±4.08	83.00±3.27
120	20.67±2.49	45.33±5.44	80.67±2.87	80.33±6.02

Figure 1: Relationship between ammonium nitrogen concentration and abundance of AOB in control tanks

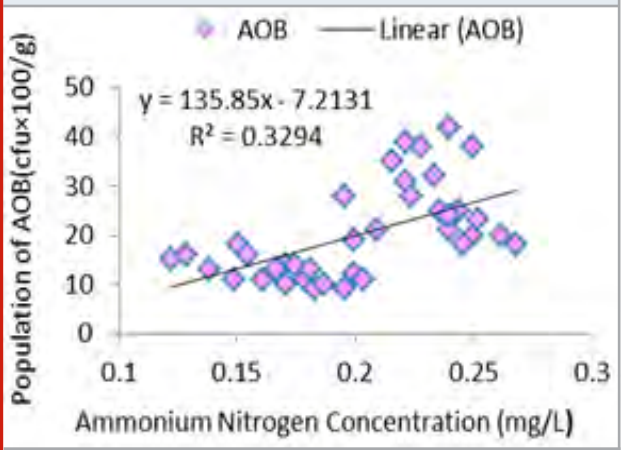


Figure 2: Relationship between ammonium nitrogen concentration and abundance of AOB in Treatment 1

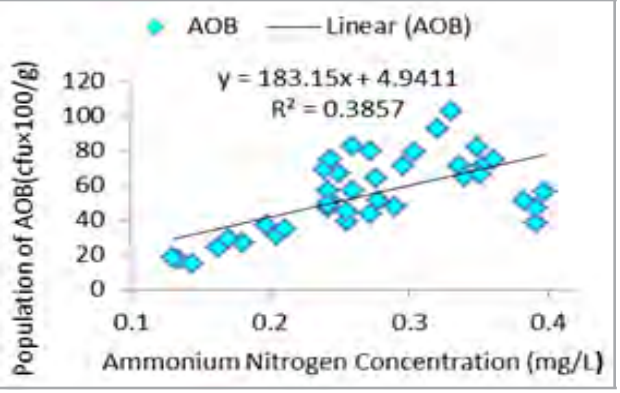
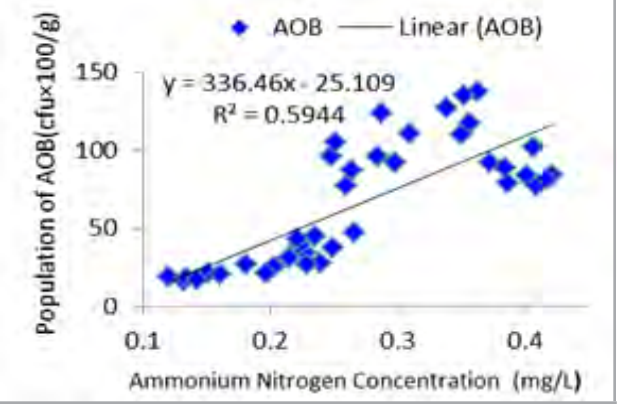
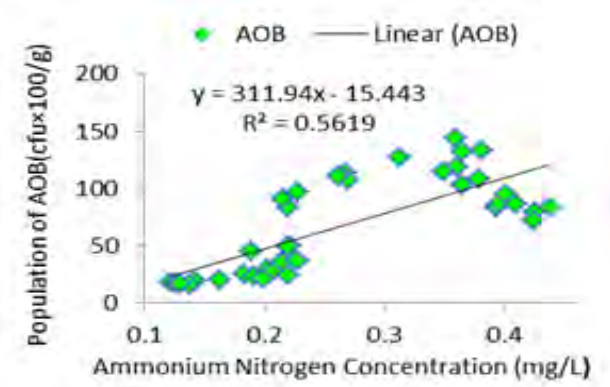


Figure 3: Relationship between ammonium nitrogen concentration and abundance of AOB in Treatment 2



Fish growth: Absolute growth rate was significantly different (P<0.001, ANOVA) among control and treatments with highest specific growth rate in T_2 followed by T_3, T_1 and lowest in control (Table: 4, Figure: 5). Specific growth rate of the fish also followed the similar pattern (Figure: 6). Specific growth rate of *Labeo bata* was observed within the range of 1.24±0.07 to 1.64±0.09 in an investigation with

Figure 4: Relationship between ammonium nitrogen concentration and abundance of AOB in Treatment 3



formulated fish feed (Mondal et al., 2015). The present study also postulates the similar specific growth rate except the control system. Average specific growth rate of the C, T_1, T_2 and T_3 were 0.75±0.08 %.d^{-1}, 1.30±0.09 %.d^{-1}, 1.43±0.10 %.d^{-1} and 1.42±0.10 %.d^{-1} respectively. The control system was devoid of nutrient supply as no organic input was there, which affected the fish growth. While T_2 and T_3 were mostly alike just like as the other parameters. Specific growth rate of *Labeo bata* was observed to be positively correlated (Figure: 7) with the abundance of AOB. That clarifies the fact of considerably higher growth rate in treatments according to their bacterial abundance and other nutrient parameters.

Figure 5: Absolute growth rate of fish in control and treatments tanks

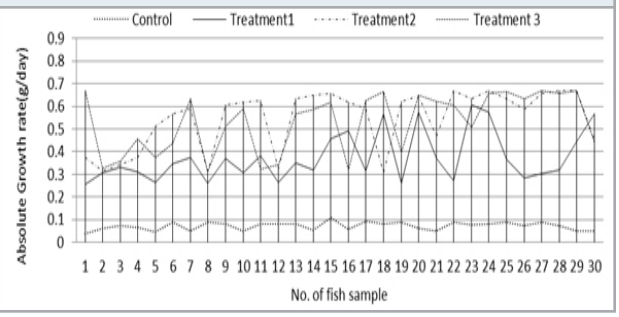


Table 4. Growth performance of *Labeo bata* in treatment and control tanks

Parameters	Control	Treatment 1	Treatment 2	Treatment 3
Mean initial weight (g)	5.23±0.18	5.23±0.18	5.23±0.18	5.23±0.18
Final weight (g)	9.87±2.09	46.11±12.92	66.79±15.35	64.84±15.59
Mean initial length (cm)	1.23±0.15	1.23±0.15	1.23±0.15	1.23±0.15
Final length (cm)	10.52±0.82	16.71±1.37	18.68±1.49	18.49±1.51
*AGR (d^{-1})	0.07±0.02	0.37±0.11	0.55±0.13	0.53±0.13
*SGR (%.d^{-1})	0.75±0.08	1.30±0.09	1.43±0.10	1.42±0.10
Survival rate (%)	82.22%	88.89%	93.33%	91.11%

*SGR=Specific growth rate, *AGR=Average growth rate.

Figure 6: Specific growth rate of fish in control and treatments tanks

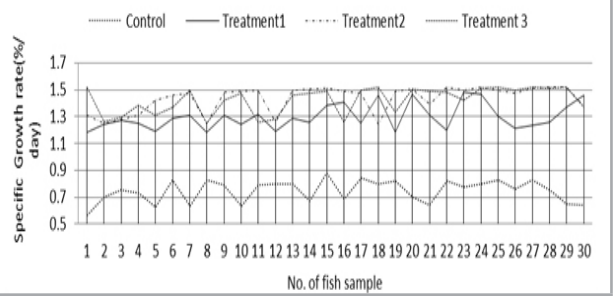
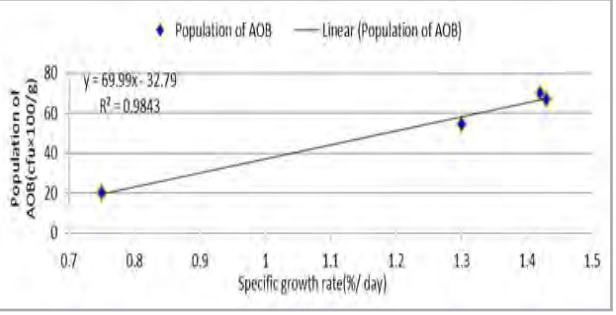


Figure 7: Relationship between treatment specific AOB abundance and Specific growth rate of *Labeo bata*



CONCLUSION

It is evident from the investigation that AOB population developed maximally when all the Species of nitrogen (Ammonium-N, Nitrate-N and Nitrite-N) is relatively high. That is because of substrate dependency of AOB on ammonia and subsequent productions of nitrite which can be further get utilized by the nitrite oxidizing bacteria of the system. As a result increment of nitrite in the system can cause a better growth of planktons thereby enhancing fish growth. Previously it was seen that split dose of fertilizer is beneficial over a single annual dose (Jana et al., 2001a). The present study also revealed that split application of poultry litter at weekly intervals is beneficial over the application of poultry litter at monthly intervals. No remarkable difference regarding fish growth, water quality and AOB abundance were seen between daily and weekly application as both the systems had a continuous nutrient supply. In case of monthly application of poultry litter the nutrient gets exhausted after certain time, that results into a sudden drop after 21 day in each monthly cycle.

On the other hand, as AOB is highly temperature sensitive , the density of AOB drops in high temperature even after having plenty of substrate in the systems. Excess ammonia can be removed from the system by using nitrifying bacteria (Krishnani et al., 2009) thereby maintain the nitrogen budget of the system. In the present experiment positive relation between the abundance of AOB and specific growth rate of *Labeo bata* contributes to the similar finding of maintaining the nitrogen budget thereby enhancing fish growth. After all AOB needs our special attention for further use in aquaculture to increase biological productivity. In addition to that optimum dose and mode of poultry litter application in fish farming ponds by means of integrated farming can be taken as a useful tool for sustained development and income generating practice for village people. So beside scientific findings this study also have a good social impact for betterment of the economic status of rural people.

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Statistical Optimization and Partial Purification of Laccase from A Novel Fungal Strain *Peyronellaea pinodella* BL-3/4

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ABSTRACT

Peyronellaea pinodella BL-3/4, an ascomycete was isolated from the humus of municipal solid waste. The novelty regarding the present study is that, to date the isolated fungal strain has not been explored for laccase production and statistical optimization of medium parameters for enhanced laccase production. Efficient laccase production from this fungal strain was carried out by optimizing fermentation medium using the design of experiments through submerged fermentation. Initially, the medium components were screened using Plackett Burman design. A five-level-four factor central composite design was applied to statistically specify the effect of important process variables, namely glucose, orange peelings, peptone and copper sulphate. The significance of the factors and their interactions were verified by using the analysis of variance with 95% confidence level ($p<0.05$). Among the variable screened, orange peelings, glucose, peptone and copper sulfate were found significant in laccase production. The central composite design of response surface methodology revealed that the best combination of fermentation medium for maximum laccase production is 2% glucose, 1% orange peelings, 0.5% peptone and 0.001 mg% copper sulphate with maximum laccase production of151.5 U/mL. Statistical optimization leads to 2 fold higher laccase production than the unoptimized media in the present study. Purification by ammonium sulphate precipitation followed by dialysis and gel filtration chromatography leads to 17.5 fold purification with 14.1% yield of pure laccase. Purified enzyme was identified as 60 kDa monomeric protein by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The production of laccase by *P.pinodella* BL-3/4 was also confirmed by the evaluating presence of copper in the purified fraction. Presence of copper in structure of purified laccase was confirmed by UV-visible spectroscopy, atomic absorption spectroscopy and scanning electron microscopy coupled with energy dispersive X-ray analysis. Use of orange peelings as valuable substrate by *P. pinodella*, make the fungi a better candidate for large scale production of laccase as well as for bioremediation, when compared to all other reported fungi.

KEY WORDS: CENTRAL COMPOSITE DESIGN, LACCASE, PEYRONELLAEA PINODELLA, PLACKETT BURMAN DESIGN, STATISTICAL OPTIMIZATION.

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INTRODUCTION

The major structural component of all plant is a renewable organic material, lignocelluloses (Dashtban et al., 2009). Many industries like forestry, pulp and paper, agriculture, and food generates lignocellulosic waste during processing. Such wastes are also present in municipal solid waste (MSW), and animal wastes (Kim and Dale, 2004, Kalogo et al., 2007, Batista Meneses et al., 2020). Among all three different components of lignocelluloses, lignin is a natural heterogeneous biopolymer and highly recalcitrant in nature (Wong 2009, Anwar et al., 2014, Brenelli et al., 2018, Polo et al., 2020).

Due to complexity in structure, enzymes of most microorganisms are not able to degrade lignin. Ligninolytic enzymes are the group of enzymes that degrade lignin efficiently. Laccase (EC 1.10.3.2, para-diphenol: oxygen oxidoreductase) is one of the most important enzyme among group of ligninolytic enzymes. Having diversity in substrate specificity as well as catalytic active site of copper atom (Pointek et al., 2002), laccases non specifically catalyze oxidation of wide range of phenolic compounds, aromatic amines as well as non phenolic compounds with the four-electron reduction of molecular oxygen to water (Vishwanath et al., 2014, Jaber et al., 2017, Agrawal et al., 2018, Janusz et al., 2020).

The non specific catalytic ability makes laccase highly suitable biocatalysts for various Biotechnological applications. Such application includes effluents treatment and waste detoxification, food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation, pesticide or insecticide degradation, organic synthesis, biosensor and analytical applications. Fungal laccases also play an important role in spore formation, pigment production, fruiting body formation, and plant pathogenesis (Sadhasivam et al., 2008). Laccase was first extracted and described by Yoshida (1883) from the sap of the Japanese lacquer tree, *Rhus vernicifera*. Laccases mostly been isolated and described from the white rot fungi, including *Trametes versicolor, Agaricus bisporus, Coriolus spp., Pleurotus ostreatus, Phlebia radiata, Pycnoporus cinnabarinus and Coprinus cinereus*. and few from the ascomycete group. Basidiomycetes are known laccase producers under both sub merged fermentation (SmF) (El-Batal et al., 2015) as well as solid state fermentation (SSF) (Patel and Gupte, 2016). Though SSF is preferred over SmF in terms of higher production yield, robust control of physical process parameters is difficult thus imposing problem in product recovery and scale up of laccase production.

