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EDITORIAL

NANOBIOLOGY IS THE FUTURE!

Biological systems are inherently nano in scale. Unlike nanotechnology, nanobiology is characterized by the interplay between physics, materials science, synthetic organic chemistry, engineering and biology. Nanobiology is a new discipline, with the potential of revolutionizing medicine: it combines the tools, ideas and materials of nanoscience and biology; it addresses biological problems that can be studied and solved by nanotechnology; it plans ways to construct molecular devices using biomacromolecules; and it attempts to build molecular machines utilizing concepts seen in nature. Its ultimate aim is to be able to predictably manipulate these, tailoring them to specified needs (1). Inspection of the protein structure database illustrates the breadth of scaffolds, shapes and properties that protein molecules and their building blocks can provide. Nanobiology is a field where interdisciplinary collaborations are essential and disciplines converge (2), which should enable the quantitation, leading to a better understanding of the regulatory networks within cells and between cells of an organism. These networks dictate how a cell responds to external stimuli, which in turn activates signalling cascades. It should allow the addressing of a broad range of questions on the structure and function of the cytoskeleton; the nuclear envelope; signal transduction by membrane embedded receptors; the nanomechanical properties of the extracellular matrix; nuclear transport; and voltage induced channel gating (3).

Nanobiology, as a field of study between nanotechnology and biology, encompasses a wide range of research topics that can be divided into two basic categories: 1. nanotechnologies applied to biological systems, and 2. the development of biologically inspired nanotechnologies. It acts as the merger of biological research with nanotechnologies such as nanodevices, nanoparticles, or unique nanoscale phenomena with wide range of applications in areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, single electron transistors, light emitters, nonlinear optical devices, and photo-electrochemical applications. Moreover, selection of solvent medium and selection of eco-friendly, nontoxic reducing and stabilizing agents are the most important issues, which serves as an imperative technique in the development of environmentally and economically friendly processes (4-9).

Although molecular biologists have been working with nano-sized biomolecules (Nano is a unit prefix meaning "one billionth"), for the last few decades, nanobiology was not defined as a discipline until researchers started making a focused effort to use our knowledge of nanotechnology in tackling biological problems. Nano-biological structure and system research mainly focuses on using nanotechnology to detect, measure, or probe biological systems. For instance, nanotechnology can be used to create nanochips or nanopatterned devices to screen large number of biological targets. Because of the small size of these systems, researchers can use smaller sample sizes, perform faster analyses, or use smaller amounts of expensive chemicals and reagents. In addition, unique physical phenomena at the nanoscale can be harnessed for sensing, detection and analytical purposes (10).

To sum up, advanced research in nanobiology can be a promising future to scientific world because of its various applications, but still there are some health hazard concerns due to their uncontrollable use and discharge to natural environment, which should be pondered to make it more suitable and environmentally responsive.

References

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Review articles

A novel biomimetic approach in re-mineralizing enamel and dentine - A review

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ABSTRACT

This study aims at reviewing the various methods on biomimetic remineralization of enamel and dentine. Remineralization of the dentin that is demineralized is important for improving dentin bonding stability and controlling primary and secondary caries. Remineralization of enamel plays a crucial role in the progression of carious process and the management of early caries lesion. This has implications for the management of non-caries tooth loss resulting from dental erosion, attrition, and abrasion. Nevertheless, conventional dentin remineralization strategy is not suitable for re-mineralizing completely demineralized dentin within hybrid layers created by etch-and-rinse and moderately aggressive self-etch adhesive systems, or the superficial part of a caries-affected dentine lesion left behind after minimally invasive caries removal. Biomimetic remineralization represents a different approach to this problem by attempting to backfill the demineralized dentin collagen with liquid-like amorphous calcium phosphate nano-precursor particles that are stabilized by biomimetic analogues of non-collagenous proteins.

Keywords: Biomimetic; dentine; remineralization; enamel; calcium; phosphate.

INTRODUCTION

The human tooth is composed of two layers, the outer layer enamel and the inner dentine-enamel complex (1). Enamel is the hardest mineralised tissue composed of about 95% of mineral content (2). The enamel is composed of Hydroxyapatite crystals of nanoscale level (3). Amelogenin secreted by ameloblasts, are unique set of proteins found in enamel during its formation. The ameloblasts undergo apoptosis after the enamel is formed (4). Processes like remineralization and demineralization coexist during the entire life in the teeth. In pathological conditions, demineralization outweighs remineralization; Dental hard tissue, both the enamel and the underlying dentin, cannot self-heal if damaged. These are treated with synthetic rigid restorative materials. However, this demands removal of healthy surrounding tooth tissues. They have their own disadvantages like hypersensitivity to the dentin, interface microleakage between filling materials and dental tissue. These have also resulted in financial problems an example being that in the U.S., more than 100 million dollars is spent annually on dental service (5). Currently it is difficult to remineralize remaining demineralized dentine. Dentine is more difficult to remineralize than enamel. This could be due to the fact that remineralization occurs by growth of residual crystals in the lesions.

Biomimetic approach

The biomimetic process is to regenerate destroyed enamel and it is an emerging field in regenerative dentistry and the subject of the work of several research groups (6).

Remineralisation of dentine

Dentine remineralisation is a lot of complicated and fewer effective than enamel remineralization as a result of their square measure residual seed mineral crystals on enamel, however these square measures absent in dentine lesions (7,8). It was reported that underneath a similar re-mineralizing condition, remineralization occurred on the surface of acid-etched enamel however not on the surface of acid-etched dentine (2). This difference could be attributed to the fewer amounts of residual mineral crystals and the exposure of organic matrix on the acid-etched dentine surface. Biomimetic remineralization, a technique that imitates the process of mineralization, represents a special approach to the current downside by making an attempt to backfill the demineralized dentine scleroprotein with liquid-like ACP nano-precursor particles. This does not rely on seed crystallites. This paper may be a systematic review of the various revealed ways that with success achieved biomimetic remineralization of human dentine further because the enamel which might be mentioned later. It is a known fact that the collagen matrix serves as a scaffold for crystal deposition but does not provide a mechanism for nucleation of hydroxyapatite. The biomineralization process is usually modulated (9) by a series of NCPs (non-collagenous proteins). In dentine biomineralization, NCPs with a high affinity for Ca ions and albuminoid
fibrils square measure to blame for control the nucleation and growth of minerals, like dentine matrix super-molecule (DMP1) and dentine phosphophoryn (DPP, DMP2). These NCPs can regulate biomineralization of dentine in vivo by working as nucleator or inhibitor. But it is difficult to extract and purify natural NCPs. Thus, many researchers focus on developing the analogues that can play the role of NCPs in the biomineralization process. NCP analogues like Polyacrylic acid (PAA) and polyvinylphosphonic acid (PVPA) were used in the biomimetic mineralization of dentine. PAA may act like the calcium phosphate binding sites of DMP1, and PVPA simulates the collagen-binding function of DMP1 in guiding the nano-precurors’ recruitment to the collagen matrix. STMP, which is used as a chemical phosphorylating reagent in the food industry, can absorb type I collagen via an electrostatic mechanism and a chemical phosphorylation mechanism. The phosphorylated dentine collagen matrix act as a template-molecule to attract ACP nano-precurors and to nucleate apatite within the collagen fibrils resulting in the formation of intrafibrillar and interfibrillar remineralization of dentine. Apart from the PVPA and STMP, phosphorylated chitosan, peptide/oligopeptide, and PAMAM dendrimer also functioned as the template-anaologues for the biomimetic re-mineralization of dentine collagen matrix.

Silva classified the synthesis of biomaterials as top-down or bottom-up approaches (10). The top-down approach or classical ion-based mineralization strategy occurs by epitaxial growth over existing seed crystallites, which cannot occur by spontaneous nucleation of minerals on the organic matrix, such as demineralized dentine. In contrast to the top-down approach, the bottom-up approach starts with one or more defined molecular species, which undergoes certain processes that result in a higher-ordered and organized structure. This non-classical particle-based crystallization pathway involves a multistage process. The calcium and phosphate ions would self-assemble into prenucleation clusters. These prenucleation clusters would aggregate into amorphous ACP Nano-precurors in the presence of NCP analogues. Then these precursors penetrate into the gap zones of collagen fibrils and further grow into apatite crystals along the intrafibrillar space of collagen. The intrafibrillar remineralization of collagen fibrils would lead to inter-fibrillar remineralization between adjacent collagen fibrils.

**Remineralisation of enamel**

The critical pH-value for enamel dissolution is around 5.5. Depending upon the amount of phosphate and calcium ions in plaque and saliva; the solubility of the enamel changes. Supersaturation of the saliva is generated by the abundance of phosphate and calcium; and thus, the HAP constitutes the enamel. By means of precipitating calcium phosphate, the enamel surfaces are re-mineralized. However, this remineralization from the saliva cannot resemble the enamel’s complex microstructure.

Fluorides are for the rescue of such issues. Fluoride compounds like Sodium Fluoride (NaF) or Stannous Fluoride (Sn (II) F2) are currently the most prominent remineralization systems in oral care (11). Fluoride ions, F-, are believed to enhance the natural enamel remineralization process and to inhibit de-mineralization (12).

Caries preventive property of topically applied fluorides is based on accelerating the reintegration of calcium phosphate mostly derived from saliva into demineralized surface lesions at the tooth-bacterial biofilm interface. However, exposure to extraordinary high fluoride levels can cause side effects like fluorosis and bone weakening. Therefore, the concentration of fluoride in oral care creations is strictly regulated worldwide. For example, in the EU, toothpastes are classified as cosmetic products and a maximum of 1500 ppm fluoride is allowed, and on average toothpastes with fluoride usually sold over the counter contain levels of around 1000 ppm (13). While the safety of the proper use of fluoridated toothpastes has been firmly established by numerous studies, dosage and toxicity aspects always have to be considered, particularly in children, where fluoride overdosing may result in the manifestation of mottled enamel or other signs of chronic fluorosis (14, 15).

Fluorides require salivary calcium and phosphate ions to improve the natural remineralization. Hyposalivation therefore, a common problem in elderly subjects, may significantly impair the preventive efficacy of topically applied fluorides (16). For xerostomia patients (e.g. induced by medications), oral care goods with the addition of calcium and phosphate are suggested in order to recompense a calcium phosphate deficiency (17). To overcome these drawbacks, increasing attention is given to the development of alternative non-fluoride agents that improve remineralization without having any possible side effects on the human body (18,19). In the recent years, biomimetic concepts along these lines have been developed in oral care with the aim to address these issues.

**Hydroxyapatite (HAP)**

Out of all calcium phosphate phases, HAP, Ca$_5$(PO$_4$)$_3$(OH), has the highest similarity to the natural enamel (20,21). It also has the lowest solubility of all calcium phosphates. HAP is synthesized in different
crystallite morphologies and particle sizes, i.e. from nanometre to micrometre size. Commonly used HAP particles in oral care applications are organized in micro clusters. Synthetic HAP particles were shown to interact both with enamel and dentin surfaces where they can unfold their effects such as the reduction of initial bacterial colonization. HAP particles show equivalent performance compared to the standard use of fluorides in oral care.

B-Tricalcium phosphate (B-TCP)

There are two different tricalcium phosphates, Ca$_3$(PO$_4$)$_2$, known, both of which cannot be found in pure form in nature (22). While α-tricalcium phosphate can only exist at high temperatures (above 1125 °C), β-TCP is stable at room temperature. β-TCP shows merely a moderate solubility in water (25 mg/L at 25°C). The size of β-TCP powders differs depending on the milling-procedures, but mostly ranges between 0.01-5 µm. β-TCP is the bio form of tricalcium phosphate that is used in inventions for medicine and oral care.

Re-mineralization of corroded enamel is performed by calcium and phosphate originating from saliva. By using β-TCP, the concentration of calcium in the saliva can be increased (β-TCP is soluble at pH < 6). An in vivo study showed an increase of calcium in the saliva as consequence of acidic attacks and an acidic plaque-pH, when 2.5% β-TCP is used as additional compound in chewing gums. Compared to the control (conventional gum, without an additional calcium source), the pH increased (buffering of acidic attacks) as well as the concentrations of free calcium and free phosphates. Both can be used for remineralization, when the enamel is damaged by acidic attacks. This study also showed a deposition of β-TCP in plaque and saliva becoming available as soon as an acidic attack occurs (23, 24).

Amorphous calcium phosphate (ACP)

Amorphous Calcium Phosphates (ACP), Ca$_x$(PO$_4$)$_y$·n H$_2$O, are mostly synthesized from the aqueous precipitation of calcium phosphates. ACP mostly occurs as spherical particles with an average diameter of 20-200 nm that can be visualized by SEM. It is suggested that ACP has apatite-like structures. ACP is well studied and largely combined with Casein Phospho peptides (CPP), which stabilize calcium in aqueous solutions. CPP is a natural peptide which can be obtained from bovine milk by tryptic digestion of casein containing the protein-sequence Pse-Pse-Pse-Glu-Glu. Furthermore, CPP has been shown to increase the plaque-pH due to enzymatic breakdown of casein and a resulting increase of ammonia.

ACP seem like to be a promising challenger for re-mineralizing initial caries lesions and enamel corroded by acidic assaults, especially if applied directly before, during or after acid-intervention. However, the conditions of ACP synthesis/precipitation and consequently the use in toothpastes, mouth rinses and chewing gums need to be standardized to have the same quality in each application.

Calcium phospho silicate (CSPS)

Calcium phospho silicate (CSPS) is a bioactive glass comprising 45% SiO$_2$, 24.5% Na$_2$O, 24.5% CaO and 6% P$_2$O$_5$. This mineral was originally developed as bone-regenerative material due to its high bio-compatibility and the ability to release calcium and phosphate ions. Besides occluding dentinal tubules and consequently desensitizing effects of this calcium phosphor silicate, there are also studies describing remineralizing potential as well as caries prevention and antiplaque characteristics. The active mechanism seems to be the delivery and deposition of calcium- and phosphate-ions that form a crystalline carbonated-apatite layer.

Tai et al., investigated the Plaque Index (PLI) and Bleeding Index (BILI) within a study with 100 subjects in a RCT over a six-week time period (25). In this study, CSPS was tested against a placebo-control and showed significant improvement of oral health measured by a reduction of PLI and GBI. Nevertheless, the mode of action was not clarified by this study.

Additionally, two in situ studies both from Parkinson et al., in 2017 used sodium mono-fluorophosphate with different concentrations as positive control and 5% CSPS as test dentifrice (26). They also utilized different concentrations of sodium mono-fluoro-phosphate in both studies (ranged from 0% to 0.15%). The authors conclude in both the studies no detectable developments of 5% CSPS (alone or in combination with sodium mono-fluorophosphate) compared to the positive (0.15 ppm sodium mono-fluorophosphate, 0% CSPS) or the negative control (0 ppm sodium mono-fluoro-phosphate, 0% CSPS). CSPS as active compound in toothpastes seems to act as a calcium-reservoir that can be used for remineralization of demineralized enamel or dentine. Clinical studies with CSPS alone (not in combination with any fluorides) are needed to test the outcome for caries prevention in vivo. Plaque reduction and clear improvement of oral (gingival) health can be noticed, when CSPS is used. In-situ studies were able to show non-inferiority to fluorides in case of re-mineralization.

Unlike the bio-glass explained above, which required the addition of fluoride compounds such as sodium monofluorophosphate into the toothpaste formulation,
a fluoride-containing bioactive glass was recently introduced as a caries re-mineralizing and preventive additive in toothpastes. Fluoridated bioglass (f-BG) has fluoride, strontium, potassium and zinc incorporated within the glass itself, thus enabling simultaneous delivery of $\text{Sr}^{2+}$, $\text{Ca}^{2+}$, $\text{PO}_4^{3-}$ and $\text{F}^-$ ions into the initial caries lesions to promote remineralization by formation of a partially fluoridated crystal structure, zinc ions for bactericidal function, and potassium as a desensitizing agent. Having the fluoride incorporated within the glass prevents the risk of premature reaction of fluoride and calcium ions to Calcium Fluoride ($\text{CaF}_2$), which reduces the bioavailability of the two ions. However, the lack of clinical studies does not permit any firm conclusions on their effectiveness.

**Calcium glycerophosphate (CGP)**

Calcium Glycerophosphate, $\text{C}_3\text{H}_3\text{CaO}_4\text{P}$, (CGP) is a salt of glycerol phosphoric acid. It is typically used as a food ingredient and a nutrition supplement. The first studies from 1972 evaluating the cariostatic impact of this organic calcium phosphate by Bowen used CGP as a nutrient supplement. Brook et al., (27,28) used CGP as a nutrient supplement too. Within a cohort of 14 children, the mean plaque levels were estimated between a group of children receiving 1% CGP 4 times daily and a group without any specific treatment. While plaque levels were increased in the experimental group, $\text{Ca}^{2+}$ was not different. An even smaller study sample (n=8) did not clean their teeth for 18 days and rinsed with a 50%-sucrose solution. Optical changes were characterized as demineralization and could not be reduced by the addition of CGP (1%). Even topical applications of sodium fluoride (2%) were not able to inhibit these changes. Another study determined the accumulation of CGP in the dental plaque by having three different mouth rinse-interventions: (1) No CGP, (2) 0.5% CGP and (3) 1.5% CGP. The concentration of phosphate was significantly greater in the plaque in (3) compared to (1), indicating a higher potential for buffering acidic attacks.

**DISCUSSION**

Hydroxyapatite, in enamel and in bone, is accountable for the mechanical behavior of the calcified tissues. Unlike bone, when the enamel hydroxyapatite is dissolved or scratched, it cannot spontaneously re-mineralize, because enamel contains no cells. Biomimetic carbonate hydroxyapatite (CHA) nanocrystals have been synthesized with a stoichiometric Ca/P molar ratio of around 1.7 $\pm$ 0.1, containing 4 $\pm$ 1 wt.% of carbonate ions, prevalently replacing phosphate groups, while 1% of $\text{Ca}^{2+}$ions are substituted by Zn$^{2+}$. The nanostructured Zn-CHA microcrystals, made in laboratory according to the original methodology, represent the active component of the experimental Zn-CHA toothpaste. The micrometric dimension of the crystal clusters allows avoiding any suspicion about the *in vivo* utilization of Nano-dimensioned particles. Nevertheless, the nanostructured surface of the micro clusters is responsible for the high surface area that is crucial for their chemical reactivity.

Synthesized biomimetic CHA nanocrystals and human enamel apatite not only contain a similar carbonate amount, but also have been shown to promote carbonate substitution to the phosphate and/or hydroxyl group, which is very similar to the synthetic and biological CHA nanocrystals. The synthetic experimental CHA nanocrystals have a plate-like morphology and a structure very close to that of the enamel, dentine the use of a toothpaste containing Zn-substituted CHA nanocrystals can produce a biomimetic coating on the enamel surface, thus mimicking the composition, structure, morphology and surface reactivity of the biological enamel hydroxyapatite. the other hand, the re-mineralizing/repairing effect of the enamel surface treated using synthetic nanostructured CHA microcrystals is consistent with a mineral biomimetic apatitic deposition, which does not alter the chemical-physic properties of the enamel. The biomimetic CHA coating can appear of different thickness, probably due to the underside different enamel surface morphology, which can change in function of the degree of enamel damage. However, the EDAX analysis reveals that the Ca/P molar ratio of CHA crystals (about 1.7) is homogeneously constant on the enamel surface. This finding assures a uniform enamel protection against the enamel wear and loss phenomena, thus preventing dentine exposure. Results of the first clinical randomized trial by Orsini et al., (2010) have already demonstrated the efficacy of Zn-CHA toothpastes in reducing DH (29). Moreover, a further very recent randomized trial by the same authors showed that this effect could be exerted after only 3 days of treatment (30). The results of this *in vivo* morphological and chemical-physic study might in part explain the beneficial effect of Zn-CHA toothpastes in reducing DH, since the deposition of a synthetic nanostructured CHA microcrystals-rich coating could lead to a re-mineralizing/repairing effect of the enamel surface, in the teeth treated using Zn-CHA toothpaste. Therefore, the principal finding of this study is that: the re-mineralizing mechanism of the nanostructured CHA microcrystals, largely documented by previous *in vitro* reports can be also confirmed *in vivo*. Moreover, it can be suggested that this synthetic CHA deposition mainly occurs on the enamel areas characterized by enamel loss and/or damage (probably due to erosion effects), thus being considered as a real enamel repair.

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contrast, the use of a toothpaste containing KNO3/NaF may form only a deposition, consisting of silica, as an abrasive phase on the enamel surface, which, however, does not remineralize the damaged enamel area, but it is generally deposed in correspondence of natural concavities proper of the natural tooth morphology. Furthermore, no deposition on the enamel surfaces has been observed after treatment using the fluoride and the KNO3/NaF toothpastes, which may lead only to a partial substitution of the hydroxyl groups with fluoride ions in the native enamel hydroxyapatite. The CHA formed coating is generally insoluble in physiological mouth pH, but it may undergo to solubilization when, for instance, a bacterial biofilm covers the teeth and its products decrease the pH value. During the CHA coating solubilization, Ca ions, phosphates, and Zn ions are released, allowing the Zn to exploit a strong antibacterial effect, which interferes with the plaque formation, thus preventing further solubilization processes of the newly deposited Zn-CHA coating. Therefore, it may be suggested that the coating formed by Zn-CHA toothpastes may exploit not only a remineralizing effect of the dental surface, but also a beneficial effect toward bacterial plaque attacks. The main limitation of this work is that the in vivo remineralizing effect exploited by the Zn-CHA toothpaste was morphologically demonstrated only on the enamel surfaces, since the analysed extracted teeth did not present areas of dentine exposition. Therefore, further studies will be carried out in vivo to analyse whether a stable biomimetic CHA deposition and a repairing mechanism can be demonstrated also in dentinal surfaces. In conclusion, the present study shows that only the use of a toothpaste containing Zn-substituted CHA nanocrystals can produce a biomimetic coating on the enamel surface, thus mimicking the composition, structure, morphology and surface reactivity of the biological enamel hydroxyapatite.

CONCLUSION

The human tooth, though being a hard structure is subject to damage and erosion resulting from dental caries. Once the tooth enamel and dentine are eroded, it does not heal naturally. Dentine remineralization is more complex and less effective than enamel remineralization because there are residual seed mineral crystals on enamel, but these are absent in dentine lesions (7, 8). Biomimernalization process is an organic, matrix particle-mediated, non-classical crystallization pathway. Biomimetic remineralization, a methodology that imitates the natural process of mineralization, represents a different approach to this problem by attempting to backfill the demineralized dentine collagen with liquid-like ACP Nano-precursor particles. This bottom-up remineralization strategy does not rely on seed crystallites. Biomimernalization process is usually modulated by a series of NCPs (9), it is difficult to extract and purify natural NCPs. PAA and PVPA were used as the NCP analogues in biomimetic mineralization of dentine. The phosphorylated dentine collagen matrix functions as a template-molecule to attract ACP Nano-precursors and to nucleate apatite within the collagen fibrils resulting in the formation of intrafibrillar and interfibrillar remineralization of dentine. Different concentrations (20%–37%) of PA were also used to expose the dentine collagen matrix. It is suggested that treatment of dentine with 37% PA for less than 1 min does not denature the dentine collagen matrix. In the presence of NCP analogues, these prenucleation clusters aggregate into amorphous ACP Nano-precursors. Fluoride-based dentin remineralization strategies also result in hyper-mineralization of the lesion surface and prevent effective remineralization of the deeper parts of the carious lesion. The remineralisation of the enamel; Supersaturation of the saliva is generated by the abundance of phosphate and calcium; and thus, the HAP constitutes the enamel. By means of precipitating calcium phosphate, the enamel surfaces are remineralized. When the phosphate and calcium ions pass through the protein rich pellicle layer and reach the enamel surface; remineralization occurs. Fluorides require calcium and phosphate ions from saliva to improve the natural remineralization process. Non-fluoride agents that improve remineralization. Out of all calcium phosphate phases, HAP, Ca₅(PO₄)₃(OH), has the highest similarity to the natural enamel. Others are β-TCP, ACP, Cax(PO₄)₉ · nH₂O, are mostly synthesized from the aqueous precipitation of calcium phosphates. The structure of ACP is mostly nanocrystalline, i.e. it has a short-range order of very small dimensions in the range of a few interatomic distances; ACP seems to be a promising candidate for remineralizing initial caries lesions and enamel eroded by acidic attacks, especially if applied directly before, during or after acid-intervention. CSPS as active compound in toothpastes seems to act as a calcium-reservoir that can be used for remineralization of demineralized enamel or dentine.

Many attempts at re-mineralization with fluorides have been made in this regard, but it turns out that this method requires special conditions, and the process of remineralization is too long, making it unsuitable for application in clinical settings. Ca(OH)₂ has also been used for the same purpose, in both in vitro and in vivo experiments, and its re-mineralizing properties have been demonstrated. However, its effect on collagen fibres has not been fully investigated. One group of researchers dealing with deep carious lesions used an
innovative approach, applying clusters of ACP, which have outstanding re-mineralizing potential for collagen (2). An analogue was made with PAC and PASC which stabilize ACP, because of their ability to chelate calcium ions (property due to their many carboxyl groups), and the composition of carboxymethyl chitosan was chosen. This derivative of chitosan also has many carboxyl groups, which may delay or inhibit the spontaneous precipitation of calcium phosphate and have chelating capabilities. The carboxymethyl chitosans recommended as an indirect pulp agent in combination with ACP. Moreover, it can be converted into a matrix (scaffold) through lyophilization for further remineralization. Furthermore, the authors focus on its biological compatibilities and degradation, antibacterial properties and lack of toxicity. These examples of the application of dentin remineralization prove that this relatively new approach can be quite promising in various areas of clinical application. This is so because biomimetic nanomaterials are used. They are completely new as a concept, and thanks to their nanostructure, they come close to natural tooth tissues. However, in order for them to be implemented clinically and for practical recommendations to be offered, fundamental research studies on dentin and enamel remineralization and regeneration are still needed.

CONFLICTS OF INTEREST: There are no conflicts of interest.

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A review on the influence of insertion torque on the primary stability of mini implants in orthodontics

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ABSTRACT

Absolute anchorage is an integral part of successful orthodontic treatment and mini implants have been used widely in orthodontic practice to provide absolute anchorage. The primary stability of these implants is influenced by various factors and one among them is insertion torque, which represents the resistance offered by bone during implant placement. The purpose of this review is to summarize the influence of insertion torque on the primary stability of mini implants used in orthodontics.

Keywords: Implant design; insertion torque; mini implants; primary stability.

INTRODUCTION

Absolute anchorage is commonly used nowadays for successful orthodontic treatment. Osseo-integrated implants cannot be used for orthodontic purposes due to certain limitations such as cost, extensive surgical approach and increased time (1-4). Therefore, mini implants are used commonly in orthodontics as anchor units (4-6). Mini implants are also known as micro-screws and are used as temporary anchorage devices. The major advantage of these implants in the field of orthodontics is due to its ease of use and insertion, and its ability to provide absolute anchorage.

There are many factors, which determine the success of mini implants. The screw factors include screw diameter, length, pilot hole diameter and screw thread form (7). Patient related factors which affect the stability of these implants includes, cortical bone thickness, quality of bone, bone density, site of placement, anatomical landmarks, systemic diseases and placement factors include insertion torque, punch hole and loading of mini implants. In order to improve the stability of mini implants these factors should be considered before placement (1).

One of the factors that influence the primary stability of mini implants to a greater extent is the insertion torque. It represents the resistance offered by the osseous tissue during implant placement (7, 8). It is used to evaluate the mechanical stability of implants. Studies conducted earlier have shown that a certain value of insertion torque is necessary to obtain primary stability of mini implants. An insertion torque between 30Ncm – 50Ncm is the reported range between which mini implants have to be placed to obtain primary stability (8). An increase or decrease in insertion torque compromises the primary stability of these mini implants (7, 8).

Insertion torque

Insertion torque results from frictional resistance between the screw thread and its surrounding bone and is a standard parameter to evaluate mechanical stability of mini implants (9). Maximum insertion torque is expressed in Newton centimeters (Ncm) which is the maximum torque value recorded during the insertion of orthodontic mini implants. The stability of implants can be divided into primary and secondary. The former is mechanical stabilization achieved immediately after insertion, and the latter is attained when new bone forms at the implant interface (10). To achieve initial stability, a certain level of maximum insertion torque is necessary (7, 10). However, excessive stress to the bone can cause necrosis and local ischemia and might impede osseointegration and hence secondary stability (7, 11). The orthopedic literature has shown that over tightening can damage and cause stripping of the bone, and this can lead to diminished holding strength (12) with losses in pullout strength up to 40% to 50% (13). To control excessive stress during the insertion of orthodontic mini-implants, torque ratchets or torque sensors have been developed. Maximum insertion torque values in the range of 5 to 10 Ncm have been presented as the gold standard in several articles (7, 11). Clinicians want to know whether specific maximum insertion torque values are associated and if a range of safe torque levels can be identified to attain maximum stability.

The aim of this review is to emphasize on the influence of insertion torque on the primary stability of mini implants in orthodontics.
Factors that influence insertion torque required for primary stability of mini screws

The following factors influence the maximum insertion torque required for the stability of mini implants. These broadly include patient factors, screw factor and technical or procedural considerations. The patient factors include the age, sex, density of bone, the site of placement of mini screws. The screw factors that influence insertion torque include the length, diameter, shape, surface treatment, threads and thread design. The procedural factor includes self-drilling and self-tapping technique of implant insertion.

Age

Motoyoshi et al., found significantly higher insertion torque values with increasing age (14). This was expected, because bone densities in adolescents are lower than those of young adults. Older individuals have increased bone density than young adults due to increased mineralization and calcium deposition. Due to an increase in the density of bone, the amount of force required to insert the mini implant also increases proportionally (14). However, in another article from the same research group, no differences in the insertion torque values were recorded between age groups. These opposing associations were both rejected, because variables related to patient and location were not controlled. Another study conducted by Motoyoshi et al, states that there is a reduction in the insertion torque as the age advances. This is influenced by the factors and conditions like osteoporosis, hypocalcaemia, vitamin D deficiency that is usually prevalent in older individuals (15,16). Hence, there still lies a controversy into determining if insertion torque is influenced by age.

Sex

Evaluation of changes in insertion torque between males and females, proved to be not significant in a study conducted by Motoyoshi et al., In his study he described that sex of an individual does not affect the insertion torque (15).

Bone Density

One important factor that determines the success of mini implants is the thickness or density of the bone into which these mini implants have been placed. According to Misch et al., bone can be classified into 5 types or classes depending on the density of the bone (2). D1 indicates dense cortical bone, D2 indicates Thick dense to porous cortical bone on crest and coarse trabecular bone within, D3 indicates Thin porous cortical bone on crest and fine trabecular bone within, D4 indicates Fine trabecular bone and D5 indicates immature, non-mineralized bone. Insertion torque is directly proportional to the density of the bone. The denser the bone is, the more is the insertion torque. Insertion torque increases as the density of the bone increases. In studies conducted by Motoyoshi et al., he concluded that there is a significant correlation between cortical bone thickness and placement torque in the maxilla. In contrast, no correlation was found between cortical bone thickness and placement torque in the mandible. But in contrary, Motoyashi et al., has disproved his own study results wherein another study he had concluded that the insertion torque is maximum in mini implants placed in the mandible than the maxilla. This is due to the increased density and thickness of the cortical bone in the mandible when compared to maxilla. However, these variables where also limited due to certain factors such as location of placement of mini implants, varied bone drills and uncontrolled patient related factors (8, 14, 15). In Orthodontics, these mini implants have been used as anchorage units during active treatment period and hence it is important that the mini implant is stable throughout the course of procedure. This can be achieved if the primary stability is adequate which is influenced by the density of bone and the torque required for mini implant placement.

Site of implant placement

The most common sites for placement of orthodontic mini implants are the buccal alveolar processes of maxilla and mandible and the mid palatine suture. Table 1 gives the maximum insertion torque as suggested by Reint et al., (16).

<table>
<thead>
<tr>
<th>Site</th>
<th>Maximum insertion torque</th>
</tr>
</thead>
</table>
| Alveolar bone in maxilla and mandible and in mid-palatal suture area (PDI- PRE-DRILLING IMPLANT) (SDI- SELF DRILLING IMPLANT) | PDI maxilla: 7.2 +/- 1.4 Ncm  
PDI palate: 14.5 +/- 1.6 Ncm  
PDI mandible: 12.4 +/- 1.2 Ncm  
SDI maxilla: 12.1 +/- 3.1 Ncm  
SDI palate: 21.1 +/- 2.2 Ncm  
SDI mandible: 15.7 +/- 2.3 Ncm |
In relation to torque values in maxilla and mandible, as discussed earlier, the insertion torque is maximum in the mandible than maxilla due to increased density and thickness of the cortical bone (14). Considering the side in the jaw where the implant has been placed, it has been concluded by Motoyashi et al., that there is no alteration in the insertion torque (15). The insertion torque values are the same when the implant is placed on either right or left side of the jaw (14, 15). In relation to the maxilla, mini implants when placed in the palatal side have higher insertion torque than those placed along the buccal aspect (17). In a study conducted by Suzuki and Suzuki, they concluded that the insertion torque is higher in the mid palatal suture of the maxilla than the dentoalveolar region of the same jaw (18). In comparison to the mandible, the insertion torque is not as high as that observed in mini implants placed along the mid palatal suture (18). Due to certain discrepancies in relation to uncontrolled implant factor, uncontrolled patient factor and location factor, these study results are not accepted.

**Screw factors: Length**

In studies conducted by Lim et al., it has been proved that the length of the mini implant has a positive influence on the insertion torque. They conducted a study using various lengths of cylindrical and taper screw implants which was tested in a 1.5 mm block of bone with various levels of insertion torque. Results showed that the maximum insertion torque of the implant increased with increasing screw length (1).

**Diameter**

Implants have an outer and inner diameter which influences the insertion torque during implant placement. In accordance to studies conducted by Lim et al., it has been concluded that a change in the outer diameter had the most significant effect on the torque (1). In his study, he used implants of a standard length but varied outer diameter and inserted them in the test block of artificial bone whose density was similar to that of normal bone. From the results obtained, it was concluded that the outer diameter of the screw is the most influential factor in determining the insertion torque. This also corresponds to the results from finite element analysis of the difference in a bone’s stress and distribution according to the screw design. Previous studies reported that among the various mini screw designs, the change in diameter caused the greatest change in stress. All the screws showed that the amount of maximum implant torque increased with increasing cortical bone thickness (1).

**Surface treated implants**

Numerous surface engineering methods have been developed to create featured implant surfaces in order to improve the clinical performance of implants and to guarantee a stable mechanical bone implant interface. Nowadays, there are several methods to modify the implant surface characteristics with the main objective of improving the bio-mechanical properties of the implant. These include Turned surface (machined dental implants), Grit blasting or sand blasting, Calcium phosphate coatings on titanium implants, Laser modified micro- and nano-structured surface, Anodized surface, Grit-blasting and acid-etching, Titanium Plasma Spraying, etc., (19). According to Chaddad et al., the insertion torque is the same in machine drilled and surface treated mini implants. But due to certain limitations in the study, such as uncontrolled patient related, implant related factors, number of implants and location of implant placement, the results have not been accepted (20-22). There is not much literature for the other surface treated implants.

**Implant design**

Various systems with different implant designs are available that include, the Orthosystem implant, Straumann ortho implant, Aarhus implant, Mini implant system, Micro- implant, C – implant, Spider screw, Infinitas, Vector TAS, Dual Top, Tomas Pin and Ortho-Easy.