Scale up needs right choice of the nutritive substrate in the culture medium that significantly decreases the total production costs and reduces the time period for expression of enzyme. Carbon, nitrogen and copper sources are the main nutritional parameters that regulate the level of gene transcription for laccase expression. Strain improvement to obtain higher laccase yield by single parameter approach is simple but laborious and time consuming and often do not tell about interaction effects between the medium parameters. Statistical optimization by design of experiments (DOE) concepts is the only solution to search such key factors and study interaction between medium parameters in a very few experiments. Plackett-Burman design (PBD) [Plackett and Burman, 1946] is most widely used experimental design for initial screening of such significant factors from multiple nutritional parameters and optimizes only the positive and main effects on laccase production. The important and positive factors obtained from the screening experiments could be further optimized by employing response surface methodology (RSM) that enables the study of interaction effects between different variables.

Few reports are available on the statistical optimization of media components for the production of laccase in different fungal strain of division ascomycota i.e. *Trichoderma harzianum* strain (Gao et al, 2013, Bagewadi et al., 2017), *Aspergillus flavus* (Ghosh and Ghosh, 2017). Moreover, laccase production during dye degradation has only been reported from *Peyronellaea prosopidis* (Bankole et al., 2018). To the best of our knowledge there are no reports on laccase production and use of statistical approach for its optimization from novel fungal strain *Peyronellaea pinodella* BL-3/4. The main objective of the study is to statistically optimize laccase production by fungal strain *Peyronellaea pinodella* BL-3/4 using DOE concept.

MATERIAL AND METHODS

A newly isolated fungal strain *Peyronellaea pinodella* BL-3/4 (Gene bank Accession Number: KT833620) prescreened (using lignin model compounds) from the humus soil of composted MSW was used in this study. This genus of ascomycetes has not been explored for any enzyme production specifically laccase production. The fungal strain was grown and maintained on Mineral salt- glucose peptone (MS-GP) medium according to the method of Patel and Bhaskaran, (2020). Ground orange peelings were employed as support-substrates as it is the best source for improving laccase productivity during single parameter optimization studies performed with *Peyronellaea pinodella* BL-3/4. The enzyme production was performed in MS-GP medium supplemented with 0.5% orange peelings (Patel and Bhaskaran, 2020). The enzyme was extracted by filtering fermentation broth and the filtrate was used as the crude enzyme preparation. Extracellular laccase activity of crude enzyme preparation was determined spectrophotometrically with 2.5 mM 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (SIGMA) by method of Silva et al. (2007) and Patel and Bhaskaran, (2020).

Screening of factors by PBD is commonly employed to select significant factors in a production medium with lesser experimentations (Rajendran et al., 2007). For laccase optimization by Peyronellaea pinodella BL-3/4, factors considered for screening by PBD were

orange peelings (A), glucose (B), peptone (C), ammonium acetate (D), KH2PO4 (E), MgSO (F), CaCL2 (G) MnSO4(H) and CuSO4 (J). Selected factors were experimentally screened with 12 trials in triplicates at 2 stages, high (+1) and low (- 1) (Table 1). The laccase activity is mean of 3 independent experiments. PBD is based on the first-order polynomial model shown in equation 1.

$$Y = \beta 0 + \Sigma\, \beta i Xi \qquad (1)$$

Where Y is the response (laccase production U/mL), β0 is the model intercept, βi is the linear coefficient, and Xi is the level of the independent factor (i = A, B, C, D, E, F, G, H, J).

The significant factors were identified by the analysis of the PBD experiments and their levels were further optimized by Central Composite Design under RSM. Each selected factor was studied at five different levels coded as $-\alpha$, -1, 0, $+1$, and $+\alpha$ in a total of ($\alpha = (2^4)^{1/4} = 2.000$) 30 runs, with two blocks (Bhamare et al., 2018). The laccase yield U/mL as the measured response (Y) was fitted by second-order polynomial equation 2.

$$Y = \beta o + \beta 1A + \beta 2B + \beta 3C + \beta 4D$$

$$+\beta 11A2 + \beta 22B2 + \beta 33C2 + \beta 44D2$$

$$+\beta 12AB + \beta 13AC + \beta 14AD + \beta 23BC + \beta 24BD + \beta 34CD \qquad (2)$$

Where, Y is the measured response (laccase production U/mL), A, B, C and D are independent factors, β1, β2, β3, β4, are linear coefficients, β 11, β 22, β 33, β 44 are quadratic coefficients and β 12, β 13, β 14, β 23, β 24, β 34 are cross product coefficients of the model.

This design was used to evaluate the main effects, interaction effects and quadratic effects to optimize the levels of parameters for enhancing laccase production. The fitted polynomial equation was expressed as three-dimensional response surface plots and counter plots to find the concentration of each factor for maximum laccase production (Sondhi and Saini, 2019). The statistical significance of the model terms was studied using analysis of variance (ANOVA). The significance of the model was assessed using Fisher's 'F' test and its corresponding probability 'p'. Design-Expert Design-Expert version 10.0.6.0 software Version 10.0.6.0, Stat-Ease, Minneapolis, USA. was used as a tool to design experiments of statistical optimization and all statistical analysis.

Purification of laccase was carried out by growing the fungal culture in statistically optimized medium. The fermentation broth was centrifuged at 3000 x g for 10 min at 4^0C for crude laccase preparation. Obtained supernatant was precipitated by ammonium sulphate in the range of 0-70% (w/v) at low temperature. Precipitated protein was dialyzed overnight with 0.1 M sodium acetate buffer using dialysis membrane of 10 kDa (Hi-Media Laboratories, India). Total protein content (method of Lowry et al., 1951) and laccase activity of the precipitated samples and dialyzed samples were determined according to method mention in laccase enzyme assay.

Concentrated dialyzed protein sample (1.5 mL) was applied to sephacryl s-100 HR (Amersham biosciences, USA) column (1.8x30 cm) pre-equilibrated with sodium phosphate buffer (pH 6.0). Protein was eluted with the same buffer having 0.15 M NaCl at a flow rate of 0.4 ml/min. A total of 30 fractions were collected and assayed for protein content and laccase activity. The purity of the laccase enzyme was confirmed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) according to the method of Laemmli (1970). Prestained protein molecular weight marker (Genei, Bangalore, India) [Phosphorylase b (97.4 kD_a), bovine serum albumin (66 kDa), ovalbumin (43 kD_a), carbonic anhydrase (29 kDa), lacto globulin (18.4 kD_a)] was loaded along with crude, dialyzed and gel filtered protein samples to know the approximate molecular mass of laccase enzyme.

Protein bands on SDS-PAGE gels were stained with coomassie brilliant blue G-250 and compared with standard protein. The presence of Cu^{2+}, Zn^{2+}, Fe^{2+} and Mn^{2+} in purified laccase were determined and quantified by Atomic absorption spectroscopy (AAS) (SL 194; ELICO, India). Spectroscopic characterization (Schimadzu UV 1800) of purified laccase was performed to confirm type of Cu centers. The presence of Cu2+ in purified laccase was confirmed by scanning electron microscopy coupled with energy dispersive X-ray (SEM/EDAX) analysis (Model: ESEM EDAX XL-30; Philips, Netherlands).

RESULTS AND DISCUSSION

The design matrix generated by design expert statistical software for the screening of variables and corresponding responses in terms of laccase enzyme yield is shown in Table 1. Highest laccase production (132.77 U/mL) was observed in a 9th run with a high level of glucose. Variation in laccase production among the different combinations occurred due to the influence of the factors at high and low levels as shown in Table 1. Parameters with statistically significant effects were identified using Fisher's test for ANOVA. ANOVA for laccase production indicated 'F-value' of 95.33, which implied that the model was appropriate. Model terms having 'Prob>F' values less than 0.05 are considered to be significant and Prob>F' values greater than 0.1 indicates the insignificant model terms (Niladevi et al., 2006, Sondhi and Saini, 2019).

Factors having a confidence level greater than 95% were considered to have a significant effect on the response and were selected for further studies. In present study glucose was found to be the most influencing factor ($p<0.0008$), followed by CuSO4 ($p<0.0013$), orange peelings ($p<0.0018$) and peptone ($p<0.0065$) in to the medium (Table 2). Positive effect of glucose and copper on laccase production has been reported recently

(Karp et al., 2015, Ghosh and Ghosh, 2017, Bhamare et al., 2018). Although ammonium acetate and $MnSO_4$ were significant model terms, they exerted a negative effect on laccase response. According to model KH_2PO_4, $MgSO_4$ and CaCl2 had no significant effect on laccase production as shown in Figure 1.

The first-order model was fitted to the experimental results with the following final equation 3 in terms of coded factors:

Laccase activity = +65.91 +9.97* A +13.00* B +6.35* C -6.42* D - 3.37* H +11.05 * J ..... (3)

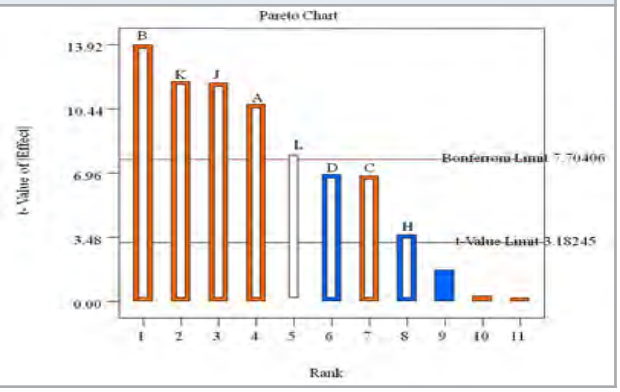
Where A, B, C, D, H and J are coded value of orange peelings, glucose, peptone, ammonium acetate, $MnSO_4$ and $CuSO_4$ respectively.

The positive effects of the four factors namely glucose, orange peelings, peptone and $CuSO_4$ on laccase production were studied using CCD of RSM to optimize their levels for maximum enzyme yield. The levels of other factors kept constant during experiments. Experimental study based on a CCD experimental design was performed according to Table 3 and 4. These factors and their levels were chosen based on the preliminary experiments. The 30 experimental trials reveal the different combinations of the factors. Maximum laccase response (125.0 U/mL) was obtained in run 30 having a maximum concentration of glucose (2.9%). Fisher's F test for the analysis of variance of data indicated that the model was highly significant with Prob>F' value of less than 0.0001 and F-value of 17.14 (Table 5). A not significant lack of fit showed that the quadratic model was valid for the present study. Among all factors and interactions considered in the experimental design, A, B, D^2 and AD were statistically significant at 95% confidence level. The value of R^2 (0.8903) indicated a good agreement between the experimental and predicted values of laccase yield.

Table 1. PBD matrix of nine variables (A-H and J) and two dummy variables (K and L) along with observed response.

	Factor A	Factor B	Factor C	Factor D	Factor E	Factor F	Factor G	Factor H	Factor J	Factor K	Factor L	Response
Run	Orange Peel (gm%)	Glucose (gm%)	Peptone (gm%)	Ammonium Acetate (gm%)	KH_2PO_4 (gm%)	$MgSO_4$ (mg%)	$CaCl_2$ (mg%)	$MnSO_4$ (mg%)	$CuSO_4$ (mg%)	Dummy	Dummy	Laccase activity
1	0.2	1	0.5	0.1	0.05	0.5	0.5	0.2	0.25	1	1	59.16
2	0.2	0.5	0.5	0.05	0.1	1	0.5	0.2	0.5	1	-1	84.16
3	0.2	0.5	0.2	0.05	0.05	0.5	0.5	0.1	0.25	-1	-1	32.91
4	0.5	1	0.2	0.05	0.05	1	0.5	0.2	0.5	-1	1	79.58
5	0.5	0.5	0.2	0.05	0.1	0.5	1	0.2	0.25	1	1	50.41
6	0.5	0.5	0.5	0.1	0.05	1	1	0.2	0.25	-1	-1	43.33
7	0.2	0.5	0.2	0.1	0.05	1	1	0.1	0.5	1	1	46.66
8	0.5	1	0.2	0.1	0.1	1	0.5	0.1	0.25	1	-1	89.16
9	0.5	1	0.5	0.05	0.05	0.5	1	0.1	0.5	1	-1	132.77
10	0.2	1	0.2	0.1	0.1	0.5	1	0.2	0.5	-1	-1	58.6
11	0.5	0.5	0.5	0.1	0.1	0.5	0.5	0.1	0.5	-1	1	59.99
12	0.2	1	0.5	0.05	0.1	1	1	0.1	0.25	-1	1	54.16

Figure 1: Positive and negative effect of different factors on laccase production by *Peyronellaea pinodella* BL-3/4 as screened with a PBD.



To evaluate the main effects, interaction effects and quadratic effects of the selected factors on the laccase yield, second-order polynomial equation was derived equation 4:

Y= 58.8112 + 15.7583 A + 19.825 B + 1.39917 C + -4.15833 D + 2.60875 AB + -11.4312 AD + -0.18 BD + -0.9975 CD + -10.1603 D^2

Where Y is the predicted response and A, B, C, D are coded factors.

The equation can be used to make predictions about the response for given levels of each factor. A positive linear coefficient value for A and B indicates laccase production was increased with increased concentrations of orange peelings (up to 1%) and glucose (up to 2.9%)

as shown in Table 3 and 4. The response obtained at different level of orange peelings clearly indicate that growth and enzyme yield was high at high concentration of orange peelings (1%) and very low when orange peelings concentration was low (0.1%) or absent in the medium. However results of present study disagree with report of the Ire and Ahuekwe (2016) on use of 0.1% orange peelings for maximum laccase production by *Pleurotus ostreatus*. Moreover, laccase activity was high at high level of glucose (2.9%) and low when glucose concentration was low (0.2%) Ghosh and Ghosh (2017) studies have revealed that although glucose supports the highest specific growth rate, specific rate of laccase production was significantly reduced. This is not supported by the findings of the present study where maximum laccase activity was obtained at 2.9% glucose with good mycelial growth. Bhamare et al. (2018) reported less laccase production even at at the 9th day of incubation. More yield in less incubation period (4 days) indicated the metabolic potential of fungus and its suitability for cost efective and economized production of enzyme at industrial scale.

The interactive effect of various factors on laccase production by *Peyronellaea pinodella* BL-3/4 was investigated by plotting the contour plots and three-dimensional response surface curves against any two independent variables while keeping the third independent variable at the '0' level. Studying the interaction among two variables provides knowledge of the optimum concentration of individual factor for highest laccase yield. The interactive effect of response surface quadratic model reveals that interaction among factors AB, BD and CD are insignificant whereas AD is significant (Table 5). The response surface curve for interactive effect of AD is shown in Figure 2. The yield was found to be increasing with the increase in orange peelings (A) concentration with limited level of peptone (D). But increasing the concentration of peptone inhibits laccase production even in the presence of higher concentration of orange peelings.

Table 2.ANOVA table for selected factorial model in PBD.

Source	F Value	p-value Prob > F	
Model	95.33	0.0016	Significant
A-Orange Peel	113.89	0.0018	
B-Glucose	193.72	0.0008	
C-Peptone	46.30	0.0065	
D-Ammonium Acetate	47.32	0.0063	
H-$MnSO_4$	13.00	0.0366	
J-$CuSO_4$	140.08	0.0013	
K-Dummy 1	142.46	0.0013	
L-Dummy 2	65.90	0.0039	

The model R^2 Value: 0.9961, The Predicted R^2 Value: 0.9373 and the adjusted R2 value: 0.9856; Coefficient of Variance (CV): 4.91

Table 3. Central composite experiments design matrix (Block 1) for laccase production from *Peyronellaea pinodella* BL-3/4.

Block	Factor A Run	Factor B Orange Peel gm%	Factor C Glucose gm%	Factor D $CuSO_4$ mg%	Peptone gm%	Actual Response Laccase activity U/ml
Block 1	1	0.55	1.1	0.5625	0.55	52
Block 1	2	1	2	1	1	56
Block 1	3	1	2	0.125	1	51.24
Block 1	4	0.1	0.2	1	0.1	1.33
Block 1	5	0.1	2	0.125	0.1	27.49
Block 1	6	0.55	1.1	0.5625	0.55	50.83
Block 1	7	1	0.2	0.125	1	14.16
Block 1	8	1	0.2	1	0.1	45
Block 1	9	0.1	0.2	1	1	11.8
Block 1	10	0.1	2	1	0.1	35
Block 1	11	0.55	1.1	0.5625	0.55	53.74
Block 1	12	0.1	2	1	1	45
Block 1	13	1	0.2	1	1	17.49
Block 1	14	0.1	0.2	0.125	1	9
Block 1	15	0.1	2	0.125	1	45.41
Block 1	16	1	2	1	0.1	99
Block 1	17	0.1	0.2	0.125	0.1	8
Block 1	18	1	2	0.125	0.1	86.66
Block 1	19	0.55	1.1	0.5625	0.55	43.33
Block 1	20	1	0.2	0.125	0.1	48.74

This is in accordance with Hammel (1997), who confirmed that the ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen. Optimum concentration of each factor was revealed by performing confirmation run in triplicates. Actual mean laccase activity of 151.5 U/mL was obtained with optimum concentration of orange peelings-1.0%, Glucose-2.0%, Peptone-0.5% and CuSO4-1.0 mg% against predicted laccase activity (139.0 U/mL) by design expert software. Extracellular laccase produced by Peyronellaea pinodella BL-3/4 was purified by ammonium sulphate precipitation followed by dialysis and gel filtration chromatography. Laccase purification at different steps is summarized in Table 5. Enzyme was purified to 6.01 fold with 85.1% yield after dialysis. Final purification with sephacryl s-100 HR gel filtration chromatography leads to 17.5 fold purification with 14.1% yield of pure laccase.

Table 4. Central composite experiments design matrix (Block 2) for laccase production from *Peyronellaea pinodella* BL-3/4.

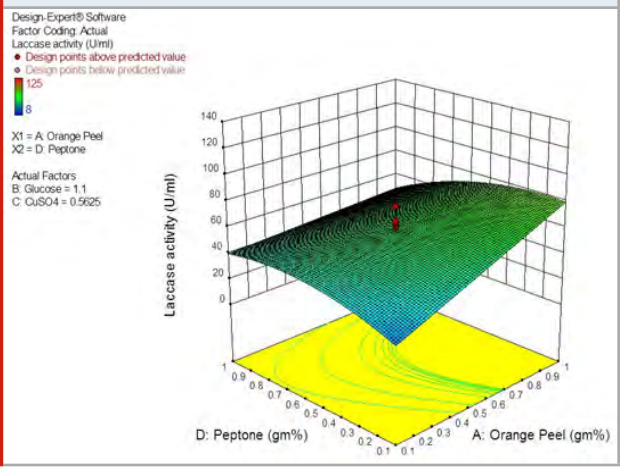
Block	Run	Factor A Orange Peel gm%	Factor B Glucose gm%	Factor C $CuSO_4$ mg%	Factor D Peptone gm%	Actual Response Laccase activity U/ml
Block 2	21	0.55	1.1	0.5625	0.55	76.66
Block 2	22	1.45	1.1	0.5625	0.55	99.16
Block 2	23	0.55	1.1	0.5625	-0.35	35.83
Block 2	24	-0.35	1.1	0.5625	0.55	39.19
Block 2	25	0.55	1.1	1.4375	0.55	64.16
Block 2	26	0.55	-0.7	0.5625	0.55	23.74
Block 2	27	0.55	1.1	-0.3125	0.55	75.83
Block 2	28	0.55	1.1	0.5625	0.55	64.99
Block 2	29	0.55	1.1	0.5625	1.45	24.99
Block 2	30	0.55	2.9	0.5625	0.55	125

Table 5: Analysis of variance for response surface quadratic model

Source	F Value	p-value Prob > F	
Model	17.14	< 0.0001	significant
A-Orange Peel	43.68	< 0.0001*	
B-Glucose	69.13	< 0.0001*	
C-CuSO4	0.34	0.5643a	
D-Peptone	3.04	0.0973a	
AB	0.80	0.3829a	
AD	15.32	0.0009*	
BD	3.799E-003	0.9515a	
CD	0.12	0.7364a	
D2	21.79	0.0002*	
Lack of Fit	0.83	0.6517	not significant

R^2 = 0.8903; adjusted R2 = 0. 8384; Predicted R^2=0.7630; probability P *(P<0.05) corresponds to Significance; P^a corresponds to insignificance

Figure 2: Response surface curve showing the interactive effect of orange peel (A) and peptone (D) on laccase production.



AAS studies showed presence of copper in laccase active fraction (6.8 mg/mL) where as iron, zinc and manganese were absent. The type of copper catalytic centre was investigated spectrophotometrically by UV-Visible spectrum. The UV-Visible spectrum (Figure 4) shows presence of a shoulder at 330 nm. Shoulder at 330 nm indicates type III binuclear copper (Solomon et al., 1994, Solomon et al., 1996) having two electron accepting site, which is characteristic to the yellow laccases. Absorption peak around 600 nm confirms presence of type 1 copper, which is characteristic of blue laccases (Bertrand et al., 2002, Morozova et al., 2007, Madhavi and Lele 2009). Type III Cu exhibits a weak absorption at 600 nm (Palmieri et al., 1997). In present study, absence of peak around 600 nm (Figure 4) conferred absence of type 1 copper in purified laccase and

presence of type III copper in purified laccase. Evidence was also provided for instability of type I copper in all fungal laccases (Rogalski and leonowicz, 2004). Most of the laccases are blue containing four copper atoms per enzyme molecule. Reports of Giardina et al. (2009) suggests that formation of yellow laccase is due to altered oxidation state of active copper centre during binding of the lignin degradation aromatic products which in turn results in the reduction of type 1 copper and loss of the characteristic blue copper of laccase. SEM-EDAX analysis was performed to confirm presence of copper. Figure 5 shows scanning electron microscopic image of purified laccase. Peak at 8 KeV in SEM-EDAX spectrum confirmed presence of copper in structure of laccase (Figure 6).

Table 6. Purification of laccase from *Peyronellaea pinodella* BL-3/4.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude filtrate	13500	633	21.3	1	100
Ammonium sulfate precipitation and Dialysis	11491	89.7	128.1	6.01	85.1
Gel filtration chromatography	1900	0.85	2235.3	17.5	14.1

Figure 3: SDS-PAGE of purified laccase from *Peyronellaea pinodella* BL-3/4. Lane 1: Standard Protein molecular weight marker, Lane 2: Crude Laccase, Lane 3: Dialyzed Laccase and Lane 4: Purified Laccase

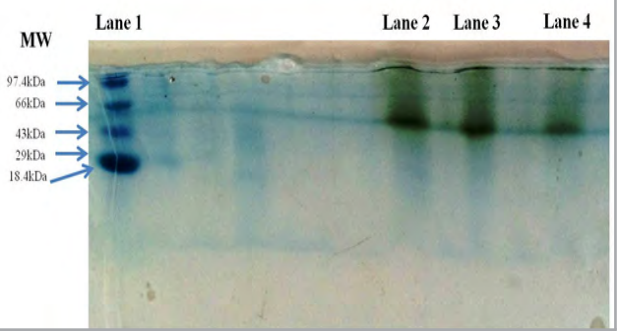


Figure 4: UV-Visible spectrum of purified laccase

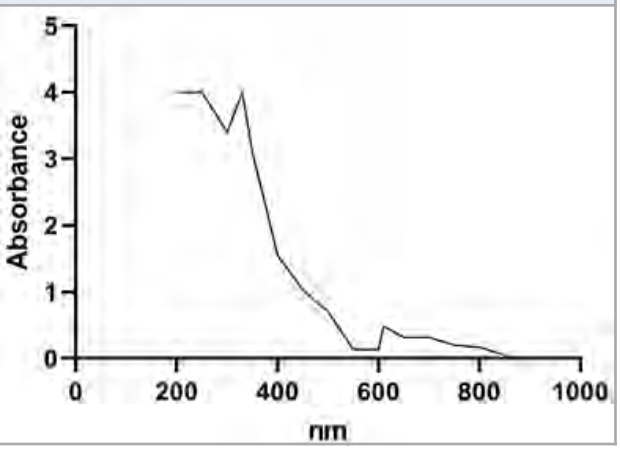


Figure 5: Scanning electron microscopy of purified laccase from *Peyronellaea pinodella* BL-3/4.

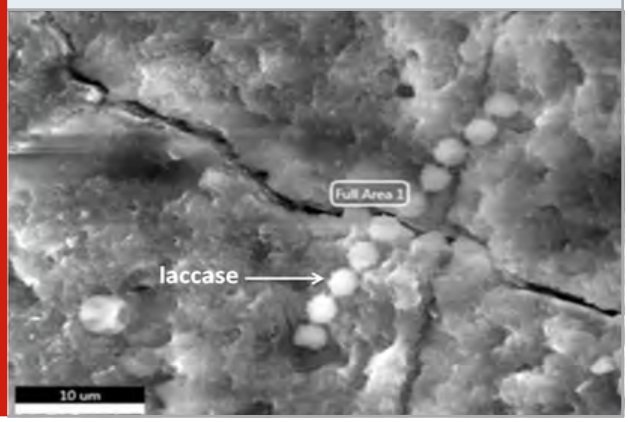
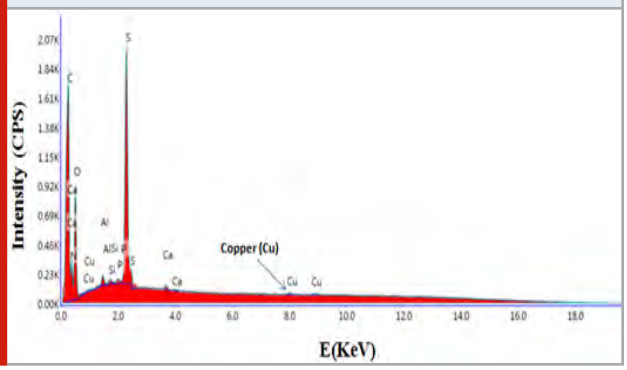


Figure 6: Energy dispersive X-ray (SEM/EDAX) spectrum showing presence of copper in purified laccase from *Peyronellaea pinodella* BL-3/4



CONCLUSION

The present study has explored the potential of Peyronellaea pinodella BL-3/4, a newly isolated ascomycetes to produce laccase under optimal medium components designed by statistical software through submerged fermentation. Statistical optimization has provided best combinations of medium components while considering interaction between medium components

studied. The usage of design expert software reduces the resources required and also saved time. Optimization leads to two fold increases in laccase production compared to control using orange peelings (1%) as a lignocellulosic substrate. Laccase yield of 14.1% was achieved in final purification with sephacryl s-100 HR gel filtration chromatography. The production of laccase by *Peyronellaea pinodella* BL-3/4 was also confirmed by the evaluating presence of copper in the purified fraction. SEM-EDAX analysis confirms the presence of copper in purified laccase. Further research on *Peyronellaea pinodella* BL-3/4 can be explored to scale up the laccase production for its vivid industrial applications.