Table 2: Various implant designs by different manufacturers and the average insertion torque recommended for placement of orthodontic mini implants.

<table>
<thead>
<tr>
<th>Aarhus implant(22)</th>
<th>Nguyen, Melissa et al stated that the minimum insertion torque for primary stability should be &gt;24Ncm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini spider screw (23)</td>
<td>Average torque value 6.5Ncm</td>
</tr>
<tr>
<td>Infinitas (23)</td>
<td>Average torque value 12.4 Ncm</td>
</tr>
<tr>
<td>Vector TAS (23)</td>
<td>Average torque value 30.9 Ncm</td>
</tr>
<tr>
<td>Dual Top (23)</td>
<td>Average torque value 29.4 Ncm</td>
</tr>
<tr>
<td>Tomas Pin (23)</td>
<td>Average torque value 25.4 Ncm</td>
</tr>
<tr>
<td>Ortho-Easy (23)</td>
<td>Average torque value 24.8 Ncm</td>
</tr>
</tbody>
</table>

**Technical/procedural factors:**

**Pre-drilling and self-drilling insertion of mini implants.**

Self-drilling mini screws induce tighter bone tissue contact; therefore, they might provide improved primary stability. Self-drilling insertion have shown to have higher torque when compared to pre-drilling insertion. They allow simple surgical procedures for placement without the need of a predrilled pilot hole; although they provide significantly superior primary stability compared with pre-drilling mini screws, the strength of the osseointegration is significantly lower.
This is of clinical importance, since mini screw implants are not designed to remain in the bone (18, 24).

**Discussion of animal studies**

Animal studies have shown that pre-drilling or self-drilling surgical techniques and the diameter of the pilot hole can significantly influence maximum insertion torque values. By modifying these variables, clinicians can insert orthodontic mini implants with desired maximum insertion torque levels and thereby obtain appropriate primary stability in sites with either stiff or fragile bone. Surgical procedures can also be modified to lower insertion torque values to prevent fractures of orthodontic mini implants. Only 1 surgery-related association with maximum insertion torque was presented, but it was rejected because of confounding (24, 25)

**Complications due to excessive insertion torque application**

Inflammation, pain, discomfort, bone necrosis, fracture of the implant, long term consequences such as root or nerve damage, sinus cavity perforation, nasal cavity perforation, trauma to the periodontal ligament (26).

**CONCLUSION**

Currently, recommended specific maximum insertion torque levels to obtain higher success rates of orthodontic mini implants have not been scientifically proven. Associations were proposed to correlate maximum insertion torque levels with implant, patient, location, and surgery related factors, but none proved to be significant. Hence studies should be performed where the insertion torque is measured using Digital torque sensors and not mechanical devices. This would give us an insight on the influence of insertion torque on the primary stability of mini implants used in orthodontics.

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Special article

HIV and ways to maintain a meaningful life

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ABSTRACT

Perhaps, HIV (human immunodeficiency virus) infection is the end for many people. For those who have a positive test for HIV, their life is similar to be condemned to death. Not only does it have bad effect on someone’s life mentally and physically, but it also causes acute anxiety for the entire world, as medical communities have been completely incapable of killing the virus so far. Besides, the HIV-infected patients would find themselves confronted by massive difficulties related to their family and society. However, above all, there is nothing more frightening than the estrangement and discrimination of the people around them. Anyway, HIV does not mean life’s end. In reality, many people infected with HIV have been still living healthily for decades thanks to simple tips. This research aims to verify common causes of HIV and propose recommendations based on previous studies in order to help people have a good prevention as well as avoid this such chronic disease. By conducting semi-structured in-depth interviews with forty adults suffering for HIV, the current study discovered common reasons for HIV infection including homosexual intercourse, drug using and having sex with prostitutes. Besides, some recommendations are given due to the symptoms caused by the disease in terms of eating habits, daily activities, using medicine, sexual activities, ARV testing and mental care.

Keywords: ARV; HIV; life; prevention.

INTRODUCTION

There has been a decreasing trend in the number of people infected with HIV in the world since 2011. There were 2.1 million patients in 2011 and the number was the same in 2012, followed by 2 million infections in 2013 and 2 million in 2014. From 2015 to 2017, the number of infected people dropped to 1.8 million patients (Our World in Data). The number of infected people was proportionate to the number of deaths by AIDS, that is, 1.54 million were dead in 2011 and 954,492 were in 2017 (Our World in Data). "The number of persons living with HIV worldwide reached approximately 35.3 million in 2012" (1).

With the data taken online from The General Statistics Office of Vietnam website, there were the same trends in the number of new infection and the number of deaths during the same period. There were 14,113 new HIV infections and 2413 people dead due to the disease while these numbers dropped to 9,920 and 2,233 in 2017, respectively (Fig. 1).

According to the statistics provided by the Ministry of Health of Vietnam, in the first nine months of 2018, the whole country detected nearly 7,500 HIV cases; the number of patients turning to AIDS was over 2,500, the number of patients died reached 1,436 cases. The number of new HIV infections was mainly at the ages of 16-29 (38%) and 30-39 (36%). It is reported that the transmission routes were mainly unsafe sexual activities (63%) and blood transfusion (23%). Among new HIV infections, 36% were women infected from husbands, partners, 24% were men who had sex with men, and transgender women, 23% were injecting drug users, 10% were sex buyers,
5% were men who are infected with HIV from their wives and partners and 2% were prostitutes.

Content

It is said that 2018 was the fifth year in a row; Vietnam continued to pursue and implement the goal of 90-90-90. In recent years, Vietnam has achieved many achievements in HIV/AIDS prevention and control as 11 years of continuous HIV epidemic control in all three criteria including reducing the number of new HIV infections every year, reducing the number of people turning to AIDS and reducing the number of deaths from HIV/AIDS. Vietnam has continued to control HIV prevalence among the population at less than 0.3%. However, Vietnam's current targets are still quite far from the 90-90-90 targets set by the United Nations.

How HIV-infected patients can be aware of the importance of treatment to be able to reintegrate into the community and have a meaningful life is what many people care about. Some patients know they are sick and want to live long enough until scientists can cure their chronic disease without knowing what to do (2). There are lot of secrets that help us stay healthy normally for more than 30 years after HIV infection even at the stage of AIDS. However, few people know them. Mastering the most basic knowledge about the disease helps patients to have more optimistic views.

The spread of HIV disease

HIV is a disease of the century without any cure yet. Most patients are often spread through blood, sex and from mother to child. So far, HIV has been found in semen, vaginal fluid, blood, blood products, tears, saliva, cerebrospinal fluid, and breast milk (3).

However, only three routes of HIV infection have been identified including the use of needle-injecting equipment that is not sterile. The risk of infection is associated with the number of injections and the use of injecting equipment for many people. Caregivers of HIV/AIDS patients can also become infected through contacts with a patient's blood and biological fluids. In addition, HIV can be transmitted through unprotected sex. The estimated rate of HIV infection for each unprotected sex is 0.1-1%. This rate increases with the frequency of relationships. Meanwhile, having sex with people with HIV protected with condoms will be safe up to 90-95% if done properly. Oral sex is less likely to transmit disease. However, if there are scratches or bleeding teeth in the mouth without knowing it is still possible to spread HIV. A mother infected with HIV will have about 30% of the chance of giving birth. Infants with HIV usually do not live for more than 3 years.

The stages of HIV/AIDS progress in different stages. The primary stage of infection usually lasts 1-2 months after HIV enters the body, 40-90% of patients experience flu-like symptoms such as fever 38-40°C, sweating, sore throat, joint pain, swollen glands, etc. This is the time, the virus moves into the blood, replicating in large numbers. Symptoms of swelling, inflammation are inflammatory reactions of the immune system. Approximately 2-3 months after the primary infection of the patient's body produces specific antibodies and at this time tests for HIV positive. The HIV-positive stage lasts about 5 - 10 years (4).

In the stage associated with AIDS, the body gradually weakens the patient presents with tonsillitis, sinusitis, stomatitis, pharyngitis, rashes, rash, nail fungus, etc. After several months to several years, the patient may experience weight loss, persistent fever, lymphadenopathy, night sweats, diarrhea, and so on. The stage of AIDS is considered as the final stage of HIV infection, manifested as disorders related to immune deficiency including fever (lasting more than a month), long diarrhea, severe weight loss (approximately 10% of body weight). Patients easily die from opportunistic infections such as pneumonia, meningitis, enteritis, lymphoma, vascular cancer, etc. In terms of transmission risk, HIV virus can be transmitted at any stage of infection (3).

There is some possibility that HIV will not spread to others. HIV can be transmitted through mosquito bites or saliva. Healthy people will not become infected with HIV if they shake hands, kiss, and eat with sick people because the composition of body fluids such as saliva of HIV carriers has only a very small amount of virus, so it is not enough for destroy the human’s body.

METHODOLOGY

Semi-structured in-depth interviews were used with the support of 40 adult patients who are suffering from HIV/AIDS and are living in Ho Chi Minh City, Vietnam. The interview transcripts were analyzed by using comparative methodology in order to find out ways to have a better life for the patients.

Three main criteria can be classified including (a) homosexuals; (b) drug users; (c) prostitutes. These criteria are based on data we collected from the ODP department and the support of some patients who are treating the disease. The other data was gathered from interviews with chat tools like Ola, Blueed for gay people.

Men with homosexual tendencies are those who are prone to HIV infection. This incident had been said since the 1980s before the first AIDS patients were discovered to be homosexual.

The first ten participants were assessed to make it clear the reasons why they have been suffering from HIV. Interviews began with a list of questions ranging from easy to difficult ones. The author administered these interviews at same time in order
to find out the answers for their experiences of living with HIV. Then, questions are intended to identify their concerns and thoughts about their future, family and society (Table 1).

### Table 1. Questions about the patient's response to HIV disease

<table>
<thead>
<tr>
<th>Content</th>
<th>Questions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opening questions</td>
<td>How are you feeling about your health these days?</td>
</tr>
<tr>
<td></td>
<td>How long have you been infected with HIV?</td>
</tr>
<tr>
<td>Health status</td>
<td>Do you eat well and gain/lose weight?</td>
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<tr>
<td></td>
<td>Has your CD4 count been improved?</td>
</tr>
<tr>
<td>Mental impact</td>
<td>Are you sad or depressed?</td>
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<td></td>
<td>Have you ever thought that your life is deadlocked?</td>
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<tr>
<td>Social support</td>
<td>Have you ever received any support from the society?</td>
</tr>
<tr>
<td></td>
<td>Have your family and friends helped you?</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

The below chart shows the percentage of infected-HIV groups in which homosexuals occupied approximate a half of the patients, at 45 percent. The second common group was prostitutes, at 30 percent. There were 22 percent of drug users infected with the virus while there were still other 3 percent from other cases. Due to the findings, it can be seen that homosexual activities can be prone to HIV transmission the highest among other groups. In general, although the three groups are different from each other in their forms, they are blood transfusion in common in which healthy people can get infected easily (Fig. 2).

![Fig. 2: Groups of people infected with HIV](image)

This study was continued with the discovery in the action of the HIV-infected patients. Accordingly, the first reaction of the person who was positive for HIV test got shocked, then become angry and desperate. Others were stunned and speechless. When the results were confirmed, most people infected with HIV / AIDS turned to anger (42%) and wanted to find out the cause of the infection. HIV infection could cause them to suffer from a bad reputation and be rejected by others, including relatives and friends (5). There were some patients who wanted to hide the disease, manifest as self-abuse, do not want to interact with surrounding people or even want to die (6). More dangerously, some people committed violent acts, or gave a "revenge for life" by intentionally transmitting HIV to others (Fig. 3).

![Fig. 3: Reaction of infected-HIV patients](image)

At a certain stage, the permanent feelings of people with HIV were anxiety and angry. The anxiety was about death, losing jobs, discriminatory attitudes of surrounding people and financial problem for future
treatment. The patients mentioned about the potential other feelings such as depression or panic if they continuously maintained the anxiety without the suitable support from the community, especially when they kept negative thoughts in the minds (5). More than a quarter of patients said they felt angry, but they did not really know where the anger was from. They easily turned to this feeling when there was something a bit unpleasant happing to their life or any of their activities. The other percentages are shared for tired and other feelings. The others have unidentified feelings because they sometimes felt angry or worried, but sometimes they were depressed seriously. Moreover, they also expressed their extreme worry as they might unintentionally transmit the disease to their family members or partners. The disease doesn’t cause positive impact on the patients. It makes the patients lose faith in their life as well as the burden for the patient’s family and society. That why there have been patient committing suicide (4).

By asking the patients about what they hope for their life, then it can be recognised that if HIV-infected patients receive enough care and support from relatives and community, they will gradually accept their situation and rekindle their hope into the future. How to live the last remaining days helpfully to benefit families and society was the expectation from most patients as well. The world always hopes that science will find the cure for such dangerous disease in the near future (2).

In fact, HIV-infected patients are not near the death sentences like cancer, diabetes, high blood pressure, etc. When being positive for HIV, patients will be assigned ARV treatment (7). The drug is distributed free in hospitals in Vietnam. If they want to use better quality-specific medicine, the patients have to spend about one million dongs (50 USD) per month. Besides, the happiness in life and optimism of patients are much better than the recovery process. "Many HIV patients who are treated properly can live to the end of their lives with a very high life expectancy.”

SOME RECOMMENDATIONS

There are several effective measures to prevent HIV transmission that are of great interest to many people. We must live healthy, faithful monogamy, not indiscriminately having sex. In the case of sexual intercourse with others, we need to use condoms properly. We only transfuse blood and blood products when absolutely necessary. Patients only receive blood and blood products that have been tested for HIV. Medical personnel should wear protective gloves when transfusing blood and when coming in contact with vomit, blood, etc. of the sick person.

We do not share needles and syringes, that is, we only use sterilized equipment when having surgery, tattoos, acupuncture, etc. HIV-infected women should not become pregnant, if pregnant, should not give birth. If the mother wants to have a baby, she should go to a reputable health facility for advice on how to avoid infecting her children. And one thing we should keep in mind is not to share toothbrushes, razors, etc. with sick people.

Thus, the most accurate time for an HIV test is 2-3 months after exposure to the HIV virus. If we suspect that we have been exposed, or if there are early manifestations of HIV disease, early testing should be done during this time. As we begin to treat HIV and our viral load begins to decline, the number of CD4 cells is likely to increase. The growth rate of CD4 cells is dependent on the individual. During the first months of treatment for HIV, the CD4 count will continue to be monitored regularly. However, the viral load in the blood is a more important indicator of health and effectiveness compared to the number of CD4 cells. After some time, the time to check for CD4 cell count will be more spaced, depending on the state of health.

Due to various causes, there will be a number of symptoms related to eating habit and nutrition such as anorexia, dry mouth, diarrhea, nausea, frequent vomiting, mouth infection, and anemia. From unsafe eating diet, the patient's life is shortened very quickly. Doctors introduced some tips to help HIV infected people overcome the above symptoms as follows:

**For diarrhea:** Milk, alcohol, coffee, fried foods, butter should be restricted. People with HIV and diarrhea should not drink soft drinks or eat some kinds of vegetables such as cabbage and onions.

**For nausea:** Patients should limit spicy foods, greasy foods, sweet foods. They need to avoid hungry stomachs and heavy smells that will eliminate the feeling of anorexia.

Oral fungal infection is a common disease in HIV-infected people. If it is left untreated, it can cause gastrointestinal infections. Therefore, sugar, salt, sour, sticky foods and alcohol must be avoided. Similarly, HIV patients with symptoms of anemia must limit the intake of tea, coffee, milk and meals because it will inhibit iron absorption.

HIV patients should follow the suggestions of nutritionists: Eating red meat 1-2 times / week; replacing red meat with white meat like chicken, turkey without eating the animals' skin; eating less fat meat; replacing meat with fish as much as possible. If the patient has high cholesterol or heart disease, they must not eat more than four eggs a week. An adult need to add about 50 grams of protein every day or from 220 to 250 grams of meat, fish, beans or eggs to his or her body. People with HIV should also limit...
contact with dogs and cats because they will be susceptible to brain fungus.

Family members who take care of an AIDS patient (A following period after HIV infection when the immune system of HIV patients becomes weak and CD4 cells are low) must pay attention to safety (8). At this time, patients will have clear symptoms such as: ulcers, rapid weight loss, and opportunistic diseases. The career needs special precautions to avoid the risk of infection. Specifically, clothes and personal items with blood sores (due to ulcers). They need to be soaked in soap and antiseptic for about 20 minutes before washing and it should be washed separately by wearing Gloves by the career while washing. If the patient suffers from opportunistic diseases, they should focus on treatment for these diseases first and then treat AIDS (5).

Patients must absolutely adhere to ARV treatment, as this is the most important issue. Non-compliance with right HIV treatment will result in drug resistance leading to treatment failure. After every 6-month period, doctors will test patient’s CD4 count to assess the patient's immune system. If the CD4 are above 500, they are safe.

Next, what patients have to do is to remain their healthy living, to exercise regularly and to avoid working too hard. They should not stay up late, drink alcohol. When having sex, they must use safe ways in order not to infect HIV with others (3). By relieving anxiety, stress, patients will live better. Scientists have found that HIV-infected people who died quickly were mainly depressed, hopeless because of social stigma and discrimination (7). In addition, body enhancement is important to increase the immune system's resistance. This is the most fundamental problem that makes the difference in the health of patients. Moreover, the patients should be optimistic about a brighter future. They need to live long enough until scientists find a cure for the HIV / AIDS (7). Certainly, with the world's knowledge, energy and good way of life, patients will live a normal, healthy life for over 30 years and increase CD4 to above safety (2).

In order for make the HIV/AIDS prevention and control effective and deep, the local government should go into the reality of people's life. In the past years, in addition to promoting communication and changing the behavior of the province, the government has actively been implementing enough policies for people who have been infected with HIV / AIDS, setting up a system for monitoring and supervising translation assessment, establishing HIV care and treatment services and focusing on achieving goals 90 - 90 - 90. At the same time, all resources have been mobilized to invest in facilities, procurement of equipment, HIV testing and counseling rooms to ensure that districts and cities have HIV testing rooms (1). Every year, free testing and preventive treatment with free ARV drugs are done and necessary to be continuously maintained. In addition, the hospitals should focus on implementing good harm reduction interventions by distributing, syringes and condoms through the network of medical staffs (3). To improve the health of people with HIV infection, the government should keep encouraging patients to participate in health insurance to get the most appropriate treatment.

CONCLUSION
In summary, to help people in general and HIV patients in particular understand the importance of using medicines to treat diseases is essential. For patients, they must realize that HIV infection is not a death sentence. Diligent practice, adding nutrients and living well will prolong the patients’ lives. Society therefore also needs to join hands to build a better supporting community. Science will grow and will surly push back this disease into the past.

ACKNOWLEDGEMENT
The authors would like to express our deep thanks to our universities since we have always been encouraged to complete our study.

LIMITATIONS OF THE RESEARCH
This article has not been done in depth to get the best results. Taking the results from the survey is still mainly based on ODP rooms of some districts in Ho Chi Minh City in Vietnam. However, the disease is still considered an infectious disease and the direct access to patients is difficult by some causes. The scope of this study is rather small as well.

ETHICAL CLEARANCE
We are ensuring the quality and integrity of our research. The ideas and opinions expressed in this paper are of our effort. By writing this research paper, we surely respect the confidentiality and anonymity of our research respondents since they participated in our work voluntarily.

CONFLICTS OF INTEREST
No conflicts of interest noted in the paper.

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REFERENCES


Research articles

**In vitro antioxidant and antidiabetic properties of *Eryngium foetidum* Linn.**

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**ABSTRACT**

**Introduction and Aim:** *Eryngium foetidum* Linn. belongs to the family Apiaceae, is an aromatic herbaceous plant with culinary importance, that is used worldwide as substitute for coriander. The aim of the present study was to extract the phytochemicals of *E. foetidum* leaves and evaluate the *in vitro* antioxidant and antidiabetic properties.

**Materials and Methods:** *E. foetidum* plants were collected, shade dried, coarsely powdered and defatted. The defatted material was subjected to Soxhlet extraction using methanol as solvent. The extract was subjected for phytochemical screening by qualitative analyses by UV-vis spectroscopy and TLC and RP-HPLC. Quantitative analyses of phenolics, flavonoids and saponins was carried out. The extract was evaluated for antioxidant activity by DPPH and ABTS radicals scavenging assays, and for antidiabetic activity by performing α-amylase inhibition and α-glucosidase inhibition assays.

**Results:** Results of the investigations suggest that *E. foetidum* leaves are rich source of phytochemicals like flavonoids and saponins. The phytochemicals present in the methanolic extract of *E. foetidum* leaves possess antioxidant and antidiabetic properties.

**Conclusion:** The present study reveals that *E. foetidum* leaves possess therapeutically important phytochemicals that could be further exploited as nutraceuticals considering the culinary importance of the plant.

**Keywords:** Phytochemicals; *Eryngium foetidum* Linn.; flavonoids; saponins; antioxidant activity; antidiabetic activity.

**INTRODUCTION**

*Eryngium foetidum* Linn., is an aromatic biennial herb, belonging to Apiaceae family, native to Central America and Mexico, introduced or cultivated in both tropics (1). It is widely known as Culantro or wild coriander, Mexican coriander or long coriander. It is a popular condiment widely used in continental cuisine as food flavoring and seasoning herb like soups, salads, sauces, salsa, noodles and curries among the ethnic populations (2, 3). *E. foetidum* grows wild and also now a days cultivated in all parts of the world for its culinary purposes. In Karnataka, *E. foetidum* grows in Hassan and Coorg Districts of the state as naturalized weed. It’s common vernacular name in Kannada - Kadu Sambaru, in Hindi - Ban Dhaniya.

The plant *E. foetidum* is used as an ethno-medicinal plant for the treatment of various ailments. It is been used in the treatment of fever, ear pains, diarrhea, hypertension, oedema, sinusitis, snake or scorpion bite, and decoction prepared from leaves of *E. foetidum* is used in traditional medicine as a vulnerary, and hypotensive and for digestive troubles (2,4,5). The crushed leaves are placed in the ear to treat pain, and are used for the local treatment of arthritic processes (6). It has showed the topical anti-inflammatory activity and myeloperoxidase activity (7) and antimalarial and antibacterial activity (2, 3 and 8). Aerial parts have been reported to show anthelmintic activity and anti-convulsant activity (3, 4). Methanolic extract of irradiated *E. foetidum* fresh plantlets yielded phenolic compounds - coumarin, caffeic acid, p-coumaric acid, salicylic acid, benzoic acid, and apigenin (9). Commonly the plant extract is used for anti-inflammatory activity (10) and antimicrobial activity (3). The earlier studies focused on the extraction of leaf and other parts of the plant using solvents of different polarity (11). In view of the culinary and pharmacological properties of the present study has been carried out for the evaluation of antioxidant and antidiabetic properties of *E. foetidum* leaf extract that contains flavonoids and saponins under *in vitro* conditions.

**MATERIALS AND METHODS**

**Collection of plant materials**

*E. foetidum* plants were collected from different parts of Hassan and Coorg Districts of Karnataka state during the monsoon and post-monsoon seasons, identified and authenticated with the help of Flora of Karnataka (http://florakarnataka.ces.iisc.ac.in/).

**Chemicals and Reagents**

Chemicals and reagents were procured from either Sigma-Aldrich (USA) or HIMEDIA (Mumbai, India).
or Sisco Research Laboratories (Mumbai, India) and were of either analytical grade. Millipore (Mili-Q) water was used throughout all the investigations.

**Preparation of leaf methanolic extract**

Leaves of *E. foetidum* were excised, shade dried and coarsely powdered, defatted using n-hexane. The defatted material was subjected to Soxhlet extraction using methanol as solvent. The extract was concentrated using Rotary evaporator (Medica Instruments, Mumbai) and dried in the oven at 40°C. A greasy, viscous material, semi-solid in nature and aromatic in odor, soluble in methanol was obtained, stored in the air tight bottles at 4°C till further use.

**Preliminary phytochemical screening**

Leaf methanolic extract of *E. foetidum* was subjected for the preliminary phytochemical screening tests to find the presence of the active chemical constituents using standard procedures (11, 12).

**Thin layer chromatography (TLC) analysis**

Leaf methanolic extract of *E. foetidum* was subjected for thin layer chromatography using silica gel G and mobile phase. For flavonoids, methanol: ethyl acetate: chloroform (1:1:8) was used as solvent and detected under UV-light exposure at 25-260nm. For saponins chloroform: ethanol: water (60: 40: 5 v/v) was used as solvent and 5% vanillin-sulphuric acid was used as spraying reagent.

**UV-visible spectroscopy analysis**

Leaf methanolic extract of *E. foetidum* was subjected for UV-Visible spectroscopy analysis (13), scanned through 200 to 800 nm wavelength range using UV-Vis spectrophotometer (Systronics, India). The characteristics peaks were detected. The peak values of the UV-Vis analysis were recorded.

**RP-HPLC analysis**

Leaf methanolic extract of *E. foetidum* was subjected for reverse phase high performance liquid chromatography (RP-HPLC) analysis using Agilent 1260 system equipped with quaternary pump and diode array detector (DAD). Separation was carried out in a Zorbax SB-C18 reverse phase column (5 mm, 4.6x250 mm), methanol: water (35:65) used as mobile phase, flow rate was monitored at 1 ml/min, column temperature was maintained at 35°C, and analytes in the effluent were detected at 330 nm for flavonoids and at 210 nm for saponins.

**Determination of total phenolics and flavonoids**

Total phenolic content of the extract was determined using Folin–Ciocalteu reagent, absorbance was measured at 670 nm, and a calibration curve was generated using the gallic acid standard. Total phenolic content was expressed as gallic acid equivalent (GAE) mg/g tissue extract (14). Total flavonoids content was determined by the aluminum chloride colorimetric method, absorbance was measured at 510 nm, and a calibration curve was generated by using the catechin standard (15). Total flavonoid content was expressed as catechin equivalents (CE mg/g tissue extract).

**Determination of total saponin content**

Total saponin content of the extract was determined using vanillin-sulphuric acid reagent method (16) *Quillaja* bark saponin was used as standard, absorbance was measured at 540 nm using UV-Visible spectrophotometer (Systronics, India). Total saponin content was expressed as *Quillaja* bark saponin equivalent (QSE) mg/g tissue extract.

**DPPH radical scavenging assay**

Leaf extract of *E. foetidum* containing saponins was subjected for 1, 1-diphenyl-2 picryl hydrazyl (DPPH) radical scavenging assay (17). Gallic acid was used as the positive control. The per cent (%) inhibition of absorbance (scavenging activity) was calculated using the following formula,

\[
\% \text{ scavenging activity} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Test)}}{\text{Absorbance (Control)}} \times 100
\]

**ABTS radical inhibition assay**

Leaf extract of *E. foetidum* containing saponins was subjected for 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay

\[
\% \text{ scavenging activity} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Test)}}{\text{Absorbance (Control)}} \times 100
\]

**α-amylase ((EC 3.2.1.1) inhibition assay**

Leaf extract of *E. foetidum* containing saponins was subjected for α-amylase inhibition assay (19) using pancreatic α-amylase (EC 3.2.1.1) and 2-chloro-4-nitrophenol-α-D-maltotrioside (CNP-G3) as substrate. The amount of product 2-chloro-4-nitrophenol formed was measured at 405 nm using a microplate reader (BioRad, Germany). Acarbose was used as positive control. Inhibition per cent (%) of α
amylase activity was calculated using the following formula.

\[
\text{% inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Test)}}{\text{Absorbance (Control)}} \times 100
\]

The concentration of the extract required to inhibit 50% of α-amylase activity under the assay conditions was defined as the IC\textsubscript{50} value.

\textbf{α-glucosidase (EC 3.2.1.20) inhibition assay}

Leaf methanolic extract of \textit{E. foetidum} containing saponins was subjected for α-glucosidase (EC 3.2.1.20) inhibition assay using \textit{Saccharomyces cerevisiae} α-glucosidase (Merck-Millipore formerly Sigma-Aldrich) and p-nitrophenyl-α-D-glucopyranoside as substrate (20). The amount of product p-nitrophenol released was measured at 405 nm using a microplate reader (BioRad, Germany). Acarbose was used as positive control.

Inhibition per cent (%) of α-glucosidase activity was calculated using the following formula.

\[
\text{% inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Test)}}{\text{Absorbance (Control)}} \times 100
\]

The concentration of the extract required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC\textsubscript{50} value.

\textbf{RESULTS}

The results of qualitative analysis for phytochemicals revealed the presence of active principles like phenolic compounds, flavonoids, tannins, alkaloids, saponins and steroids in leaf methanolic extract of \textit{E. foetidum}. Methanol that has the high polarity was found to extract the maximum active principles. The dry weight of extract was found to be 1.1197 g. Results of the UV-visible spectroscopy and chromatography analyses (TLC and RP-HPLC) confirm the presence of both flavonoids and saponins in the leaf methanolic extract of \textit{E. foetidum}. Results of UV-visible spectroscopy analysis showed the absorption peaks at 200 – 230 nm and 270-330 nm (Fig. 1). Thin layer chromatography (TLC) analysis showed the presence of flavonoids and saponins (Fig. 2 a & b), this was also confirmed further by reverse-phase HPLC analysis confirmed the presence of both flavonoids (330 nm) and saponins in the leaf extract of \textit{E. foetidum} (Fig. 3 a & b).

![Fig. 1: UV-visible spectrum of \textit{E. foetidum} leaf extract scanned through 200 nm to 800 nm](image1)

![Fig. 2: TLC separation of \textit{E. foetidum} leaf extract (a) Flavonoids (b) Saponins](image2)
Results of the quantitative analyses suggest that, *E. foetidum* leaf extract is a rich source of polyphenols, flavonoids and saponins (Table 1).

**Table 1: Total phenolics, flavonoids and saponin content in *E. foetidum* leaf extract**

<table>
<thead>
<tr>
<th>Phytochemical in the leaf extract</th>
<th>Quantity determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolics</td>
<td>378 mg GAE/g</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>196 mg GAE/g</td>
</tr>
<tr>
<td>Total saponins</td>
<td>292 mg QSE/g</td>
</tr>
</tbody>
</table>

Total phenolic content of extract was 378 mg GAE/g, total flavonoid content was 196 mg GAE/g, and the total saponin content of was 292 mg QSE/g leaf extract was the concentration of total saponin (Table 1).

**DPPH radical scavenging activity of *E. foetidum* leaf extract**

The free radical scavenging activity of *E. foetidum* leaf extract was evaluated (5-500 µg/ml) by DPPH radical scavenging assay, and results are depicted in Table 2. DPPH radical scavenging activity of *E. foetidum* leaf extract was observed to be increased with increasing concentration and showed 94.2% inhibition at 500 µg/ml. The *IC*$_{50}$ value for *E. foetidum* leaf extract was determined as 31.45 µg/ml, while positive control gallic acid showed 97.38% inhibition at 5 µg/ml and its *IC*$_{50}$ value was found to be 1.14 µg/ml (Table 2).

**Table 2: DPPH inhibitory activity of different concentrations of *E. foetidum* leaf extract**

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration tested (µg/ml)</th>
<th>% inhibition</th>
<th>IC*$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. foetidum</em> leaf extract containing flavonoids</td>
<td>5</td>
<td>32.8±0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>46.6±1.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>68.8±2.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>89.6±2.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>94.2±1.96</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.5</td>
<td>28.42±1.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>62.82±3.08</td>
<td>31.45</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>83.46±3.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>97.38±2.72</td>
<td>1.14</td>
</tr>
</tbody>
</table>

**ABTS radical scavenging activity of *E. foetidum* leaf extract**

The free radical scavenging activity of *E. foetidum* leaf extract was evaluated (5-500 µg/ml) by ABTS radical scavenging assay, and results are depicted in Table 3. The free radical scavenging activity of *E. foetidum* leaf extract was evaluated (5-500 µg/ml) by ABTS radical scavenging assay, and results are depicted in Table 3. ABTS radical scavenging activity of *E. foetidum* leaf extract was observed to be increased with increasing concentration and showed 90.44% inhibition at 500 µg/ml. The *IC*$_{50}$ value for *E. foetidum* leaf extract was determined as 40.82 µg/ml, while positive control gallic acid showed 84.42% inhibition at 5 µg/ml and its *IC*$_{50}$ value was found to be 1.32 µg/ml (Table 3).
Table 3: ABTS radical inhibitory activity of *E. foetidum* leaf extract

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration tested (µg/ml)</th>
<th>% inhibition</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. foetidum</em> leaf extract containing flavonoids</td>
<td>5</td>
<td>22.62±1.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>40.32±1.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>62.16±2.24</td>
<td>40.82</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>79.82±2.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>90.44±2.96</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.5</td>
<td>22.38±2.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>38.16±1.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>56.54±2.16</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>68.88±2.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>84.42±2.98</td>
<td></td>
</tr>
</tbody>
</table>

Effect of *E. foetidum* leaf extract on α-amylase activity

Leaf extract *E. foetidum* (5-500 µg/ml) was evaluated for α-amylase inhibitory activity and, the results could be extrapolated as the antidiabetic property/potential. The percentage of inhibition displayed by *E. foetidum* leaf extract at different concentrations are depicted in Table 4. *E. foetidum* leaf extract was found to have inhibitory effects on α-amylase in a dose dependent manner with maximum 78.62% inhibition at 500 µg/ml, and the IC<sub>50</sub> value was 88.64 µg/ml. While the positive control acarbose (1 µg/ml) has shown significant inhibition of enzyme activity (92.44%), with IC<sub>50</sub> value 0.48 µg/ml (Table 4).

Table 4: α-amylase inhibitory activity of *E. foetidum* leaf extract

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration tested (µg/ml)</th>
<th>% inhibition</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. foetidum</em> leaf extract containing flavonoids</td>
<td>5</td>
<td>10.28±1.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.32±1.38</td>
<td>88.64</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>35.46±1.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>57.82±2.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>78.62±1.98</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>0.1</td>
<td>22.64±2.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>52.86±2.72</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>92.44±3.46</td>
<td></td>
</tr>
</tbody>
</table>

Effect of *E. foetidum* leaf extract on α-glucosidase activity

Leaf extract *E. foetidum* (5-500 µg/ml) was evaluated for α-glucosidase inhibitory activity and, the results could be extrapolated as the antidiabetic property/potential. The percentage of inhibition displayed by *E. foetidum* leaf extract at different concentrations are depicted in Table 5. *E. foetidum* leaf extract was found to have inhibitory effects on α-glucosidase in a dose dependent manner with maximum 62.82% inhibition at 500 µg/ml, and the IC<sub>50</sub> value of 312.4 µg/ml. While the positive control acarbose (2 µg/ml) has shown significant inhibition of enzyme activity (88.14%), and IC<sub>50</sub> value 0.82 µg/ml (Table 5).

Table 5: α-glucosidase inhibitory activity of *E. foetidum* leaf extract

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Concentration tested(µg/ml)</th>
<th>% inhibition</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. foetidum</em> leaf extract containing flavonoids</td>
<td>5</td>
<td>10.28±1.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>16.12±1.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>22.34±1.82</td>
<td>312.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>32.42±2.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>62.82±2.88</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>0.5</td>
<td>42.62±2.28</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>58.28±1.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>88.14±2.16</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

Results of the present study suggest that the phytochemicals of *Eryngium foetidum* L. possess antioxidant and antidiabetic properties. The findings for the present study are in agreement with the earlier studies (21). Distress during the day-today life results in the increased generation of free radicals of both oxygen (ROS) and nitrogen (RNS), which may exhaust antioxidant defenses thus leading to the oxidative stress leading to oxidative damage of vital biochemicals (22). One of the strategies for alleviating the oxidative damage is the usage of natural antioxidants. During the present study it has been found that, *E. foetidum* phytochemical fractions present in the leaf extract possess significant antioxidant activity as confirmed by both DPPH and ABTS radicals scavaging activities comparing with positive control gallic acid (Tables 2 & 3). The antioxidant potential of *E. foetidum* leaf extract could be attributed to the presence of flavonoids.

The best therapeutic approach that has been suggested for diabetes is to decrease postprandial hyperglycemia is to retard absorption of glucose through inhibition of carbohydrate hydrolyzing enzymes in the digestive organs (23). During the present study, *E. foetidum* leaf extract showed significant inhibition of both αamylase and α-glucosidase activities as compared to positive control acarbose (Tables 4 & 5). Slowing of carbohydrate absorption is associated with improved glycemic control, and so α-glucosidase inhibitors (AGIs) have been developed to delay intestinal absorption of carbohydrates (24). Results of the present study suggest that, *E. foetidum* leaf extract inhibits both α-amylase and α-glucosidase activities in a dose dependent manner at a concentration of 500 µg/ml (Table 4 & 5). From the results of the present study it could be concluded that, *E. foetidum* leaf extract may interfere with transit, digestion or absorption of carbohydrates like starch, sucrose and maltose, and may delay carbohydrate digestion and causing decreased rate of glucose absorption into blood stream. The α-amylase and α-glucosidase inhibition activity by *E. foetidum* leaf extract could be attributed to the presence of inhibitory substances like saponins in the extract for both the enzymes. Similar findings of *in vitro* antioxidant and antidiabetic potential of phytochemical extracts of other medicinal plants were also reported (25).

CONCLUSION

Leaves of *Eryngium foetidum* Linn. are the rich source of valuable phytochemicals such as flavonoids and saponins with antioxidant and antidiabetic properties. Further studies are warranted for the isolation, purification and characterization and also to explore the therapeutic potentials of these flavonoids and saponins of *Eryngium foetidum* as nutraceuticals.

**REFERENCES**

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Role of baseline biochemical work up in the diagnosis of possible respiratory chain disorder in clinically suspected children

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ABSTRACT

Introduction and Aim: Respiratory chain disorder is a genetic defect in electron transport chain which involves ATP generation. There is no single screening or confirmatory test for its diagnosis, hence combination of clinical symptoms and biomarkers are utilized despite the advancement of techniques. The present study conducted to investigate the usefulness of baseline biochemical metabolic workup in clinically suspected children for diagnosis of possible respiratory chain disorder.

Materials and Methods: Clinical and baseline metabolic workup results of 385 children with clinically suspected inborn errors of metabolism was collected from their medical records. These results were utilized to classify them as possible respiratory chain disorder.

Results: Of the 385 children with clinically suspected to have inborn errors of metabolism, 99 were classified as possible respiratory chain disorder based on the applied criteria. Nearly 50 % of the cases had positive family history however; the majority of them visited the hospital only after the appearance of symptoms such as developmental delay, seizures, hypotonic, etc.

Conclusion: Baseline metabolic work up may be used to identify possible respiratory chain in children with family history and or initial clinical symptoms to initiate further diagnosis and timely intervention. Educating parents and primary care physician in this regard may be useful.

Keywords: Mitochondrial disorder; liquid chromatography; elevated lactate; lactate: pyruvate ratio.

INTRODUCTION

The mitochondrial disorder occurs due to the genetic defects of energy metabolism and includes pyruvate dehydrogenase complex, the Kreb’s cycle and the respiratory electron transport chain- involved in the final steps of ATP generation. The occurrence of respiratory chain disorder is 1 in 5000 to 10,000 births (1-3). The first case of respiratory chain disorder was identified about 56 years ago by Luft and later Theoder Leber in the year 1981 described in adult-onset blindness unknowingly. In the subsequent years, a tremendous development has occurred in the field of mitochondrial disorder which has led to the detection of more than 200 mutations of the mitochondrial genome (2, 4, 5). Mitochondrial respiratory chain disorder may be due to acquired or mutations in mitochondrial DNA or nuclear DNA (6).

As the knowledge increased in the area of mitochondrial disorders, the spectrum of clinical symptoms also has expanded dramatically. They usually involve multisystem, progressively disturbing the total functioning of the organism. Being the powerhouse, pathologic changes in the mitochondria deplete the adenosine triphosphate (ATP) and affects the activity of the cell. As metabolic rate is high in skeletal muscle, brain, and heart they are more vulnerable organs for ATP depletion. Depletion in ATP may also accelerate alternative pathways, resulting in the formation of elevated lactate, which may be injurious to the organism.

Respiratory chain disorders (RCD) manifest with signs and symptoms of a highly variable pattern of organ dysfunction. Detection requires a knowledge of their characteristic signs and symptoms as well as mitochondrial biochemistry and genetics. Studies found that serum lactate is elevated in most of the cases with RCD (3, 7-9) and it is also evident that sometime it varies (10-13). Preprandial and postprandial measurement of lactate (14), serum lactate: pyruvate ratio, alanine, carnitine profile, urine and plasma amino
acids and organic acids are the useful biochemical markers for diagnosis and differential diagnosis of mitochondrial disorder (15-17). There is no single diagnostic marker hence work-up may be difficult. Studies have used mitochondrial disorder criteria for diagnosing the definite mitochondrial disorder using scoring system. The scoring system was established and utilized several clinical symptoms, laboratory investigation and MRI findings for this purpose. The Walker criteria modified by Bernier et al considered family history such as stillbirth related with a decreased intraterine movement, neonatal death, pediatric features such as movement disorder, neonatal hypertonia, neonatal hypotonia, weakness, muscle wasting, exercise intolerance, severe failure to thrive, ptosis and cardiomyopathy as clinical criteria for respiratory chain disorder. One or more metabolic markers of respiratory chain dysfunction are considered the minor laboratory criteria for respiratory chain disorder. G.Oliviera used Bernier’s major and minor criteria for the classification of the Mitochondrial disorder. Mitochondrial respiratory chain (MRC) disorder was considered ‘definite’ if two major or one major and two minor criteria were present. It was considered as ‘probable’ if one major and one minor or at least three minor criteria were present and as ‘possible’ if one major or two minor criteria (one of which must be laboratory) were present (7,8,18). In our present study, we utilized these criteria partially and attempted to detect possible respiratory chain disorder based on clinical symptoms and baseline metabolic work-up which includes lactate, lactate:pyruvate ratio, ammonia and arterial blood gas analysis.

MATERIALS AND METHODS

This retrospective study was conducted after obtaining Institutional ethical committee clearance. Children of age group 0 to 14 years with clinical suspicion of IEM who attended Paediatric outpatient department of Kasturba Hospital, Manipal, Manipal Academy of Higher Education were included for this study. Symptoms included weak cry at birth, poor suck at the breast, history of sibling death, early breath holding spells, seizures, regression of milestones, excessive lethargy, hypotonia, developmental delay. Children in the above age group with perinatal brain injury, brain trauma/tumor, infections to central nervous system and chromosomal anomalies were excluded from the study. During the 3 ½ years period from July 2010 to December 2013, we have included data from 385 children as per our criteria, using convenient sampling method. Results of baseline biochemical metabolic workup which included arterial lactate, pyruvate(done on perchlorate sample), ammonia and arterial blood gas analysis were collected from the medical record of selected children and analysed. Results were considered abnormal if lactate: pyruvate ratio was more than 25 (normal <25), arterial lactate level as more than 22 mg/dL (normal 5-20 mg/dL) and ammonia as more than 90 μg/dL (normal 20-70 μg/dL).

We have defined children as possible respiratory chain disorder based on clinical symptoms, history, and baseline metabolic workup result in two categories. a. Clinical symptoms or history suggestive of IEM with elevated lactate, elevated lactate: pyruvate ratio, normal ammonia with or without metabolic acidosis. b. Clinical symptoms or history suggestive of IEM with elevated lactate, elevated lactate: pyruvate ratio, elevated ammonia with or without metabolic acidosis.

Statistical analysis

Statistical analysis included descriptive statistics was done using SPSS version 15.

RESULTS

Of the 385 children with clinical symptoms suggestive of inborn errors of metabolism 99 were considered as possible respiratory chain disorder based on our criteria. Among them 74 cases with possible respiratory chain disorder and 15 cases with possible RCD associated with organic aciduria (Table 1).

Table 1: Baseline biochemical metabolic work-up in possible respiratory chain disorder: n= 99

<table>
<thead>
<tr>
<th>Lactate</th>
<th>L: P ratio</th>
<th>Ammonia</th>
<th>Metabolic acidosis</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>73 (73.7%)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>11 (11%)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>12 (12%)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>03 (3%)</td>
</tr>
</tbody>
</table>

Table 2: Demographic characteristics of children with possible RCD: n=99

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-30 days</td>
<td>23 (23%)</td>
</tr>
<tr>
<td>1 month to 6 months</td>
<td>08 (8%)</td>
</tr>
<tr>
<td>6 months to 1 yr.</td>
<td>30 (30%)</td>
</tr>
<tr>
<td>1 yr. to 4 yrs.</td>
<td>29 (29%)</td>
</tr>
<tr>
<td>&gt;4 years</td>
<td>09 (9%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender-wise distribution</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>82 (82.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>17 (17.2%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Region-wise distribution</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Udupi District</td>
<td>29 (29.3%)</td>
</tr>
<tr>
<td>Outside Udupi within Karnataka</td>
<td>53 (53.5%)</td>
</tr>
<tr>
<td>Outside Karnataka</td>
<td>17 (17.2%)</td>
</tr>
</tbody>
</table>
The data presented in the above table 2 shows that the majority of children are males and 61% of children with possible RCD reported before one year of age.

**Table 3:** Family history, physical signs and symptoms at presentation in possible RCD: n=99

<table>
<thead>
<tr>
<th>Symptoms at presentation</th>
<th>Birth history</th>
<th>Family history</th>
<th>Physical signs</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>22</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>2</td>
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<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1</td>
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<tr>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>8</td>
</tr>
</tbody>
</table>

Of the 99 cases, 48 cases of possible RCD had a positive family history. Of them, 8 cases visited the hospital only because of family history suggestive of IEM, the remaining 40 cases who had a positive history, visited the hospital only after the appearance of physical signs and symptoms.

**Table 4:** Detailed family history in children with possible RCD: n=99

<table>
<thead>
<tr>
<th>Family history</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected sibling</td>
<td>25 (25.3%)</td>
</tr>
<tr>
<td>Consanguinity</td>
<td>22 (22.2%)</td>
</tr>
<tr>
<td>Abortion</td>
<td>12 (12.1%)</td>
</tr>
<tr>
<td>Other family members affected</td>
<td>06 (6.1%)</td>
</tr>
</tbody>
</table>

The family history of sibling affection was obtained in 25 cases of possible RCD. Of them, 8 cases of possible RCD were asymptomatic with a positive work-up.

**Table 5:** Symptoms at presentation in children with possible RCD: n=99

<table>
<thead>
<tr>
<th>Symptoms at presentation</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental delay</td>
<td>52 (52.5%)</td>
</tr>
<tr>
<td>Seizures</td>
<td>50 (50.5%)</td>
</tr>
<tr>
<td>Poor feeding at the breast</td>
<td>15 (15.2%)</td>
</tr>
<tr>
<td>Weak cry at birth</td>
<td>10 (10.1%)</td>
</tr>
<tr>
<td>Respiratory distress</td>
<td>11 (11.1%)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>09 (9.1%)</td>
</tr>
<tr>
<td>Regression</td>
<td>07 (7.1%)</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>08 (8.1%)</td>
</tr>
<tr>
<td>Early breath holding spells</td>
<td>02 (2%)</td>
</tr>
</tbody>
</table>

**Table 6:** Physical signs in children with possible RCD: n=99

<table>
<thead>
<tr>
<th>Physical signs</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonia</td>
<td>32 (32.3%)</td>
</tr>
<tr>
<td>Hypertonia</td>
<td>20 (20.2%)</td>
</tr>
<tr>
<td>Absent DTRs</td>
<td>10 (10.1%)</td>
</tr>
<tr>
<td>DTR brisk</td>
<td>20 (20.2%)</td>
</tr>
<tr>
<td>Muscle weakness</td>
<td>27 (27.2%)</td>
</tr>
<tr>
<td>Truncal weakness</td>
<td>09 (09.1%)</td>
</tr>
<tr>
<td>Lethargy</td>
<td>05 (05.1%)</td>
</tr>
<tr>
<td>Calf muscle Firm</td>
<td>05 (05.1%)</td>
</tr>
<tr>
<td>Ptosis</td>
<td>02 (02.0%)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The mitochondrial disorder is associated with decreased energy production, which is heterogeneous by genetically, clinically and biochemically; respiratory chain disorder is one among this. By applying partial diagnostic criteria of this disorder in our study, we found that 22% (99 out of 385) of the studied cases belonged to possible respiratory chain disorder. A retrospective study from Australia reported that 20% of the referred cases were definite respiratory chain disorder, according to Bernier criteria (8). We could not further classify our cases as definite respiratory chain disorder for lack of further confirmatory results. In our study of the 99 cases of possible RCD, 15 cases had elevated plasma ammonia level with associated metabolic acidosis in 12, which may be due to the association of organic aciduria along with respiratory chain disorder.

Almost all our cases with possible RCD were born at term. The majority of (90.9%) cases reported below four years of age, which is in accordance with other studies, which found that age at diagnosis for most of the cases ranges from 0 to 4 yrs. A study by Scalgi et al. found the mean age at presentation of the mitochondrial disorder as 3.3 years and by Kim et al. as 1.5 yrs. A study by Debray et al., documented the median age at presentation to be seven months (3, 19, 20).

We found male predominance in children diagnosed with possible RCD which is similar to other studies (9, 20, 21). In our cases with possible RCD, about 50% cases had a family history suggestive of IEM. Among them, an affected sibling was the most common (25) followed by consanguinity, history of abortion and history of other affected family members. Eight of them with positive biochemical workup were
asymptomatic and screened in view of the affected sibling, which suggests the importance of screening children for baseline metabolic workup when there is the presence of strong positive family history and initiation of therapy before the appearance of symptoms. Studies showed that positive family history in children RCD varies from 10% to 47.4 % (3, 9). Our finding suggests that whenever there is family history it warrants the requirement of further metabolic workup.

Skeletal muscle, brain and heart are the major organs—highly energy dependent and susceptible to energy deficiency. Based on the underlying pathogenesis of the diseases, the mitochondrial disorder may show a variety of clinical symptoms. Neurological and neuromuscular symptoms are most common in mitochondrial disorders, and many studies have reported a high incidence of these symptoms (3, 20-27).

In our study, nearly 70% of cases were symptomatic at presentation. Developmental delay was most common followed by seizures, poor feeding, weak cry at birth, respiratory distress, vomiting, neuro-regression and early onset of breath holding spells. 75% of the cases had physical signs such as hypotonia, DTR brisk/absent DTRs, muscle weakness, truncal weakness, lethargy, calf muscle firmness and ptosis. Identifying the clinical symptom is one of the markers to undertake further biochemical workup and initiate early intervention—some disorders can cause irreversible damage to the brain.

A significant number of children were diagnosed as possible respiratory chain disorder in our study based on clinical criteria and baseline metabolic workup. Some of them showed signs such as weak cry at birth, poor sucking at the breast early breath holding spell which are easily identified by the parents. Establishing guidelines and creating awareness about basic information of disorders among parents, relatives and health care provider may be useful for timely detection and initiation of therapy if available.

Limitation of the study: We could not get the data about these investigations in healthy children as the collection of arterial blood gas is invasive, and routinely these tests are not done in healthy children. Most of the children in our study never underwent definitive testing hence we could not confirm the diagnosis.

ACKNOWLEDGEMENT

I acknowledge Dr. Pragna Rao, Former Professor in Biochemistry and Former Dean, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal for the support given during the study.

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Green synthesis of silver nanoparticles (Ag-NPs) from Olea dioica Roxb., leaf extracts and its biological activity

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ABSTRACT

Introduction and Aim: The silver nanoparticles have attained a special place in the area of nanotechnology because of their different biological applications. Fabrication of nanoparticles using green synthesis is done because of its wide applications in different fields such as biomedical, medicine, agriculture and food engineering. This study is to develop an easy and eco-friendly method for the synthesis of Ag-NPs using leaf extracts of the medicinal plant.

Materials and Methods: The medicinal plants are rich sources of various medicinal properties. Olea dioica Roxb., leaf extract was used to investigate the effects of Ag-NPs having antibacterial activity and antioxidant capacity. The plant leaf extract contains flavonoids, alkaloids, saponins, and phenolic compounds which acts as reducing and stabilizing agents. The green synthesized silver nanoparticles were characterized by various techniques like UV-visible spectrophotometer, FTIR spectroscopy, and SEM analysis.

Results: The synthesis of silver nanoparticles from plant source, and analysis of nano particles by UV-Vis spectra, SEM and FTIR. The biological evaluations of Ag-NPs indicated an excellent inhibitory efficacy, antioxidant and antimicrobial activity for their future applications in medicine.

Conclusion: The synthesized silver nanoparticles exhibited potent antioxidant and antimicrobial activities against Gram-positive and Gram-negative bacteria. The silver (Ag-NPs) nanoparticles synthesized by the pot green synthesis method proves its potential use in various medical applications.

Keywords: Silver nanoparticles; Medicinal plants; Ag-NPs; Olea dioica Roxb.,

INTRODUCTION

Nanoscience is based on the manipulation of individual molecules to manufacture materials from them for implementation well below the sub-microscopic level (1). Nanobiotechnology is a well-growing technology as an interdisciplinary eco-friendly research area today and used in broad research sections such as biology, biochemistry, chemistry, physics, biomedicine, nanomedicine, and material engineering (2). It deals with various shapes and size of nanoparticles in the range of 1 to 100 nm. Materials in the nano-dimensions have a high surface to volume ratio that gives them some unique or similar properties that are varied from the same material in bulk which are helpful in various fields such as electronics, biomedical, photonics, etc.. The nanoparticles are also utilized in the field of solar energy conversion, and water treatment. Among the various fine metals, silver is preferred as a nanoparticle because of its antibacterial catalytic activity and it has no toxicity towards human beings which is similar to other metals (4). Previously several methods have been used for the synthesis of Ag-NPs, which can be either, chemical, physical or biological methods.

Green synthesis of nanoparticles has been considered as one of the hopeful methods for synthesis of nanoparticles because of their low toxicity, biocompatibility and eco-friendly nature (3). Thus, synthesis of silver nanoparticles by eco-friendly processes using the plant materials like root, leaf extract, bark, stem, fruit latex, bud is being carried out (5).

Medicinal plants have been used from ancient times to attempt cures for various diseases. The therapeutic power of traditional herbal medicines has been realized and familiar since Rigveda and Atharvaveda. Medicinal plants are potentially renewable natural resources and are generally considered to play a beneficial role in human health care (6-8). In view of this in the present study silver nanoparticles were synthesised using leaves of Olea dioica Roxb.

Olea dioica Roxb., is an important ethno medicinal plant medicinal plant belong to Oleaceae family. It grows in open evergreen forests up to 1100-1200 m and is distributed throughout the Western Ghats region of Kodagu, Dakshina Kannada, Udupi and Hassan. The plant parts such as roots, bark, and leaves are used for anti-cancer, antioxidant, and anti-AChE activity. Although vast amount of literature is available on the green synthesis of Ag-NPs, to the best of our knowledge no information is available on the synthesis of silver nano particles using the leaves of Olea dioica Roxb.. In the present investigation a
A stable and eco-friendly method has been developed for the synthesis of silver nanoparticles using methanolic extracts of *Olea dioica* Roxb. Thus, synthesised Ag-NPs were evaluated for their antimicrobial activities.

**MATERIALS AND METHODS**

**Chemicals**

Silver nitrate, (AgNO₃) was purchased from Himedia Ltd., India. All other chemicals used in this study were of analytical grade.

**Preparation of plant extract**

*Olea dioica* Roxb., leaves were collected from Pilikula Mangalore, Dakshina Kannada, Karnataka, India. Freshly collected leaves were washed 3-4 times with tap water and finally washed with distilled water, and shade dried at room temperature for 23 days and mechanically made a fine powder. Fifty grams of powder was extracted with methanol (12 hours) using Soxhlet extractor. The extracts were evaporated at room temperature and stored in brown bottles at room temperature until screened. This methanolic extract was used for the synthesis of silver nanoparticles.

**Synthesis of silver nanoparticles**

The methanol leaf extracts of *Olea dioica* Roxb., was used in the bio-reduction of silver ions. Five ml of leaf extract was taken in BOD bottle separately and was mixed with 95 ml of 1mM AgNO₃ solution. The silver nanoparticles synthesized were separated by centrifugation at 10,000 rpm for 10 minutes. The pellets were washed thoroughly at least three times with methanol, ethanol, and water to remove any biological contaminants. The particles were then dried and stored for further analysis (6, 12, 13).

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**Flow-chart:** Change in colour of the solution with time when silver salt was added to leaf extract.

**Characterization of silver nanoparticles**

**UV-visible spectral analysis**

The bio-reduction of silver nanoparticles was observed by UV-spectroscopy of the solution between 200 and 700 nm using (Beckman Coulter) UV-Vis spectrophotometer (14).
Effect of different contact time on Ag-NPs concentration

The effect of different contact time on silver nanoparticles concentration in the reaction mixture at different time intervals is exhibited with a colour change of the solution. The intensity of UV-absorption peaks is gradually increased and then becomes stable because the Ag-NPs concentration is initially increased with increase in different contact time which is due to the effect of outside Plasmon character of Ag-NPs (16).

FTIR (Fourier-transform infrared spectroscopy)

For FTIR analysis, the sample was prepared by dispersing the silver nanoparticles uniformly in a matrix of KBr (potassium bromide). The characterization of functional groups on the silver nanoparticles the spectra was scanned in the range of 4000–500 cm\(^{-1}\). The intense bands were compared with standard values to identify the functional groups (15).

SEM analysis

The sample for SEM analysis was prepared by dissolving nanoparticles in 0.1 ml of deionized water. This was placed on a glass coverslip and air-dried. The coverslip itself was used during SEM analysis (Carl Zeiss, Germany, and Model No. EVO LS 15). The images of Ag-NPs were obtained (16) in a scanning electron microscope at different nanometers (25, 50 nm).

Microbial cultures

The microbial cultures used in the present study were obtained from Department of Microbiology, Mangalore University, PG Centre, Chikka Aluvara, Kodagu, Karnataka, India. The cultures used were pathogenic bacteria such as Escherichia coli, Staphylococcus aureus, Streptococcus sp., Proteus vulgaris, Klebsiella and Salmonella sp. The pure bacterial cultures were sub-cultured on nutrient agar media. Different concentrations of silver nanoparticles in distilled water were used for antibacterial activity. The zone of bacterial inhibition was recorded after incubating the plates for 24 hours at 37°C (9).

Antioxidant assay

DPPH assay

Antioxidant activity was determined by Brand-Williams method (11) with a slight modification. The absorbance of the reaction mixture was measured at 517 nm after incubating at room temperature in dark condition for 30 min. using ascorbic acid as a standard. The percentage of antioxidant activity was calculated using the following formula.

\[
\% \text{ inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Test)}}{\text{Absorbance (Control)}} \times 100
\]

UV–visible spectroscopy

The synthesis of silver nanoparticles from plant leaf extracts

The colour changed to brown due to the addition of 1mM AgNO\(_3\) to the plant extracts. There was no colour change observed in 0 min. After 2-4 hours, colour of the solution changes from green to brown, the UV-Vis absorption spectra were studied at 300 to 700 nm, which confirmed the presence of Ag-NPs (Fig. 2). The absorption peak of silver nanoparticles is at 438 nm.

Different contact time and Tm (temperature) affect the synthesis of nanoparticles (Graph 2) and show the UV- absorption spectra of Ag-NPs at different time in the range of 20-60 min and 20-60°C. The temperature increased the change in the colour of the solution rapidly. When the Tm of the reaction mixture is increased, an increase in the synthesis of silver nanoparticles is logical which is due to the amplification in a decreased rate of Ag\(^+\) ions and the solution colour turning from brown to dark brown within 3 min and decrease in particle size. An increase in Tm above 40-60°C increases the absorbance peak (15).

![Graph 1: UV-Vis spectra of silver nanoparticles at different time intervals](image1)

![Graph 2: Absorption spectra of silver nanoparticles at 1mM concentration. The above figure shows an increase of absorption intensity as a function of Ag-NP concentration](image2)
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**FTIR**

FTIR measurement was used to find out the interaction of silver ions with the bioactive components present in the plant leaf extracts that are responsible for stabilizing of Ag-NPs. The absorption bands in the FTIR spectrum indicate the occurrence of functional groups in the silver nanoparticles (Fig. 2). FTIR results show the band in the range of 3500-500 cm⁻¹; different band range represents different functional groups (Table 1).

![FTIR Spectrum](image)

**Fig. 2:** FTIR spectrum of Ag-NPs

**Table 1:** FTIR peak values and functional groups of Ag-NPs

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Peak Value</th>
<th>Functional Group</th>
<th>Functional Group Name</th>
<th>Vibrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of nanoparticles</td>
<td>639.20</td>
<td>C-I</td>
<td>Haloalkane</td>
<td>Stretch</td>
</tr>
<tr>
<td>970-799</td>
<td>C-C</td>
<td>Alkane</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1121.21</td>
<td>C-F</td>
<td>Haloalkane</td>
<td>Bending</td>
<td></td>
</tr>
<tr>
<td>2921-2852</td>
<td>C-H</td>
<td>Alkane</td>
<td>Stretch</td>
<td></td>
</tr>
<tr>
<td>1463-1382</td>
<td>C-O, C-F</td>
<td>Alcohols, ethers, esters, and haloalkane</td>
<td>Bend out-of-plane</td>
<td></td>
</tr>
<tr>
<td>1633.57</td>
<td>C=C</td>
<td>Alkene</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1721.07</td>
<td>C=O</td>
<td>Acid, saturated</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2921-2852</td>
<td>C-H</td>
<td>Alkane</td>
<td>Stretch</td>
<td></td>
</tr>
<tr>
<td>3449.61</td>
<td>O-H</td>
<td>alcohols and Phenols</td>
<td>Stretch</td>
<td></td>
</tr>
</tbody>
</table>

**SEM**

The morphology of silver nanoparticles was examined using SEM and observed the reduction of AgNO₃ with plant leaf extract. The SEM results also depicted the morphology and size details of Ag-NPs with high-density. An assembly of spherical and uniform Ag-NPs were observed (Fig. 3).

![SEM Image](image)

**Fig. 3:** SEM image of the biosynthesized silver nanoparticles

**Antimicrobial activities**

Silver nanoparticles were tested for antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria. Green synthesized silver nanoparticles exhibited good antibacterial activity against the pathogenic bacteria (Table 2 and Fig. 4). The silver nanoparticles synthesized using methanolic leaf extract showed good antibacterial activity compared to those synthesised using water extract of leaf.
Pratap and Shantaram: Green synthesis of silver ..... its biological activity

Table 2: Zone of inhibition (in mm)

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Name of the Bacteria</th>
<th>Methanol extract</th>
<th>Water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E. coli</td>
<td>4 mm</td>
<td>2 mm</td>
</tr>
<tr>
<td>2</td>
<td>S. epidermis</td>
<td>2 mm</td>
<td>1 mm</td>
</tr>
<tr>
<td>3</td>
<td>B. subtilis</td>
<td>3 mm</td>
<td>1 mm</td>
</tr>
</tbody>
</table>

Antioxidant activity

The antioxidant activity of silver nanoparticles was evaluated by DPPH radical scavenging method. A potent antioxidant activity was observed, and the activity was increased in a dose dependent manner (Graph 3).

DISCUSSION

Green synthesis of nanoparticles has been considered as one of the hopeful methods for synthesis of nanoparticles because of their low toxicity, biocompatibility and eco-friendly nature (3, 17). The colour of silver nanoparticles changed to brown due to the addition of 1mM AgNO₃ to the plant extracts. The absorption peak of silver nanoparticles is at 438 nm.

UV-vis spectra were used to observe the effect of contact time and optimum temperature on Ag-NPs concentration in the reaction mixture at altered optimum intervals of the time period. The solution was observed for colour change (Fig. 1 and Flow chart 1). The intensity of UV-vis absorption peaks was increased gradually and then became constant because the concentration of silver nanoparticles was initially increased with increase in temperature and different contact time. Different contact time was obtained for this experiment between 40-60 minutes (Graph) which showed a very broad absorbance peak showing an increasing particle size (10, 18).

FTIR spectrum showed different peak positions at 639.20, 961.20, 1121, 1522.04, 1646.15, 1739.98, 2359.98, 2927, 3369 cm⁻¹. The similarities between the spectra with some marginal shifts in peak position clearly indicates the presence of the residual plant extract in the sample as a capping agent to the silver nanoparticles. The peak located at the different range and functional groups of each peak (Fig 3 & Table 1). Antimicrobial activity of Ag-NPs was carried out against pathogenic bacteria (Fig. 4 & Table 2). The synthesized Ag-NPs exhibited excellent antibacterial activity against both Gram-positive and Gram negative such as S. aureus and E. coli bacteria (19, 20). The nanoparticles are also utilized in the field of solar energy conversion, and water treatment. Further, in biomedical application, silver nanoparticles are being used for anticancer, anti-inflammatory activity.

CONCLUSION

The green synthesis of Ag-NPs from Olea dioica Roxb., leaf extract is simple, cost-effective, fast, environment-friendly and the plant leaf phytochemical acts as the reducing agent. The silver (Ag-NPs) nanoparticles synthesized by the one-pot green synthesis method are proved to be potential reagents in various medical and industrial applications.
REFERENCES


A comparative study on levels of renal and lipid profile in type 2 diabetic and diabetic nephropathy patients - a case control study

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ABSTRACT

Introduction: Diabetic nephropathy is a leading cause of end-stage renal failure worldwide. Its morphologic characteristics include glomerular hypertrophy, basement membrane thickening, mesangial expansion, tubular atrophy, interstitial fibrosis and arteriolar thickening. All of these are part of micro vascular complications of diabetes.

Objective: The present study is one such attempt to find the relation between renal and lipid profile in diabetic nephropathy in ethnic south Indian population.

Materials and Methods: In the present study, 60 cases presenting with diabetic Nephropathy and 60 age and sex matched controls with type 2 diabetes were included in the study.

Results: In the present study FBS, PPBS, Urea, Creatinine, Total cholesterol, Triglycerides; LDL levels are increased whereas, HDL levels are decreased in diabetic nephropathy when compared with type 2 diabetes mellitus patients.

Conclusion: The present study shows LDL mass without major compositional changes suggests that the elevation of LDL in incipient and established diabetic nephropathy is primarily due to the increased number of LDL particles.

Keywords: Diabetes; Nephropathy; Urea; Creatinine; Total cholesterol; Triglycerides; HDL.

INTRODUCTION

Diabetes mellitus continues to be the leading cause of chronic kidney disease (CKD) and end-stage renal disease in the United States, Japan, and Europe, accounting for up to 45% of all cases in Western societies. Diabetic nephropathy (DN) is a clinical syndrome found in both type 1 diabetes and type 2 diabetes that is characterized by heavy proteinuria, renal failure, and arterial hypertension (1), with its hallmark being persistent albuminuria (> 300 mg/24 hours). Risk factors for DN include age, race, systemic hypertension, hyperglycaemia, male gender, race, smoking, genetic susceptibility, and dyslipidaemia. These variables have also been positively linked to the increase in cardiovascular events (2). In turn, abnormal renal function and albuminuria may independently predict cardiovascular risk. There is increasing evidence linking dyslipidaemia as an independent contributing factor in the development and progression of glomerular injury, although the underlying mechanisms are currently debated (3). Diabetic dyslipidaemia over two decades ago, the recent study shows suspected that the persistent filtration of lipids and lipoproteins promote progression of chronic renal injury. Many subsequent observational studies supported the role of elevated levels of serum lipids in the development of albuminuria and in the progression of glomerulosclerosis. The lipid profiles of individuals with DN have been characterized to have higher plasma concentrations of very low density lipoprotein cholesterol (VLDLC), low-density lipoprotein cholesterol (LDLC), intermediate-density lipoprotein cholesterol, and triglycerides but lower levels of HDLC (4) also noted are elevated plasma concentrations of Apo lipoprotein (apo) B, apo C-III, and apo (a). The aforementioned lipid profile has been termed “diabetic dyslipidaemia”; it is mostly seen in individuals with type 2 diabetes (5).

Moreover, this is further characterized by a preponderance of dense, small-diameter LDLC and HDLC particles along with excessive postprandial lipemia, which results from increased concentrations of VLDLC and chylomicron remnants. An increase in hepatic lipase activity and a reduced post hepamin plasma lipoprotein lipase (LPL) ratio have also been documented. Interestingly, the aforementioned lipoprotein and HDLC particles may independently predict cardiovascular risk. There is increasing evidence linking dyslipidaemia as an independent contributing factor in the development and progression of glomerular injury, although the underlying mechanisms are currently debated (3). Diabetic dyslipidaemia over two decades ago, the recent study shows suspected that the persistent filtration of lipids and lipoproteins promote progression of chronic renal injury. Many subsequent observational studies supported the role of elevated levels of serum lipids in the development of albuminuria and in the progression of glomerulosclerosis. The lipid profiles of individuals with DN have been characterized to have higher plasma concentrations of very low density lipoprotein cholesterol (VLDLC), low-density lipoprotein cholesterol (LDLC), intermediate-density lipoprotein cholesterol, and triglycerides but lower levels of HDLC (4) also noted are elevated plasma concentrations of Apo lipoprotein (apo) B, apo C-III, and apo (a). The aforementioned lipid profile has been termed “diabetic dyslipidaemia”; it is mostly seen in individuals with type 2 diabetes (5).

Role of dyslipidaemia in renal injury pathogenesis of glomerular and tubulointerstitial injury in the human glomerulus, one often finds the formation of mesangial foam cells via the expression of scavenger receptors for modified, glycosylated and oxidized...
LDLC. Accumulation of these substances in the mesangial matrix triggers the activation of monocytes into macrophages. It is believed that this pathophysiologic mechanism of renal injury is related to dyslipidemia via three stages. First, exposure to oxidized lipoproteins stimulates the mesangial cell secretion of chemotactic agents and adhesion molecules further enhancing the recruitment of macrophages.

The monocyte infiltration results in glomerulosclerosis and tubular fibrosis. Secondly, the uptake of oxidizing LDLC by recruited macrophages stimulates the release of reactive oxygen species and the expression of proinflammatory cytokines (transforming growth factor [TGF]-β1 and platelet-derived growth factor-AB). Finally, these cytokines stimulate the production of extracellular matrix proteins subsequently promoting mesangial expansion. In the tubule-interstitium, the renal injury due to hyperlipidemia has been suggested to be a prognostic indicator because tubulointerstitial lesions may precede glomerular changes and correlate better with renal disease progression. In studies of hyperlipidemic, the tubular injury was ascribed to interstitial macrophage infiltration and an increase in TGF-β1 gene expression. It is believed that this is mediated via cytokine reactions and reactive oxygen species. These phenomena are similar to those in vivo studies where the tubular uptake and metabolism of filtered lipoproteins resulted in the expression of cytokines and subsequent local inflammation. The Present study is to find the relation between renal and lipid profile in diabetic nephropathy in South Indian population.