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Conflict of Interests: None

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Sensory Evaluation of Value Added Products and Quantification of Ascorbic Acid of Ash Gourd (*Benincasa hispida*, Thumb.) Cong. Germplasm by Volumetric Method

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ABSTRACT

Ash gourd is an important, under-exploited vegetable that is immensely used in ayurvedic medicine preparations. It has a long storage life and good scope for value addition. Petha (Candy) and Badi (Nugget) prepared from ash gourd are much preferred in India. But ash gourd varieties differ significantly as regards the taste, acceptability and nutritive value of petha and badi prepared from them. Ideal varieties for badi and petha preparation have not been identified yet. Therefore, sensory and quality evaluation of petha and badi made from ash gourd pulp was done by taking fruits of eight promising ash gourd genotypes grown during the rainy season. Sensory quality attributes were evaluated in nine points Hedonic scale by 10 trained panelists for petha and badi. On the basis of scores given by the panel, BAGS-11 (7.54) was found to be the best for petha with a TSS of 2.03 0 Brix closely followed by Pusa Sabji Petha (6.27). In the case of badi, the local line BAGS-6 scored the highest overall acceptability (7.2) followed by BAGS-1(6.7). The range of ascorbic acid contents of eight parents was from 14.67 to 40 mg $100g^{-1}$. The released variety Kashi Dhawal had maximum ascorbic acid content of 40 mg $100g^{-1}$.

KEY WORDS: ASH GOURD, ASCORBIC ACID, CANDY, NUGGET. SENSORY EVALUATION, VALUE ADDITION.

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INTRODUCTION

The Indian subcontinent has a rich diversity of cucurbits and is believed to be the primary and secondary center of origin of several gourds and melons (Choudhury 2017). Ash gourd (*Benincasa hispida*) belonging to the Cucurbitaceae family, is a single species of tender annual vines. Indo-China region being a center of diversity is endowed with great variability in terms of morphological characters especially, growth habit, maturity including shape, size and flesh thickness of fruits (Rubatzky and Yamaguchi 1997 Flores et al. 2019).

Ash gourd (*Benincasa hispida*; syn. white gourd, wax gourd, white pumpkin, Chinese preserving melon, tallow gourd and Chinese water melon (Tindall 1986; Pandey et al. 2015) is an important vegetable mainly valued for its long storage life and having good scope for value addition. The fruits are consumed as baked, fried, boiled, pickled or candied/preserved (Robinson and Decker-Walters 1999). World-famous confectionery known as Petha (Candy) is prepared using ripe flesh of ash gourd in sugar syrup. Apart from fresh and processed produce, the fruits are immensely used in ayurvedic medicine preparations (Nagaraju et al.2016).The famous Ayurvedic preparation 'Kusumanda rasayanam' used as a nerve tonic and health rejuvenator, is prepared using well matured ash gourd fruit. The methanolic extract of the fruit is reported to possess anti-ulcer (Grover et al., 2001), antihistaminic and antidepressant activities (Anil and Ramu, 2002). Fruits of *Benincasa hispida* are used in folk medicine for the treatment of weak nervousness and debility (Nadhiya et al. 2014).

Ash gourd is composed a of higher amount of moisture (96.50 %), low calories 10 K cal, protein 0.40g, fat 0.1g, carbohydrates 1.9 g, ascorbic acid 1mg, fiber 0.80g, ash 0.39, iron 0.8 mg and 30 mg of calcium (Gopalan et al. 1989). Its mature fruits are mostly used by confectionaries for sweet making and in villages it is used for 'Badi' making, which is a popular ingredient of vegetable curry. Petha is a very popular sweet dish of western parts of Uttar Pradesh. The delicacy of sweet can be judged that it can be prepared and served in many forms depending upon the choice of consumers. On the compositional basis, petha based sweets contain on an average basis of total fat 0.4%, total carbohydrate 65%,dietary fibre 3%, protein 0.6% and sugar 40% (Pandey et al. 2015).

Flores et al. (2019) confirmed that there are genuine differences among vegetable varieties as regards sensory parameters. There are several varieties of ash gourd which are yet to be evaluated. Under the present scenario, it is believed that the future of agriculture is safe in increasing the productivity, diversification and value addition to horticultural crops. The value addition to ash gourd products fetches higher prices, extends the shelf life and can be made available throughout the year (Singh and Singh, 2015). Petha (candy) is a popular sweet dish made from ash gourd pulp in Uttar Pradesh in India. Badi (nugget) prepared from ash gourd is much preferred in several Indian states. Research involving varietal differences in sensory parameters of petha and badi is very limited. Therefore, the present investigation was undertaken for a comparative analysis on physic-chemical and sensory quality was carried out on a different variety of ash gourd to determine overall acceptability.

MATERIAL AND METHODS

The research work was carried out at the All India Coordinated Research Project on Vegetable Crops, Orissa University of Agriculture and Technology, Bhubaneswar and Department of Food and Nutrition, College of Community Science, Orissa University of Agriculture and Technology, Bhubaneswar, India.

Selection of suitable ash gourd variety: During the rainy season of 2015-16, eight genotypes of ash gourd (BAGS-1, BAGS-2, BAGS-6, BAGS-7, BAGS-11, Kashi Dhawal, Kashi Surbhi and Pusa Sabji petha) were grown in the randomized block design with 3 replications. The BAGS lines have been developed from local landraces of ash gourd collected from different parts of Odisha state and the rest of the genotypes are Nationally released varieties of India.The soil of the experimental plot was sandy loam having pH 5.1. The chemical analysis of soil indicated, the nitrogen content of 100 kg/ha, phosphorous content of 35.7 kg/ha and potassium content of 413.9 kg/ha. The organic carbon content of soil was 0.31%. The sowing was done on 31st July, 2015 as well as 2016 in kharif season. The crop was harvested on 20th of January. The experiment Randomized Block Design and replicated thrice. In each replication, each genotype was grown in four pits with four plants in each pit in a plot size of 3.0 m x 2.7m. The recommended package of practices was followed to raise a successful crop. Fully matured fruits of the 8 genotypes were obtained for the preparation of Petha (candy) and Badi (nugget).

Physico-chemical analysis: The Physico-chemical parameters were analyzed with fruit samples of 8 genotypes. The edible portion of ash gourd was analyzed for ascorbic acid content in triplicates by using standard procedures (Dinesh et al. 2015). Total soluble solids (TSS) were measured by using Erma hand refractometer and were expressed as degrees Brix.

Ascorbic acid analysis: The analysis was performed by taking a sample from each genotype. Ascorbic acid content in fruit was estimated by the volumetric method. Five milliliters of standard ascorbic acid (100 µ g/ml) was taken in a conical flask containing 10 ml 4% oxalic acid and was titrated against 2, 6-dichlorophenol indophenol dye. The appearance and persistence of the pink colour were taken as an end point. The amount of dye consumed (V1ml) is equivalent to the amount of ascorbic acid. 5 ml of sample (prepared by taking 5 g of fruit in 100 ml 4% oxalic acid) was taken in a conical flask having10 ml of 4% oxalic acid and titrated against the dye (V_2 ml). The amount of ascorbic acid was calculated using the formula (Ashwah et al, 1980).Ascorbic acid (mg/100 g) = (0.5 mg/V_1ml)×(V2/5 ml)×(100 ml/Wt. of sample)×100 (Ranganna, 1997)

Sensory evaluation: The sensory attributes of ash gourd petha and badi were analyzed for colour, flavor, texture, taste and overall acceptability by a trained panel of judges consisting of ten members by using a nine point hedonic scale (1 = dislike extremely; 9 = like extremely) (Amerine et al. 1965).

Procedure of preparing Badi: This is one of the most popular sun dried items. These badis are either deep fried and eaten or they can be used in many differentdishes/ curries.

Ingredients: 1 cup black gram, 1 cup grated ash gourd, salt. To prepare badi, black gram was first soaked in water overnight. Ash gourd was grated and the gratings were kept in colander to remove any water from it. This water was reserved as it would be used later for grinding. The soaked black gram was then grinded with reserved water to a very smooth paste. The mixture should not be too watery and therefore, ash gourd gratings should be squeezed to drain out excess water. These gratings were added to the paste of black gram. Salt was mixed in this paste and then small balls of paste were made which were kept under sun on a plastic paper which should be oiled so that badis can be taken out easily. Enough space was kept between two badis. After drying for 2-3 days, badis came off the paper easily. They were dried till they became crispy. Then the badis were cooled at room temperature and stored in air tight containers.

Petha preparation: Petha is a popular Indian sweet. It is soft, chewy and candy-like, eaten dry or dipped in sugar syrup (*Chashni*). It is known to have originated in Agra, India.

Ingredients of petha: 1 kg white ash gourd fruit, 2 tsp chemical lime, 3/4 kg (3 cups) sugar and 3 cups of water, 2 tbsp milk mixed with 2 tbsp water,1 tbsp lemon juice, 3-4 green cardamoms (peeled and crushed) and1 tsp rose water.

Procedure for making petha: The ash gourd fruit was peeled and the seeds and the soft fibrous portions were removed. Then fruits were cut into large thick slices and pricked well with a fork. 1 tsp of chemical lime was dissolved in enough water to cover the ash gourd pieces, soaked and washed well. Lime water solution was made with the remaining tsp of chemical lime and the pieces were again soaked for 2 hours. The cubes were then drained and washed thoroughly, squeezing out water and rinsing again so that no trace of lime remained. The cubes were cooked in boiling water until they became soft and transparent. Meanwhile, 3 cups of water and the sugar were filled in a pan; place over low heat, stirring till sugar was dissolved and brought to boil. The lemon juice and the cardamoms were added and cooked till it reached 'one thread' consistency. Foam, that might get collected along the sides of the pan, was skimmed off, Cooked pieces were drained with a slotted spoon and transferred into the warm syrup. After simmering for a couple of minutes, rose water was added and mixed well on the cooked ash gourd pieces. Then the pieces were cooled and served (Pandey et al. 2009).

Statistical analysis: Analysis of variance and critical difference for triplicate data of each observation (n=3) was performed after logarithmic transformation of all values (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

Sensory quality attributes of fresh ash gourd for TSS and petha were evaluated in nine point Hedonic scale (Peryam and Pilgrim, 1957) by 10 trained panelists (Table 1). The overall acceptability scores for petha showed a wide range from 4.81 to 7.54. The highest overall acceptability score for petha was obtained in the line BAGS-11 (7.54) closely followed by Pusa Sabji Petha (6.27) and Kashi Surbhi (5.72). As evaluated by 10 trained panelists, overall acceptability scores for badi had a wide range from 4.3 to 7.2. The local line BAGS-6 had the highest overall acceptability score of 7.2 for badi followed by BAGS-1(6.7). BAGS-7 and Kashi Dhawal had the same score (6.1).It is noteworthy that the highest acceptability scores for both petha and badi were obtained by selections from local landraces which were superior to even the released varieties of ash gourd in this regard. This indicates that there are very valuable attributes in our local landraces that are yet to be thoroughly explored. Hence, we should conserve our local germplasm and evaluate them in the best possible way.

Table 1. Overall acceptability scores of Badi (nugget) and Petha (candy) based on genotypes of Ash Gourd (*Benincasa hispida*)

Genotypes	Badi	Petha
BAGS-1	6.7	5.27
BAGS-2	5.1	4.81
BAGS-6	7.2	5.0
BAGS-7	6.1	4.81
BAGS-11	4.3	7.54
Kashi Dhawal	6.1	5.72
Kashi Surbhi	6.3	5.36
Pusa Sabji Petha	6.0	6.27

A wide range of variation was recorded among 8 genotypes of ash gourd with respect to total soluble solids (TSS) and ascorbic acid content of mature fruits (Table 2).As regards TSS of mature fruit, there was a wide range from 1.58 to 2.850 Brix. The genotype BAGS-6 (2.850 Brix) was the best followed by BAGS- 2 (2.030 Brix) and BAGS-11(2.030 Brix). In general, the TSS of fresh fruit of ash gourd juice is 2.5° Brix as reported by Kalpeshwar (2010). Significant variations were observed among the tested genotypes with respect to ascorbic acid content of fruits. The range of ascorbic acid content of 8 genotypes was from 13.33 to 40 mg $100g^{-1}$. It conforms to the range of ascorbic acid reported by Gopalan et al. (1989) and Pandey et al. (2009). The released variety

Kashi Dhawal recorded maximum ascorbic acid content of 40 mg 100g-1 which was significantly superior to that of the rest of the genotypes. Pusa Sabji Petha, BAGS-6, BAGS^{-1} and BAGS-11 were statistically at par and recorded moderately high TSS. Patil (1991) in fresh wood apple and Panesar et al. (2000) in Kinnow fruit found relatively higher amounts of ascorbic acid content i.e 44.75 mg 100g^{-1} and 28.84 mg100g-1 respectively.

Table 2. Total soluble solids and ascorbic acid contents of fruits in 8 genotypes of Ash Gourd (*Benincasa hispida*)

Sl. no	Genotypes	TSS (^{0}Brix)	Ascorbic acid content (mg/100g)
1.	BAGS-1	1.8	20.00
2.	BAGS-2	2.03	14.67
3.	BAGS-6	2.85	21.33
4.	BAGS-7	2.0	13.33
5.	BAGS-11	2.03	20.00
6.	Kashi Dhawal	1.9	40.00
7.	Kashi Surbhi	1.9	16.00
8.	Pusa Sabji Petha	1.58	26.67
SE(m)+		-	3.59
CD(0.05)		-	10.13

BAGS-7 and BAGS-2 recorded very low ascorbic acid contents of 13.33 and 14.67 mg 100g^{-1}. This is in conformity with the findings of Baber et al. (1998) in bottle gourd. The moderately high ascorbic acid content of BAGS -11(20 mg/100g) combined with moderately high TSS (2.030 Brix) may have contributed to the high acceptability score of the genotype for preparation of petha. The moderately high ascorbic acid content of BAGS-6 (21.33 mg/100g) combined with the highest TSS (2.85 0 Brix) may have contributed to the high acceptability score of the genotype for preparation of badi.