MATERIALS AND METHODS

The present study was conducted in the Department of Biochemistry in collaboration with Department of Nephrology in Saveetha medical College, Thandalam, Chennai. The study was conducted on patients with type-2 diabetes mellitus and diabetic Nephropathy admitted in the nephrology unit in Saveetha Hospital and Medical College. This study was approved by Institutional Human ethics Committee. Study population consisted of 60 patients with diabetic nephropathy (Age range 40-75 yrs) and control group consisted of 60 patients with type-2 diabetes mellitus who are on medical treatment without any complications.

Inclusion criteria: Cases were taken who are diagnosed patients of diabetic nephropathy attending the department of nephrology of saveetha medical college (defined as patients having arterial hypertension less than 200/160, eGFR > 45 and <90 mL/min/1.73 m2 and/or urinary albumin: creatinine ratio >3 mg/mmol (11). Controls: Known diabetes mellitus patients who are on medical treatment without any complications as controls and Age Group of 40-70yrs for both cases & controls.

Exclusion criteria

Patients will be excluded if they have any of the following: a history of cardiovascular disease, defined as having a clinical record of ischemic heart disease (angina, myocardial infarction, coronary artery revascularization and or heart failure), peripheral vascular disease (intermittent claudication or peripheral artery revascularization) or cerebrovascular disease (transient ischemic episodes or stroke). A history of malignancy or any other life threatening illness, current pregnancy, systolic blood pressure >200 mmHg, diastolic blood pressure >160 mmHg, hemoglobin Alc > 10 %. Significant renal impairment (eGFR< 45 mL/min 1.73 m2) and nephrotic range urine protein excretion (total protein excretion rate >3 g/day or albumin to creatinine ratio >300 mg/ mmol) and patients with age <40 and >70 are excluded. Sample collection and storage 5ml of venous whole Blood were collected from both type-2 diabetes mellitus and diabetic Nephropathy.

Biochemical analysis

Fasting Blood Sugar (FBS), Urea, Creatinine, Total Cholesterol, Triglycerides, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL) were estimated in VITROS 240. Statistical analysis were done using student ‘t’-test and p-value significance. P-value <0.01 were considered as significant.

RESULTS

In the present study, a total number of 120 subjects comprising of 60 type-2 diabetes mellitus patients (control) and 60 diabetic nephropathy cases were included. Here, we have grouped 120 subjects into four groups based on gender. In the present study, we identified that association between renal and lipid profile in diabetic nephropathy. In the present study, there was significant increase in the Total cholesterol, triglycerides, LDL, Urea, Creatinine and decrease of HDL in the diabetic nephropathy patients when compared with diabetic patients (Table 1).

Table 1: Comparision of fasting blood sugar (FBS), post prandial blood sugar (PPBS), urea, creatinine, total cholesterol, triglycerides, HDL, LDL, VLDL in type 2 diabetes mellitus (T2DM-Controls) and diabetic nephropathy (DN- Cases)
DISCUSSION
Table 1 shows the mean value of Fasting Blood Sugar of male in type-2 diabetes mellitus and diabetic nephropathy are 123.82 and 163.30 respectively. Its P value is < 0.001. It is found to be statistically significant (12).

Similarly, the mean value of Fasting Blood Sugar of female in type-2 diabetes mellitus and diabetic nephropathy are 119.00 and 154.64 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of Fasting Blood Sugar in diabetic nephropathy in both male and female is observed.

The mean value of Postprandial blood sugar of male in type-2 diabetes mellitus and diabetic nephropathy are 201.00 and 235.09 respectively. Its P value is <0.001. It is found to be statistically significant. Similarly, the mean value of Postprandial Blood Sugar of female in type-2 diabetes mellitus and diabetic nephropathy are 198.94 and 247.23 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of post-prandial blood sugar in diabetic nephropathy in both male and female is observed.
values of Creatinine in diabetic nephropathy in both male and female is observed.

Table no 1 shows the mean value of total Cholesterol of male in type-2 diabetes mellitus and diabetic nephropathy are 205.52 and 228.55 respectively. Its P value is < 0.001. It is found to be statistically significant. Similarly, the mean value of total Cholesterol of female in type-2 diabetes mellitus and diabetic nephropathy are 207.05 and 228.47 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of total Cholesterol in diabetic nephropathy in both male and female is observed (15).

Table no 1 shows the mean value of triglycerides of male in type-2 diabetes mellitus and diabetic nephropathy are 172.95 and 189.51 respectively. Its P value is < 0.001. It is found to be statistically significant. Similarly, the mean value of triglycerides of female in type-2 diabetes mellitus and diabetic nephropathy are 174.81 and 189.47 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of triglycerides in diabetic nephropathy in both male and female is observed (15).

Table no 1 shows the mean value of HDL of male in type-2 diabetes mellitus and diabetic nephropathy are 41.261 and 30.860 respectively. Its P value is < 0.001. It is found to be statistically significant. Similarly, the mean value of HDL of female in type-2 diabetes mellitus and diabetic nephropathy are 40.838 and 30.647 respectively. Its P value is < 0.001. It is found to be statistically significant. Decreased values of HDL in diabetic nephropathy in both male and female is observed (16).

Table no 1 shows the mean value of LDL of male in type-2 diabetes mellitus and diabetic nephropathy are 134.696 and 143.00 respectively. Its P value is < 0.001. It is found to be statistically significant. Similarly, the mean value of LDL of female in type-2 diabetes mellitus and diabetic nephropathy are 135.514 and 143.941 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of LDL in diabetic nephropathy in both male and female is observed (17).

Table no 1 shows the mean value of VLDL of male in type-2 diabetes mellitus and diabetic nephropathy are 34.591 and 37.902 respectively. Its P value is < 0.001. It is found to be statistically significant. Similarly, the mean value of VLDL of female in type-2 diabetes mellitus and diabetic nephropathy are 34.962 and 37.89 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of VLDL of female is statistically significant. Increased values of VLDL of male in diabetic nephropathy are 34.962 and 37.89 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of total Cholesterol of female in type-2 diabetes mellitus and diabetic nephropathy are 207.05 and 228.47 respectively. Its P value is < 0.001. It is found to be statistically significant. Increased values of total Cholesterol in diabetic nephropathy in both male and female is observed (15).

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CONCLUSION
The present study shows LDL mass without major
compositional changes suggests that the elevation of LDL in incipient and established diabetic nephropathy is primarily due to the increased number of LDL particles. The net result consists of enhanced lipolysis with a subsequent increase in free fatty acids and VLDLC synthesis, a defect in LPL activity leading to the increased life span of chylomicrons and VLDLC in circulation, an increased transfer of cholesterol esters resulting in triglyceride-rich LDLC, and finally the elevation of plasma triglycerides and the reduced ratio of LPL to hepatic lipase causing the accelerated breakdown of HDLC.

ACKNOWLEDGEMENT
The author is thankful to Department of Biochemistry and Nephrology Departments of Saveetha Hospital and Medical College for providing facilities to carry out this research work.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest in this study.

REFERENCES


Evaluation of synergistic potential of *Tridax procumbens* and *Boerhavia diffusa* in isoproterenol induced myocardial injury

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ABSTRACT

Introduction and Aim: Combinative action of medicinal plants has earned an important place in the herbal medicinal field. The combination therapeutics may provide higher activity against various diseases in a synergistic manner. In the present research work the ethanolic leaf extracts of *Tridax procumbens* and root extracts of *Boerhavia diffusa* were studied for the assessment of synergistic cardioprotective activity through animal model.

Materials and Methods: Wistar male albino rats were treated with combinative ethanolic extract (150 +150 mg/kg BW) to isoproterenol-induced myocardial infarcted rats. Cardio-protection was investigated by estimating the activities of inflammatory markers like CRP, myeloperoxidase, homocysteine, xanthine oxidase and HMG CoA reductase in serum, plasma and tissue sample.

Results: The activities of CRP in serum and tissue myeloperoxidase were significantly elevated in isoproterenol-induced rats. Parameters like plasma homocysteine, tissue xanthine oxidase and HMG CoA reductase of isoproterenol administered rats also showed significant elevation. Combinative ethanolic leaf extract of *Tridax procumbens* and root extracts of *Boerhavia diffusa* treatment showed a marked difference in the altered parameters by preventing myocardial necrosis.

Conclusion: The present study concludes that co-treatment with ethanolic extract of *Tridax procumbens* and *Boerhavia diffusa* confirmed the protective and inhibitory effect against isoproterenol induced myocardial infarction and proved to be more beneficial.

Keywords: Isoproterenol; myocardial infarction; *Tridax procumbens*; *Boerhavia diffusa*; ethanol extract.

INTRODUCTION

Myocardial infarction (MI) is the most important killer disease responsible for death and disability throughout the world (1). It is characterized by inadequate oxygen due to low supply of blood, which leads to myocardial injury. Oxidative stress was found to play a major role in the development of myocardial infarction (2). Development of free radicals leads to hemo-dynamic, biochemical and histopathological alterations which results in membrane damage, diminished endogenous antioxidants and escape of cardiac markers into systemic circulation (3). Even though various medications are available to treat MI, nature has provided excellent source of medicinal agents that find applications in various field. Phytochemicals present in medicinal plants play a vital role in fighting against deadly diseases in particular MI. Medicinal plants are the best choice for a preventive approach that is cost effective with fewer side effects (4). Several reports are focused on medicinal plants individually and in combination. Bioactive compounds present in different plants exert synergistic functions in combination by interacting with one another (5).

*Tridax procumbens* is a weed that is found throughout India along fields, roadside etc., (6). *T. procumbens* belongs to Asteraceae family called as “Vettukayalthalai” in Tamil that has significant wound healing activity (7). It is well known for excellent medicinal properties like antidiabetic, antioxidant and cardiovascular role. Its leaves are well known medicine for liver disorders (8), hypocholesterolemic, hypotensive and weight reducing properties (9).

*Boerhavia diffusa* belongs to Nyctaginaceae family called as “Mookerettai keerai” in Tamil, “Punarnava” in Hindi, “Varshabhu” in Sanskrit (10). It is a creeping weed found in fields and wastelands (11). Various parts of *B. diffusa* provide significant medicinal activity. The roots of *B. diffusa* have numerous medicinal properties. It is used to treat kidney disorders, cardiac disorders, and cancer (12).
The present research work is focused on the evaluation of the cardioprotective efficacy of *T. procumbens* and *B. diffusa*, individually and in combination, against isoproterenol induced MI.

**MATERIALS AND METHODS**

**Collection on medicinal plants**

Fresh leaves and roots of *T. procumbens* and *B. diffusa*, respectively, were collected from the local villages around Tiruchirappalli and was authenticated by John Britto Rapinat Herbarium, Department of Botany, St. Joseph’s College, Tiruchirapalli. The collected parts of the plants were washed and shade dried. The shade dried plant materials were ground into coarse powder separately and stored in clean containers at room temperature.

**Preparation of the plant extracts**

*T. procumbens* leaves (100 g) and *B. diffusa* root (100 g) powder were mixed with 500ml of ethanol for 24 hours. It was filtered and the filtrates were then evaporated with the help of rotary evaporator. The extracts were stored in sterile glass bottle at room temperature.

**Selection and maintenance of experimental animals**

Male Wistar albino rats weighing 150-200 g were selected for the study. The animals were housed in well-ventilated neat cages, lined with sterile husk and fed with standard pellet rodent diet and water ad libitum. The rats were acclimatized to laboratory conditions for 10 days before the commencement of the experiments. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

**Grouping of experimental animals**

The animals were divided into nine groups. Each group consisted of 6 rats (n=6).

*Group I:* Control (normal rats received 0.9 % saline)

*Group II:* received isoproterenol subcutaneously twice (85 mg/kg SC) at an interval of 24 hours.

*Group III:* received isoproterenol (85 mg/kg SC) and *T. procumbens* (100 mg/kg body weight orally) for 15 days

*Group IV:* received isoproterenol (85 mg/kg SC) and *T. procumbens* extract (200 mg/kg body weight orally) for 15 days

*Group V:* received isoproterenol (85 mg/kg SC) and *T. procumbens* extract (300 mg/kg body weight orally) for 15 days

*Group VI:* received isoproterenol (85 mg/kg SC) and *B. diffusa* (100 mg/kg body weight orally) for 15 days

*Group VII:* received isoproterenol (85 mg/kg SC) and *B. diffusa* extract (200 mg/kg body weight orally) for 15 days

*Group VIII:* received isoproterenol (85mg/kg SC) and *B. diffusa* extract (300 mg/kg body weight orally) for 15 days

*Group IX:* received isoproterenol (85 mg/kg SC) in combination with *T. Procumbens* (150 mg/kg body weight orally) and *B. diffusa* extracts (150 mg/Kg body weight orally) for 15 days.

The animals were sacrificed by cervical decapitation and blood was collected and separated. The heart was dissected and washed in ice cold saline, homogenized and used for experiments.

**Assessment of biochemical parameters in serum**

Blood samples from animals were taken. Serum and plasma were separated for the analysis of CRP, myeloperoxidase and homocysteine by standard procedure (13, 14).

**Assessment of biochemical parameters in tissue**

The heart tissue of the experimental animals was taken and homogenized in 10% ice-cold phosphate buffer and the mixture was centrifuged. The homogenate thus obtained was used for the estimation of xanthine oxidase (15).

**Estimation of HMG-CoA reductase in rat liver**

The ratio between 3-hydroxy-3-methylglutaryl-CoA and mevalonate concentrations in tissues in terms of absorbance was taken as an index of the activity of HMG-CoA reductase. The level of HMG-CoA reductase was estimated in rat liver homogenate (16).

**Statistical analysis**

Statistical significance was evaluated by One-way analysis of variance (ANOVA) using SPSS version (17.0) and the individual comparisons were obtained by the Duncan’s multiple range test (DMRT). A value of p<0.05 was considered as a significant difference between groups.

**RESULTS**

Development of atherosclerosis is considered to be a significant inflammatory event (17). The effect of plant extracts on serum / tissue levels of inflammatory markers (CRP, myeloperoxidase) in normal and isoproterenol-injected rats are shown in figure 1 and 2. Rats injected with isoproterenol showed a significant (p<0.05) increase in serum CRP and tissue MPO activity as compared to the control group. Treatment with the leaf extracts of *T. procumbens* and the root extracts of *B. diffusa* individually and in combination...
in isoproterenol injected rats showed a significant (p<0.05) decrease in serum CRP levels, and MPO activity.

Values are mean ± SD; n = 6; Statistically significant difference at P < 0.05 + group I Vs group II, * group II Vs group III, IV, V, VI, VII, VIII, IX.

**Group descriptions:**
- Group I - Control rats;
- Group II - isoproterenol induced rats;
- Group III - isoproterenol with *T. procumbens* (100mg);
- Group IV - isoproterenol with *T. procumbens* (200mg);
- Group V - isoproterenol with *T. procumbens* (300mg);
- Group VI - isoproterenol with *B. diffusa* (100mg);
- Group VII - isoproterenol with *B. diffusa* (200mg);
- Group VIII - isoproterenol with *B. diffusa* (300mg);
- Group IX - isoproterenol with *T. procumbens* and *B. diffusa* (150 mg + 150 mg).

**Group descriptions:**
- Group I – Control rats;
- Group II – isoproterenol induced rats;
- Group III - isoproterenol with *T. procumbens* (100mg);
- Group IV - isoproterenol with *T. procumbens* (200mg);
- Group V - isoproterenol with *T. procumbens* (300mg);
- Group VI - isoproterenol with *B. diffusa* (100mg);
- Group VII - isoproterenol with *B. diffusa* (200mg);
- Group VIII - isoproterenol with *B. diffusa* (300mg);
- Group IX - isoproterenol with *T. procumbens* and *B. diffusa* (150 mg + 150 mg).

Homocysteine, a non-essential thiol containing amino acid is derived from methionine metabolism may generate partially reduced ROS that are able to stimulate the LPO in the atherosclerotic process (18). Significant (p<0.05) elevation in the level of homocysteine was noted in plasma of Group II isoproterenol-administered rats compared to Group I control rats (Fig. 3). *T. procumbens* and *B. diffusa* treated rats individually in increasing concentrations and in combination normalized the level of homocysteine.
Fig. 3: Level of homocysteine in plasma of experimental groups

Values are Mean ± SD; n = 6; statistically significant difference at p< 0.05 + group I Vs group II, * group II Vs group III, IV, V, VI, VII, VIII, IX.

**Group descriptions:**

- Group I – Control rats;
- Group II – isoproterenol induced rats;
- Group III - isoproterenol with *T. procumbens* (100mg);
- Group IV - isoproterenol with *T. procumbens* (200mg);
- Group V - isoproterenol with *T. procumbens* (300mg);
- Group VI - isoproterenol with *B. diffusa* (100mg);
- Group VII - isoproterenol with *B. diffusa* (200mg);
- Group VIII - isoproterenol with *B. diffusa* (300mg);
- Group IX - isoproterenol with *T. procumbens* and *B. diffusa* (150mg + 150mg).

Table 1 shows the level of HMG-CoA reductase in the liver of experimental rats. A significant (p<0.05) increase in HMG-CoA reductase in isoproterenol administered rats were observed when compared to control groups. Isoproterenol administered rats treated with plant extracts showed significant decrease in the activity of HMG-CoA reductase.

**Table 1:** Comparison of HMG-CoA activity in the liver tissue homogenate of the experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>HMG CoA reductase (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.50 ± 0.01</td>
</tr>
<tr>
<td>II</td>
<td>6.24 ± 0.02*</td>
</tr>
<tr>
<td>III</td>
<td>5.07 ± 0.03*</td>
</tr>
<tr>
<td>IV</td>
<td>4.45 ± 0.02*</td>
</tr>
<tr>
<td>V</td>
<td>4.00 ± 0.02*</td>
</tr>
<tr>
<td>VI</td>
<td>5.13 ± 0.04*</td>
</tr>
<tr>
<td>VII</td>
<td>5.01 ± 0.05*</td>
</tr>
<tr>
<td>VIII</td>
<td>4.15 ± 0.01*</td>
</tr>
<tr>
<td>IX</td>
<td>3.83 ± 0.03*</td>
</tr>
</tbody>
</table>

Values are Mean ± SD; n = 6; statistically significant difference at p < 0.05 + group I Vs group II, * group II Vs group III, IV, V, VI, VII, VIII, IX.

**Group descriptions:**

- Group I - Control rats;
- Group II - isoproterenol induced rats;
- Group III - isoproterenol with *T. procumbens* (100mg);
- Group IV - isoproterenol with *T. procumbens* (200mg);
- Group V - isoproterenol with *T. procumbens* (300mg);
- Group VI - isoproterenol with *B. diffusa* (100mg);
- Group VII - isoproterenol with *B. diffusa* (200mg);
- Group VIII - isoproterenol with *B. diffusa* (300 mg);
- Group IX - isoproterenol with *T. procumbens* and *B. diffusa* (150 mg + 150 mg).

Xanthine oxidase is a vital cause of free radical generation. Xanthine oxidase act on xanthine and hypoxanthine with the resultant production of oxygen free radicals and increased uric acid level. Figure 4 depicted the level of xanthine oxidase in heart tissue of experimental rats. There was a significant increase in xanthine oxidase level in heart tissue of
isoproterenol-injected rats compared to control rats. Plant extract treated rats in combination significantly reversed the increased levels of xanthine oxidase.

Values are Mean ± SD; n = 6; statistically significant difference at p < 0.05 + group I Vs group II, * group II Vs group III, IV, V, VI, VII, VIII, IX.

**Group descriptions:**
Group I - Control rats;
Group II - isoproterenol induced rats;
Group III - isoproterenol with *T. procumbens* (100mg);
Group IV - isoproterenol with *T. procumbens* (200mg);
Group V - isoproterenol with *T. procumbens* (300 mg);
Group VI - isoproterenol with *B. diffusa* (100 mg);
Group VII - isoproterenol with *B. diffusa* (200 mg);
Group VIII - isoproterenol with *B. diffusa* (300 mg);
Group IX - isoproterenol with *T. procumbens* and *B. diffusa* (150 mg + 150 mg).

**DISCUSSION**
C-reactive protein is frequently studied as an inflammatory marker and sensitive predictor of cardiovascular events. Myeloperoxidase (MPO) has emerged as a new inflammatory marker, which is involved in oxidative stress and is a leading marker for the assessment of atherosclerosis (19).

Myeloperoxidase cause oxidative modification of low density lipoprotein (LDL) is considered as a key event in the promotion of atherogenesis and in the initiation and progression of cardiovascular diseases (20). An elevated concentration of C-reactive protein is an indicator of inflammation. C-reactive protein is predominantly made in the liver and is secreted in increased amounts within 6 hours of an acute inflammatory stimulus. Co-treated plant extracts based on different dosage and in combination prevented the inflammation and modification caused by LDL (21).

Elevation of homocysteine is used as an independent risk factor for MI (22). The plant extracts might have inhibited the production of homocysteine from methionine that are vital for free radicals inducement. Investigations by Subashini and Rajadurai (2011) indicated that *Nelumbo nucifera* leaf extracts effectively decreased the levels of homocysteine in isoproterenol treated rats (23).

HMG-CoA reductase is the rate limiting enzyme in the cholesterol biosynthesis. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase are the most effective and best-tolerated drugs to treat elevated levels of low-density lipoprotein cholesterol. Increase in the level of HMG CoA might be due to higher demand for energy by the β-adrenergic receptor stimulated myocardium and lipid peroxidation in isoproterenol-induced rats. The plant extracts in combination significantly decreased the activity of this enzyme in the liver of myocardial infarcted rats due to the regulation of β-adrenergic receptor stimulation and its anti-lipid peroxidative activity. The results of present study are in accordance with the previous reports of Shameela et al., (2015), who reported that ethanolic extract of *B. diffusa* significantly decreased the activity of HMG-CoA reductase in isoproterenol treated myocardial infarcted rats (24).

Increased xanthine oxidase level indicates that myocardial ischemia has a specific relationship with xanthine oxidase activity. During ischemic conditions, the adenosine nucleotide group is catabolized to hypoxanthine and xanthine, in association with the conversion of xanthine dehydrogenase to xanthine oxidase (25). In the present study, rats treated with the combination of plant extracts had significant decrease in the myocardial necrosis. Studies also showed that serum uric acid concentrations are higher in patients with established coronary heart disease compared with healthy control.

**CONCLUSION**
Screening the synergistic combinations of medicinal plants is an ongoing challenge in research field. The present study clearly concludes that *T. procumbens* and *B. diffusa* in combination exhibited significant cardioprotective activity when compared to individual
extracts. The combinative treatment altered the changes exerted by isoproterenol by proving as powerful cardioprotective agent. Thus, the combination of *T. procumbens* and *B. diffusa* can be used as an alternative effective drug for treating MI due to the excellent synergistic potential and due to enriched polyphenolic contents.

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Association of different co-morbidities among patients of chronic obstructive pulmonary disease and its exacerbations in a tertiary care hospital

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ABSTRACT

Introduction and Aim: Chronic obstructive pulmonary disease (COPD) and its exacerbations related mortality pose a major socio-economic burden to the community. Along with exacerbation, it is also associated with a number of comorbid systemic diseases, which in turn increases the risk of morbidity and mortality in all stages of COPD. Therefore, the present study aims to find out commonly prevalent extra-pulmonary comorbidities that will help in understanding the real burden of the disease and also help in proper management and improving clinical outcome of COPD and its exacerbations.

Materials and Methods: The prevalence of comorbidities was compared between 209 subjects of COPD and 146 subjects of COPD with acute exacerbations (COPD with AE) for a period of one year. COPD was diagnosed basing upon the Global Initiative for Obstructive Lung Disease (GOLD) criteria. Age of presentation of the disease, duration of hospital-stay and comorbidities associated with COPD were analyzed. Statistical analysis was done using standard statistical software Stata 15.1.

Results: The mean age of presentation of COPD and COPD with AE did not differ significantly. Duration of hospital stay was more in COPD than COPD with AE (8.53 ± 6.65 Vs 6.95 ± 3.78 days, respectively, p< 0.001). COPD subjects had higher level of creatinine than COPD with AE (1.37 ± 1.38 vs.1.13 ± 0.85mg/dL respectively, p=0.06). Hemoglobin level shows significant difference between two groups (p< 0.05). Hypertension, coronary artery disease, anemia, renal diseases were more frequent comorbidities associated with COPD and its exacerbations.

Conclusion: Extra pulmonary comorbidities increase morbidity, mortality, healthcare cost and make management of COPD difficult. Therefore, there is a need for evaluation and adequate treatment of comorbidities which can avoid future exacerbation.

Keywords: GOLD criteria; Comorbidities; Prevalence; DM; HTN.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive disabling illness, characterized by chronic inflammation and irreversible obstruction of the airways due to inhalation of noxious particles (1). Inflammation and inflammatory cytokines plays major role in the development of the disease process. Localized airway inflammation gradually progresses to systemic inflammation (2, 3). COPD is reported to be a major cause of chronic morbidity and mortality (4). Near about 30 million of people are suffering from COPD, the number is more than western population and mortality of this disease is rising faster (5). The burden of COPD is increasing in developing countries due to exposure to risk factors like biomass, fumes, smoke, air pollution and history of chronic asthma (6, 7).

Various extra-pulmonary comorbidities [Coexistence of other chronic conditions along with COPD] like hypertension (HT), diabetes mellitus (DM), coronary arterial disease (CAD), skeletal muscle dysfunction, osteoporosis, depression, renal insufficiency and malignancy of lungs are common and significant in COPD (8-10). The concomitant existence of comorbidities has significant clinical implications in disease progression and considerable impact on overall management of such patients (11). It may also be worth mentioning that comorbidities in COPD are associated with poor clinical outcome and even increase risk of mortality (12). Importantly, inclusion of many drugs to treat COPD and associated comorbidities, may also contribute to worsen some of the comorbidities (13). Several studies, around the world were conducted to estimate the prevalence of COPD along with associated comorbidities (14, 15). However, many comorbidities are often undiagnosed and hence untreated. Therefore, objectively identifying these comorbidities and their prevalence is an important step towards comprehensive therapy approach. This necessitates the need of understanding COPD as a systemic disease. Although a number of studies have documented the prevalence of
comorbid conditions of varying degree of severity, yet the reliable estimation of COPD prevalence remains a big concern in many parts of the world, as many of these estimates are based on varying definition and diagnostic criteria of COPD. Moreover, little is known about the interaction of comorbidities with the burden of the disease process. To study the growing prevalence of COPD worldwide and its adverse impact on clinical implication, the present study was planned to explore these issues and to adapt health policy measures to increase life expectancy and to reduce the burden of increasing COPD. Our objective was to assess the prevalence of co-morbidities in patients with COPD and to correlate the prevalence to the severity of the disease process, which ensures better management policies.

MATERIALS AND METHODS

This retrospective study was conducted in a tertiary care hospital for a period of twelve months [Sep 2017 to Sep 2018]. Waiver of consent was obtained as the study involved less than minimal risk. In the present study, data sheets of diagnosed cases of COPD (n=355) were evaluated. The patients were in the age group of 35-70 years. They were categorized into two groups. Group I: COPD cases (n=209) and Group II: Cases of COPD with acute exacerbation [AE] (n=146). Data sheets of patients diagnosed, evaluated and confirmed by respiratory physicians, as per internationally accepted standardized Global Initiative for obstructive lungs disease (GOLD) criteria, were included in the study.

GOLD Criteria is based upon spirometry, airflow limitation was used for categorizing COPD cases as mild, moderate, severe and very severe grades (GOLD 1-mild, GOLD 2- moderate, GOLD 3-severe, GOLD 4- very severe). Post bronchodilator ratio of forced expiratory volume (FEV1) to forced vital capacity FVC, i.e., FEV1/ FVC < 70% was used to determine airflow limitation, along with oxygen saturation >90%, which is followed to classify severity of COPD (1). Grading of severity as per GOLD criteria is as follows: GOLD 1: mild (FEV1 ≥ 80% predicted), GOLD 2: moderate (50%≤ FEV1 < 80% predicted), GOLD 3: severe (30% ≤ FEV1 < 50% predicted) and GOLD 4: very severe (FEV1 < 30% predicted).

Patients suffering from lung diseases other than COPD, and pregnant women were excluded from the study. Detailed epidemiological data were collected from medical records as follows: Age, gender, socioeconomic status, duration and severity of the disease, smoking history, tobacco use, alcohol consumption, exposure to biomass fuel, other occupational exposure, number of exacerbations of COPD, duration of hospital stay, associated co-morbidities, and the medicines prescribed to treat COPD were collected.

Statistical analysis

The data was analyzed by SPSS software version 15.1. Normally distributed quantitative variables were expressed as mean ± SD. Fischer’s exact test was used to compare categorical parameters between two groups. Comparison of continuous variables between two groups was done by Man Whitney U test. A p-value of less than p<0.05 was considered as statistically significant.

RESULTS

Among the 355 subjects, of both the study groups, included in the study, all most all individuals had at least one co-morbidity. Table 1 shows that the most frequent co-morbidities were diabetes mellitus (52.1%), followed by hypertension (38%), and chronic kidney disease (11.5%). Lung diseases, other than COPD, contributed to 28.2% of all comorbidities. The present study included 209 subjects of COPD and 146 subjects of COPD with AE. The mean age of COPD and COPD with AE was 68.30 ± 11.72 Vs 68.36 ± 12.16 years (p=0.065). In both the study groups, COPD and COPD with AE, males outnumbered the females (p=0.002). Mean duration of hospital stay was more in case of COPD. There was no significant difference (p>0.05) between serum urea, creatinine, sodium, potassium levels of of the two study groups. However, hemoglobin (Hb) level was significantly different between two group (p< 0.001). COPD (55.14%) and COPD with AE (56.85%) were suffering from diabetes mellitus (DM). COPD (66 %) and COPD (34 %) with AE had hypertension as comorbidities. COPD with AE were having more underlying kidney diseases in comparison to COPD subjects alone. The prevalence of other diseases like depression, dyselectrolytemia, pneumonia, malignancy of lungs, bronchiectasis, cardiomyopathy, and hypothyroidism was contributed as 73.7% in COPD and 26.32% in AE cases (Table 2).

Table 1. Prevalence of different co-morbidities in the study population

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Co-morbidities</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>DM</td>
<td>185 (52.1%)</td>
</tr>
<tr>
<td>2.</td>
<td>HTN</td>
<td>135 (38.0%)</td>
</tr>
<tr>
<td>3.</td>
<td>CKD</td>
<td>41 (11.5%)</td>
</tr>
</tbody>
</table>
4. Other lung diseases except COPD 100 (28.2%)
5. Other comorbid diseases 190 (53.5%)

Group II was COPD with acute exacerbation. Abbreviations used: COPD – Chronic obstructive pulmonary disease, DM- Diabetes Mellitus, HTN- Hypertension, CKD- Chronic kidney disease. The data represented is frequency with percentage in parenthesis.

Table 2: Comparison of study parameters between COPD and COPD with AE

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Parameters</th>
<th>Group I (n=209)</th>
<th>Group II (n=146)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>General parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Age (y) mean ± SD</td>
<td>68.30 ± 1.72</td>
<td>68.36 ± 12.16</td>
<td>0.065</td>
</tr>
<tr>
<td>2.</td>
<td>Gender n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>60 (35.93)</td>
<td>31 (34.07)</td>
<td>0.002**</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>56.44</td>
<td>115 (78.77)</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Hospital stay (days) mean ± SD</td>
<td>8149.53 ± 6.65</td>
<td>6.96 ± 3.87</td>
<td>0.011*</td>
</tr>
<tr>
<td></td>
<td>Biochemical parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Serum urea (mg/dL)</td>
<td>44.54 ± 32.6</td>
<td>46.71 ± 44.67</td>
<td>0.598</td>
</tr>
<tr>
<td>5.</td>
<td>Serum creatinine (mg/dL)</td>
<td>1.38 ± 1.39</td>
<td>1.13 ± 0.87</td>
<td>0.063</td>
</tr>
<tr>
<td>6.</td>
<td>Serum Na* (meq/L)</td>
<td>134.59 ± 7.55</td>
<td>134.18 ± 7.94</td>
<td>0.622</td>
</tr>
<tr>
<td>7.</td>
<td>Serum K* (meq/L)</td>
<td>4.08 ± 0.69</td>
<td>4.14 ± 0.76</td>
<td>0.453</td>
</tr>
<tr>
<td>8.</td>
<td>Hb (g/dL)</td>
<td>11.11 ± 2.04</td>
<td>11.92 ± 2.09</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td></td>
<td>Comorbidities n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>DM</td>
<td>107 (62.94%)</td>
<td>63 (37.06%)</td>
<td>0.135</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>102 (55.14%)</td>
<td>83 (56.85%)</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>HTN</td>
<td>120 (54.55%)</td>
<td>100 (45.45%)</td>
<td>0.034*</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>89 (65.93%)</td>
<td>46 (34.07%)</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>CKD</td>
<td>183 (58.28%)</td>
<td>131 (41.72%)</td>
<td>0.530</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>26 (12.44%)</td>
<td>15 (36.59%)</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Lung diseases</td>
<td>No</td>
<td>139 (54.5%)</td>
<td>116 (45.49%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>70 (70.0%)</td>
<td>30 (30.0%)</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Other diseases</td>
<td>No</td>
<td>69 (41.8%)</td>
<td>96 (58.18%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>140 (73.7%)</td>
<td>50 (26.32%)</td>
<td></td>
</tr>
</tbody>
</table>

Group I: COPD – Chronic obstructive pulmonary disease and group II COPD with AE (acute exacerbation) Abbreviations used: DM- Diabetes mellitus, HTN- Hypertension, CKD- Chronic kidney disease. # Lung diseases other than COPD. Statistical test using standard statistical software Stata 15.1, Level of significance: p<0.05* was considered as significant, p<0.01** highly significant and p<0.001*** very highly significant, p>0.05 was considered non-significant.

DISCUSSION

COPD is a complex, heterogeneous disease. In the present study, it is recognized that acute exacerbations and associated comorbidities contribute to the overall severity of the disease process. It has a major impact on quality of therapeutic approach. It is essential to identify specific contributors in order to group the heterogeneous COPD population for a patient oriented therapeutic approach, which remains a challenge. The analyzed 355 patients of COPD and COPD with AE, shows that, DM, CKD, HT, other lung diseases are the most common prevalent diseases. Majority of the study participants suffer from at least one comorbid condition, which agrees to evaluation by Schnell et al., (12). That 80% of the COPD population have at least one co-morbidity is already reported (16, 17). In a similar kind of study, Dal Negro & coworkers reported one comorbidity of clinical relevance in 78.6%, two comorbidities in 68.8% in their study (18). In particular, this evidence supports the detrimental impact of relevant comorbidities on clinical outcome, which is also imperative for future management of COPD patients. There is growing evidence that, more than 50% of COPD patients have 4 or more comorbidities (11). So it is difficult, to outline COPD as a separate entity from its comorbidities or whether these are part of the spectrum of COPD manifestations. Overall prevalence of comorbidities in our study was observed to be higher in male than females, which confirms the gender dependency of comorbidities in COPD. Many comorbidities frequently predominate particularly in elderly patients but the relationship between gender, age and disease prevalence is still questionable (19). The mean age of COPD and COPD with AE was noted to be 68.30 ± 11.72 Vs 68.36 ± 12.16 years respectively, (P =0.065) in the present study. As exemplified in our study, increase in COPD among the aged population represents a problem for public health care (20). The present study reports that the duration of hospital stay in COPD with AE group
was lesser than the COPD patients in group I. The reason behind this might be due to more aggressive treatment in cases of AE cases in comparison to exclusive COPD. Also found out that, hemoglobin level was more in AE cases of COPD. However, occurrence and severity of comorbidities confirm their role in socio economic impact and mortality of COPD.