The research findings are novel. To date, there were no research findings identifying the most ideal varieties/ genotypes of ash gourd for making badi and petha with high overall acceptability. Ash gourd itself has not been much exploited earlier for meeting such objectives of value addition. Both for making badi and petha, the landraces BAGS-6 and BAGS-11 respectively, have been found to be best instead of the nationally released varieties of ash gourd. By proving that there are very promising local landraces that have not been adequately exploited for such a purpose till date, we have upheld the importance of our valuable indigenous genetic diversity which may become extinct soon if we fail to collect, evaluate and utilize them promptly. The superior genotypes which have been identified from this research work can also be used as parents in future breeding programs to transfer the desired genes and develop many potent varieties of ash gourd meant for preparing badi and petha with highly superior sensory parameters.

CONCLUSION

BAGS-11 (7.54) was found to be the best for petha followed by Pusa Sabji Petha (6.27). In the case of badi, the local line BAGS-6 scored the highest overall acceptability (7.2) followed by BAGS-1(6.7). The moderately high ascorbic acid content of BAGS -11(20 mg/100g) combined with moderately high TSS (2.030 Brix) may have contributed to the high acceptability score of the genotype for preparation of petha. The moderately high ascorbic acid content of BAGS-6 (21.33 mg/100g) combined with the highest TSS (2.85 0 Brix) may have contributed to the high acceptability score of the genotype for preparation of badi. As regards TSS of mature fruit, there was a wide range from 1.58 to 2.850 Brix. The range of ascorbic acid contents of eight genotypes was from 14.67 to 40 mg 100g^{-1}. The released variety of Kashi Dhawal recorded the maximum ascorbic acid content of 40 mg 100g^{-1}.

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Irrigation Performance Assessment of Janpad Major of Nagarjuna Sagar Project Using Remote Sensing and GIS

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ABSTRACT

An Investigation on irrigation performance assessment of Janpad major, Left bank canal, Nagarjuna Sagar Project (NSP), Andhra Pradesh, India using remote sensing and GIS was conducted during rabi (2008-09) at Water Technology Center, ANGRAU, Rajendranagar, Hyderabad. Multi temporal remote sensing (RS) data-based crop inventory and generation of NDVI was carried out over the Janpad major command of NSP, using Indian Remote Sensing Satellite (IRS-P6) and Linear Imaging and Self Scanning –III (LISS-III) data. In this study, six comparative indicators for the assessment of irrigation system performance were applied to Janpad major command area, during rabi season (2008-09). The irrigation intensity (%), out put per cropped area (t/ha), out put per unit cultivable command (t/ha), water utilization index (ha/M.m3), water productivity (kg/m3) were computed. Overall consumption rate (Efficiency) was computed by comparing the crop water requirement with the water release data. Irrigation intensity, output per unit cropped area and out put per unit cultivable command was 96 %, 4.44 t ha^{-1} and 4.27 t ha^{-1} respectively for the Janpad major command. Water utilization index, overall consumption rate (Efficiency) and Water productivity was found as 124 ha/M.m3, 67.7 per cent and 0.64 kg m^{-3}, respectively Above set of performance indicators describes the performance of an irrigation project for decision making. With satellite remote sensing and calculating actual ET of the crop and overall consumption rate (OCR) helps the policy makers in irrigation water supply as per crop. By knowing the supply and demand of irrigation water of a project is necessary for better irrigation efficiencies and improving the water productivity of irrigation project.

KEY WORDS: SATELLITE DATA, IRRIGATION INTENSITY, WATER UTILIZATION INDEX, WATER PRODUCTIVITY.

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INTRODUCTION

India is amongst the largest irrigator countries in the world. In most of the existing irrigation schemes there is a serious lack of reliable and adequate information on system performance. Periodical assessment of irrigation project is needed to find out irrigation potential utilized vis-à-vis the potential created. System performance monitoring, evaluation, and diagnostic analysis are keys for the improvement of irrigation projects. A set of performance indicators describes the system performance of irrigation schemes in different dimensions for the benefit of decision-makers. The temporal and spatial variations of such indicators will help in improving the system performance. Remote sensing provides an opportunity to measure the crop yield without field measurements and this opens the possibility to study the variability of water productivity within an irrigation system. Neelima et al.,2015 and Kumar et al. 2015 assessed the irrigation performance of Jurala irrigation project and Nagarjuna Sagar Project in 2008-10 using LISS III and AWiFS data using RS based crop inventory and NDVI values. Ayat Elnmer et al. (2018) assessed the irrigation performance of the Nile Delta using remote sensing using indicators like adequacy index (AI), equity index (EI) and Dependability (PI), Water productivity (WP), water depleted fraction (DF), relative evapotranspiration (RET) with LANDSAT ETM+ ETM+ images and SEBAL approach.

Remote sensing is now playing a major role in acreage estimation and to forecast crop yields primarily based upon statistical– empirical relationships between yield and vegetation indices (Thenkabail et al., 2002, Casa and Jones, 2005). Wheat acreage estimation for Karnal district using the LISS – III satellite data (Urmil Verma and Haryana, 2011), Boro rice in Nagaon district of Assam (Raihan Ahmed and Haroon Sajjad, 2015), and yield of soybeans crop in some districts of M.P (Abhishek Kumar Maurya, 2016) and area, production and productivity of potato for 5 major potato growing states of India i.e. Bihar, Gujarat, Punjab, Uttar Pradesh and West Bengal was done using RS and GIS, (Kumar 2019).

The Area under Janapad major located at 90.5 km (160 56' 12.8" E& 790 42' 0.7" N) on Left Bank canal of Nagarjunasagar project with the length of 24 km and designed discharge of 555.13 cusecs was selected for the study of. The ayacut area spreads in Nereducherla, Garidepally and part of Mattampally mandals of the Nalgonda district (Fig 1). It has a total geographical area of 13216 ha with 11 Water Users Associations. It is located in Southern Telangana Agro-Climatic Zone of Andhra Pradesh. It comes under Agro-Eco region 7-Hot Semi-arid eco region with red and black soils (K6 D2) (NBSS&LUP, 1992). By using IRS P6 WiFS (Wide Field Sensor) and IRS-1C WiFS and LISS3 which have a good periodicity, it is possible to construct growth profiles and retrieve yield related parameters at region level (Menon, 2012). Satellite remote sensing and Geographic Information System (GIS) offer great promise for natural resources management (Shanmugapriya et al., 2019).

Irrigation performance assessment is the systematic spatial and temporal evaluation of irrigation systems to diagnose problems and is considered as the most critical element for improving irrigation management (Abernethy and Pearce, 1987).Irrigation performance assessment was carried out for various projects in India. The irrigation performance assessment of Jurala (Neelima et al., 2013) and Krishna Western Delta in Andhra Pradesh was carried by using satellite data indicated that there was reduction in the total cropped area by 17,383 ha in 2005-06 compared to1998-99 (Prasad et al., 2006). However, such information is not available intensively at irrigation major of Nagarjuna Sagar multipurpose irrigation project. Hence, the research project on irrigation performance assessment of Janpad major left bank canal, Nagarjuna Sagar Project using Remote Sensing and GIS was taken up to assess the irrigation potential utilized vis-à-vis the potential created, agricultural production and productivity achieved and how efficiently the irrigation being managed.

MATERIAL AND METHODS

The performance indicators are computed for the Janpad major command area. The main output considered is crop production and the major inputs are water and land. Six basic comparative performance indicators namely irrigation intensity, overall consumption rate, water utilization index, output per unit cropped area, output per unit command area, and water productivity were computed. Computation of these indicators require data on cropped area, command area, potential evapotranspiration, water diverted by the canal, rainfall and production. The multispectral data from LISS-III sensor of IRS –P6 (Resourcesat-1) were acquired on 21-2-09, 17-3-09 and 10-4-09 and geo-coded false colour composites (FCC) were utilized for delineation of major crops grown in the study area. The Supervised classification for the images taken on Feb, Mar and April 2009 was done.

The training windows were defined for various crops and other land use classes based on the ground truth information. Multiple training sites for each class were identified in order to represent the variability with in the same class. The image was classified using maximum likelihood algorithm. Rice mask map was generated based on classified image. The pixels thus generated were multiplied with 24 m x 24 m to estimate paddy area. Crop cutting experiments were conducted (5 m × 2 m) randomly at selected points in the command area and the corresponding GPS reading were taken. Paddy grain weight at 14 per cent moisture was taken for developing yield model. Normalized Difference Vegetation Index (NDVI) proposed by Rouse et al., (1974) was used in this study. This index is very sensitive to the presence of green vegetation. It permits the prediction of agricultural crops in semi arid areas.
NDVI can be defined by following equation

$$NDVI = \frac{NIR - R}{NIR + R}$$

NIR and R are the reflectance in the near infra red and red regions, respectively.

The red and infra red channel data pertaining to the IRS P6 LISS III image were transformed in to NDVI image in ERDAS IMAGINE Modeler panel by running the NDVI model. The resultant NDVI image of the paddy crop was used for yield estimation. Maximum NDVI image derived using 2008-09 rabi season satellite data was used to establish the relationship between NDVI and paddy yield observed at CCE plots through the development of regression model. Data on command area and water diverted were collected from Divisional executive engineer, Left bank canal, Nagarjunasagar project, Miryalguda, Nalgonda district, A.P., India. For computation of PET and water requirement, the weather data of ARI Rajendranagar was considered. Potential Evapotranspiration was computed using the Penman-Monteith method of CROPWAT computer model. These indicators were calculated as shown below:

$$\text{Irrigation Intensity (\%)} = \frac{\text{Area irrigated}}{\text{Culturable command area}}$$

$$\text{Overall Consumption Rate (Efficiency)} = \frac{ET_p - P_e}{V}$$

ETp is the potential evapotranspiration (m^3) Pe is the effective rainfall (m^3)
V is the total volume of water supplied to the command area (m^3)

$$\text{Water Utilization Index (ha/M.m}^3\text{)} = \frac{\text{Area irrigated}}{\text{Unit of water supplied}}$$

$$\text{Output per cropped area (t/ha)} = \frac{\text{Production}}{\text{Irrigated cropped area}}$$

$$\text{Output per unit command (t/ha)} = \frac{\text{Production}}{\text{Command area}}$$

$$\text{Water productivity (kg/m}^3\text{)} = \frac{\text{Yield of harvested crop}}{\text{Volume of water supplied}}$$

RESULTS AND DISCUSSION

Initial observations from field visits indicate that paddy constitute more than 96 per cent of the total cropped area. Other crops grown in the command area are chilies, cotton and citrus. The satellite derived crop estimates in Janpad major in NSP left canal command indicate that paddy occupies 11,750 ha while water bodies, settlements, barren lands and shrubs occupy 9630.6 ha (Table 1). The area under other crops is negligible and this area was considered under shrubs. The paddy area is more in middle reach followed by head and tail end. Paddy is the major crop in Janpad major command area, left bank canal, Nagarjuna sagar. Out of 4493.5 ha of head region of the command area, 3179 ha is paddy. Compared to paddy area, other crops are negligible. The water bodies, settlement, barren land and shrubs occupied 3.7, 508.5, 11.3 and 791.0 ha respectively.In middle reach out of 10255.1 ha, the paddy crop occupied 7460.90 ha. The water bodies, settlement, barren land and shrubs occupied 92.0, 1221.2, 2.9, and 1892.0 ha respectively.

Table 1. Satellite derived crop area (ha) in head, middle and tail reaches of Janpad major, NSP left canal during *rabi* 2008-09

Crop	Head	Middle	Tail	Janpad Major command
Paddy	3179.0	7046.9	1525.0	11750.9
Water body	3.7	92.0	17.2	113.0
Settlement	508.5	1221.2	2988.2	4718.0
Barren land	11.3	2.9	568.6	583.9
Shrubs	791.0	1892.0	1532.8	4215.9
Total	4493.5	10255.1	6631.9	21381.5

In tail end area, out of 6631.9 ha, the paddy occupied 1525.0 ha while considerable area was occupied by settlement (2988 ha), followed by shrubs. The water bodies occupied considerably less area. Neelima et al., (2014) estimated the irrigated area under Jurala Project command area and found that there was an increase in the paddy area from 70.7 to 72.4% and from 76.3 to 83.5% in the right main canal and left main canal respectively. Ahmed and Sajjad, (2015) estimated the crop acreage of Boro rice using NDVI, RVI and supervised classification in Nagaon district of Assam in single date Landsat 5 TM digital data coinciding with maximum crop growth (March, 2009) has been used for identification and estimation of crop acreage. Maurya, (2016) estimated the acreage and yield of soybeans crop in some districts of M.P. in the Kharif season 2007. NDVI was performed on all the October images (2007, 2006, 2005) using hybrid classification techniques.

Table 2. Irrigation intensity (%) at Head, middle and tail reach and entire Janpad major of NSP left canal during *rabi* 2008-09.

Head	Middle	Tail	Janpad major command
97	97	90	96

Irrigation intensity was estimated based on the satellite derived crop estimates as percent of the total culturable command area (CCA) i.e., ayacut in the command. The irrigation intensity of the head, middle and tail reach was observed to be 97, 97 and 90 % respectively (Table 2). On the other hand the irrigation intensity was only 96 % in Janpad major during rabi 2008-09. Further, in tail reach, decrease in irrigation intensity (90 %) was observed, this may be due to large area in the tail reach was under shrubs and rocky land. Prasad et al., (2006) has reported the irrigation intensity of 83 per cent under NIZ east canal command of Krishna western delta. Kumar, et al.., (2014) reported that the irrigation intensity varied from 92.39 to 123.19 for against target of 100 per cent in Nagarjuna sagar left canal command area during rabi 2008-09 and 2009-10, respectively.