A comprehensive overview of comorbidities, commonly associated with COPD in our study obtained from 355 patients revealed a prevalence of 52.1% in DM, 38% HT, 28.2% lung diseases and 11.5% CKD. These results confirm a high prevalence of comorbidities, which are consistent with reports showing significant burden of comorbidities associated with COPD (21, 22). It is agreed that these comorbidities should be given equal importance like the parent disease and early multidisciplinary treatment including psychological therapy should be started for the benefit of the individual. Recent studies have identified the association between comorbidities and specific COPD phenotype (23).

Comprehensive assessment of COPD, based on FEV1 and risk of exacerbation and existence of comorbidities can be considered to have overall impact on patient’s quality of life. Our results support DM as key driver of total health care cost in patients with COPD. It may also be worth mentioning that metabolic syndrome is a risk factor often associated with increased risk of developing DM and atherosclerotic cardiovascular disease. This may be attributed to chronic systemic inflammation, contributing to significant extra pulmonary complications (24). Thus, comorbidity should be given more importance in COPD control strategies. Several mechanisms are proposed to explain the link between COPD and metabolic syndrome and / or type 2 DM, which are still poorly understood. Nevertheless, all the mentioned comorbidities should be identified early, to allow early treatment. Assessment of disease severity as in GOLD Criteria does not seem adequate, as it does not reflect disease progression or mortality risk (25). According to the growing body of evidences, most frequent comorbidities associated with COPD are those related to cardio vascular system. It should be noted that hypertension seems to be the most prevalent cardiovascular comorbidity across all gold stages (26).

The strength of the present study is that, spirometry was done in all cases to assess functional impairment of lungs. Detailed history of confounding factors was taken into consideration. In inclusion criteria, a wide variety of COPD severity, and AE cases were included. The present study has several limitations. Prospective information was not included in the study, more importantly different phenotypes of COPD has not provided with the present data, and no control group was compared.

CONCLUSION

COPD and its comorbidities are the major causes of morbidity and mortality worldwide. There is need for comprehensive, personalized, multi-disciplinary treatment approach for preventing AE and disability of the disease process. The prognostic value of these comorbidities remains to be evaluated in longer observational studies.

REFERENCES

Studies on antibacterial activity of protease inhibitors from the seeds of *Caesalpinia mimosoides*

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ABSTRACT

Introduction and Aim: Antimicrobial proteins/peptides have anchored its presence in the innate host defense mechanism in a wide variety of living organisms including plants, insects, amphibians and mammals. The incidence of resistant antibiotics to microbial pathogens is one of the greatest challenges in modern medicine, which created considerable interests among the researchers for the isolation and investigation of novel potent antimicrobial proteins/peptides from different biological sources. This study is aimed to examine the antibacterial activity of protease inhibitors against a few pathogenic bacterial strains.

Materials and Methods: In the present study, protease inhibitors were isolated from the soaked seeds of *Caesalpinia mimosoides* and tested against pathogenic bacterial strains.

Results: The maximum protein (83.6 mg/g) and protease inhibitory activity (5491 TIU/g) were extracted in 50 mM Sodium Phosphate buffer pH 7.0. Electrophoretic analysis indicated the presence of one major and two minor protease inhibitor activity bands, three major and seven minor protein bands. The extracts of differential extractions were tested for antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* & *Salmonella typhii*. Zone of inhibition is observed for water, Sodium phosphate buffer, pH 7.0 and Tris-HCl buffer, pH 8.0 extractions.

Conclusion: The inhibition of microbial growth by protease inhibitor factors evidenced that the protease inhibitors can be used as the lead compound for the development of novel antimicrobial drugs.

Keywords: Protease inhibitors; differential extractions; anti-bacterial activity; *Caesalpinia mimosoides*.

INTRODUCTION

Protease inhibitors were small proteins or peptides inhibiting the hydrolytic activity of proteases, which were ubiquitous in nature. They occur abundantly in storage tissues such as tubers, seeds and also in the aerial parts of plants (1, 2). These were found in plants belonging to a variety of systematic groups, although high levels of inhibitor activity of proteases were often found in many plants belonging to the *Solanaceae, Fabaceae, and Poaceae* families (3). Serine protease inhibitors were more extensively studied inhibitor proteins. A wide array of antimicrobial peptides were isolated and characterized from living entities, which includes animals, bacteria, insects and plants during the past two decades (4, 5). The inhibitor proteins of proteases block the amino acids necessary for growth and development in pests and thereby acting as plant defensive agents (6). Antimicrobial activity possessing protease inhibitors are namely, Serpins, pepstatins, cystatins, and metalloproteins of proteases inhibitors (2). Evidences suggested that cationic, hydrophobic peptides containing 15 to 40 amino acids in length were involved in innate immunity and were said to provide protection against bacteria, fungi and virus, invasion of pathogens by depolarization of plasma membranes (7, 8). The interest on inhibitor proteins is increased due to their involvement in the inhibition of carcinogenesis in a wide variety of in-vivo and in-vitro systems (9, 10). The inhibition growth of variety of pathogenic bacterial and fungal strains by these inhibitor proteins are exclusively excellent candidates used as the forefront compounds for the development of novel antimicrobial agents.

*Caesalpinia mimosoides*, is a small spiny perennial tropical climbing shrub belonging to the family Fabaceae (subfamily: *Caesalpinioideae*) distributed throughout southern and northern parts of India and its uses have been seen in folk medicines. Younger shoots and leaves were consumed, showing that plant compounds could be safely used as therapeutics. The roots were used for the treatment of arthritis, ulcer, wound management and skin diseases by the folk practitioners of Udupi district of India. The root extracts of *C. mimosides* possess antipyretic, anti-inflammatory and antimicrobial activities (7, 8, 11, 12). The shoot tips were used as digestive tonics and the roots along with ginger paste have found to be effective
for anti-helminthic property and leaves for epilepsy (13). Anti-proteolytic activity studies in the species Caesalpinia mimosoides is still in its onset and a very few reports were available. In view of the biological importance of protease inhibitors as defensive agents and seeds of Caesalpinia mimosoides being a potential source of protease inhibitors, this study was aimed to examine its antibacterial activity against a few pathogenic bacterial strains.

**MATERIALS AND METHODS**

**Materials**

Seeds of Caesalpinia mimosoides were collected from local areas of Madikeri Taluk, Kodagu district, Karnataka, India.

**Chemicals**

Bovine serum albumin, Trypsin, Acrylamide, N N methylene Bisacrylamide were obtained from Sigma Chemical Company, USA. All other chemicals used were of technical grade.

**Preparation of seed powder in butanol**

A 10% soaked, (10 g of seeds were dehulled with 100 ml butanol) dehulled seeds were blended using a homogenizer with butanol, filtered, and the cake obtained was dried, powdered and stored at 4°C until further use. This was carried out according to Chandrashekaraiah et al., with a slight modification (14).

**Preparation of crude inhibitor extract**

Crude extract of inhibitor was prepared using water, 0.05M sodium phosphate buffer with pH 7.0, 0.05M sodium acetate buffer with pH 5.0, 0.5% NaCl, 1.0% NaCl, 0.05M Tris-HCl buffer with a pH 8.0, 0.1 N HCl, 0.1 N NaOH as per the method of Klomklaa et al., (15). It was mixed over a magnetic stirrer for 2 hours at 4°C, followed by centrifugation at 10,000 rpm for 20 minutes. Supernatant obtained was collected separately and were subjected to quantitative and qualitative analysis of proteins and protease inhibitor activity.

**Protein estimation**

Quantitative analysis of proteins were carried out according to the method of Lowry et al., (16).

**Determination of trypsin activity**

The trypsin activity is determined using casein as the substrate according to the method of Kakade et al., (17). 120μg/ml of trypsin was dissolved in 0.001M HCl containing 20mM CaCl2. The assay mixture contained 60μg/ml of trypsin, 1ml of 1% casein and it was incubated at 37°C. The reaction was terminated by 5ml of 6% trichloroacetic acid after 20 minutes, and allowed to stand for 1 hour, the suspension was filtered through Whatman no. 1 filter paper. The filtrate was collected, and the absorbance was read at 280nm. One trypsin unit is arbitrarily defined as an increase in absorbance by 0.01 at 280 nm under conditions of assay.

**Determination of trypsin inhibitor activity**

Enzyme solution (60 μg of trypsin) was pre-incubated with known aliquots of the inhibitor extract, incubated for 15 minutes. The enzyme activity was determined as described above. Trypsin inhibitory unit is defined as the number of trypsin units inhibited under the assay conditions.

**Polyacrylamide gel electrophoresis**

Anionic gel electrophoresis is carried out essentially according to the method of Laemmli (18). Gel system consisting of 10% separating gel and 0.5% stacking gel was used. The electrophoresis has been carried out in cold condition, applying a current of 50-100 volts for 3 hours using tris-glycine (pH 8.3) as electrode buffer and bromophenol blue as the marker dye. After the electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 for 1 hour and de-stained using 10% acetic acid and 25% methanol.

**Gel-assay of protease inhibitors**

Protease inhibitory activity is visualized by reverse zymogram on gelatin PAGE performed by copolymerizing 1% gelatin within the polyacrylamide matrix, which was carried out according to the method of Felicioli et al., (19). After the electrophoretic run, the gel was treated with 100μg/ml trypsin. The gel was washed with distilled water and stained. Protease inhibitory activity appears as coloured bands against a transparent background after staining with Coomassie brilliant blue R-250.

**Antibacterial activity by agar well diffusion method**

Antibacterial activities of the different extraction samples on different bacterial strains (Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli and Salmonella typhii) were determined using agar well diffusion assay method (19). A 100 ml nutrient broth culture of each bacterial organism was used to prepare bacterial lawns. Agar wells of 5mm diameter were prepared with the help of a sterilized cork borer. Four wells were prepared in each of the agar plates. The wells were labelled, and samples were loaded to each of the wells, kept in an incubator at 37°C. After 24 hours, the plates were examined for growth and zones of inhibitions.
RESULTS AND DISCUSSION

Extraction of protease inhibitors from C. mimosoides seeds

The protein and inhibitor potential of C. mimosoides crude extracts of various solvents, against standard trypsin were estimated and presented in Table 1. All the extraction solvents showed suitable stability except a few. The protein content and inhibitory activity of various solvents showed variations like water (87.12 mg/g, 1584 TIU/g), 50mM sodium acetate buffer, pH 5.0 (44.88 mg/g, 2856 TIU/g), 50mM sodium phosphate, pH 7.0 (79.74 mg/g and 5491 TIU/g), 0.5% NaCl (60.98 mg/g, 1742 TIU/g), 1% NaCl (52.8 mg/g, 2682 TIU/g), 50mM Tris-HCl buffer pH 8.0 (42.5 mg/g, 1020 TIU/g), 0.1N HCl (39.6 mg/g, 2682 TIU/g), 0.1N NaOH (118 mg/g, 3720 TIU/g). The maximum protein content was found in 0.1N NaOH (118 mg/g). Water (87.12 mg/g), and 50mM sodium phosphate buffer, pH 7.0 (79.74 mg/g). The maximum inhibitory activity was obtained in 50mM sodium phosphate buffer, pH 7.0 (5491 TIU/g), 1.0% NaCl (5100 TIU/g), 0.1M NaOH (3720 TIU/g). The protein content and trypsin inhibitor activity of sodium phosphate buffer, pH 7.0 was in a suitable range and there was no maximum decrease in protein as well as inhibitor content when compared to the other differential extractions. Hence, we considered 50mM sodium phosphate buffer, pH 7.0 as a suitable extraction medium and the same extraction medium was used for further purification procedures later on. A good amount of protein as well as trypsin inhibitor units were found in C. mimosoides which had a protein content of 118 mg/g and trypsin inhibitor units of 3720 TIU/g in 0.1M NaCl. This was found to be more greater when compared to the studies reported by Pesoti et al., wherein they obtained about 53.291 mg/g of protein in Chenopodium quinoa seeds and the trypsin inhibitory units was 804.28 TIU/g in 0.1M NaCl as the extraction medium (20). When assessment with some known legumes like peas and beans, had a total protein of 17-20%, whereas C. mimosoides was found to contain 11.8% of protein, which means protein content is less when compared to the commonly consumed legumes. Earlier studies conducted by Guillamon et al., showed a protein content of 79.32 mg/g in Phaseolus indicating C. mimosoides as a good source of protein (21).

Table 1: The total protein content and total inhibitor units

<table>
<thead>
<tr>
<th>Extraction medium</th>
<th>Total protein content mg/g</th>
<th>Total inhibitory units TIU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.12</td>
<td>1584</td>
</tr>
<tr>
<td>0.005M Sodium Acetate buffer, pH 5.0</td>
<td>44.88</td>
<td>2856</td>
</tr>
<tr>
<td>0.05M Sodium phosphate buffer, pH 7.0</td>
<td>79.74</td>
<td>5491</td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>60.98</td>
<td>1742</td>
</tr>
<tr>
<td>1.0% NaCl</td>
<td>52.8</td>
<td>5100</td>
</tr>
<tr>
<td>0.05M Tris-HCl buffer, pH 8.0</td>
<td>42.5</td>
<td>1020</td>
</tr>
<tr>
<td>0.1N HCl</td>
<td>39.6</td>
<td>2682</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>118</td>
<td>3720</td>
</tr>
</tbody>
</table>

NATIVE-PAGE electrophoresis

Electrophoretic analysis of the crude inhibitor extract on NATIVE-PAGE is represented in the Fig. 1 and Fig 2. The results indicate the protein as well as inhibitor bands in different crude extracts. No protein as well as inhibitor bands were observed in 0.1N HCl this literally means that the crude inhibitor is unstable at acidic conditions. The protein bands of water extract indicated the presence of (4 minor and 3 major bands), while the 1% NaCl showed (3 minor and 2 major bands), 50mM sodium acetate buffer, pH 5.0 showed (2 minor and 2 major bands), Tris-HCl buffer, pH 8.0 indicated (4 minor and 3 major bands), sodium phosphate buffer, pH 7.0 revealed (5 minor and 3 major bands), 0.1N NaOH showed the presence of (2 minor and 2 major bands). The presence of the inhibitor was determined by zymogram analysis. The inhibitor bands of water extract indicated the presence of (2 major bands), while the 1% NaCl showed (1 minor and 2 major bands), 50mM sodium acetate buffer, pH 5.0 showed (1 minor and 2 major bands), Tris-HCl buffer, pH 8.0 indicated (3 major bands), sodium phosphate buffer, pH 7.0 revealed (1 minor and 2 major bands), 0.1N NaOH showed the presence of (1 minor and 3 major bands). A dark blue band due to the complex of the non-hydrolyzed gelatin with staining determined this. The stability of the inhibitor was found to be less in 0.1N NaOH and 0.1 N HCl indicated the presence of a band having very less intensity when compared to the other extractions.
Antibacterial activity

The inhibitor activity of the crude inhibitor in different extraction solvents were tested against a few proteases of bacterial origin (Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli and Salmonella typhii) against a standard control amoxicillin. Crude inhibitor protein in the range of 200 µg/ml showed zone of inhibition in Tris-HCl buffer, pH 8.0, 50mM sodium phosphate buffer, pH 7.0, 50mM sodium acetate buffer, pH 5.0 and water. We could not observe any zone of inhibition against the 4 different bacterial strains in 0.1N NaOH, 1% NaCl, 0.5% NaCl and 0.1N HCl which indicates that highly acidic and basic pH is blocking the inhibitor to show its effect on bacterial proteases. The minimum zone of inhibition was observed in 50mM sodium phosphate buffer, pH 7.0, 50mM sodium acetate buffer, pH 5.0 about 1mm in all the bacterial strains. The crude extract of Tris-HCl , pH 8.0 strongly affected the growth of Staphylococcus aureus, (4 mm) Klebsiella pneumoniae (6 mm), Escherichia coli (4 mm), and Salmonella typhii (5 mm), the zone of inhibition was found to be nearly 50% when compared to the control (13mm). Similarly, the crude water extract indicated a zone of inhibition in Salmonella typhii (3mm), Staphylococcus aureus (4 mm), Klebsiella pneumoniae (4 mm), Escherichia coli (3 mm). Micro-organisms synthesize proteases into the extracellular medium for their anchorage in the host, at this particular point protease inhibitors are said to bind to these proteases employing antimicrobial effect. Experimental observations done by Rakashanda et al., against few bacterial strains showed strong antibacterial activity which is in agreement with the inhibition of the strains in C. mimosoides (22). Not all the isolated inhibitors show antibacterial activities. This is in account with the studies conducted by Sawano et al., were the inhibitor extract did not exhibit any antimicrobial activity (23). Earlier studies report that bactericidal proteins form a channel on cell membrane and the cell dies because of the out flowing cellular contents, this particular effect of inhibitors, are still under observation.
### Table 2: The zone of inhibition for different organisms in different extraction mediums.

<table>
<thead>
<tr>
<th>Pathogenic organisms</th>
<th>Different extraction medium</th>
<th>50mM Sodium phosphate buffer pH 7.0</th>
<th>50mM Sodium acetate buffer pH 5.0</th>
<th>Water</th>
<th>Tris-HCl pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhii</em></td>
<td></td>
<td>1 mm</td>
<td>1 mm</td>
<td>3 mm</td>
<td>5 mm</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>1 mm</td>
<td>1 mm</td>
<td>3 mm</td>
<td>4 mm</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td>1 mm</td>
<td>1 mm</td>
<td>4 mm</td>
<td>6 mm</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>3 mm</td>
<td>1 mm</td>
<td>4 mm</td>
<td>4 mm</td>
</tr>
</tbody>
</table>

### CONCLUSION

The protein profile of *C. mimosoides* recommends that the ripe seeds can be used as food source and this particular bean seed has been widely used by the tribes of Kerala and Udupi district that are located in western coast of India. Protease inhibitors from the seeds of *C. mimosoides* was isolated using a few solvents, suitable solvent was selected comparing protein as well as inhibitor profile. Presence of the protease inhibitors was visualized by gelatin zymography. Protease inhibitors were found to be active against a few bacterial strains likely to provide direct applications for agricultural, food and pharmaceutical industries.

### ACKNOWLEDGEMENT

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### REFERENCES

Synthesis, anticancer and antimitotic activity of analogues of podophyllotoxin on B16F10 melanoma cell lines and Allium cepa L.

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ABSTRACT

Introduction and Aim: As a timely need for potent anticancer drugs, we attempted to synthesize analogues of podophyllotoxin which are related to etoposide and tenosposide presently in the market.

Materials and Methods: Compounds 8-13 were synthesized at standard condition by modifying the ring C structure of the parent podophyllotoxin and characterized by IR, NMR, Mass spectra and elemental analysis. Anticancer (MTT assay) activity for synthetic compounds was carried out on B16F10 mouse melanoma cell lines and antimitotic activity (cytotoxic) assay was on mitotic cells of root tips of Allium cepa L.

Results: Analogues 8 and 9 exhibited greater anticancer activity with the IC50 values of 1.6 and 1.75mM respectively, and strong inhibition of mitosis with the ID50 values of 1.85mM and 2.10mM respectively, whereas the analogues 10, 11, 12 and 13 showed moderate anticancer activity.

Conclusion: Analogues 8 and 9 would become the novel anticancer drugs in future for cancer chemotherapy after further investigations.

Keywords: Synthesis; podophyllotoxin; anticancer; antimitotic.

INTRODUCTION

At present, the deadly disease cancer remains one of the major causes for the increasing death toll over the globe. Currently, the discovery of anticancer drugs became an extraordinary challenge to the current researchers over the course of years. The number of plant origin and synthesized products having potent antimitotic and anticancer, cytotoxic (1, 2), cathartic, antiviral, antibacterial, antiangiogenic activity have been reported worldwide. Camptothecin, an alkaloid from Camptotheca acuminate as an anticancer drug having a unique mode of action that is inhibition of DNA topoisomerase I (3, 4). Podophyllotoxin 1 (5), otherwise known as Podofilox (Fig. 1, Podophyllum species), is a well-known naturally occurring aryltetralin lignin, and it shows strong cytotoxic activity against various cell lines (6). It is effective in the treatment of Wilms’s tumors, various genital tumors and in non-Hodgkin and other lymphomas and lung cancer (7, 8). Recently, several synthetic analogues of podophyllotoxin and its related compounds reported antiviral and cytotoxic activity (9, 10). The attempt to use podophyllotoxin in the treatment of human neoplasia is restricted due to its complicated side effects (11, 12) such as nausea, vomiting, damage of normal tissues etc., Because of this reason, podophyllotoxin as such is not used as a drug. Extensive structural modifications were done to obtain more potent and less toxic anticancer agents, which resulted in two semisynthetic glycosidic cyclic acetals of epipodophyllotoxin, etoposide and tenosposide (6). These are the most widely used derivatives for the treatment of lymphoma, leukemia, testicular cancer, small cell lung cancer, ovarian, bladder, brain cancer, etc., (13). Analogues of podophyllotoxin etoposide and tenosposide (Fig. 2) are also potent antitumor agents which are clinically in use still retaining side effects (14). This observation has prompted the authors to prepare new analogues and examine them for their antimitotic (cytotoxicity) and anticancer activity.
2) \( R = H, \ R' = R'' = OH \)
3) \( R = H, \ R' = OCH_3, \ R'' = OH \)
4) \( R = O\text{-Glucosyl}, \ R' = OCH_3, \ R'' = OH \)
5) \( R = R' = H, \ R'' = O\text{-Glucosyl} \)
6) \( R = H, \ R' = OCH_3, \ R'' = O\text{-Glucosyl} \)
7) \( R = O\text{-Glucosyl}, \ R' = R'' = H \)

![Scheme 1](image1)

8, 9 and 10

![Scheme 2](image2)

11, 12 and 13
MATERIALS AND METHODS

General: All the chemicals and reagents required were purchased from Sigma Aldrich and Merck Company. Melting points were determined on a SONAR melting point apparatus and are uncorrected. Reactions were monitored by TLC on 0.2mm precoated silica gel 60 F254 plates (E. Merck). Infrared spectra were recorded on a Perkin Elmer spectrum-1000 (450-4000cm-1) spectrometer on KBr discs or Nujol mull. The 1H NMR spectra were recorded with a Varian T-60 and Bruker DRX-300 (300MHz FT NMR) spectrometer using tetra methyl silane (Me4Si, δ=0) as an internal standard in CDCl3, J values are given in Hz. The mass spectra were recorded on a JEOI SX102A spectrometer.

Elemental analysis data were recorded on Elemental Analyser Vario EL III. The preparation of hydroxy methylene tetralone esters 15a-c and hydroxy methylene tetralone acids 16a-c; Formylation of the sodium hydroxide extract was acidified with 2N H2SO4 to give a yellow crystalline solid 16c in 3.0% yield (0.15g). M.p. 96-97°C, (0.16g). M.p. 102°C. IR (KBr): 3500 – 3200 (OH), 1738 (ester C=O), 1690 (tetrалone C=O), 1635 (conjugated C=C), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 4.1 (q, J=4Hz, 2H, ester CH2), 0.9 (t, J=4Hz, ester CH3), 3.5 (d, J = 4Hz, 1H, C3-H), 3.9 (d, J = 4Hz, 1H, C4-H), 8.3 (s, 1H, vinyl), 5.6 (s,1H, Vinyl OH), 6.7 (m, 6H, Ar-H), 6.0 (s, 2H, OCH2O); Anal. calcd. for C12H16O3: C, 65.24; H, 4.46%. Found: C, 65.59; H, 4.44%. The bicarbonate extract was acidified with 2N H2SO4 to give a yellow crystalline solid of 15b in 3.6% yield (0.18g). M.p. 98-100°C. PMR (CDCl3): 6.99 (bs, 1H, COOH) and no ester group signal. Anal. calcd. for C10H17O4F2: C, 64.05; H, 3.68%. Found: C, 64.03; H, 3.67%.

A typical procedure is described for the preparation of 4-(p-chlorophenyl)-1-oxo-2-hydroxy methylene-3-ethylcarboxy-6,7-dioxymethylene-1,2,3,4-tetrahydro naphthalene 15a (6).

4-(p-Fluorophenyl)-1-oxo-2-hydroxy methylene-3-ethylcarboxy-6,7-dioxymethylene-1,2,3,4-tetrahydro naphthalene 15b.

Prepared from 14b (5g, 0.0140 mole), sodium hydride (0.0336g, 0.0140 mole) and ethyl formate (10 ml) as a yellow crystalline solid in 83.40% yield (4.53g). M.p. 102°C. IR (KBr): 3500 – 3200 (OH), 1738 (ester C=O), 1690 (tetrалone C=O), 1635 (conjugated C=C), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 4.1 (q, J=4Hz, 2H, ester CH2), 0.9 (t, J=4Hz, ester CH3), 3.5 (d, J = 4Hz, 1H, C3-H), 3.9 (d, J = 4Hz, 1H, C4-H), 8.3 (s, 1H, vinyl), 5.6 (s,1H, Vinyl OH), 6.7 (m, 6H, Ar-H), 6.0 (s, 2H, OCH2O); Anal. calcd. for C12H16O3: C, 65.24; H, 4.46%. Found: C, 65.59; H, 4.44%. The bicarbonate extract was acidified with 2N H2SO4 to give a yellow crystalline solid of 15b in 3.6% yield (0.18g). M.p. 98-100°C. PMR (CDCl3): 6.99 (bs, 1H, COOH) and no ester group signal. Anal. calcd. for C10H17O4F2: C, 64.05; H, 3.68%. Found: C, 64.03; H, 3.67%.

4-(p-Nitrophenyl)-1-oxo-2-hydroxy methylene-3-ethylcarboxy-6,7-dioxymethylene-1,2,3,4-tetrahydro naphthalene 15c.

Prepared from 14c (5g, 0.0130 mole), sodium hydride (0.3120g, 0.0130 mole) and ethyl formate (10 ml) as a pale yellow solid in 78.48 % yield (4.21g). M.p. 88-90°C. IR (KBr): 3500–3200 (OH), 1740 (ester C=O), 1695 (C=O), 1630 (conjugated C=C), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 3.8 (q, J = 4Hz, 2H, ester CH2), 1.0 (t, J = 4 Hz, 3H, ester CH3), 3.6 (d, J = 4Hz, 1H, C3-H), 4.1(d, J = 4Hz, 1H, C4-H), 8.2 (s, 1H, Vinylic), 5.7 (s, 1H, vinyl OH) , 6.7 (m, 6H, Ar-H), 5.9 (s, 2H, OCH2O); Anal. calcd. for C12H16O3N: C, 61.32; H, 4.17; N, 3.41%. Found: C, 61.29; H, 4.15; N, 3.37%. The bicarbonate extract was acidified with 2N H2SO4 to give a yellow crystalline solid 16c in 3.0% yield (0.15g). M.p. 96-98oC. PMR (CDCl3): δ 9.8 (bs, 1H, COOH) and no ester group signal. Anal. calcd. for C10H17O4N: C, 59.54; H, 3.42; N, 3.65%. Found: C, 59.52; H, 3.44; N, 3.64%.

To a solution of 15a (4g, 0.01 mole) in absolute methanol (60 ml), sodium borohydride (0.38g, 0.01 mole) in absolute methanol (60 ml) was added during 1h at room temperature. At an hourly interval, a solution of sodium borohydride (1.0g) in methanol (10 ml) was added three times. The reaction mixture, after stirring at room temperature for 5h, was concentrated to 40 ml, acidified with 2N HCl and then the pH of the solution was adjusted to 8 by adding 1% aqueous ammonium hydroxide solution. The product was extracted into ether (3x50 ml), the ether layer was washed with cold 1% sodium.
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hydroxide solution (2x40 ml), water (2x40 ml) and then dried over anhydrous Na2SO4. A pink colored semi solid was obtained in 72.77 % yield (2.94g), after evaporating the solvent. IR (Nujol): 3550-3200 (OH), 1735 (ester C=O), 1600 (aromatic C=C) cm−1; PMR (CDCl3): δ 4.2 (q, J = 3Hz, 2H, ester CH2), 1.0 (t, J=3Hz, 3H, ester CH3), 3.3 (m, 2H, C1-H, C3-H), 6.8 (m, 6H, Ar-H) , 2.4 (m, 3H, C2-H, & CH2OH), 4.1 (d, J = 3Hz, 1H, C4-H), 5.3(s, 1H, CH2OH), 5.9 (s, 1H, OCH2O); Anal. calcd. for C13H21O3Cl: C, 62.30; H, 5.23 %. Found: C, 62.28; H, 5.22%.

1-Hydroxy-2-hydroxymethyl-3-ethylcarboxy-4-(p-fluorophenyl)-6,7-dioxymethylene-1,2,3,4-tetrahydroaphthalene 17b.

Prepared from 15b (4g, 0.0104mole) and sodium borohydride (0.3934g, 0.0104 mole) in methanol (80 ml) as a reddish yellow colored semi solid in 76.69 % yield (3.10g). IR (Nujol): 3560 – 3200 (OH), 1742 (ester C=O), 1590 (aromatic C=C) cm−1; PMR (CDCl3): δ 4.2 (J = 4Hz, 2H, ester CH2), 0.9 (t, J=4Hz, ester CH3), 6.8 (m, 6H, Ar-H), 2.4 (m, 3H, C2-H, & CH2OH), 4.0 (d, J=4Hz, 1H, C4-H), 3.6 (m, 2H, C1-H & C3-H), 5.9 (s, 2H, OCH2O), 5.6 (s, 1H, CH2OH ); Anal. calcd. for C13H19O2F : C, 64.94; H, 5.45%. Found: C, 64.91; H, 5.43%.

1-Hydroxy-2-hydroxymethyl-3-ethylcarboxy-4-(p-nitropheno)-6,7-dioxymethylene-1,2,3,4-tetrahydroaphthalene 17c.

Prepared from 15c (4g, 0.01 mole) and sodium borohydride (0.3783g, 0.01 mole) in methanol (80 ml) as a yellow colored semi solid in 71.30 % yield (2.88g). IR (Nujol): 3500 – 3200 (OH), 1735 (ester C=O), 1595 (aromatic C=C) cm−1; PMR (CDCl3): δ 4.3 (q, J = 4Hz, 2H, ester CH2), 1.0 (t, J=4Hz, ester CH3), 6.7(m, 6H, Ar-H), 2.5 (m, 4H, C2-H & CH2OH), 4.0 (d, J = 4Hz 1H, C4-H), 3.5 (m, 1H, C3-H) , 6.0 (s, 2H, OCH2O), 5.7(s, 1H, CH2OH); Anal. calcd. for C13H17O3N: C, 60.72; H, 5.10; N, 3.37%. Found: C, 60.7; H, 5.08; N, 3.34%.

1-Hydroxy-2-hydroxymethyl-3-carboxy-4-(p-chlorophenol)-6,7-dioxymethylene-1,2,3,4-tetrahydroaphthalene 18a.

A solution of 17a (3g, 0.0074 moles) in methanol (30 ml) and 5% sodium hydroxide (40 ml) was refluxed for 3h. After removing the methanol under reduced pressure, the alkaline solution was acidified with 2N HCl to give a yellow colored solid which on recrystallization from methanol gave a pale yellow colored crystalline product in 73.06 % yield (2.04g). M.p. 87-89°C. IR (KB): 3520-3200 (OH), 1715 (carbonyl C=O), 1600 (aromatic C=C) cm−1; PMR (CDCl3): δ 9.9 (s, 1H, COOH); Anal. calcd. for C19H10O5Cl : C, 60.57; H, 4.55%. Found: C, 60.53; H, 4.53%.

1-Hydroxy-2-hydroxymethyl-3-carboxy-4-(p-fluorophenyl)-6,7-dioxymethylene-1,2,3,4-tetrahydroaphthalene 18b.

Prepared from 17b (3g, 0.0077 mole) in methanol (30 ml) and 5% sodium hydroxide (40 ml) as a pale yellow crystalline solid in 76.80% yield (2.85g). M.p. 91-92°C. IR (KB): 3500-3200 (OH), 1710 (carbonyl C=O), 1600 (aromatic C=C) cm−1; PMR (CDCl3): δ 9.8 (s, 1H, COOH); Anal. calcd. for C19H10O5F : C, 63.33; H, 4.76%. Found: C, 63.32; H, 4.72%.

1-Hydroxy-2-hydroxymethyl-3-carboxy-4-(p-nitropheno)-6,7-dioxymethylene-1,2,3,4-tetrahydroaphthalene 18c.

Prepared from 17c (3g, 0.0072mole) in methanol (30 ml) and 5% sodium hydroxide (40 ml) as a yellow crystalline solid in 75.07 % yield (2.80g). M.p. 92-94°C. IR (KB): 3500-3200 (OH), 1708 (carbonyl C=O), 1595 (aromatic, C=C) cm−1; PMR (CDCl3): δ 9.9 (s, 1H, COOH); Anal. calcd. for C19H10O5N : C, 58.92; H, 4.42; N, 3.62%. Found: C, 58.90; H, 4.41; N, 3.59%.

A typical procedure is described for the preparation of 6,7-dioxymethylene-9-p-chlorophenyl naptho[2, 3-C] furan-1-(3H, 4H, 9H) one 8a.

A mixture of 18a (2g, 0.0053 mole), p-toluene sulfonyl chloride (1.01 g, 0.0053 mole) and dry pyridine (30 ml) in dry benzene (60 ml) was refluxed for 3h. The reaction mixture was cooled to room temperature, washed with 2N HCl (3x50 ml) and then with water (2x40 ml). The solvent was removed by distillation under reduced pressure to give a thick brown residue. The crude product was column chromatographed over silica gel in 10x30 cm column using benzene as an eluent. The solvent was removed and evacuated at 50°C on a rotary evaporator to give an orange red crystalline solid in 71.32 % yield (1.29g). M.p. 76-78°C. IR (KB): 1775 (lactone C=O), 1670(shoulder tetra substituted C=C) 1605 (Ar C=C, )cm−1; PMR (CDCl3): δ 4.0 (s, 1H, C3-H), 6.8 (m, 6H, Ar-H), 3.8 (s, 2H, C4-H) , 4.4 (s, 2H, C9-H) , 5.9 (s, 2H, OCH2O); Mass (m/z, % abundance): 341(M+, 83), 339 (28), 112 (11), 245 (18), 228 (36); Anal. calcd. for C19H10O5Cl: C, 66.97; H, 3.85%. Found: C, 66.95; H, 3.82%.

6,7-Dioxymethylene-9-p-fluorophenyl naptho[2, 3-C] furan-1-(3H, 4H, 9H) one 9 was prepared from 18b (2g, 0.0056 mole), p-toluene sulfonyl chloride (1.07g, 0.0056 mole) and dry pyridine (25 ml) in dry benzene (60 ml) as orange red colored crystalline colored compound in 73.33 % yield (1.32g). M.p. 74- 76°C. IR (KB): 1756 (lactone C=O), 1666 (shoulder tetra substituted C=C), 1595 (aromatic C=C) cm−1. PMR (CDCl3): δ 4.3 (d, J=3Hz, 1H, C9-H), 6.8 (m, 6H, Ar-H), 3.7 (s, 2H, C4-H) , 4.1 (s, 2H, C3-H), 6.0 (s, 2H, OCH2O); Mass (m/z, % abundance): 324 (M+, 93), 322 (19), 229
A typical procedure is described for the preparation of 6,6-dihydro-2,3-dioxymethylene-9-chloro-11bH benzo[c]fluoren-5,7-dione 11. A mixture of benzhydryl succinic acid 19a (2g, 0.0076 mole) and thionyl chloride (40 ml) was refluxed for 2h. The excess thionyl chloride was distilled off. A pale yellow residue was obtained as a gummy product 20a in 85.32 % yield (1.88g). IR (KBr): 1777 (C=O of acyl chloride) cm⁻¹; PMR (CDCl₃): δ 4.3 (s, 1 H, C₄-H), 5.9 (s, 2H, C₆a-H), 6.9 (m, 5H, Ar-H), 5.9 (s, 2H, OCH₂O); Mass (m/z, % abundance): 351 (M⁺, 24), 271 (18), 243 (29), 219 (44), 218 (44), 37 (100); Found: C, 64.95; H, 3.71; N, 4.0%.

Anticancer assay

The newly synthesized analogues were screened for anticancer activity by determining the ability of compounds to inhibit the proliferation of B16F10 mouse melanoma cells following the in-vitro antiproliferative method (17). The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x10⁵ cells/ml using medium containing 10% fetal bovine serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell culture was added and the cell count was adjusted to 1.0x10⁵ cells/ml using medium containing 10% fetal bovine serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with PBS pH 7.4.2mM and 100 μl of different drug concentrations were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was done. After 72 h, the drug solutions in the wells were discarded and 50 ml of MTT in HBSS was added to each well. After 3 h, the plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 50 μl of propanol was added and the solvent was removed and evaporated at 50°C on the rotary evaporator to give a dark yellow solid. It was recrystallized from ethanol in 73.79 % yield (0.91g). M.p.166-168°C. IR (KBr): 1713 (Indanone C=O), 1670 (tetalone C=O), 1590 (aromatic C=CH) cm⁻¹; PMR (CDCl₃): δ 2.7 (d, J=4Hz, 2H, C₂-H), 3.6 (q, J=4Hz, 1H, C₆a-H), 4.5 (d, J =4Hz, 1H, C₁₁b-H), 6.8 (m, 5H, Ar-H), 5.9 (s, 2H, OCH₂O); Mass (m/z, % abundance): 337 (M⁺, 22), 309 (38), 281 (23), 253 (28), 132 (62); Found: C, 64.10; H, 3.29; N, 4.15%.

% of inhibition = \frac{\text{Test} - \text{Control}}{\text{Test}} \times 100
Antimitotic assay

The antimitotic activities of the synthesized analogues of podophyllotoxin were examined using onion root tip method (18). The ID$_{50}$ (concentration for 50% inhibition of mitosis) was determined. Test solution prepared by dissolving exactly known weight (0.001 to 0.003g) of synthetic analogue in 3ml of absolute ethanol and diluted with distilled water to 250 ml in a standard flask. All the tested synthesized products gave a clear solution in the above process. Onion base was immersed to an extent of about half a centimeter in a sample tube (7x3 cm) after removing the old roots from it and immersion was continued for two days for germination. After two days, germinated root tips were removed and placed on the sample tube containing fixing solvent (absolute ethanol-glacial acetic acid, 3:1v/v). After 24hrs, fixing solvent was decanted carefully and the root tips were washed with preserving solvent (70% ethanol) and kept immersed in the same solvent. An onion root tips were also allowed to germinate in a control solution (3 ml of absolute ethanol diluted with distilled water to 250 ml) without the synthetic analog in exactly the same way as done in preparing solution of synthetic analogues. Root tips were placed on a clean watch glass containing stain solution (orcein solution-HCl solution 7:1 v/v) and heated on the flame until fumes come out. It was then cooled to room temperature. Root tips were then placed on the micro slide, a drop of stain solution was added and the root tips were squashed by a razor blade and slides were prepared. The prepared slide was mounted for observation under a compound microscope. The total number of cells and the number of dividing cells were counted. The percent of the number of dividing cells compared to the control and the percent inhibition of mitosis by the test antimitotic agent at a given concentration against a control were calculated. The inhibition studies for each synthetic product were done for three different concentrations. The statistical data are presented in the table. A graph of concentration, verses percent inhibition for each test compound was drawn. The concentration needed for 50% inhibition (ID$_{50}$) was extrapolated from the graph as per the method of Thomas et al., (19). ID$_{50}$ values for the synthetic derivatives for antimitotic activity were calculated individually.

RESULTS AND DISCUSSION

Chemistry of synthetic compounds, 8-13

The Podophyllotoxin analogues 8-13 were synthesized by formylation of the tetralone esters 14a-c previously (15) with ethyl formate using sodium hydride as the base, afforded the hydroxy methylene tetralone esters 15a-c and hydroxy methylene tetralone acids 16a-c by following walker’s method (16). Reduction of 15a-c and 16a-c with sodium borohydride in methanol gave the dihydroxy esters 17a-c and dihydroxy acids 18a-c respectively in excellent yield (6). Saponification of the dihydroxy esters 17a-c with 5% aqueous sodium hydroxide and methanol gave the dihydroxy acids 18a-c which is refluxed with p-toluene sulfonyl chloride in pyridine gave the podophyllotoxin analogues 8, 9 & 10 in excellent yield (Scheme 1).

The diketones 11, 12 & 13 were also synthesized by Friedel-Crafts intramolecular acylation reaction of 19a-c (6). The benzhydryl succinic acids 19a-c were converted into the benzhydryl succinyl chlorides 20a-c which were then cyclized by using anhydrous aluminium chloride in dry dichloromethane to yield the diketones 11, 12 & 13 - Scheme 2.
The IR spectra of 15a-c showed characteristic absorptions in the region of 3500–3200 cm⁻¹ and 1635-1625 cm⁻¹ assigned to vinylic hydroxyl and conjugated double bond groups respectively. The IR absorptions for the tetralone carbonyl group and the ester carbonyl were not much displaced when compared to that of the tetralone esters 14a-c. The compounds 16a-c showed broad peaks at 3600 – 3200 cm⁻¹ and a sharp peak in the range 1630-1620 cm⁻¹ due to the vinylic hydroxyl as well as carboxylic hydroxyl groups and conjugated double bonds respectively. The carbonyl group of the carboxylic acid absorbed in the range of 1720-1710 cm⁻¹ and the tetralone carbonyl at 1695-1710 cm⁻¹.

The PMR spectra of 15a-c resembled each other except the differences due to the substituents. A broad singlet centered in the range δ 5.6-5.8 due to vinylic hydroxyl proton, which was exchangeable with D₂O and a broad singlet in the range of δ 8.2-8.4 due to the vinylic proton. The compounds 16a-c showed a similar type of PMR spectra due to the absence of ethyl group, but a broad singlet in the range of 9.9 assigned to carboxylic OH proton(6).

Sodium borohydride has been extensively used to reduce ketones as well as α, β unsaturated ketones to 1, 3-diols without affecting the ester functional group. The hydroxy methylene tetralone esters 15a-c were reduced to corresponding dihydroxy esters 16a-c by sodium borohydride. Compounds 15a-c were dissolved in absolute methanol and then excess sodium borohydride in absolute methanol was added during 1h at room temperature. The reaction mixture was stirred for 5h at room temperature, which on usual work up gave a brown pasty mass in good yields (6). Based on Walker’s work (16) in a similar reduction, it was assumed that a mechanism involving 1,4 attack on the keto enol system which involved in the sodium borohydride reduction of 15a-c to 17a-c. The substituent groups at positions 1 and 2 in 17a-c are assumed to be cis to each other, similar to the views of walker. The IR spectra of 17a-c showed a broad absorption in the region 3600 – 3200 cm⁻¹, assigned to the OH groups, and a sharp absorption at 1737 cm⁻¹ assigned to the ester carbonyl group (6).

Hydrolysis of the esters 17a-c with 5% aqueous sodium hydroxide in methanol was affected smoothly at reflux temperature to give the dihydroxy carboxylic acids 18a-c in 73-78% yield. During alkaline hydrolysis of 17a-c inversion of the carboxyl group did not occur under these conditions since the compounds 16a-c on reduction with sodium borohydride in absolute methanol gave the identical products 18a-c. The hydroxy methylene tetralone acids 16a-c was dissolved in methanol and then sodium borohydride in absolute methanol was added during 1h at room temperature. The excess of sodium borohydride was decomposed by dilute HCl and the separated solid on recrystallization from methanol gave white feathery crystals. The IR spectra of 18a-c from both routes were identical. An absorption in the region of 3550 – 3200 cm⁻¹ was assigned to carboxylic hydroxyl groups and other primary and secondary hydroxyl groups (6).

The p-toluene sulfonyl chloride in pyridine has been used as a dehydrating agent in many organic syntheses. The same reagent was used to convert podophyllotoxin (1) to β-apopiciprodophyllin in a single step by Murthy and Rai (20). Following the same procedure, the dihydroxy acids 18a-c were successfully dehydrated with concomitant isomerization to the lower corresponding podophyllotoxin analogues 8, 9 and 10 respectively (6).

Compounds 18a-c in dry benzene were mixed with p-toluene sulfonyl chloride in pyridine and refluxed for 3 hr. After the usual workup, the crude products were column chromatographed over silica gel using chloroform as the eluent. The products 8 & 9 are orange red solids and 10 is yellow solid from the eluents. They showed the absence of OH group in their respective IR spectra, but a strong absorption in the region of 1750 – 1740 cm⁻¹ due to the presence of an α,β, unsaturated-γ-lactone carbonyl group and a shoulder at the range 1665 – 1635 cm⁻¹ due to the tetra-substituted C=C bond. These observations corresponded very well to those of β-apopiciprodophyllin as observed by Gensler (21) and Murthy (20). The PMR spectra of the analogues 8, 9 & 10 showed singlets at 3.3 and 4.5 for C4 & C3 protons, a singlet at δ 5.9 due to the 1, 3-methylenedioxy protons and a singlets at δ 7.2 and 7.4 for C5 & C8 protons respectively (6). The mass spectra of 8, 9 and 10 showed molecular ion peaks respectively at 341, 324 and 351.

The diketones 11, 12 & 13 were synthesized by Friedel-Crafts intramolecular acylation reaction of 20a-c previously reported by Sathisha et al., (6). This method has two steps, first, the benzhydryl succinic acids 19a-c were converted into the benzhydryl succinyl chlorides 20a-c, which were then cyclized by using anhydrous aluminium chloride in dry dichloromethane. The products formed were found to be the diketones 11, 12 & 13, which were characterized by IR, PMR, Mass spectra and elemental analysis data (Scheme 2). The IR absorption in the region of 1710-1700 cm⁻¹ is assigned to the six membered carbonyl group and 1745-1730 cm⁻¹ assigned to the five membered carbonyl group (6). PMR of 11, 12 & 13 showed a doublet of a double in the region of δ 2.6 assigned to Cα-H, a doublet at 4.1and a quartet at δ 3.7 to 6a-H. The mass spectra of 11, 12 & 13 showed molecular ion peaks (M⁺) at their respective mass numbers m/z 327, 310 and 337 respectively.
Anticancer activity: As any new molecules that are developed for the treatment of cancer, should be subjected to the preliminary investigations. In line to this, there are several methods known to carry out the anticancer activity. Among these, in-vitro MTT assay (17) to check the cellular viability was used in our study and was the most convenient as per the facility available in our laboratory. We have used the mouse melanoma B16F10 cell line for conducting anticancer activity experiments.

The antiproliferative (anticancer) assay was carried out for the synthesized podophyllotoxin analogues 8-13. Fig. 3 by modifying the ring C of parent compound, using the in-vitro MTT assay method. The analogues 8 and 9 have chloro and fluoro groups respectively showed a greater antiproliferative activity compared to control at low concentration whereas the remaining analogues showed moderate inhibition of cancer cell growth Fig. 3.

![Fig. 3: Dose dependent antiproliferative assay (MTT assay)](image)

The compounds were screened individually in the different dose range from 0-2.5µM with mouse melanoma B16F10 cell line, incubated for 3 days, absorbance was read at 570nm, against the control and percentage inhibition and IC50 values were calculated. The standard mean ±SEM, for n= 4.

The dose dependent assay was carried out for all the analogues to monitor the IC50 values. The analogues 8, 9, 10, 11, 12 and 13 have given the IC50 values 1.6, 1.75, 2.0, 2.15, 1.82 and 2.0µM respectively (Table 1).

Antimitotic activity (cytotoxicity): The cytotoxic effect of the new analogues 8-13 were studied in terms of inhibiting the cell cycle (mitosis) using the onion root tip method (18). The analogues 8 and 9 showed the maximum antimitotic activity, 10 and 11 moderately, whereas the rest of the analogues 12 and 13 showed comparatively very less activity (6). The podophyllotoxin analogues earlier reported were known to potent antimitotic activity (22) in which the ring C was modified. The above analogues 8-13 were synthesized by modifying the ring C of parent compound using chloro, fluoro and nitro groups and these analogues showed maximum antimitotic activity by arresting the cell cycle Fig. 4. The analogues 8, 9, 10, 11 and 12 gave the ID50 values in 1.85, 2.1, 2.25, 2.5 and 2.42mM respectively when dose dependent assay was carried out (Table-1).

![Fig. 4: The dose dependent antimitotic activity](image)
CONCLUSION
Currently, there is an immediate need of potent anticancer drugs. Keeping that view, after an extensive literature survey, the podophyllotoxin analogues 8-13 were synthesized and characterized. These analogs were subjected to a preliminary screening where they rendered significant anticancer and antimitotic activity. Among these, Compounds 8 and 9 were having strong growth inhibitory effects on mouse melanoma cancer cell lines as well as mitotic cells of onion root tips. These experimental observations conclude that compound 8 and 9 would become novel drugs in future cancer chemotherapy after further investigations.

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Biological synthesis and characterization of silver nanoparticles using stem extract of \textit{Lagenaria siceraria} and their antibacterial activity against \textit{Escherichia coli} and \textit{Staphylococcus aureus}

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\section*{ABSTRACT}

\textbf{Introduction and Aim:} Nanoparticle synthesis using plants extract has been considered ecologically innocuous. In this study we have reported the synthesis of stable silver nanoparticles (AgNPs) using the stem extract of \textit{Lagenaria siceraria} under two different conditions viz. room temperature and sunlight irradiation.

\textbf{Materials and Methods:} The silver nanoparticles were synthesized using 90 ml of \(10^{-3} \text{ M AgNO}_3\) added to 10 ml of the aqueous extract of \textit{L. siceraria}. The solutions were kept under two different conditions viz. sunlight irradiation and room temperature. The color change of the solutions was monitored periodically using UV-Vis spectroscopy. The synthesized AgNPs were further characterized using XRD, FTIR, DLS, EDX and SEM.

\textbf{Results:} The UV-Vis spectroscopy result of the synthesized AgNPs under the influence of sunlight irradiation showed highest peak with shorter reaction time compared to AgNPs synthesized at room temperature. The XRD analysis of the AgNPs synthesized using sunlight irradiation were crystalline in nature. In addition, the SEM image revealed the AgNPs were spherical in shape with average particles size of 105 nm. Moreover, the AgNPs showed antibacterial activity against \textit{Escherichia coli} (MTCC 739) and \textit{Staphylococcus aureus} (MTCC 96).

\textbf{Conclusion:} From the above study, we can conclude that the biosynthesis of AgNPs using stem extract of \textit{Lagenaria siceraria} is a cost effective and eco-friendly way to produce AgNPs and can be exploited in the field of biomedicines as well as industries.

\textbf{Keywords:} Silver nanoparticles; \textit{Lagenaria siceraria}; stem extract; antibacterial; biomedicine.

\section*{INTRODUCTION}

Recently nanotechnology has emerged as the most attractive area of research due to its unique physiochemical properties and wide range of applications (1). Nanoparticles are extensively used in the field of therapeutics, electronics, catalysis, sensor (2), forensic science, biomedicine and waste management (3). Silver nanoparticles have a broad spectrum of antibacterial activity even at a very low concentration (4). Furthermore, silver nanoparticles have been reported to have various properties such as anti-inflammatory, anti-plasmodial activity, antiviral, anti-cancer and antimicrobial (5). The silver nanoparticle is highly toxic towards microorganisms and cause structural changes in the bacterial cell membrane, DNA damage, mitochondrial damage and also produce ROS that interferes with the cellular constituents of microorganisms (6). Different routes are available for the synthesis of silver nanoparticles, which includes physical, chemical, photochemical and biological methods. Though physical and chemical method are most commonly used but many disadvantages are associated with these two methods which includes the use of toxic and expensive chemicals. In addition, are high energy dependent and are not eco-friendly (7). Biological method for the synthesis of silver nanoparticles has proved to be the most cost effective and environmentally benign process (8). The biological method for synthesizing silver nanoparticles includes fungi, yeast, bacteria, extract of different parts of plants, bacteria, yeast, Fungi etc. (9).

Synthesis of silver nanoparticles by using microorganisms are costly and labor intense compare to plant mediated biosynthesis of silver nanoparticles as microbes require maintenance of culture media and also maintenance of sterile environment (10).

In the present study, silver nanoparticles has been synthesized from stem extract of \textit{Lagenaria siceraria} under two different conditions viz. room temperature and sunlight irradiation. The stem extract of \textit{Lagenaria siceraria} acts as a reducing as well as capping agent and reduce the silver ions to stable silver nanoparticles. \textit{Lagenaria siceraria} is a soft pubescent, climbing herb belonging to the family of Cucurbitaceae and is used traditionally for the treatment of various diseases. The plant \textit{Lagenaria siceraria} also known as bottle gourd, is a common fruit vegetable used by Indian people (11).
Many Literatures suggests that the stem extract of *Lagenaria siceraria* is diuretic and have antibacterial activity (12). The synthesized nanoparticles were tested to evaluate the inhibitory effect against the two bacterial strains viz. *Escherichia coli* and *Staphylococcus aureus*.

**MATERIALS AND METHODS**

**Materials**

Nutrient agar and Silver Nitrate were purchased from Himedia (Mumbai). All the working stocks were made freshly before experiment using double distilled water. *L. siceraria* stems were collected from Guwahati, Assam, India. *Escherichia coli* (MTCC 739) and *Staphylococcus aureus* (MTCC 96) strains were used to evaluate antibacterial activity of biosynthesized AgNPs along with plant extract and standard antibiotic (gentamycin).

**Preparation of stem extract of *L. siceraria***

The *L. siceraria* stems were washed thoroughly under tap water and then finally washed twice with double distilled water. 25g of *L. siceraria* stems were crushed in 100 ml of double distilled water and boiled at 60°C for 10 minutes. The extract was cooled down to room temperature and then filtered using Whatman filter paper No.1 (pore size 25µm). The filtered extract was then stored at 4°C for further use.

**Biosynthesis of silver nanoparticles by stem extract of *L. siceraria* under room temperature**

For the biosynthesis of the silver nanoparticles, 10 ml of the stem extract of *L. siceraria* was added to 90 ml of 10-3 M AgNO3. The reaction mixture was incubated at room temperature under dark condition and the color change of the reaction mixture was checked periodically. The color change of the reaction mixture from light green to dark brown was observed after 48 hours of incubation at room temperature. The color change was then monitored by using UV-vis spectroscopy.

**Biosynthesis of silver nanoparticles using stem extract of *L. siceraria* under sunlight irradiation**

10 ml of the of stem extract of *L. siceraria* was added to 90 ml of 10^{-3} M AgNO3. In order to initiate the formation of AgNPs, the reaction mixture was exposed to bright sunlight. The color change of the solution from light green to dark brown started within a few minutes of the exposure and remained unchanged after 32 minutes from the time of exposure. The change in the color confirmed the formation of silver nanoparticles and were monitored by using UV-vis spectroscopy.

**Production and recovery of the biosynthesized silver nanoparticles by centrifugation**

Among both the methods used for the biosynthesis of silver nanoparticles using stem extract of *L. siceraria*, sunlight irradiation method showed maximum production of silver nanoparticles. Furthermore, it has been selected for bulk production of silver nanoparticles. The biosynthesized AgNPs were then subjected to centrifuge using Eppendorf AG Model No. 5430R at 12,000 rpm for 20 minutes. The pellets were collected and washed 3 times with ethanol to remove any water-soluble biomolecules. The pellets thus obtained was dried at room temperature and were used for XRD, SEM and FTIR analysis.

**Characterization of biosynthesized silver nanoparticles**

The formation of stable silver nanoparticles under both the conditions viz. room temperature and sunlight were recorded using UV-Vis spectroscopy between 350 to 600 nm. The UV-vis spectral analysis was done using an Analytikjena SPECORD 50 PLUS spectrophotometer. The obtained dried pellets after centrifugation were subjected to XRD analysis by using Bruker AXS, Germany, D8 Advance, operated at a voltage of 40kV and a current of 40mA with Cu Kα radiation. The Scanning Electron Microscope (SEM) analysis has been performed to determine the shape and size of the biosynthesized silver nanoparticles by using ZEISS EVO 18 Special Edition. The crude stem extract of *L. siceraria* (without AgNO3) and the dried pellets of silver nanoparticles were subjected to FTIR (Perkin Elmer FTIR Spectroscopy Spectrum Two) spectroscopy analysis in the range of 500 – 4000 cm⁻¹ with KBr pellets. Furthermore, the EDX analysis of the biosynthesized silver nanoparticles were done using Oxford instrument X act “PentaEF” Precision”. The average size distribution of the biosynthesized silver nanoparticles was measured using Dynamic Light Scattering (DLS) in the range of 0.1 – 1000 µm at 25°C using Nano plus (Micromeritics, USA). The sunlight induced AgNPs were tested for their antibacterial potential against *E. coli* and *S. aureus*. The evaluation of the antibacterial potential of the synthesized silver nanoparticles were done by disc diffusion method on nutrient agar plates. The bacterial culture was grown overnight in nutrient broth having (1 × 105) CFU/ml. The culture was then spread onto the nutrient agar plates. Gentamycin was used as positive control. The crude stem extract of *L. siceraria* were also used to evaluate a comparative analysis of antibacterial activity along with AgNPs against the two bacterial strains. The cultured petri-plates were incubated at 37°C for 24 hours. After 24 hours of incubation, the zone of inhibition was measured.

**RESULTS AND DISCUSSION**

**UV-VIS Spectroscopy**

In this study, the stem extract of *Lagenaria siceraria* (Fig. 1a) were used to synthesize stable silver nanoparticles. 10 ml of the stem extract of *Lagenaria siceraria* was added to 90 ml of 10^{-3} M AgNO3 and were incubated under two conditions viz. room temperature and sunlight irradiation. The color changes from light...
green to dark brown was observed in both the conditions as shown in Fig.1 (b) and (c), thus indicating the formation of silver nanoparticles. The colour change takes place due to the excitation of surface Plasmon resonance (SPR) exhibited by the silver nanoparticles (13, 14).

The AgNPs formed under both conditions viz. room temperature and sunlight irradiation were further characterized by using UV-Vis spectroscopy (Fig. 2) in the range between 350-700 nm. The room temperature mediated silver nanoparticles synthesis using stem extract of Lagenaria siceraria showed characteristic absorbance peak at 425 nm whereas the sunlight irradiated reaction mixture showed a strong characteristic absorbance peak at 428 nm. Among both the conditions sunlight irradiated nanoparticle synthesis showed highest and sharp peak and were used for further studies.

**Fig 1:** Biosynthesis of AgNPs: (a) Stem extract of L. siceraria, (b) Reaction mixture of AgNO$_3$ and plant extract (before reaction), (c) Reaction mixture incubated at room temperature after 48 hours, (d) Reaction mixture exposed to sunlight irradiation after 32 minutes.

**Fig 2:** UV-Vis absorption spectra of: (a) Stem extract L. siceraria (control), (b) Biosynthesized AgNPs under room temperature, (c) Biosynthesized AgNPs under sunlight irradiation.

**X-Ray diffraction (XRD) analysis**

The XRD result (Fig. 3) confirms the synthesis of AgNPs using stem extract of Lagenaria siceraria. Silver nanoparticle synthesized showed four intense and sharp peak at 2θ = 38.10º, 44.33º, 64.56º and 77.61º and can be assigned to (111), (110), (200) and (311). Thus, from the XRD pattern clearly represents the crystalline nature confirming the formation of silver nanoparticles from the stem extract of Lagenaria siceraria (15).
Scanning electron microscopy (SEM) and energy dispersive X-Ray analysis (EDX):

The biosynthesized AgNPs using stem extract of *Lagenaria siceraria* were subjected to SEM analysis for determination of shape of the particles (Fig. 4 a). The spherical shape of the synthesized silver nanoparticles is confirmed through SEM micrograph. The EDX spectrum (Fig. 4 b) recorded a strong signal of elemental silver, which thus confirms the synthesis of AgNPs using the stem extract of *Lagenaria siceraria*. Other weak signals (C, O, Cl) have also been noted which may be due to the presence of other compounds in the stem extract of *Lagenaria siceraria* (16).

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR absorption spectra of control dried stem extract of *Lagenaria siceraria* (as shown in Fig. 5a) showed peak at 3368 cm\(^{-1}\), 2932 cm\(^{-1}\), 1598 cm\(^{-1}\), 1386 cm\(^{-1}\), 1121 cm\(^{-1}\), 1084 cm\(^{-1}\), 1047 cm\(^{-1}\), 927 cm\(^{-1}\), 855 cm\(^{-1}\), 824 cm\(^{-1}\), 777 cm\(^{-1}\), 621 cm\(^{-1}\), 666 cm\(^{-1}\). The peak at 3368 cm\(^{-1}\) is due to the O–H stretching, the band at 1598 and 2932 indicates C–H group. The peak at 1386 could be assigned to (-COO-) carboxylate ions. The peak at 1121 cm\(^{-1}\) is due to the carbonyl group. The band at 1084 cm\(^{-1}\) could be due to the presence of phenolic group. The peak at 1047 cm\(^{-1}\) is due to the presence of C–OH stretching. The peak in between 400 – 800 cm\(^{-1}\) could be due to the presence of aromatic groups.

The FTIR absorption spectra of biosynthesized AgNPs showed prominent peaks at 3432 cm\(^{-1}\), 2925 cm\(^{-1}\), 2100 cm\(^{-1}\), 1636 cm\(^{-1}\), 1547 cm\(^{-1}\), 1402 cm\(^{-1}\), 1241 cm\(^{-1}\), 1081 cm\(^{-1}\) and 696 cm\(^{-1}\). The broad band at 3432 cm\(^{-1}\) is due to the strong O-H stretching intermolecular bond of alcohol, the band at 2925 cm\(^{-1}\) corresponding to C-H stretching bands, the band at 2100 cm\(^{-1}\) are due to the N- H stretching band in the free amino groups of AgNPs, the band that appeared at 1636 cm\(^{-1}\) represented carbonyl (C=O) group. The band at 1547 cm\(^{-1}\) is due to the amides, 1402 cm\(^{-1}\) can be assigned to C=C aromatic.
The band at 1241 cm$^{-1}$ and 1081 cm$^{-1}$ may be due to the C–N stretching.

The above study revealed the interaction between the Ag$^+$ ions with the aqueous stem extract of *Lagenaria siceraria*. The following data also explains the multifunction of the stem extract of *Lagenaria siceraria* as both the reducing and stabilizing agent (17).

**Dynamic light scattering (DLS):**

From the dynamic Light Scattering (DLS) analysis, the size distribution of the synthesized silver nanoparticles was obtained by measuring the dynamic variation of the light scattering intensity caused due to the Brownian movement of the synthesized particles. The measurement provide the hydrodynamic diameter that is the particles diameter along with the ion or molecule attached with it (18, 19). The average particle size of the synthesized AgNPs were found to be 105 nm and the polydispersity index was 0.160 as shown in Fig. 6.

**Fig. 5:** Fourier transform infrared spectroscopy (FTIR) image of: (a) Control dried stem extract of *L. siceraria* (without AgNO$_3$) and (b) Biosynthesized AgNPs (after reaction with AgNO$_3$)

**Fig. 6:** Particle size distribution of synthesized AgNPs.
Antibacterial activity of AgNPs

The antibacterial activity of the biosynthesized silver nanoparticles was evaluated against gram positive (Staphylococcus aureus) and gram-negative (Escherichia coli) strains of bacteria using disc diffusion method (20). The antibacterial activity of the synthesized AgNPs was found to be effective against both the strains of bacteria. The result of the study are depicted in [Fig. 8 (a) and (b)] and the zone of inhibition are showed in Table 1. The biosynthesized AgNPs showed satisfactory inhibition activity than the crude stem extract of Lagenaria siceraria itself. The exact mechanism behind the antibacterial activity of the silver nanoparticles is still not known. According to literatures silver nanoparticles binds to the thiol group of the cellular enzymes, also report suggests that silver ion interact with the cell membrane and increasing its permeability and the respiration. In addition, AgNPs interact with the DNA by reacting with the sulfur and the phosphorus group (21-23).

In the present study, it has been shown that the biosynthesized silver nanoparticles from stem extract of L. siceraria has antibacterial activity against both S. aureus and Gram-negative Escherichia coli bacterial strains.

![Fig. 7: Anti-bacterial activity of synthesized AgNPs](image)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Species</th>
<th>Antibiotics</th>
<th>Stem Extract</th>
<th>AgNPs</th>
</tr>
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<tbody>
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<td>1.</td>
<td>S. aureus</td>
<td>1 cm</td>
<td>0.7 cm</td>
<td>1.2 cm</td>
</tr>
<tr>
<td>2.</td>
<td>E. coli</td>
<td>2.4 cm</td>
<td>1.1 cm</td>
<td>1.5 cm</td>
</tr>
</tbody>
</table>

CONCLUSION

In the present study, silver nanoparticles were biosynthesized by using the stem extract of L. siceraria under two different conditions viz. room temperature and sunlight irradiation. The colour change of the reaction mixtures containing (AgNO₃ + stem extract of L. siceraria), visually confirmed the formation of silver nanoparticles. In addition, UV-Vis spectroscopy further confirmed the formation of AgNPs using stem extract of L. siceraria between 350 -700 nm. UV-Vis analysis results thus obtained showed characteristic absorbance peak at 425 nm and 428 nm for both the conditions. The synthesis of silver nanoparticles by sunlight irradiation was found to be faster and effective method in terms of reaction time as compared to silver nanoparticle synthesized at room temperature. The synthesized AgNPs via sunlight were characterized further using X-ray diffraction (XRD) analysis (FTIR), FTIR spectroscopy, Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) and Energy Dispersive X-ray analysis (EDX). SEM and DLS analysis revealed that the AgNPs were spherical in shape with the average particle size of 105 nm and the polydispersity index was 0.160 respectively. The XRD pattern obtained thus clearly proves that the biosynthesized AgNPs are crystalline face centered cubic (fcc) in nature. Additional to this, The EDX analysis showed strong signal of Ag (Silver) that confirmed the AgNPs formation and FTIR analysis revealed various bands shifting which is due to the biomolecules present in the crude extract involved in reducing and capping of the Ag ions. Furthermore, the biosynthesized AgNPs showed antibacterial activity against both the Gram positive (S. aureus) and Gram-negative (E. coli) bacterial strains.

From the above results, it can be concluded that biosynthesis of silver nanoparticles using stem extract of L. siceraria is an energy efficient process for producing silver nanoparticles and can be exploited in production of biomedicine in near future.

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Green synthesis of silver nanoparticles using Enteromorpha compressa and its in vitro anticancer potential against human colon cancer cells

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ABSTRACT

Introduction and Aim: According to recent statistics, cancer is the highest cause of death in worldwide. The drugs utilized to treat cancer are not fully effective and are ultimately susceptible to resistance. Thus, there exists a need to discover more effective therapeutic agents to treat this disease. The newly emerging field of nanobiotechnology is gaining importance owing to its wide application in fields such as agriculture, medicine and industry. The present study aims at the investigation of anticancer efficiency of synthesized silver nanoparticles (AgNPs) from Enteromorpha compressa, a marine alga.

Materials and Methods: The brown algae E. compressa was collected from Chilika, Odisha, India, for experimental analysis. The AgNPs were synthesized from E. compressa and then characterized through UV-VIS Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS). The cell viability study, survival and DAPI staining method were conducted to ensure the anticancer activity of the synthesized AgNPs.

Results: We found a distinctive peak at 446 nm owing to surface plasma resonance characteristics of synthesized AgNPs, was observed in the UV-Vis spectra. Simultaneously, the FTIR spectra confirmed the presence of various phytochemicals in E. compressa, which plays a crucial role in the formation of silver nanoparticle. Again, the XRD and TEM analysis validate the crystallinity feature of the synthesized AgNPs with an average particle size of 44.3 ± 0.12 nm. The green synthesized AgNPs exhibited an excellent cytotoxic property against HCT-116 cell lines with >80% and >20% death of HCT-116 and FHM cells respectively occur at an AgNP dose of 200 µM.

Conclusion: This study shows the E. compressa extract can be used as a reductant in the formation of spherically shaped AgNPs with an average particle size of 44 nm size and potential source for anticancer agent against HCT-116. Thus, synthesized AgNPs in this study can be a solid base for forthcoming research in the synthesis of a new medicine.

Keywords: Green Synthesis; AgNPs; E. compressa; anticancer activity; colon cancer.

INTRODUCTION

The nanomaterials owing to its unambiguous characteristics viz. chemical, mechanical and optical, imparted its use in human day-to-day life (1). The nanomaterials also become a choice for its application in the field of catalysis and biomedical because of its encouraging and optimistic features (2). In accordance with this, various noble nanoparticles (NPs) such as gold, silver, titanium, zirconium, strontium emerged for their extensive application. Among these, AgNPs extended its boundary of application owing to its distinctive physicochemical characteristics, wide spectrum of antibacterial and antimicrobial activity (3). Various physical and chemical means were employed for the synthesis of AgNPs, however, the expensive nature and employment of toxic chemicals during synthesis hinders their commercialization (4). To overcome the issues of physical and chemical method, a safe environment-friendly and cost-effective method i.e. biological method emerged for the green synthesis of AgNPs. Biosynthesis of nanoparticles is good because it is nontoxic, biocompatible, eco-friendly, cost effective, antimicrobial and anticancer activities. Furthermore, the synthesis of AgNPs using biological method does not require high temperature, pressure and toxic chemicals (5), thus become energy intensive, safe and easily scalable.