Overall Consumption Rate was derived by dividing water requirement estimated using Potential Evapotranspiration (ETp) minus Pe (effective rain fall) by volume of water applied to the Janpad command area. The water requirement was derived through CROPWAT model. The PET for paddy was 544.1 mm and for shrubs is 325 mm. The effective rainfall was 9.9 mm. The gross water requirement for paddy was observed to be 1584.4 mm.The Overall Consumed Rate (efficiency) estimated was 0.67 (Table 3). This is the Overall Consumed Rate (efficiency) which was derived without considering the conveyance and application losses. The higher OCR values in the selected distributary implies that insufficient water was supplied to that particular distributary. The OCR values in the Jural project command area ranged from 0.06 to 0.15and 0.02 to 0.12 during rainy season 2009 and 2010 respectively and thus there was no shortage of water supply to the command area (Neelima et al., 2015) and for Janpad and Mudimanikyam majors in Nagarjunasagar left canal area during 2010 was 0.52 and 0.40 (Kumar et al., 2014).

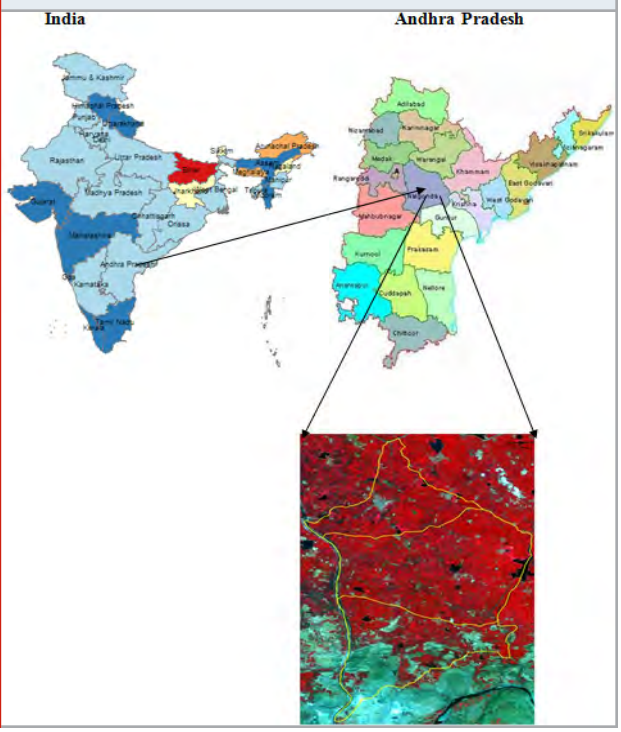
Water Utilization Index (WUI) defined as area irrigated per unit volume is a measure of water delivery performance and constitutes one of the important spatial performance indicators of an irrigation system. WUI also forms basis for evaluating the adequacy of seasonal irrigation supplies in an irrigation system (Table 3). It is estimated based on the equivalent wet area and amount of water released in to the various irrigation units. The paddy irrigated area was 11750.9 ha and the water delivered to the major was 94.752 M.m3. The water utilization index was 124 ha/M.m3 or 3512 ha per one TMC which is less than the normal expected irrigation of 4000 ha of paddy irrigation (wetland) per TMC. Neelima et al. (2014) reported that in Jurala project command area in Telangana during 2009-10, area irrigated was much less per unit of canal irrigation water supplied (68.8 ha irrigated million m-3 water supplied) for the left main canal, compared to right main canal when better utilization of canal irrigation water was achieved (80.2 ha irrigated million m^{-3} water supplied).

Table 3. Water Utilization Index, Overall Consumption Rate and Water Productivity of Janpad command area.

Indicators	Janpad major command area
Water Utilization Index (ha/Mm^3)	124
Overall Consumption Rate	0.67
Water Productivity (kg/m^3)	0.647

In Janpad major of NSP left canal command during rabi the paddy yields were highly variable at different sites obtained through crop cut experiments (CCE) and the yields ranged from 4.30 to 9.25 t/ha. Maximum NDVI image derived using Feb 21, 2009 satellite image was used to establish the relationship between NDVI and paddy yield observed at CCE plots through the development of linear regression model (Fig. 3). Yield prediction model Y= 8.5076(NDVI) + 2.8125 Y = Estimated yield (t ha-1) NDVI: NDVI of paddy crop.

Figure 1: Location map of Janpad command area, Nagarjuna Sagar left canal, Andhra Pradesh



The yield prediction model was found to be significant R^2 = 0.7095, which explain 71 % of the variability in yield estimation which is reasonable for crop yield estimation studies. The Paddy yield model generated for Janpad major of NSP left canal command for rabi 2008-09 was applied to generate paddy yield map. Using the model, the average yield of paddy in Janpad command was

estimated to be 4.44 t/ha. The total production of paddy in Janpad major command was calculated by using the relationship Production= area (ha) × yield (t/ha). The estimated production of paddy in Janpad major was 52,174 tons.

Figure 2: Supervised classification image of Janpad major, NSP left canal, India of Feb21, Mar17 and Apri10, 2009

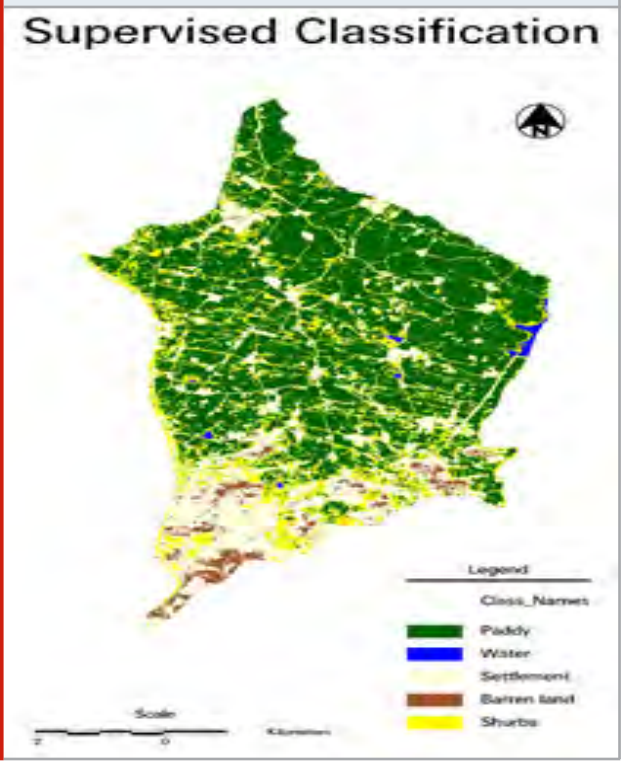
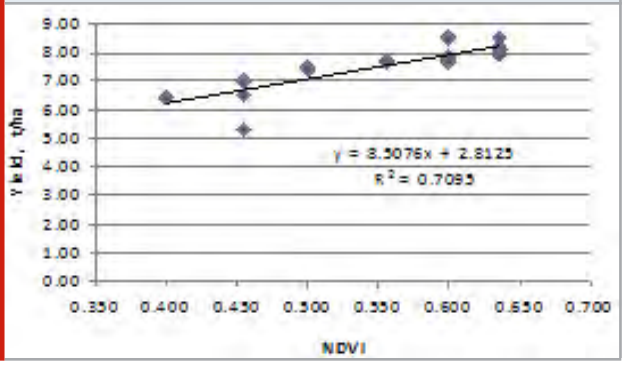


Figure 3: Paddy yield model developed for Janpad major command area, NSP, India



The out put per cropped area for Janpad command area was computed by dividing the total production obtained by multiplying satellite derived yield (4.4 t/ha) with paddy area (11750.9 ha). The area under other crops (4 %) which are included in shrubs were not considered because it was scattered and negligible. The overall production of Janpad major was found to be 51704 tons. The out -put per culturable command area for Janpad command was computed by dividing the total production obtained by multiplying satellite derived yield (4.44 t/ha) with paddy area (11750.9 ha) of the command. The culturable command area is 12194 ha. The output per culturable command area for Janpad major was 4.27 t/ha. Similar result was observed by Upadhyaya (2005) for Patna Main Canal Command. Productivity of water is one of the irrigation performance indicator used in irrigation performance assessment as it can establish the relation between the amount of water supplied to irrigation command and the production of paddy realized from the command. The water productivity observed in command area was 0.647 kg/m3. A similar trend was reported by Prasad et al., (2006) (0.7 kg/m3) for Krishna western delta during 2005-06.

Taghvaeian et al., (2018) reported that by using Remote Sensing and GIS Techniques for assessing Irrigation Performance indicators based on the magnitude and uniformity of ground water depth was found to be functioning at an optimal level. This suggests that a set of performance indicators like Supply Demand Ratio, Over all Consumption Rate (OCR) and Water Utilization Index (WUI) can describe performance of irrigation schemes in different dimensions for the benefit of decision-makers. . It is clear that the Janpad major supposed to irrigate more area, however it is falling short of 12 % of less area than expected. The estimated indicators provided information the performance of the Janpad major irrigation canal is under utilizing the irrigation water. Understanding the performance of irrigation by irrigation managers at farm levels has the potential to improve irrigation water use efficiently and contribute to more efficient use of water resources

CONCLUSIONS

A set of performance indicators describes the system performance of irrigation schemes in different dimensions for the benefit of decision-makers. Information generated on irrigation performance of the project can be used for improving better irrigation management. Further, such periodical studies are required for taking remedial measures which ultimately help in efficient management of the most important and scarce natural resource water in increasing the water productivity. Satellite measurements provide near real time data and can be revived and updated.

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A Report on Wild Edible Fruits Used by the Tribal Communities Inhabiting Near Katepurna Wildlife Sanctuary, Maharashtra, India

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ABSTRACT

Wild edible plants (WEPs) especially bearing edible fruits are considered as a rich source of nutrition for the aboriginal tribal and local communities residing in the forest catchment area since ancient time. The wild fruits are refreshing, delicious and cheap supplements of vitamins, minerals and proteins for the users. However, current scenario indicates that their use and knowledge is declining day by day. In this study, we reported wild edible fruits belonging to 26 angiospermic plants from 23 genera and 23 families from protected area of Katepurna wildlife sanctuary (MS) India. Of these identified wild edible fruit plants, 69% were trees, 23% were shrubs and 8% were climbers. Most of the fruits were eaten raw, some cooked and few were used to make pickles or chutney. Further, it was noted that most of these fruits were used by the tribals of this area for their ethnomedicinal potential. This knowledge about duel application of wild edible fruits should be preserve and utilize for the benefit of mankind.

KEY WORDS: WILD EDIBLE PLANTS, ETHNOMEDICINE, KATEPURNA WILDLIFE SANCTURY AND TRIBAL.

INTRODUCTION

Wild edible plants (WEPs) are those plant species which are not cultivated or domesticated but could be accessible from various natural habitats and used as food (Beluhan and Ranogajec, 2010). It was estimated that, humans might have utilized more than 7000 WEPs globally (Grivetti and Ogle, 2002) but most of these remain under-utilized (Mohan Ram, 2000).In many developing countries, WEPs produce specially fruits play a vital role in the livelihoods of Tribal and rural communities residing in forest catchments (Patole and Jain, 2002; Pundir and Singh, 2002). These plants serve as an alternative to staple foods during times of seasonal food scarcity and also could be used as valuable supplement for a nutritionally balanced diet. Currently, some Tribal and rural peoples use to sell these fruits in local market, providing a source of income for poor communities. Further, these species could be used as new potential sources for domestication (Prasad et al., 2003; Shrestha and Dhillon, 2006). Currently there is decline in the use of wild edible fruits by the native community people, (Mallick et al., 2017, Sardespande and Shalckleton, 2019).

This might erode the traditional knowledge about using these fruits as healthcare supplements. Therefore, present study was planned to focus on documentation of wild edible fruits/ fruit plants from Katepurna wild life

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sanctuary (KWS), Maharashtra, India. The most tribal populated villages near KWS are Kurankhed Dhotarkhed, Fetra, Kasmar, Vastapur, Deodari, Yedsi, Khopdi , Wai and Wagha. The total population of tribals in this area is about 12,000 (as per 2011 census) which is mostly depends on forest products for their livelihood.

The notified spread area of KWS is 73.69 Sq. km. which lies between the meridians of longitudes '77^{0}7'41" and 77^{0}12'36" East and between the parallels of latitude 19^{0}22'14" and 19^{0}29'77" North. It is situated in Akola District, West Vidarbha region of Maharashtra State, India (fig. 1). The climate is dry deciduous with an average maximum temperature of 45 ^{0}C and minimum of 21 ^{0}C. The vegetation of the sanctuary is dry deciduous type, while the slopes have thickly forested dense vegetation patches harboring rich fauna. The Andh, Bhill, Pawra and Halba are the prominent Tribal communities inhabiting this forest catchment area. The study area was divided into five sub-centers and these sub-centers were visited twice a month post-monsoon to mid- summer during 2015 to 2018. To assess the traditional knowledge about WEPs, especially edible fruits, frequent interactions were made with the local Tribal experts and villagers, including farmers, shepherds, housewives and village heads.

Table 1. List of collected wild fruit plants from Katepurna wildlife sanctuary

Sr. No.	Botanical Name of Plant	Local Vernacular Name	Family
1	*Aegle marmelos* (L.) Corr.	Bel	Rutaceae
2	*Alangium salvifolium* (L.f.) wang, Engl.	Ankol	Alangiaceae
3	*Annona squamosa* L.	Sitaphal	Annonaceae
4	*Azadirachta indica* Juss.	Neem	Meliaceae
5	*Buchanania cochinchinensis* (Lour.) Almeida	Charoli	Anacardiaceae
6	*Capparis spinosa* L.	Kabar/ Kalavari	Capparaceae
7	*Capparis decidua* (Forssk.)Edgew.	Nepti/ Karel	Capparaceae
8	*Canthium coromandelium* (N.Burm.) Alst	Kirma	Rubiaceae
9	*Carissa carandus* L. Mant.	Karavand	Apocyanaceae
10	*Celastrus paniculatus* Willd	Kanguni	Celastraceae
11	*Cordia dichotoma* Foret. f.	Bhokar	Boraginaceae
12	*Diospyros peregrine* L.	Tembruni	Ebnaceae
13	*Emblica officinalis* Gaertn. Fruct.	Awala	Euphorbiaceae
14	*Erythrina variegata* L.	Pangara	Fabaceae
15	*Ficus recemosa* L.	Umber	Moraceae
16	*Garcinia indica* (Thou.) Chois.	Ratamba	Clusiaceae
17	*Grewia tiliifolia* Vahl, Symb	Dhaman	Tiliaceae
18	*Momordica dioeca* Roxb.ex Willd.	Kartuli	Cucurbitaceae
19	*Semicarpus anacardium* L.	Biba	Anacardiaceae
20	*Sterculia foetida* L.	Goldaru	Sterculiaceae
21	*Syzygium cumini* L.	Jambhul	Myrtaceae
22	*Terminalia bellirica* (Gaertn) Roxb.	Behda	Combretaceae
23	*Terminalia chebula* Retz.	Hirda	Combretaceae
24	*Trichosanthes tricuspidata* Lour.Fl.	Kaundal	Cucurbitaceae
25	*Ziziphus mauritiana L.*	Bor	Rhamnaceae
26	*Zizipus rugosa Lamk.*	Yeruni	Rhamnaceae

Table 2. Edible/ medicinal uses of the fruits collected from Katepurna wildlife Sanctuary

Sr. No.	Name of Plant	Edible uses	Medicinal uses
1	*Aegle marmelos* (L.) Corr.	The mature ripe fruit is eaten raw, also made into pickle.	The fruit pulp is digestive, also used to cure diarrhoea, dysentery and peptic ulcers.
2	*Alangium salvifolium* (L.f.) wang, Engl.	The ripe fruits eaten raw.	The fruit is said to have aphrodiastic, carminative and expectorant properties. It is also used locally as antidote for scorpion and snake bite.

Continue Table 2

3	*Annona squamosa* L.	The mature ripe fruits eaten raw for taste and nutrition.	The fruit is used as antidiabetic , anti-inflammatory and anti-tumor activity.
4	*Azadirachta indica* Juss.	The fruits are eaten raw or cooked, sometimes made into lemonade.	The seed oil is used as antiseptic agent and for its microcidal property.
5	*Buchanania cochinchinensis* (Lour.) Almeida	Fruits are eaten fresh, dried fruits are also eaten and used for other purposes. The seeds are used as condiment in different sweet recipes.	The fruits are used to treat cough and asthma.
6	*Capparis spinosa* L.	The fruits are used as condiments in vegetables and pickles.	Fruits are used against rheumatic pain, gout.
7	*Capparis decidua* (Forssk.) Edgew.	Unripe fruits eaten raw after cooking; ripe fruits eaten raw without cooking.	The fruit is given to treat rheumatic pain.
8	*Canthium coromandelium* (N.Burm.) Alst	The fruits are eaten raw or cooked.	The fruits are given to small children to remove intestinal worms
9	*Carissa carandas* L. Mant.	Raw fresh fruits eaten to strengthen cardiac muscles.	The fresh fruit is also used to prepare pickles. The fresh fruit juice is use to improve appetite and digestion.
10	*Celastrus paniculatus* Willd.	Young fresh fruits are eaten raw.	The seed oil is used as. brain tonic by tribals The dried seed powder is given with milk to improve the conditions like memory loss and dementia.
11	*Cordia dichotoma* Foret. f.	The fresh fruits eaten either raw or cooked, sweet in taste. Immature fruits use to prepare pickles.	The fresh fruits are fleshy and used as demulcent and laxative. Their paste also been use to recover skin eruptions.
12	*Diospyros peregrine L.*	Ripe fruits eaten raw for taste and nutrition.	The locals use the fruits as anticold, astringent and anthelmintic.
13	*Emblica officinalis* Gaertn. Fruct.	Mature fruits are eaten raw. Fruits are also made into pickle or murraba.	The fruits are said to have antioxidant, anti-inflammatory and antiulcer activities.
14	*Erythrina variegata* L.	Roasted fruit seeds eaten.	The boiled water with fruits/ seeds of the plant is considered as antidote against snakebite.
15	*Ficus recemosa* L.	Ripe fruits, sweet, eaten raw; sometimes made into pickle.	The fruits used as astringent and also in treatment of menorrhagia.
16	*Garcinia indica* (Thou.) Chois.	The fresh fruits eaten raw and made into sherbet of mature fruits.	The fruit juice is use to recover sunstrokes (cooant). Fresh juice is use to treat constipation. The fruit is also considered as anticancer, antidiabetic and anti-ulcer agent.
17	*Grewia tiliifolia* Vahl, Symb	The fruits are having good flavour, use to eat raw by the tribals.	Traditionally it is used as good source of natural antioxidant.

Continue Table 2

18	*Momordica dioeca* Roxb.ex Willd.	The fresh fruits used as vegetables.	The fruit is said to regulate blood pressure and reduce hypertension. respiratory disorders, reduce weight. also suggested that it works against cancer and diabetes
19	*Semicarpus anacardium* L.	The mature fruits are eaten raw.	The fruit extract said to have anti-inflammatory, antioxidant and antimicrobial
20	*Sterculia foetida* L.	The seeds roasted and eaten raw.	The fruit and seeds are said to have laxative property.
21	*Syzygium cumini* L.	The seeds are with unique taste, eaten raw.	The tribals use these fruits as best anti-diabetic agent and natural blood purifier.
22	*Terminalia bellirica* (Gaertn) Roxb.	The dried fruit seeds are eaten raw.	The fruit and fruit oil have anthelmintic, astringent, digestive and tonic.
23	*Terminalia chebula* Retz.	Green as well as mature dry fruits are eaten raw.	The fruit is digestive in nature. The fruit powder along with honey is given to improve appetite and cure cough and cold.
24	*Trichosanthes tricuspidata* Lour.Fl.	The fresh fruits mostly eaten raw.	The fruits have laxative property. It also have antimicrobial use. The soup prepared from fruit pulp cures cold and fever.
25	*Ziziphus mauritiana* L.	The fresh mature fruits are eaten raw for taste. Dried fruits boiled and added with salt also eaten as food.	The fruits given to increase muscular strength, prevent liver and bladder diseases. Fruit powder is given to cure constipation.
26	*Zizipus rugosa* Lamk.	The fresh mature and ripe fruits eaten raw	The fruits are used to improve digestion. It also used as liver tonic.

Figure 1

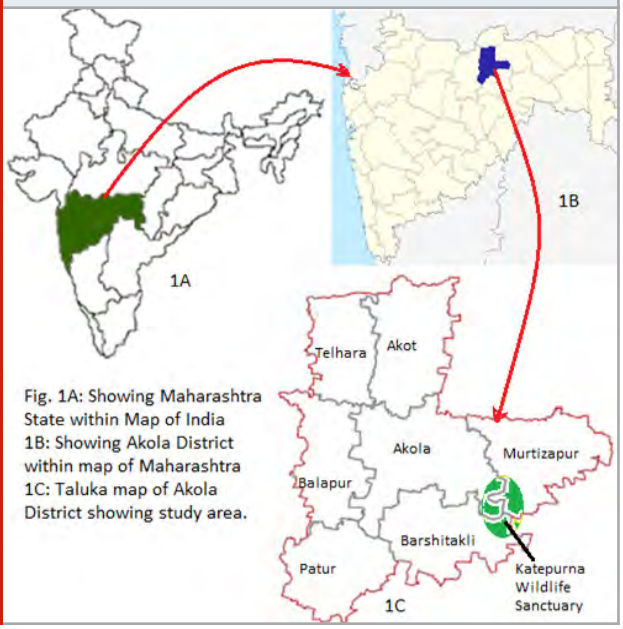
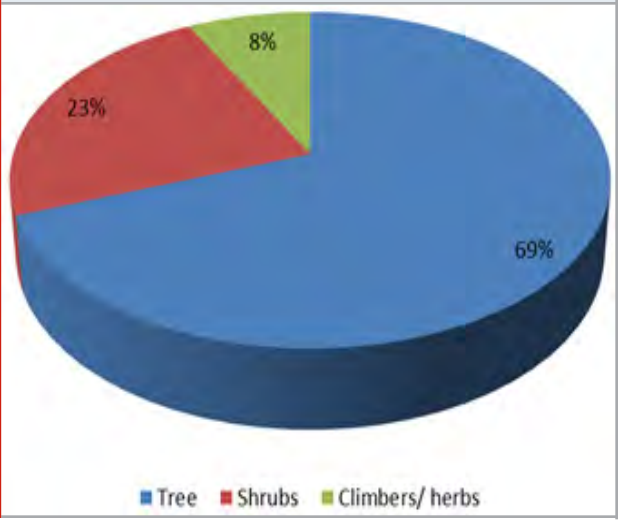


Fig. 1A: Showing Maharashtra State within Map of India
1B: Showing Akola District within map of Maharashtra
1C: Taluka map of Akola District showing study area.

Figure 2: Percentage occurance of habits of edible fruit producing plants



In total 18 informants were interviewed regarding their knowledge about use of wild fruits available in the sanctuary area. Of these informants, 4 were housewives, 3 were Tribal healers (Vaidoo's from Andh, Pawra and Bhill communities), 4 were village heads who were practicing herbal medicine, 3 were old age formers and 4 were shepherds. All the informants were aging more than 60 years except the shepherds with age ranging from 30 to 40 years. Live specimens and available photographs were shown to them for local identification. The fruits were preserved and identified with the help of available literature and floras (Naik, 1998; Singh and Karthikeyan, 2000; Singh et al., 2001; Yadav and Sardesai, 2002) and specimens were deposited in Department of Botany, Shri Shivaji College of Arts, Commerce and Science, Akola (MS) India.

The fruits WEPs used by the Tribal communities and local villagers residing in the vicinity of Katepurna wild life sanctuary were collected, identified and documented (table-1). In total 26 WEPs whose fruits were edible, were collected and identified during the study. They are presented here with their local name, botanical name along with family to which they belongs. The collected material belongs to 23 genera and 23 angiospermic families with 69% trees, 23% shrubs and 8% climbers (Fig. 2). In the modern era of urbanization and industrialization, only few peoples from different Tribal communities in the area, along with mostly elderly villagers were only noted to use these fruits seasonally in their diet indicating their declining use. Apart from the edible use, the collected plant fruits also have some medicinal properties. Most of the fruits reported here in this paper are eaten raw or cooked. Some of them use to make pickles. During the survey, it was also noted that, these fruits were also used for their medicinal potential (table- 2). Of the collected fruits, 4 were used to improve digestion, 4 were as anti-diabetic agents, 3 as anticancer agents, 3 as anti-inflammatory, 3 as antioxidants, 3 as antimicrobial agents; 2 for ulcer healing properties and rheumatism curing agents. The details are presented in table 2.

Earlier, Barua et al., (2000) investigated wild edible plants from Majuli Island and Darang District of Assam. A similar report was made by Rajasab and Isaq (2004) from north Karnataka. Aberoumand and Deokule (2008) had reported edible fruits from Iran and India. Bhogaonkar and Marathe (2010) studied the wild edible plants from Melgahat forest, Amravati District (MS). Reddy (2011) made similar report from Chandrapur District, Setiya et al., (2016) from the Gadchiroli District (MS) and Mondaragi et al., (2017) from the Southern Western Ghat of India. All these reports indicate that Tribal communities and local people use the WEPs or plant parts including fruits as nutritional supplements.

We argue that this is probably a nutraceutical approach of different tribal communities to maintain good health (Pushphagadan, 2000; Sardespande and Shalckleton, 2019), which is now a days observed to be declining alongside new assess to foods, markets and urbanization. Most of these wild edible fruits are rich in nutrients and minerals promoting their use in traditional medicine (Rothe, 2003; Kamble et al., 2010; Bhatia et al., 2018). Further, these plants could play vital role in eradication of poverty by generation of income resources, availability of food and diversification of agriculture (Thakur et al. 2017 and Bhatia et al. 2018). Our report is in analogy with earlier reports indicating that the Tribal communities in the vicinity of Katepurna wildlife sanctuary frequently use the fruits of WEPs as supplementary nutrition rich food or medicinal component. However, further study can validate each wild fruit for essential nutrients and minerals. These underexplored wild fruits have to be researched and conserved in natural habitats and if it will be possible, cultivate some of them for food security in future.

Author Contributions: RPS has surveyed the study area seasonally during 2015 to 2018 and also done the interview with local herbal healers. She has prepared initial draft of the manuscript. Later along with DKK she has finalize manuscript, interpreted and presented data after analysis to present form.

Conflict of Interest: Authors declared that there is no conflict of interest.

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Expression of Silk Sericin-Cecropin B Fusion Protein in *Pichia pastoris* and Cell-Free System

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ABSTRACT

Silk sericin has recently reported to be a promising biopolymer with wide applications in biomedicine and biotechnology. Combining sericin with other functional proteins could enable the development of novel biomaterials. Most of the functional properties of sericin, including its hydrophilicity and mechanical strength are attributed by the internal repetitive 38-amino acid motif of *Ser I* gene. Because of the unique repetitive sequence of sericin, recombinant expression of it in heterologous expression system is difficult and hence accomplished only in bacteria so far. In this study, we report an attempt to express sericin and sericin-cecropin B fusion using the cell-free and *Pichia pastoris* expression systems. Vector constructed using both the systems were successful as sericin and sericin-cecropin B recombinant proteins were expressed. SDS-PAGE followed by western blot demonstrated that the recombinant sericin-cecropin B protein of approximately 16 and 20 kDa were expressed in cell-free system and *Pichia*, respectively. However, the yield of the recombinant proteins was comparatively lower than that in bacterial expression system.