Plant-mediated biosynthesis of AgNPs had gained immense popularity owing to its abundant and cheap availability. The choice of plants depends on cells, bacteria or fungi. Considering biological potency, activity of the plants depending on the intended use of the nanoparticles to be synthesized e.g. if I need nanoparticles for use against fungi, use a plant with anti-fungal properties etc. In view of this, various
investigations were carried out for the synthesis of AgNPs using different leaf extract viz. 
'Tulsi' (6), Rosmarinus officinal (7), Minusops elengi (8), Emblica Officinalis (9), Guar gum (10), Calendula officinalis (11), Nigella Sativa (12). The mechanism of synthesis of AgNPs lied with the replacement of chemical products, thus enabling it for the removal of hazardous substances that have an antagonistic effect on human health and the environment. However, the AgNPs synthesized were unstable and agglomerate with each other because of the presence of a higher concentration of phytochemicals in the leaf extracts (13). Marine seaweeds, being regarded as an imperative bio-factory, has been emerged as a potential reducer and stabilizer for the synthesis of AgNPs owing to its less cost, extraordinary metal adsorption capacity and macroscopic structure (14). Moreover, the architecture of seaweeds is composed of the typical nanostructure with a layer of silica and calcium in its coral reefs, thus enable it for better metal reducer among all genres of bio-reductants (15). Furthermore, seaweeds possess enchanting characteristics of antibacterial, antioxidant, antimicrobial, anticancer ability owing to its inherence presence of sulfated polysaccharides. Brown algae, regarded as edible seaweeds, often used as animal feed and fertilizer, are abundantly available in the eastern coast of India. Moreover, the inherent protein, carbohydrate, vitamin, and mineral sources along with phenolic compounds (20-30% dry weight) widen its application as an effective antioxidant (16). Owing to the antioxidant characteristics, these seaweeds can be extensively used against numerous diseases. E. compressa, among the brown seaweeds, has been extensively studied owing to it's in vitro antioxidant potential which in turn due to the rich content of alkaloids, flavonoids and terpenoids and also responsible for synthesis of AgNPs (17). Meanwhile, the occurrence of phenolic compounds and flavonoids in E. compressa has an added advantage in its remarkable anticancer potential (18).

Several researchers (19) investigated antimicrobial activity of green synthesized AgNPs from various algae and seaweeds. However, to the best of author’s knowledge, the anticancer efficacy of brown seaweeds, in specific to E. compressa remains unexplored. Moreover, exploring such novel anticancer nanomaterial might become a breakthrough in the pharmaceutical field of research. To explore this, in the current investigation a low cost, easily scalable, the eco-friendly method was adopted for synthesizing the AgNPs using an extract of abundantly available E. compressa. The synthesized AgNPs were characterized by UV-Vis spectrometer, FTIR, XRD, TEM, and DLS. Further, the anticancer efficacy of the synthesized AgNPs was investigated on HCT116 human colon cancer cell lines and then characterized through cell viability, survival and DAPI staining method.

**MATERIALS AND METHODS**

**Sample collection, chemicals, source of cancer cells**

The brown algae E. compressa was collected from Chilika, Odisha, India, for experimental analysis. The samples were rinsed several times with de-ionized water to take out the impurities. Further, the washed algae were allowed to be dried in a shade for a week and then minced using a mortar and pestle. The minced samples (~ weight of 200g) were further powdered using an electric mixer. The powdered samples were collected in a muslin bag and then solvent exchange was conducted using ethanol as a solvent at 60°C for 20h. Further, the ethanol extract was separated from the E. compressa using a rotary evaporator to obtain a yield(w/w) of 25% (20). Silver nitrate (AgNO₃) was purchased from Sigma Aldrich, India. Normal FHM cell lines and HCT-116 were purchased from the National Centre for Cell Science, Pune, India. All the chemicals used for this investigation were used as received.

**Synthesis of AgNPs**

10ml of aqueous extract of E. compressa was treated with 90ml of an aqueous solution of AgNO₃ (1mM) in a range of controlled temperature of 35-45°C for 20 min. For further analysis, the solution was kept away from light to hinder the photo-activation of formed AgNPs (21).

**Characterization of AgNPs**

The biosynthesis of AgNP is characterized by UV-Vis spectrometer (UV-1800spectrophotometer, Shimadzu, Japan). The UV-visible spectra for E. compressa-AgNPs were recorded in the range of 200-900nm. The XRD analysis (D8 Advanced Brucker X-ray diffractometer) was conducted for the confirmation of the different phase formation of the AgNPs. The diffraction data were collected by thoroughly coating a thin film of formed AgNPs on the XRD grids. The potential chemical interactions between E. compressa extract and AgNO₃ were confirmed through Fourier Transform Infrared (PerkinElmer, MA, USA). The morphology of the formed AgNPs was investigated by using Transmission Electron Microscope (JEOL JEM 2100). The average particle size and zeta potential of the formed AgNPs were measured using Dynamic Light Scattering (Zetasizer Nano ZS, ZEN3600 and Malvern, UK).

**In vitro analysis and anticancer activity**

**Cell line culture and toxification with AgNPs**
The procured cell lines and HCT-116 were grown by using DMEM (Dulbecco’s Modified Eagle’s Medium) for its sustainability. The contamination in the cancer cells was avoided by enriching the cancerous cells with 10% FBS and M199 culture media supplied with penicillin and streptomycin followed by incubation in CO₂ atmosphere at standard temperature. Then the individual cells were obtained through trytspinning the cells present on the surface for 3-4 min and centrifuging at 750 rpm for 8-10 min. Then the individual cells were counted and then dispersed on a microplate (96 well) at 5000 cells/well. Finally, the convergent monolayer was obtained by further incubation in CO₂ atmosphere (22). The FHM cell line and HCT-116 cell lines were treated with varying concentration of AgNPs (10 – 250 µg/ml). In this investigation, the experiments were performed thrice to eradicate the error. After the intoxication period of 48 h, the cell colonies were calculated using Microplate Reader (2030 Muan Itilabel Processor VictorTMX3 Perkin Elmer, Waltham, MA, USA).

**Cell viability study**

For cell viability study, 200µl of MTT solution is exposed to each culture followed by incubation for 4-5 h. Then the MTT solution was taken out and further hatched for 15 min in dark by the subsequent addition of 200 µl of DMSO to each well. Further, the microplate reader is applied to determine the optical density of the product. The AgNp treated colon cancer cells and their viability was obtained through MTT (3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyl tetrazolium bromide) analysis (23).

**RESULTS**

**UV-visible spectrum of AgNPs**

The UV-visible spectroscopy analysis was conducted through measuring the concentration of the AgNPs in the solution and presented as fig. 1. The AgNPs formation was entrenched by the subsequent reduction of Ag⁺ ion with biomolecules of *E. compressa* to form Ag⁰. This reaction was also indicated by the alteration in color (brownish-yellow) owing to the surface plasmon resonance (SPR) effect and AgNO₃ reduction, as shown in fig. 2. Moreover, the intensity of color was observed to increase with an increase in the incubation period. The aqueous sol of AgNPs prepared at different pH values using hydrothermal method display different Surface Plasmon Resonance (SPR) behavior and the maximum absorption values were at pH = 9. When increasing the pH values, the result revealed the formation of larger nanoparticles cluster with more accurate crystallite sizes. Small and uniform sized nanoparticles were synthesized by increasing pH of the reaction mixture. The nearly spherical AgNPs were converted to spherical AgNP by altering pH. A peak was observed for the individual *E. compressa* extract confirmed the presence of phenolics, flavonoids, and other secondary metabolites.

![UV-visible spectrum of *E. compressa* solution, AgNO₃ solution, *E. compressa* -AgNPs in water, and AgNPs in cell culture media.](image1)

**Fig. 1:** UV-visible spectrum of *E. compressa* solution, AgNO₃ solution, *E. compressa* -AgNPs in water, and AgNPs in cell culture media.

![Image of *E. compressa* extract and synthesized silver nanoparticles](image2)

**Fig. 2:** (a) *E. compressa* (b1) *E. compressa* extract (b2) synthesized silver nanoparticles (AgNPs).
FTIR analysis

FTIR analysis (fig. 3) was conducted to investigate the various potential biomolecules in the extract of *E. compressa* responsible for reducing the Ag⁺ ion to Ag⁰. Pure *E. compressa* shows peaks at 754 cm⁻¹ corresponding to saccharide structure, 1562 cm⁻¹ (amide II), 1876 cm⁻¹ (amide I and III peaks), 3499 cm⁻¹ (amine N-H symmetrical vibration) and 2376 and 3256 cm⁻¹ (C-H stretch vibrations). Pure AgNO₃ shows peaks at 1469 cm⁻¹ corresponding to nitrate group, 3501 cm⁻¹ (amine N-H symmetrical vibration) and 2389 cm⁻¹ (C-H stretch vibrations). Eight distinctive bands were observed for synthesized AgNPs. The band at 3491 cm⁻¹ and 2370 cm⁻¹ confirms the stretching vibration of primary and secondary amides of protein. The bands at 2927 cm⁻¹ and 2370 cm⁻¹ were assigned to asymmetric C-H stretching and primary amine group. Similarly, the band at 1867 cm⁻¹ confirms the presence of carbonyl stretch owing to the amide-I band of protein. The presence of amino and amino-methyl of protein was confirmed at a band of 1512 cm⁻¹. Again, the band at 786 cm⁻¹ was attributed to the C-O stretching, which is responsible for the binding of the AgNPs. Further, the free amine and cysteine groups in proteins have also a strong ability to bind the AgNPs.

![FT-IR spectra of (a) E. compressa extract (b) AgNO₃ (c) AgNP](image)

XRD analysis

The XRD analysis of the extract of *E. compressa*, AgNO₃ and biosynthesized AgNPs was presented as fig. 4. For E-AgNPs three prominent peaks at 40, 46 and 72 were observed in the 2θ range of 20 to 75, which are pertaining to the Bragg’s reflection of (1 1 1), (2 2 0) and (3 1 1) planes. The presence of such planes confirms that AgNPs synthesized are face centric cubic (FCC) silver.

![XRD spectra of (a) E. compressa, (b) AgNO₃, (c) AgNP](image)

Dynamic light scattering (DLS) studies

The average particle size, surface charge and the stability of E-AgNPs were measured through the DLS studies. The average particle size and zeta potential of synthesized E-AgNPs were of 46nm (Fig. 5a) and -26.2 mv (Fig. 5b) respectively. Moreover, the negative value of the zeta potential with peak area intensity of 100% indicates that E-AgNPs synthesized are more stable with little agglomeration.
Morphology studies

The SEM and TEM morphology of the synthesized AgNPs are presented as Fig. 6a and b respectively. The SEM morphology reveals that individual nanoparticles are formed in clusters of spherical shape. The TEM morphology reveals the shape and size of the formed AgNPs and particle size found is 44nm.

Cell survival measurement and cell viability study

To predict the consequences of the synthesized AgNPs on the colony-forming capability of the colon cancer cells, the HCT-116 cells were treated individually with different concentrations of E. compressa, AgNO₃, and AgNPs for 3 days. The individual survival (%) of the colon cells for various concentrations of E. compressa, AgNO₃ and AgNPs are presented as Fig. 7(a). It is observed that the pure E. compressa do not possess any significant inhibition capacity towards cell growth. Although, with the increase in pure AgNO₃ concentration the survival of cells decreases significantly, but a maximum 32% of the death of cells were observed at 200µM. Further, the survival (%) of cells was found to decrease with increase in the concentration of AgNPs and the maximum death of 80% was observed at 200µM concentration. Further, to obtain the IC₅₀ value, the survival % of cells was noted with the broader range of concentrations of synthesized AgNPs and presented as Fig. 7b. It is observed that with an increase in the concentration of AgNPs the cell death % increases and the IC₅₀ value was obtained as 150 µM.

The clonogenic assay further can be confirmed by performing the MTT cell viability assay. The MTT assay was used to determine the cell viability by using yellow tetrazolium salt by analyzing the cellular metabolic activity. The details of MTT assay is represented fig, 8a describing the viability of HCT-116 cells after exposure to the extract of E. compressa, AgNO₃ solution, and AgNPs. According to the data of clonogenic cell survival assay, there is no evidence of...
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killing of the cancer cell by *E. compressa* extract, whereas 25% cancer cell were killed by AgNO₃ but a spectacular fall of cell viability i.e. more than 80% through AgNPs at the similar concentration of 200µM. The time-dependent killing by AgNPs on cancer cells is shown in Fig. 8b where three-time point has been taken to examine the dose-depended killing and it is observed that the lowest IC₅₀ is at 72 h (80µM) in contrast to 48 h (100 µM) and 24 h (310µM).

**Biocompatibility study**

Human colon epithelial cell lines, FHM were exposed with the increasing concentrations of synthesized AgNPs for 48h for evaluating its antiproliferative activity. The comparison of cell viability (%) of FHM and HCT-116 cells for varying concentrations of AgNPs is presented as Fig. 9. It is evident that the >80% and >20% death of HCT-116 and FHM cells respectively occur at an AgNP dose of 200 µM. Thus, the synthesized AgNPs has the capability for selectively killing the colon cancer cells.
Apoptosis analysis by a nuclear staining method

The fluorescent microscopy photographs of apoptosis analysis are presented as Fig. 10, where AgNPs treated cells were stained with DAPI (4, 6-diamidino-2-phenylindole) and nuclear staining method was used to assess the apoptosis. The DAPI stains the DNA of cancer cells which were treated with AGNPs. It was found that in response to gradual increase concentration of AgNPs, the nuclei of HCT-116 became fragmented, shrunken and bubble-shaped (shown by arrows) and at 200µM concentration of AgNPs, the apoptosis is maximum.

![Fig. 10: Silver-based nanoparticles caused apoptosis in HCT-116 cells](image)

DISCUSSION

The peak around 300 nm and 446 nm for AgNO₃ solution and the E-AgNPs solution respectively confirms the formation of AgNPs in UV-Vis spectroscopy analysis. The FTIR shows eight distinctive bands for synthesized AgNps. The band at 3491 cm⁻¹ and 2370 cm⁻¹ confirms the stretching vibration of primary and secondary amides of protein. The bands at 2927cm⁻¹ and 2370cm⁻¹ were assigned to asymmetric C-H stretching and primary amine group. Similarly, the band at 1867 cm⁻¹ confirms the presence of carbonyl stretch owing to the amide-I band of protein. The presence of amino and amino-methyl of protein was confirmed at a band of 1512 cm⁻¹. Again, the band at 786 cm⁻¹ was attributed to the C-O stretching, which is responsible for the binding of the AgNPs (6-12). Further, the free amine and cysteine groups in proteins have also a strong ability to bind the AgNPs. The characteristic protein peaks of *E. compressa* over the silver nanoparticle surface may act as reducing and stabilizing agent (17, 20). The extract of *E. compressa* plays as a reductant of Ag⁺ to Ag₀ (13, 20). Moreover, there is no evidence of nitrate ions and other impurities in the XRD, signifying that highly pure AgNPs were formed. Simultaneously, in the XRD pattern of *E. compressa* two distinctive peaks were found at 38 and 62, thus there is no evidence of silver. Again, for AgNO₃ the presence of silver is confirmed through the peaks at 37 and 62, but the silver is in the nitrate form. The negative value of the zeta potential with peak area intensity of 100% indicates that E-AgNPs synthesized are more stable with little agglomeration. It was found that the formed AgNPs are spherically shaped with little agglomeration with each other. The mean particle size was found to be 44 nm, which is in concurrence with the DLS study. With the increase in pure AgNO₃ concentration, the survival of cells decreases significantly, but the death of cells was observed at 200 µM. The survival (%) of cells was found to decrease with increase in the concentration of AgNPs and reaching maximum death at 200 µM concentration (12). Further, it is observed that with an increase in the concentration of AgNPs the cell death % increases and the IC₅₀ value was obtained as 150 µM. Interestingly the MTT assay data reveals, there is no evidence of killing of the cancer cell by *E. compressa* extract. 25% cancer cells were killed by AgNO₃ but a magnificent fall of cell viability (23) i.e., more than 80% through AgNPs at the similar concentration of 200µM and the dose-dependent killing is observed lowest IC₅₀ value at 72 h (80 µM) in contrast to 48 h (100µM) and 24 h (310µM). The nuclear staining with DAPI for apoptosis analysis also confirmed the maximum fragmentation of cancer cell nuclei with AgNPs at 200µM concentration.

CONCLUSION

In the present investigation, *E. compressa* extract was used as a reductant in the formation of spherically shaped AgNPs with an average particle size of 44 nm size. The presence of phenolic content in the extract plays an important role in the synthesis of AgNPs. Further, the synthesized AgNPs exhibited a strong anticancer activity. Moreover, >80% and >20% death of HCT-116 and FHM cells respectively occur at an AgNP dose of 200µM. Thus, the *E. compressa* extract can be a potential source for anticancer agent against HCT-116 and synthesized AgNPs in this study can be a solid base for forthcoming research in the synthesis of a new medicine.

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Periodontitis presage pre-diabetes – A comparative study of glycemic control in non-diabetic population with and without periodontal disease

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ABSTRACT

Introduction and Aim: The understanding of the etiology and pathogenesis of periodontal diseases and their chronic, inflammatory and infectious nature suggests that these infections may influence events elsewhere in the body. Poorly controlled diabetes is a well-recognized risk factor for developing periodontal disease. There is also ample evidence that periodontal disease can worsen a patient’s glycemic control and proper management of periodontal disease can improve the same. However, very few have determined effect of periodontitis on glycemic control, of non-diabetic population and concluded that untreated periodontitis pose a risk of pre-diabetes in systemically healthy individuals. The purpose of this study is to estimate and compare the HbA1c levels in non-diabetic subjects with periodontitis and periodontally healthy controls.

Materials and Methods: A total of 639 non-diabetic subjects were selected for the study and were divided into 2 groups based on the periodontal status: Group A (n = 324) Periodontally Healthy controls months and Group B (n = 315) subjects with Chronic periodontitis. Clinical parameters like Plaque index (PI), Modified sulcular bleeding index (mSBI), Probing depth (PD), and Clinical attachment level (CAL) were measured. Glycemic control was measured by assessing HbA1c.

Results: The mean PI for Group A was 0.99 ± 0.38 and Group B was 1.9 ± 0.59. mSBI score for the test group was 2.9 ± 0.87 and it was 1.79 ± 0.57 in the control group. The mean PD in the Group A was 2.73 ± 0.9 and in Group B was 7.16 ± 0.93. The mean CAL for Group A was 2.24 ± 0.65 and in Group B was 5.86 ± 0.75. The values for all the clinical parameters were statistically significant. The mean HbA1c% for control group was 2.94 ± 0.29 and for the test group was 5.95 ± 0.36. This value was statistically significant between the two groups.

Conclusion: In a non-diabetic systemically healthy population, the glycated hemoglobin level of the subjects with severe periodontitis is significantly greater than the subjects without periodontitis. Non-diabetic subjects with severe periodontal disease presented a pre-diabetic state reflecting that periodontal disease has created a state of insulin resistance.

Keywords: Diabetes mellitus; HbA1c levels; glycemic control; periodontitis; pre-diabetes.

INTRODUCTION

Diabetes mellitus and periodontitis are chronic diseases that affect a large number of populations worldwide. Periodontitis is a chronic inflammatory response to the subgingival bacteria, producing irreversible periodontal tissue destruction and tooth loss. Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrates, fat and protein metabolism resulting from the defects in insulin secretion, insulin action or both (1).

Years of research have established a number of mechanisms by which diabetes can influence the periodontium (2, 3). In case of diabetic patients, concentrations of oral microflora are increased due to high concentrations of glucose in saliva and gingival crevicular fluid. Diabetic patients have higher than normal levels of perio-pathogenic bacteria. Diabetic state also results in a state of exaggerated immune response to these bacteria, resulting in more rapid and severe periodontal destruction (4). There is also abundant evidence that periodontal disease can worsen a patient’s control of diabetes mellitus and that proper management of periodontal disease can improve control of diabetes mellitus (5, 6).

Several studies revealed that the degree of glycemic control is an important variable in relationship between...
diabetes and periodontitis. Studies have reported that individuals with type 1 diabetes manifested advanced periodontal diseases with a higher prevalence and severity of gingival inflammation and periodontal destruction being seen in those with a higher glycemic index (7.8). Significantly, more periodontal attachment and alveolar bone was lost in type 1 diabetic patients who had poor glycemic control than those who were well controlled or non-diabetic patients (9).

Recent research is directed towards the effect on untreated severe periodontitis on the glycemic control of non-diabetic population. It has been noted in a pilot study that non-didactic population with severe periodontitis exhibited increased levels of glycemic index than normal controls (10). Another study found chronic periodontitis to be associated with a significant increase in glycosylated hemoglobin levels in non-diabetic periodontitis subjects. Furthermore, with improvement of periodontal status by treatment, the glycemic levels return to near normal values (11). On the contrary, another study compared the glycohemoglobin levels with severity of periodontitis in non-diabetic population and concluded that there was no significant difference in fasting plasma glucose and postprandial plasma glucose in non-diabetic periodontitis (12). In lieu with the above, the purpose of this study is to estimate and compare the HbA1c levels in non-diabetic subjects with periodontitis and periodontally healthy controls.

MATERIALS AND METHODS

The study subjects were selected from the patient pool of Department of Periodontology, Meenakshi Ammal Dental College and Hospital, Chennai and the "Meenakshi institutional review board" approved the study. The participants were recruited for the study according the following inclusion and exclusion criteria: Patients within the age group of 35 to 65 years, who are non-diabetic and who had ≥ 15 remaining natural teeth were included in the study. Subjects with history of antibiotic usage in the previous 6 months; Patients with conditions that shorten erythrocyte survival (hemolytic anemia, pregnancy or recent significant blood loss); Smokers; Patients who had undergone periodontal therapy within the previous 6 months and pregnant and feeding mothers were excluded.

Study design

Out of all the patients who reported to the outpatient department, a total of 639 non diabetic subjects were selected for the study and were divided into 2 groups based on the periodontal status: Group A (n = 324), Healthy controls with no probing depth (PD) greater than 4 mm, bleeding on probing (BOP) at ≤ 15% of tooth sides and no periodontal treatment (scaling and root planing or surgery) within the previous six months; Group B (n = 315), Chronic periodontitis cases were defined as those having 5 or more teeth with PD ≥ 5mm and clinical attachment loss (CAL) >3mm or radiographic bone loss.

All the clinical periodontal parameters were measured by a trained periodontist. The following periodontal parameters were measured: Plaque index (PI), Modified sulcular bleeding index (mSBI), Probing depth (PD), and Clinical attachment level (CAL). Glycemic control was measured by assessing HbA1c using commercially available kit (Quanita HbA1c – Tulip laboratories, India. The kit works under the immunoturbidometric principle). Reference ranges: Non-diabetic – 4.0% - 5.6 %; Pre-diabetic – 5.7% - 6.4% and Diabetic - > 6.4% (13).

Statistical analysis

Mean and standard deviation were estimated for all the clinical parameters and the Hba1c values. Mean changes were compared against the null hypothesis. Student’s independent t test was used to compare the two mean values in the control and the test group.

RESULTS

On comparing the clinical parameters between the two groups, all the periodontal parameters were increases for the test group than the control group. The mean PI for Group A was 0.99 ± 0.38 and Group B was 1.9 ± 0.59. mSBI score for the test group was 2.9 ± 0.87 and it was 1.79 ± 0.57 in the control group. The mean PD in the Group A was 2.73 ± 0.9 and in Group B was 7.16 ± 0.93. The mean CAL for Group A was 2.24 ± 0.65 and in Group B was 5.86 ± 0.75. All the parameters were statistically significant between the two groups. The mean HbA1c percentage for control group was 2.94 ± 0.29 and for the test group was 5.95 ± 0.36. When both the groups were compared a statistically significant difference in the glycosylated hemoglobin levels was noted (p < 0.001). The case group showed higher HbA1c levels than control group.

<table>
<thead>
<tr>
<th>Plaque index</th>
<th>Group</th>
<th>t test value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.99 ± 0.38</td>
<td>7.79</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>B</td>
<td>1.9 ± 0.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Comparison of periodontal and glycemic parameters between two groups
DISCUSSION

The understanding of the etiology and pathogenesis of periodontal diseases and their chronic, inflammatory and infectious nature suggests that these infections may influence events elsewhere in the body (14). Poorly controlled diabetes is a well-recognized risk factor for developing periodontal disease. There is also ample evidence that periodontal disease can worsen a patient’s glycemic control and proper management of periodontal disease can improve the same (15). However, very few have determined effect of periodontitis on glycemic control, of non-diabetic population and concluded that untreated periodontitis pose a risk of pre-diabetes in systemically healthy individuals (11, 12).

The present study assessed the glycemic control of systemically healthy, non-diabetic subjects with and without periodontitis. Non-diabetic subjects without periodontal disease was enrolled into Group B and subjects with severe periodontitis were enrolled into Group B. Glycemic status of the subjects were assessed using HbA1c as the biochemical parameter. HbA1c was chosen as it provides evidence about an individual’s average blood glucose levels during the previous two to three months, which is the predicted half-life of red blood cells. The same is the recommended standard of care for testing and monitoring diabetes, specifically the type 2 diabetes (16).

The results of this study reveal that in non-diabetic systemically healthy subjects, the glycated hemoglobin level of the subjects with severe periodontitis is significantly greater than the subjects without periodontitis. (5.95 ± 0.36 Vs. 2.94 ± 0.29). Similarly, Wolfe et al., reported that adjusted HbA1c values were statistically higher in periodontitis cases than in healthy controls (10). Murrah et al., compared blood glucose levels between subjects with, without advanced periodontal, and reported significant higher glucose levels in subjects with advanced periodontal disease (17). Jananni et al., had compared the HbA1c levels in subjects with and without periodontitis and found higher levels of glycated hemoglobin in healthy subjects with periodontitis (11). Galhauth et al., compared the glycated hemoglobin levels with severity of periodontitis and reported that glycated hemoglobin levels were not significantly different with severity of the periodontal disease (12).

The mechanism how periodontitis affects glycemic control is largely studied. Periodontitis is primarily a Gram-negative bacterial infection that initiates a cascade of host inflammatory response. The systemic infection by increasing tissue resistance to insulin, prevents glucose entry into the target cells thereby in turn increasing the blood glucose levels. This triggers pancreas to secrete more insulin as an attempt to maintain normoglycemia. In diabetic subjects, who are already resistant to insulin, further tissue resistance to insulin induced by periodontal pathogens significantly exacerbates the blood glucose levels (18).

Our study also found that non-diabetic subjects with severe periodontal disease presented a pre diabetic state reflecting that periodontal disease has created a state of insulin resistance in such cases. Insulin resistance is now considered as a chronic and low-level inflammatory condition. Insulin functions by binding to the hetero tetrameric membrane receptor leading to IRS-1 phosphorylation and IRS-1-associated phosphatidylinositol 3 phosphate kinase (PI3 kinase) activation (19). This event in turn affects effectors like Akt/protein kinase B (PKB), which triggers the glucose transporter GLUT4. GLUT4 is further translocate into the membrane and induces glucose import into the cell (20). But chronic inflammation like periodontitis leads to increased expression of pro inflammatory mediators like TNF alpha that in turn results in serine phosphorylation of IRS 1. This in turn inactivates PI3 kinase and results in insulin resistance (21).

Many studies have studied the effect of periodontal therapy in controlling the glucose levels on diabetic patients and found that periodontal therapy has beneficial effect on glycemic control (22, 23). The magnitude of reported HbA1C reductions ranges from 0.27% to 0.48% at 3-4 months following periodontal therapy (24). It has also been found that non-surgical periodontal therapy reduced slight elevation in HbA1c.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSBI</td>
<td>A: 2.9 ± 0.87</td>
<td>B: 1.79 ± 0.57</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PPD</td>
<td>A: 2.73 ± 0.9</td>
<td>B: 7.16 ± 0.93</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CAL</td>
<td>A: 2.24 ± 0.65</td>
<td>B: 5.86 ± 0.75</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HbA1c%</td>
<td>A: 2.94 ± 0.29</td>
<td>B: 5.95 ± 0.36</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Level of significance set at < 0.05. Plaque index (PI); Modified sulcular bleeding index (mSBI); Probing depth (PD); and Clinical attachment level (CAL)
levels in non-diabetic population back to normal levels (12). This reflects the importance of periodontal therapy on the maintenance of glycemic control both in diabetic as well as non-diabetic group.

Within the limitation of the study, a significant relationship between periodontal disease and glycemic control of non-diabetic population is well evident. The study did not attempt to establish any dose dependent relationship between the periodontal disease extent and the glycemic levels.

CONCLUSION

The study results reveal that HbA1c levels in non-diabetic subjects with severe periodontitis was significantly greater than non-diabetic subjects without periodontitis. Moreover, the subjects with periodontitis seem to exhibit a pre diabetic state as result of insulin resistance induced by periodontal inflammation. Though this study did not attempt to establish a causal relationship between the two disease entities, a strong association between periodontal disease and pre-diabetes is observed.

REFERENCES


Histomorphometry of umbilical cord and its vessels in natural and assisted reproduction

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ABSTRACT

Introduction and Aim: Assisted reproductive technique (ART) is an invaluable gift for infertile couple who failed in various infertility treatments. However, there are many aftereffects after assisted reproduction like preterm birth, small for gestational age, congenital anomalies etc. Umbilical cord is of fetal origin, which connects the fetus to placenta. Therefore, changes in the histomorphometry of umbilical cord and its vessels may affect the fetus and its growth. Our present study aimed to analyze the histo-morphometric parameters of umbilical cord and its vessels in natural conception and in assisted reproduction.

Materials and Methods: 30 placentas (with umbilical cord) of natural conception and 30 placentas (with umbilical cord) of assisted reproduction (ICSI) were collected immediately after delivery in and around Chidambaram. Umbilical cord bits were fixed in buffered formalin and underwent routine tissue processing procedure. 5 to 10 microns of tissue sections were taken and stained in H & E stain, Verhoeff stain and MTS stain. Histomorphometric parameters of umbilical cord and its vessels were measured in Olympus microscope using ocular micrometer.

Results: There was a reduction in the areas of umbilical cord and its vessels in assisted reproduction when compared to natural conception.

Conclusion: These results would be useful for neonatologists who handle the newborns of ART mothers.

Keywords: Umbilical cord; umbilical artery; umbilical vein; histomorphometry; connective tissue.

INTRODUCTION

Assisted reproduction is a technology where both egg and sperm are handled. It involves surgically removing eggs from women’s ovaries, combining them with sperm in the laboratory and returning them to the women’s body or donating them to another woman.

Intracytoplasmic sperm injection

Spermatozoa sometimes fail to fertilize even when they were artificially placed in close proximity to eggs during conventional in vitro fertilization (IVF). Fertilization failure in IVF is particularly common where there are gross abnormal semen parameters or number of spermatozoa is insufficient. The placing of spermatozoa beneath the zona has yielded consistent result. The intracytoplasmic sperm injection procedure entails the deposition of single spermatozoon directly into the cytoplasm of the oocyte, thus bypassing the zona pellucida and oolemma. Retrieval of low number of oocytes represents a further indication for this procedure (1).

Assisted reproductive technique (ART) is a great gift for infertile couple those who are exhausted by taking various infertility treatments and failed in it. But there are many consequences in fetal growth like preterm birth, small for gestational age etc. There are very few studies about placenta and the connecting cable umbilical cord changes in assisted reproduction.

Umbilical cord is the only connecting link between embryo and the placenta. The embryo remains attached to the trophoblast only by extra-embryonic mesoderm into which the coelom does not extend. This extra-embryonic mesoderm forms the connecting stalk. This connecting stalk is only formed as umbilical cord (2).

As embryo grows, the attachment of connecting stalk moves towards the ventral aspect of the embryo. It is attached in the region of umbilical opening. The tube of amnion, two arteries, one vein and Wharton’s jelly (mesoderm) constitutes umbilical cord. Initially there are two arteries and two veins, but later right vein disappears. Umbilical cord is when fully developed, on an average some 50cm long and 1-2 cm in diameter are attained. But the length is subjected to great variation (20-120 cm). The cord usually attaches to centre of the placenta. In few cases velamentous insertion is observed (i.e. into the membranes) and may be vulnerable to injury (3). Umbilical cord plays an important role in maintaining and regulating feto-placental circulation and thus in fetal nutrition and wellbeing (4). Umbilical vessels within the umbilical cord are not supplied by vaso-vasorum and thus depend on their oxygen supply making them more vulnerable to changes in hemodynamic condition. Umbilical cord is full of fetal origin and changes in the umbilical cord morphology may affect the fetus. Our present study aimed to analyze the histomorphometry of
umbilical cord, its vessels and connection tissue in natural conception and in assisted reproduction.

MATERIALS AND METHODS
Thirty placenta (with umbilical cord) of natural conception and 30 placenta (with umbilical cord) of assisted reproduction were collected from Rajah Muthiah Medical College, OBG Department and from private fertility center in and around Chidambaram. In ART 12 cases were dichorionic, diamniotic twins. So totally the umbilical cords of ART were 42. Human ethical committee clearance was obtained for sample collection. Patient history was collected using proper proforma. Umbilical cord bits were taken five centimeters away from placental end. Cord bits were fixed in buffered formalin. Cord bits well processed and two blocks were made for each specimen. 5 to 10 microns thick sections were made and stained by hematoxylin and eosin stain, Verhoff stain and Masson trichrome stain. Various histomorphometric parameters of umbilical cord were measured by ocular micrometer in Olympus microscope.

The following parameters were measured:
1. Umbilical cord
   a. Total umbilical cord area

RESULTS

Table 1: Clinical characteristics of normal and assisted reproductive pregnancies

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Normal (mean)</th>
<th>ICSI (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (years)</td>
<td>28.33</td>
<td>32.93</td>
</tr>
<tr>
<td>1</td>
<td>Hb (g%)</td>
<td>11.07</td>
<td>11.77</td>
</tr>
<tr>
<td>2</td>
<td>Gestational age (weeks)</td>
<td>37.033</td>
<td>36.16</td>
</tr>
<tr>
<td>3</td>
<td>Weight of baby (Kg)</td>
<td>2.92</td>
<td>2.417</td>
</tr>
<tr>
<td>4</td>
<td>Mode of delivery VD/caesarean</td>
<td>30/2</td>
<td>0/30</td>
</tr>
<tr>
<td>5</td>
<td>Abgar score</td>
<td>9/10</td>
<td>8/10</td>
</tr>
</tbody>
</table>

Mean age of mother increased in assisted reproduction when compared to natural conception. But mean gestational age and weight of baby were reduced in assisted reproduction (Table 1).

Table 2: Histomorphometry of umbilical cord

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Normal mm²</th>
<th>ICSI mm²</th>
<th>Z value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases</td>
<td>Mean</td>
<td>Std</td>
<td>No. of cases</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>Total Umbilical cord area</td>
<td>30</td>
<td>57.16</td>
<td>0.95</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Connective Tissue area</td>
<td>30</td>
<td>45.63</td>
<td>15.97</td>
<td>42</td>
</tr>
</tbody>
</table>

P value <0.05 is significant

Our present study showed that the total umbilical cord area was 57.17 mm² in natural conception. It was about 49.88 mm² in assisted reproduction (table 1). Connective tissue area of umbilical cord was 45.63 mm² in natural conception. It was about 40.08 mm² in ART (Table 2).