KEY WORDS: SILK, SERICIN, FUSION, EXPRESSION, PICHIA PASTORIS, CELL-FREE SYSTEM.

INTRODUCTION

Silk, the natural fibre with its lustrous appearance has been ruling the textile industry since centuries. The silk proteins, fibroin and sericin, with highly repetitive sequences and secondary β sheet structure exhibits environmental stability, mechanical strength, biodegradability and biocompatibility making them suitable candidates as biomaterial. Both the proteins have proven to be suitable in a range of applications, cosmetics, as dietary supplement, tissue engineering and biomedicine (Holland et al., 2019). However, obtaining these proteins from their natural source is a major concern due to drawbacks like batch-to-batch variation and impurities which often limits their applications. In this regard, recombinant silk proteins are desired which can be a source of well characterized, pure proteins that can meet the stringent needs in biomedical applications and also is non-destructive. Further, recombinant expression enables hybridization of the protein with active domains to generate multifunctional proteins.

Sericin, the unutilised by-product of the textile industry is a mixture of four glycoproteins, generated by differential splicing and secreted in the middle silk glands of *Bombyx mori* (Dong et al., 2019). Among them, Ser1 encoded by *Ser1*, consists of repeats of a 38-amino acid motif that contributes towards the hydrophilicity, mechanical

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strength and other functional properties (Teramoto et al., 2006; Huang et al., 2003). In the recent years, sericin has shown potential application as, a supplement in culture media, cryoprotectant, vehicle for drug delivery, metabolic effectors in organic systems, in wound healing and tissue engineering (Kundu et al., 2008).

Recombinantly expressed spider silk (Spidroin) and *B. mori* silk proteins in general have shown to be functional. Moreover, the chimeric proteins of spidroin and fibroin with enhanced features have also been generated and found applicable in tissue engineering (Aigner et al., 2018). However, reports on successful recombinant expression of sericin have been limited to two reports (Tsujimoto et al., 2001; Huang et al., 2003), using bacterial expression system, but no report of sericin fusion protein. Recently we have recombinantly expressed functional sericin and sericin-cecropin fusion proteins in bacteria (Thomas et al., 2020, a & b). In this study we explore the possibility of expressing these proteins in cell-free and *Pichia pastoris* expression systems for the first time.

MATERIALS AND METHODS

Vector constructions: The cloning of sericin and sericin-cecropin B is described in our earlier papers (Thomas et al 2020 a). Briefly, the PCR product sericin or sericin-cecropin B fusion flanking *SgfI* and *PmeI* were introduced in to pF25A ICE T7 Flexi vector (Promega) having the corresponding restriction sites, generating plasmids pS25A and pSC25A, respectively. For *P. pastoris* expression system, sericin alone and sericin-cecropin B fusion were amplified using the above plasmid as template with compatible primers flanking restriction sites (*MlyI, KpnI* and *FseI*) for insertion to pPink-LC vector (Invitrogen). The primers were as follows:

SerFw-5'GCGAGTCGCACTAGCACAGACCTGGCCAGG ATCCAGTACA3',SerF5'GGGGTACCTTAATGGTGATGA TGGTGATGACTATATCCTTCAGTGCTGCTACCAGC3' and CecRev-5'CGGCCGGCTTAATGGTGATGATGGTGATGTTT TCCTATAGCTTAGCCGAACC3'. Sericin sense primer was designed to create fusion product with *Saccharomyces cerevisiae* α-mating factor pre-sequence (with flanking 5' *EcoRI* and 3' *MlyI* compatible sites) for secreted expression. In both the constructs, a flanking region encoding six histidines followed by a stop codon was added at 3'end. All constructs were verified by DNA sequencing.

Cell-free expression: The reactions were set up in 1.5 ml microcentrifuge tubes as per manufacturer's protocol. The plasmids, pS25A and pSC25A, served as templates for in vitro transcription and translation. Individual reactions were setup using, 40 µl TNT T7 ICE master mix, 4 µg plasmid DNA template and milli Q to a final volume of 50 µl. Reactions were gently mixed and incubated at 28°C for 4 h and analysed.

***Pichia pastoris* expression:** The plasmids were linearized and used to transform *P. pastoris* (*Pichia* Pink, strain 2, Invitrogen) using the *Pichia* Easy Comp protocol according to the manufacturer's instructions. After transformation the entire mixture was spread onto *Pichia* adenine dropout (PAD) selection plates and incubated at 30°C for 3 to 10 days, until distinct colonies were observed. The expression vector without any insert was also transformed and served as control. The transformants (white colonies) were picked and the plasmid integration in the yeast genome was confirmed by PCR. Single colonies were inoculated into 2.5 ml of BMGY medium (Buffered Glycerol-complex medium, pH 6.0, Invitrogen) and incubated at 30°C with vigorous shaking until it attained log phase growth (A_{600}= ~3). These cultures were used to inoculate 50 ml of BMGY and cells were further incubated at 30°C with vigorous shaking until A_{600} reached 3.0.

To induce expression, cultures were centrifuged at 1,500 x g for 5 min at room temperature and the cell pellets were resuspended in 10 ml BMMY medium (Buffered Methanol-complex medium, pH 6.0, Invitrogen). The cultures were incubated at 22°C with vigorous shaking continuously till 96 h, with additions of methanol (1%) every 24 h. Cells were harvested every 24h by centrifugation at 1,500 x g for 5 min. The supernatant and pellets were separated and stored at -80°C till further processing. The cell pellets and supernatants containing recombinant proteins were processed according to the manufacturer's instruction.

Western blot: Purified protein samples were separated on a 15% SDS-PAGE and electrophoretically transferred (TE77X, Hoefer) onto polyvinylidene difluoride membranes (Hybond, GE). The His_6-tagged proteins were probed using mouse anti-histidine primary antibody (1:5000). The proteins were visualized using alkaline phosphatase conjugated goat anti-rabbit IgG and 4-nitro blue tetrazolium/5-bromo-4- chloro-3-indolyl phosphate (Western MAX, Amresco).

RESULTS AND DISCUSSION

The approximately 300 bp sericin amplicon (including flanking sites) of the *Ser 1* gene and the111 bp *Cecropin B* gene were cloned into the pF25A and pPink-LC vectors under T7 and AOX1 promoters, respectively, for cell-free and *Pichia* expression systems (Figure 1 A-D). All the inserts were confirmed by colony PCR and sequencing and found in-frame with c-terminal histidine tags. Transformation of the *Pichia* strain 2 with linearized

plasmids resulted in white colonies with a few pink ones (Figure 2). The chromosomal integration of the heterologous expression cassettes in *P. pastoris* was confirmed by PCR using genomic DNA as template (Data not shown). These results show the error-free vector constructions and successful integration of the insert into *Pichia* genome.

Figure 1: Expression vector designs for sericin and sericin-cecropin B. A & B: Cell-free expression plasmid, pF25A, C & D: *Pichia* pink, pPink-LC, P: polyhedron 5'UTR, Amf: yeast alpha mating factor.

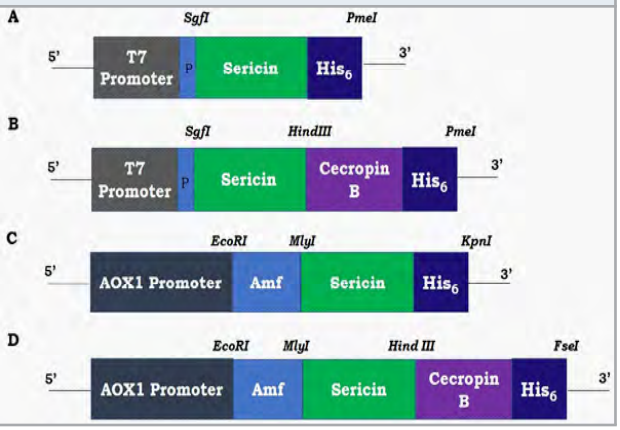
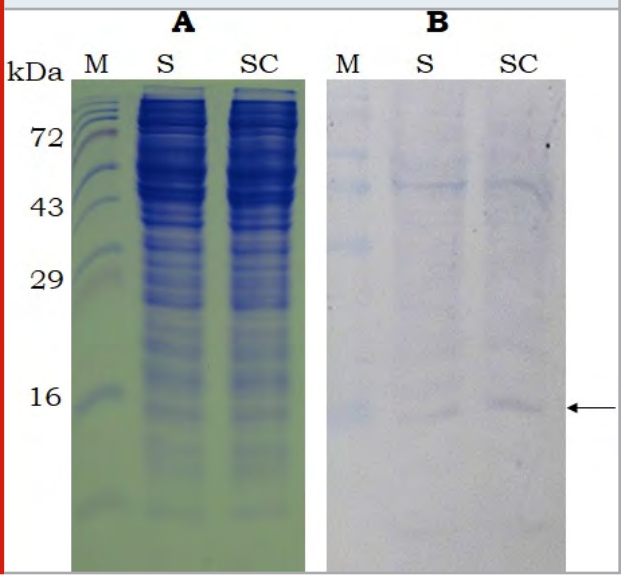


Figure 2: Colonies of *Pichia pastoris* Pink strain 2 transformed with linearized pPink-LC having sericin-cecropin B on PAD agar plate. White colonies represent cells transformed with recombinant plasmid and dark pink represent colonies without inserts.

SDS-PAGE and western analysis show the expression of sericin and sericin-cecropin B fusion proteins in both the expression systems. As observed in figure 3B, the western blot analysis of recombinant proteins expressed using cell-free expression system showed band at ~ 16 kDa for both sericin and sericin-cecropin B. However, the expression of recombinant proteins was not visible in the SDS-PAGE analysis. The effect of secreted expression (proteins extracted from culture supernatants) was analysed for various time points up to 72 h in *Pichia*. As indicated in figure 4B, the candidate protein bands were not visible up to 48 h but visible only at 72 h post-induction. No bands were visible after 72 h (Data not shown). Protein extracted from cells did not show the presence of recombinant proteins at any time points (Data not shown). Western blot showed a band of approximately 20 kDa for both proteins at 72 h. No band was observed in control having proteins extracted from *Pichia* transformed with empty vector.

Figure 3: SDS-PAGE (15%, A) and Western-blot (B, using anti-histidine antibody) analysis of the recombinant sericin and sericin-cecropin B proteins expressed using cell-free expression system. Proteins were collected after 4 h of incubation. M- Protein marker (Puregene), S- sericin, SC- sericin-cecropin B. Arrow indicates target band of ~ 16kDa.



The theoretical molecular masses and pI for sericin and sericin-cecropin protein are 9.77, 9.87 (4.73, 4.93) and 14.23, 14.04 (6.57, 6.96), respectively, in cell-free and *Pichia* vector constructs. The apparent molecular mass as noted with western blots is slightly higher than that of the calculated molecular masses in both sericin and sericin-cecropin proteins. This may be due to the unique repetitive amino acid sequence of sericin and pI of sericin and cecropin resulting in reduced electrophoretic mobility. This is in agreement with reports on recombinant sericin expressed in bacteria by Tsujimoto et al., 2001; Huang et al., 2003 and our study (Thomas et al., 2020a). Similarly, a reduction in electrophoretic mobility was observed with genetically engineered spider silk-dentin matrix protein 1 expressed in bacteria (Huang et al., 2007). The reason for the high

molecular mass of recombinant proteins in *Pichia* may be due to the post-translational modifications, especially glycosylation. Compared to the bacterial expression of sericin and sericin-cecropin B (~1.25 mg/L, Thomas et al., 2020), the level of expression of both proteins was much lesser in the present study as observed by faint bands in western blots. Unlike cell-free system, which has the limitation of producing recombinant proteins in large quantity, *Pichia* expression system can be improved by optimising culture conditions and use of fermenter. Hence, further work is required for high yield of recombinant sericin or its fusion proteins in *P. pastoris* expression system.

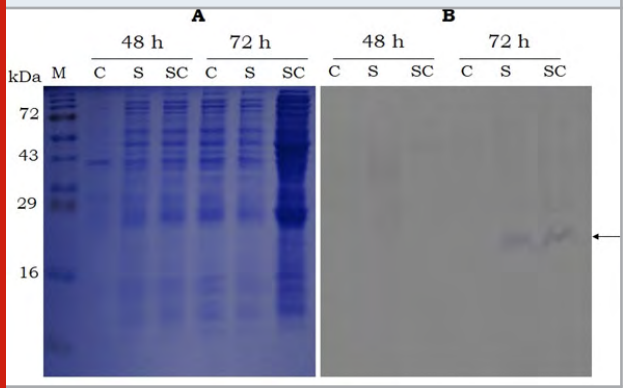


Figure 4. SDS-PAGE (15%, A) and Western-blot (B, using anti-histidine antibody) analysis of the sericin and sericin-cecropin B proteins secreted by *P. pastoris* (strain 2) in to the growth medium after 48 and 72 h of induction. M- Protein marker (Puregene), C- control (proteins from *P. pastoris* harbouring empty vector), S- sericin, SC- sericin-cecropin B. Arrow indicates target band of ~ 20kDa.

The production of recombinant spider silk proteins using various heterologous systems, including *P. pastoris* have been reported. The synthetic gene *DP-1B*, based on the partial amino acid sequence patterns of the major dragline silk protein spidroin 1 and functionalized spider silk fusion protein Z-4RepCT, were expressed in *P. pastoris* (Fahnestock and Bedzyk, 1997; Jansson et al., 2016). Further, the *in vitro* translation of synthetic genes based on MaSp 2, spider dragline silk protein, using *E. coli* S30 extract have also been reported (Lewis et al., 1996). However, till date there is no report on recombinant expression of sericin or its fusion proteins in *P. pastoris* or cell-free systems. Our results show the possibility of recombinant expression of difficult-to-express proteins like sericin or its fusion in heterologous expression systems.

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