Histological examination of umbilical cord showed that there was a well-defined single layered squamous amniotic epithelium. Deep to that was a mucoid connective tissue i.e., Wharton’s jelly within the jelly are umbilical vessels (Fig. 1). Two arteries and a single umbilical vein were present in umbilical cord. The arteries possess no elastic lamina and doubly layered smooth muscle wall vein has inner elastic lamina (Fig. 2 and 3). The umbilical vein has a larger diameter as compared to arteries and has a thin single layer of muscle coat (Fig. 2 and 4). The umbilical artery possesses thick double-layered smooth muscle coat when compared to umbilical vein (Fig. 5).
Table 3: Histomorphometry of umbilical artery (A)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Normal (mm²)</th>
<th>ICSI (mm²)</th>
<th>Z value</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases</td>
<td>Mean</td>
<td>Std</td>
<td>No. of cases</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>Total artery area</td>
<td>30</td>
<td>3.12</td>
<td>1.66</td>
<td>42</td>
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<tr>
<td>2</td>
<td>Lumen Area</td>
<td>30</td>
<td>0.28</td>
<td>0.37</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>Outer layer area</td>
<td>30</td>
<td>1.94</td>
<td>0.7</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>Inner layer area</td>
<td>30</td>
<td>0.59</td>
<td>0.42</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>Wall area (inner+outer)</td>
<td>30</td>
<td>2.84</td>
<td>1.51</td>
<td>42</td>
</tr>
</tbody>
</table>

P value <0.05 is significant

Table 4: Histomorphometry of umbilical artery (B)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Normal (mm²)</th>
<th>ICSI (mm²)</th>
<th>Z value</th>
<th>P value</th>
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<tr>
<td></td>
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<td>Mean</td>
<td>Std</td>
<td>No. of cases</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>Total artery area</td>
<td>30</td>
<td>4.05</td>
<td>2.25</td>
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</tr>
<tr>
<td>2</td>
<td>Lumen Area</td>
<td>30</td>
<td>0.411</td>
<td>0.6</td>
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</tr>
<tr>
<td>3</td>
<td>Outer layer area</td>
<td>30</td>
<td>2.28</td>
<td>1.48</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>Inner layer area</td>
<td>30</td>
<td>0.67</td>
<td>0.56</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>Wall area (inner+outer)</td>
<td>30</td>
<td>3.64</td>
<td>1.86</td>
<td>42</td>
</tr>
</tbody>
</table>

P value <0.05 is significant

Table 5: Histomorphometry of umbilical vein

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Normal (mm²)</th>
<th>ICSI (mm²)</th>
<th>Z value</th>
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<tr>
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<td>Mean</td>
<td>Std</td>
<td>No. of cases</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>Total umbilical Vein area</td>
<td>30</td>
<td>4.36</td>
<td>2.11</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Lumen Area</td>
<td>30</td>
<td>1.41</td>
<td>1.4</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>Wall area (inner+outer)</td>
<td>30</td>
<td>2.94</td>
<td>0.95</td>
<td>42</td>
</tr>
</tbody>
</table>

P value <0.05 is significant

DISCUSSION

Assisted reproductive technique is a great boon to infertile couple who are exhausted by using various infertility treatments and failed in it. There are some aftereffects in assisted reproductive techniques because of artificial manipulation of gametes. The state of vascular system of mother and of placenta has exerted great influence on intrauterine growth of fetus. The umbilical cord is a connecting cable...
between fetus and placenta. It acts as a carrier and mediator between fetus and placenta. The arteries within it return poorly oxygenated blood to the placenta while the vein carries oxygenated blood from this tissue to the fetus (5-7).

The Wharton’s jelly is a metabolically active tissue involved in fluid exchange between amniotic fluid and umbilical vessels. Torsion and fibrosis of Wharton’s jelly and thickening of vascular wall, which obstructs the fetoplacental circulation leading to anoxia and fetal death (8, 9), usually accompany the lean umbilical cords. Reduced Wharton’s jelly could be due to fetal starvation, dehydration and poor maternal nutrition. Absence of Wharton’s jelly may result in antenatal fetal death (10). These reduced Wharton’s jelly may cause compression of umbilical vessels (11). According to Barnwal et al., the total umbilical cord area was 51.56 ± 2.34 mm² and connective tissue area was 42.27 ± 2.12 mm² (12). In our present study umbilical cord area were 57.17 ± 16.77 mm² in natural conception and 49.88 ± 21.7 mm² in assisted reproduction. Connective tissue area of umbilical cord was 45.62 ± 15.97 mm² in natural conception 40.08 ± 20.52 mm² in assisted reproduction. There was significant reduction in total umbilical cord area and connective tissue area of umbilical cord in assisted reproduction (Table 2). In Kotramnavar et al., study, the total vein area was 2.16 ± 0.54 mm² in control group and 1.55 ± 0.45 mm² in IUGR cases (13). Our present study showed 4.36 ± 2.11 mm² in natural conception and 3.33 ± 1.93 mm² in assisted reproduction (Table 5). There was a significant reduction of vein area in assisted reproduction.

In Shaima et al., study, the total area of umbilical vein was 2.09 ± 1.43 mm² in control group and 3.1 ± 3.32 mm² in pre-eclamptic group, whereas lumen area of vein was 0.24 ± 0.04 mm² in control group and 5.05± 0.40 mm² in pre eclamptic group (14). In our present study umbilical vein area was 4.36 ± 2.11 mm² in natural conception and 3.33±1.43 mm² in assisted reproduction. Lumen area of umbilical vein was 1.416±1.4mm² in natural conception and 0.95±0.98 mm² in assisted reproduction. Wall area of umbilical vein was 2.94±0.95 mm² in natural conception and 2.37±1.35 mm² in assisted reproduction (Table 5). All the parameters of umbilical vein were reduced in assisted reproduction. There was a significant reduction in umbilical vein wall area and total umbilical vein area. According to Blanco et al., study, the outer layer area and Inner layer area of umbilical artery was increased in pathological conditions like IUGR, preeclampsia, gestational diabetes mellitus and antiphospholipid syndrome when compared to normal group of placentas (15). Inner layer area of umbilical artery (A) and umbilical artery (B) increased in assisted reproduction when compared to natural conception (Table 3 and 4). According to Inan et al., study, there was a significant reduction in the total umbilical vein area, wall thickness of vein and narrowing of vein lumen was observed in preeclampsia cases. The thickness of arteries was 20% increase in preeclampsia group (16). In Junek et al., study, the thickness of umbilical arteries (both intima and media) was 15% increase in preeclampsia. The thickening of tunica intima was due to the emigration of smooth muscle cells towards endothelium and splitting of internal elastic lamina. This emigration was due to rise in the sulphated glycosaminoglycan in umbilical arteries (17). In Bruch et al., study, they found that there was a reduction in the Wharton’s jelly area, luminal and wall areas of umbilical vein in Intra uterine growth restriction (IUGR) cases. Hypoblastic umbilical vessels are associated with an increase in the placental vascular resistance which in turn caused by chronic defect in the placental blood flow (18). There was a strong association between lean umbilical cord and the small for gestational age (SGA) infant (11).

We provide the histo-morphometric parameters of umbilical cord and its vessels in natural conception and assisted reproduction. Overall, there was a reduction in the areas of umbilical artery (A), umbilical artery (B), umbilical vein, lumen areas of all the vessels and areas of total umbilical cord and connective tissue. However, statistically significant changes were seen in outer layer area of artery (A), wall area of artery (B), umbilical vein area and umbilical vein wall area. There was an increase in the inner layer of umbilical artery (A) and (B) in assisted reproduction when compared to natural conception.

The changes in wall and luminal areas may be partially explained by the fact that throughout the last 2 weeks of pregnancy, the cord vessels show increasing responsiveness to mechanical irritation (19). Umbilical vessels are very sensitive to various endocrine mediators, such as serotonin, angiotensin, prostaglandins and oxytocin (20-22).

Our present study showed that there was significant reduction in areas of umbilical cord, connective tissue and its vessels in assisted reproduction. These changes may reflect over fetus and its growth. It may cause preterm birth, small for gestational age and congenital anomalies of fetus. To the best of our knowledge, this is the first study in the umbilical cord of assisted reproduction. These histo-morphometric details would be useful for neonatologists and pediatricians who handle the newborns of ART cases.

CONFLICTS OF INTEREST: None

REFERENCES


Assessment of attention concentration and memory in patients with type 2 diabetes mellitus

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ABSTRACT

Introduction and Aim: The evidence for attention concentration and memory deficits in patients with type-2 diabetes mellitus has been inconsistent. In India, very few studies have evaluated memory in the patients with type 2 diabetes mellitus. We aimed to compare the attention concentration and memory between diabetics and non-diabetics.

Materials and Methods: This cross-sectional study involved 124 type-2 diabetic patients attending the diabetology outpatient department of SRM Medical college hospital and research centre aged between 30 and 60 years and 124 age-matched non-diabetic subjects as controls. Subjects with psychiatric illness, liver dysfunction, thyroid disorder, type I diabetes mellitus, hypertension, history of previous head injury, stroke, epilepsy etc. were excluded from the study. After history taking and clinical examination, glycosylated haemoglobin (HbA1c), fasting blood sugar (FBS) and postprandial blood sugar (PPBS) levels were tested. We excluded the subjects with subsympathetic illness by using General Health questionnaire. Folstein mini mental state examination (MMSE) evaluated the cognition of the subjects and the PGI memory scale was used to investigate attention concentration and memory in all the subjects.

Results: We found that the MMSE score, attention concentration and retention for dissimilar pairs were significantly decreased among diabetics when compared to non-diabetics. The recent memory, remote memory and recognition have a negative correlation with duration of type-2 diabetes mellitus. MMSE score, attention concentration and retention for dissimilar pairs have a negative correlation with FBS, PPBS & HbA1c. Mental balance for type-2 diabetes is negatively correlated to FBS & HbA1c and immediate recall has a negative correlation with FBS.

Conclusion: Our study results indicate that attention concentration and memory were impaired in type-2 diabetic patients in comparison with the controls and most of the memory subsets of type-2 diabetes mellitus patients have a negative correlation with blood sugar levels.

Keywords: Attention concentration; Type-2 diabetes mellitus; Memory.

INTRODUCTION

Diabetes has become a major health care problem in India with an estimated 66.8 million people suffering from the condition, representing the largest number of any country in the world (1). Type-2 diabetes appears to be associated with an increased risk of cognitive dysfunction in a wide array of cognitive tests and have detrimental effects on cognitive functioning (measures of verbal and numerical reasoning, attention, concentration, verbal and visual memory, and verbal fluency) and may increase the risk of dementia (2). These findings were also borne out in larger epidemiological studies (3).

Memory is one of the most important cognitive domains with respect to everyday function and is the process of storing, encoding, and retrieving information. Different forms of memory are recognized, including sensory, short-term, long-term, and working memory (4). In sensory memory, representations of the physical features of a stimulus are stored for a very brief time (1 second), and it is difficult to distinguish from the process of perception. It seems that the principal function of sensory memory is to retain information for a period of time sufficient to allow its transfer to short-term memory. Short-term memory refers to the function that temporarily retains stimuli that have just been perceived. Its capacity is limited in terms of the number of items that can be stored and lasts for 20 seconds. Through repetition, information may be transferred from short-term memory to long-term memory. Long-term memory refers to information that is represented on a more permanent basis.

Working memory is a short-term memory system that allows concurrent retention and manipulation of information (5). It is used for thinking about what is already known and for deriving conclusions on the basis of that knowledge; therefore, working memory is fundamental to successful completion of many activities. It is essential for the calculation of mental arithmetic and allows spatial relations to be updated in our mental map as we move through a new geographical location. Earlier studies show that working memory tests are known to activate
structures in the parietal and temporal lobes and in the prefrontal cortex of humans (6).

According to some studies memory and mental processing speed are the cognitive domains most often compromised, whereas other cognitive skills for example attention, problem-solving, and general intelligence tend to be unaffected (7-9).

In those studies that examined memory, loss of verbal working memory was most consistently associated with diabetes however; this presumed association has recently been disputed (10). Whether cognitive deterioration is a direct consequence of chronically elevated blood glucose levels and HBA1C levels has not yet been determined (11-13).

If chronically elevated glucose levels are linked to poorer cognitive performance, one might predict that efforts to improve glycemic control would ameliorate cognitive function or attenuate its decline. There is only limited support for that possibility. So in this study we examined attention concentration and memory, as measured by performance in PGI memory scale in type 2 diabetes patients and age-matched non-diabetic subjects as controls.

MATERIALS AND METHODS

124 patients with Type 2 diabetes (Group 1) of age 30 to 60 years and 124 age and sex matched normoglycemic individuals (Group 2) as controls, attending the Diabetic outpatient department, SRM Medical college Hospital and Research Centre were included in this cross sectional study. Patients with psychiatric illness, liver dysfunction, thyroid disorder, type 1 diabetes mellitus, hypertension, history of previous head injury, stroke, epilepsy etc. were excluded from the study.

Institutional ethical committee approval was obtained and informed written consent was obtained from all the included study subjects. The diagnosis of Type 2 diabetes mellitus was determined according to WHO criteria, fasting blood sugar ≥126 mg/dl and 2 hour post load glucose test ≥200 mg/dl. Blood glucose levels were estimated by Glucose Oxidase-Peroxidase GOD/POD method using Beckman Coulter auto-analyzer.

All the participants were subjected to a structured interview in the out-patient department to collect demographic information such as age, sex, literacy level and occupation. Other details such as duration of diabetes, presence of co-morbidities such as hypertension, dyslipidemia, personal details such as smoking, alcoholism and treatment were recorded.

Cognitive function tests

The cognition was assessed by Folstein Mini Mental State examination (MMSE). The MMSE test scoring is for total 30 points, and impairment is identified in an individual with a score of below 24. MMSE has overall sensitivity 64% and specificity 96%. We found that the mean MMSE score was significantly decreased among diabetics when compared to non-diabetics. Also, the mean MMSE score was significantly decreased in diabetics with HbA1c levels >7%.

All participants were administered the PGI Memory Scale (14) which consists of 10 subtests standardised for adult subjects. The test for remote memory comprises of simple questions relating to personal and current information. In tests for recent memory questions were asked that assess the patient’s ability to recall information and events in the recent past. The test for mental balance gives an idea of balance over one’s mental functioning. The learned materials (alphabet and numbers) were recalled in backward and forward series. The time required to complete the recitation was noted precisely with the help of stop watch. Attention and concentration was evaluated by the test of digit span forward and backward repetition.

Digits were read out a steady rate of one digit per second. The test was started with the set of lowest length of digits. For testing delayed recall the investigator reads out the names of common objects (two series of five each) at a uniform interval. The patient was instructed to recall the same after one minute and score of correct recall recorded. The test for immediate recall included sequential reproduction of the sentence in verbatim. Patient was asked to recall the sentences immediately. For testing the verbal retention for similar pairs, a series of similar associative pairs of words (five noun-noun pairs) were administered to the patient. Patient was asked to mention the associate words in response to the stimulus word. In the test for verbal retention for dissimilar pairs, the associate pair of words was unrelated and dissimilar (five noun adjective pairs) and read at a rate of 2 seconds per pair.

For visual retention test, the investigator displayed five cards containing geometrical figure and patient was instructed to reproduce the drawing from memory. For testing recognition the investigator showed a card containing common objects. Two minutes later a second card containing another set of pictures having some picture appeared in first card was shown to the patient. Patient was asked to identify and name the picture that appeared in both the cards. Correct responses were recorded and scores allotted accordingly. The raw score of each subject was noted and then according to the education of the subjects his score was rated.

Statistical analysis

Statistical analysis was done using SPSS version 17.0. The data were expressed as mean ± standard deviation. Descriptive tables were generated, student ‘t’ test and Pearson’s correlation was used to
Mental balancing is related with FBS, and recognition have a significant variation between the diabetics and the controls in the MMSE score, attention concentration and retention for dissimilar pairs.

**RESULTS**

**Table 1:** Comparison of physical characteristics between diabetics and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Diabetics) Mean+SD</th>
<th>Group II (controls) Mean+SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in Years</td>
<td>51 ± 7.8</td>
<td>50 ± 5.6</td>
<td>0.231</td>
</tr>
<tr>
<td>Height</td>
<td>154 ± 8.2</td>
<td>159 ± 7.2</td>
<td>0.516</td>
</tr>
<tr>
<td>Weight</td>
<td>63.48 ± 8.4</td>
<td>61.12 ± 8.7</td>
<td>0.10</td>
</tr>
<tr>
<td>BMI</td>
<td>26.72 ± 3.78</td>
<td>25.76 ± 4.23</td>
<td>0.273</td>
</tr>
</tbody>
</table>

SD- Standard deviation; BMI- Body mass index

**Table 2:** MMSE score and memory test of the study groups by PGI BBD

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetes (n=124) Mean + SD</th>
<th>Controls (n=124) Mean + SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSE</td>
<td>27.16 ± 2.706</td>
<td>28.29 ± 2.083</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Remote memory</td>
<td>6.00 ± 0.312</td>
<td>6.00 ± 0.22</td>
<td>1.000</td>
</tr>
<tr>
<td>Recent memory</td>
<td>5.03 ± 0.402</td>
<td>5.00 ± 0.04</td>
<td>0.372</td>
</tr>
<tr>
<td>Mental balance</td>
<td>6.68 ± 2.336</td>
<td>7.15 ± 1.631</td>
<td>0.069</td>
</tr>
<tr>
<td>Attention/Concentration</td>
<td>7.44 ± 1.717</td>
<td>8.56 ± 1.818</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Delayed recall</td>
<td>8.02 ± 1.608</td>
<td>8.13 ± 1.331</td>
<td>0.548</td>
</tr>
<tr>
<td>Immediate recall</td>
<td>8.71 ± 2.226</td>
<td>8.42 ± 2.416</td>
<td>0.326</td>
</tr>
<tr>
<td>Retention (similar)</td>
<td>4.40 ± 0.945</td>
<td>4.45 ± 0.736</td>
<td>0.653</td>
</tr>
<tr>
<td>Retention (dissimilar)</td>
<td>11.27 ± 3.341</td>
<td>12.60 ± 2.060</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Visual retention</td>
<td>6.74 ± 3.880</td>
<td>7.26 ± 2.924</td>
<td>0.238</td>
</tr>
<tr>
<td>Recognition</td>
<td>9.35 ± 0.867</td>
<td>9.47 ± 2.1632</td>
<td>0.590</td>
</tr>
</tbody>
</table>

*P < 0.05 indicates statistical significance

There was a significant variation between the diabetics and the controls in the MMSE score, attention concentration and retention for dissimilar pairs.

**Table 3:** Correlation of memory with FBS, PPBS, HbA1c and diabetes duration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>FBS</th>
<th>PPBS</th>
<th>HbA1c</th>
<th>DM duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSE</td>
<td>r value</td>
<td>-0.369</td>
<td>-0.402</td>
<td>-0.441</td>
</tr>
<tr>
<td>P value</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.188</td>
</tr>
<tr>
<td>Remote memory</td>
<td>r value</td>
<td>-0.066</td>
<td>-0.062</td>
<td>-0.018</td>
</tr>
<tr>
<td>p value</td>
<td>0.303</td>
<td>0.328</td>
<td>0.374</td>
<td>0.003*</td>
</tr>
<tr>
<td>Recent memory</td>
<td>r value</td>
<td>-0.114</td>
<td>-0.026</td>
<td>-0.003</td>
</tr>
<tr>
<td>p value</td>
<td>0.074</td>
<td>0.681</td>
<td>0.958</td>
<td>0.006*</td>
</tr>
<tr>
<td>Mental balance</td>
<td>r value</td>
<td>-0.257**</td>
<td>-0.122</td>
<td>-0.143**</td>
</tr>
<tr>
<td>p value</td>
<td>0.000*</td>
<td>0.055</td>
<td>0.025*</td>
<td>0.928</td>
</tr>
<tr>
<td>Attention/Concentration</td>
<td>r value</td>
<td>-0.339**</td>
<td>-0.356**</td>
<td>-0.366**</td>
</tr>
<tr>
<td>p value</td>
<td>0.00*</td>
<td>0.00*</td>
<td>0.00*</td>
<td>0.837</td>
</tr>
<tr>
<td>Delayed recall</td>
<td>r value</td>
<td>-0.056</td>
<td>-0.160*</td>
<td>-0.130*</td>
</tr>
<tr>
<td>p value</td>
<td>0.381</td>
<td>0.012*</td>
<td>0.041*</td>
<td>0.176</td>
</tr>
<tr>
<td>Immediate recall</td>
<td>r value</td>
<td>-0.130**</td>
<td>-0.051</td>
<td>-0.012</td>
</tr>
<tr>
<td>p value</td>
<td>0.040*</td>
<td>0.420</td>
<td>0.857</td>
<td>0.592</td>
</tr>
<tr>
<td>Retention (similar)</td>
<td>r value</td>
<td>0.032</td>
<td>-0.116</td>
<td>-0.097</td>
</tr>
<tr>
<td>p value</td>
<td>0.611</td>
<td>0.068</td>
<td>0.127</td>
<td>0.079</td>
</tr>
<tr>
<td>Retention (dissimilar)</td>
<td>r value</td>
<td>-0.235**</td>
<td>-0.318**</td>
<td>-0.313**</td>
</tr>
<tr>
<td>p value</td>
<td>0.00*</td>
<td>0.00*</td>
<td>0.00*</td>
<td>0.455</td>
</tr>
<tr>
<td>Visual retention</td>
<td>r value</td>
<td>-0.092</td>
<td>-0.196**</td>
<td>-0.197**</td>
</tr>
<tr>
<td>p value</td>
<td>0.149</td>
<td>0.002*</td>
<td>0.002*</td>
<td>0.382</td>
</tr>
<tr>
<td>Recognition</td>
<td>r value</td>
<td>-0.091</td>
<td>-0.069</td>
<td>-0.080</td>
</tr>
<tr>
<td>p value</td>
<td>0.155</td>
<td>0.280</td>
<td>0.208</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*P < 0.05 indicates statistical significance; ** r-value shows significant correlation

The table shows that recent memory, remote memory and recognition have a negative correlation with diabetic duration. MMSE score, attention concentration and retention for dissimilar pairs have a negative correlation with FBS, PPBS & HbA1c. Delayed recall and visual retention are negatively correlated with PPBS & HbA1c. Mental balancing is
negatively correlated to FBS & HbA1c and immediate recall has a negative correlation with FBS.

**DISCUSSION**

The present study has demonstrated that in adults with type-2 diabetes, there is a significant reduction in the mean MMSE score of the diabetics when compared to the controls akin (15) to the research of Eze et al. Table 1 show that both the groups were comparable. There is a significant negative correlation between MMSE score and fasting, postprandial blood sugar levels and HbA1c (16) as shown in Table 2, which is in concordance with the results of Ebady et al.

On the contrary, Lindeman et al., compared participants having diabetes and those with normal glucose tolerance and their results did not demonstrate any cognitive impairment in diabetes after adjusting the factors like ethnic background, gender, age, literacy level and depression (17).

We found the recent memory, remote memory and recognition scores were significantly reduced with increase in the duration of diabetes as shown in Table 3. There is negative correlation between mental balance and the HbA1c and FBS, negative correlation between delayed recall, visual retention scores, post prandial blood sugar and HbA1c.

The participants of the study were evaluated extensively on ten different aspects of cognition with the help of PGI memory scale. Cognitive assessment revealed impairment on attention / concentration and verbal retention (dissimilar pairs) which tests the capability of acquiring new info. Recent memory (capability to keep in mind comparatively new info), remote memory (capability to recall past events), mental balance (order of events), and delayed recall (short duration memory), retention of similar pairs (capability to learn simple things), visual retention (capability of processing and understanding visual info), recognition etc. were in comparison with the controls as shown in table 4.

If diabetic patients typically experience chronically elevated blood glucose levels, and if this adversely affects the availability and/or utilization of glucose within the brain, then individuals with poorer metabolic control might have more difficulty performing cognitively demanding tasks, predominantly those like working memory tasks, which engage multiple cortical regions of the brain.

It is plausible that improvements that are reduction in peripheral blood glucose levels may lead to a corresponding increase in brain glucose availability as well as relative improvements in performance on certain cognitive tasks.

**CONCLUSION**

The results of the present study have clearly demonstrated the detrimental effects of diabetes on memory. Our results have shown that many individuals with type-2 diabetes are subject to substantial impairments of memory function in their everyday lives, which may have essential practical implication for daily activities, including effective working ability. Having a good control of blood glucose level in subjects with type-2 diabetes mellitus will reduce these sequelae.

**REFERENCES**

Effect of progressive resisted exercises and aerobic exercises in the management of polycystic ovarian syndrome among young women - A pilot randomized controlled trial

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ABSTRACT

Introduction and Aim: Polycystic ovarian syndrome (PCOS) is a heterogenous, multisystem endocrinopathy in women of reproductive age also called as Stein Leventhal syndrome. PCOS is a common female endocrine disorder with prevalence ranging from 2.2% to 26%. Prevalence of PCOS in Indian adolescents is 9.13%. This draws attention to the issue of early diagnosis in adolescent girls. The aim of this study was to determine the effect of progressive resisted exercises (PRE) and aerobic exercises in the management of subjects with PCOS.

Materials and Methods: This was an experimental comparative pre and post-test type study. The subjects in the age group of 18-25 years with the diagnosis of PCOS were selected based on Rotterdam criteria and with the BMI ranging between 25-29. Twenty four subjects were recruited and were randomly divided into two groups. group A was treated with PRE +aerobic exercises + diet and group B with aerobic exercises + diet. The duration of the study was 24 weeks and the outcome measures used were BMI, PCOSQ and hormonal levels (SHBG, Free testosterone, HOMA IR, Hs CRP)

Results: The result of the study showed that group A treated with PRE + aerobic exercises + diet had significant improvement in BMI at P≤0.05 and PCOSQ and specific hormonal levels at P≤0.001 when compared to group B.

Conclusion: A 24 weeks exercise intervention with a combined PRE +aerobic exercises +diet was superior to aerobic exercises +diet among young subjects with PCOS.

Keywords: BMI; quality of life; PRE; PCOSQ; Hormones.

INTRODUCTION

Polycystic ovarian syndrome is a heterogeneous clinical condition characterized by hirsutism, irregular menstruation, chronic anovulation and endocrine disorders such as hyperandrogenism that affects 7-14% of women of reproductive age (1). The aetiology of the PCOS is complex and poorly understood and both genetic and environmental factors contribute to the syndrome (2). PCOS is a condition of hormonal imbalance among women of child bearing age (15 to 44 years) and it is estimated between 2.2 and 26.7 percentage of women in this age group have PCOS.

There is an abnormally elevated level of testosterone hormone, which interferes with the ability of oestrogen to cause one of the follicles to produce a mature egg. Additionally women with insulin resistance have difficulty to lower blood sugar levels and excess blood sugar in turn triggers more insulin production. Too much insulin also increases testosterone levels. Excess LH and reduced FSH also make the condition worse. With excess LH, there could be no LH surge for the release of the egg, and it triggers more testosterone production. Reduced FSH causes poor egg development and inability to ovulate. Hence, it leads to fertility problems.

However, the consequences of PCOS go beyond the reproductive axis, with psychological and social impairments, including stress, depression, anxiety and sexual dissatisfaction. Metabolic features associated with the condition include visceral obesity, inflammation, high hypertension and elevated cardiovascular risk factors (3). Thus, the clinical manifestation of PCOS can lower self-esteem and reduce quality of life (4). The prevalence of obesity in general population is increasing, and this might result in an even higher incidence of PCOS in the future (5, 6). Studies done on the Indian population, though limited, have suggested that an abnormality of the insulin receptor is more common in Indian women with PCOS compared to white women with PCOS (7).

The treatment protocol for PCOS is divided into non-pharmacological and pharmacological approach. The former management consists of lifestyle changes, which include regular exercise for weight reduction and diet modification, preconception counselling and administering folic acids to reduce risk of neural tube defects in the foetus. The later consists of first line
drug administration with clomiphene citrate, second line treatment with gonadotropins and laparoscopic ovarian surgery and third line treatment using in vitro fertilization. Insulin sensitizing drugs, e.g., Metformin is commonly used in the management of symptoms with PCOS. Drugs possess their own side effects.

It is accepted that lifestyle modifications in the form of exercise and proper nutrition decrease the risk of developing the condition and type 2 diabetes. In addition, evidence based guidelines recommends lifestyle modification as a non-pharmacological treatment for PCOS (8).

A meta-analysis reported improved levels of FSH, sex-hormone binding globulin (SHBG), total Testosterone, androstenedione, FAI, and mFG score in women with PCOS as a result of lifestyle intervention (diet and physical activity). Similar improvements in metabolic indicators were also reported in few studies (9-11). Compared with women with other chronic conditions, including diabetes, back pain, and arthritis, women with PCOS have been shown to have similar physical health related quality of life (HRQOL) but poorer psychological HRQoL (12, 13).

Evidence suggests that a regular exercise intervention combined with a well-controlled diet had benefits in the management of physiological and psychological symptoms associated with PCOS. Published studies have demonstrated the positive effects of exercise training on maximal oxygen consumption (Max Vo2), weight and waist circumferences in PCOS subjects (14). Further weight loss may reduce pulse amplitude of luteinizing hormone (LH) in turn reducing androgen production as excess (15).

Defects within the skeletal muscle insulin signalling pathways are thought to contribute to PCOS intrinsic IR with post receptor abnormalities contributing to overall reduction in skeletal muscle responsiveness to glucose (16, 17). This lays the foundation for interventions such as strength training or progressive resistance training (PRT) recommended by the American college of sports medicine and American Diabetes Association as an integral component of daily exercise routine for healthy adults for prevention and treatment of chronic non-communicable disease (18).

Thus, PRE could be the most potent exercise modality for improving skeletal muscle mass and quality (19, 20). Two studies until date have investigated the isolated effects of PRE in women with PCOS (21, 22). There is a need to study the effects of PRE which when combined with the usually recommended aerobic exercises and diet among young PCOS subjects in Indian population. Hence, this study was intended to study the effects of progressive resisted exercise combined with aerobic exercises and diet on BMI, quality of life and hormonal profile among young subjects with PCOS.

**MATERIALS AND METHODS**

This pilot study was an experimental design comparative pre post-test type which was conducted at the Faculty of physiotherapy Dr MGR educational and research institute Deemed to be university. The institutional research and ethics committee approved the study (IRB 020/2017-2018) and the study was done strictly in accordance with the guidelines of Helsinki declaration, revised 2013 adopted by world medical association. A total of 24 subjects in the age group between 18-25 years diagnosed with PCOS based on the Rotterdam criteria and BMI range between 25-29 were recruited and divided into two groups by simple random sampling (random number tables from standard statistics book) to participate in this pilot study. Subjects with thyroid disease, prolactin excess, non-classical congenital adrenal hyperplasia, glucocorticoid dysfunction, subjects under anti hypersensitivity medications and Lipid lowering medications were excluded from the study.

All the subjects signed a written consent form before any therapy was initiated. After the demographics, recruited PCOS were randomly divided into two groups. Group A (n=12) was intervened with PRE +aerobic exercises +diet and group B (n=12) was intervened only with aerobic exercises + diet. Both the groups received the above said intervention for 24 weeks. Outcome measures used were BMI, PCOSQ and hormonal levels (SHBG, Free testosterone, HOMA IR, Hs CRP).

**Procedure**

**Intervention for group A**

PRE + aerobic exercises + diet was given to the recruited subjects in group A (n=12). Supervised PRE exercise session was carried out for 2 days in a week on consecutive days for 24 weeks at the Faculty of Physiotherapy. The exercise session lasted for 60 minutes including standardized (5 minute) warm-up and cool-down. The exercise protocol followed were lateral pull down, leg curl, seated row, calf raise, chest press, split squat, shoulder press, biceps curl, triceps extension and abdominal curl. Exercises like chest press, shoulder press, biceps curl, and triceps extension were also performed. All sets of exercise (except abdominal curl) were performed to neuromuscular fatigue i.e. 8-12 repetitions maximum. Two sets of each exercise were given in the first 2 weeks. From week 3, all exercises except split squat and shoulder press was progressed to 3 sets. The subjects also performed calisthenic exercises on non PRE days, 4 days in a week which included lying external hip rotations (‘clam shells’), side leg raises, push-ups on knees, wall squats, oblique curls and core stabilization exercises (‘bird dog’ and abdominal hollowing), performed for 3 sets x 10 repetitions.
each. The numbers of repetitions of each exercise performed were recorded. Additionally the subjects performed moderate intensity aerobic exercises in the form of brisk walking in a treadmill for 30 minutes in a day for 5 days in a week. Dietary advice from a nutritionist was given to all the subjects.

**Intervention for group B**

Aerobic exercises + diet was given to the recruited subjects in group B (n=12). The subjects performed moderate intensity aerobic exercises in the form of brisk walking for 30 minutes a day for 5 days in a week. Dietary advice from a nutritionist was given to all the subjects.

**Data analysis**

The collected data were tabulated and analysed using both descriptive and inferential statistics. All the parameters were assessed using statistical package for social science (SPSS) version 24. Descriptive paired t-test was adopted to find the statistical difference within the groups & Independent t-test (Student’s ‘t’-test) was adopted to find the statistical difference between the groups.

**Table 1:** Comparison of BMI score between group A and group B in pre and post test

<table>
<thead>
<tr>
<th>BMI</th>
<th>Group A (PRE) Mean</th>
<th>Group B (Aerobics) Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre test</td>
<td>26.65</td>
<td>26.25</td>
<td>1.03</td>
<td>28</td>
<td>0.310*</td>
</tr>
<tr>
<td>Post test</td>
<td>24.06</td>
<td>24.90</td>
<td>-2.60</td>
<td>28</td>
<td>0.015**</td>
</tr>
</tbody>
</table>

**Table 2:** Comparison of PCOS questionnaire between group A and group B in pre and post test

<table>
<thead>
<tr>
<th>PCOSQ</th>
<th>Group A Mean</th>
<th>Group B Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre test</td>
<td>21.40</td>
<td>21.50</td>
<td>0.011</td>
<td>28</td>
<td>0.999*</td>
</tr>
<tr>
<td>Post test</td>
<td>29.06</td>
<td>24.46</td>
<td>-20.18</td>
<td>28</td>
<td>0.000***</td>
</tr>
</tbody>
</table>

**Table 3:** Comparison of SHBG between group A and group B in pre and post test

<table>
<thead>
<tr>
<th>SHBG</th>
<th>Group A Mean</th>
<th>Group B Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre test</td>
<td>28.0</td>
<td>27.7</td>
<td>0.064</td>
<td>28</td>
<td>0.949*</td>
</tr>
<tr>
<td>Post test</td>
<td>34.1</td>
<td>29.8</td>
<td>-4.63</td>
<td>28</td>
<td>0.000***</td>
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**Table 4:** Comparison of free testosterone hormone value between group A and group B in pre and post test

<table>
<thead>
<tr>
<th>Free testosterone</th>
<th>Group A Mean</th>
<th>Group B Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre test</td>
<td>4.51</td>
<td>4.58</td>
<td>-3.85</td>
<td>28</td>
<td>0.703*</td>
</tr>
<tr>
<td>Post test</td>
<td>2.22</td>
<td>3.89</td>
<td>-12.0</td>
<td>28</td>
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**Table 5:** Comparison of Homeostatic model assessment insulin resistance between group A and group B in pre and post test

<table>
<thead>
<tr>
<th>HOMAIR</th>
<th>Group A Mean</th>
<th>Group B Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre test</td>
<td>3.57</td>
<td>3.55</td>
<td>0.092</td>
<td>28</td>
<td>0.928*</td>
</tr>
<tr>
<td>Post test</td>
<td>2.24</td>
<td>3.19</td>
<td>-6.87</td>
<td>28</td>
<td>0.000***</td>
</tr>
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</table>

**Table 6:** Comparison of High Sensitive C-Reactive Protein between group A and group B in Pre and Post Test

<table>
<thead>
<tr>
<th>Hs CRP</th>
<th>Group A Mean</th>
<th>Group B Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre test</td>
<td>4.30</td>
<td>4.48</td>
<td>-0.829</td>
<td>28</td>
<td>0.414*</td>
</tr>
<tr>
<td>Post test</td>
<td>2.33</td>
<td>4.09</td>
<td>-10.74</td>
<td>28</td>
<td>0.000***</td>
</tr>
</tbody>
</table>

**Table 7:** Comparison of Test Variables within group A between Pre and Post Test values

<table>
<thead>
<tr>
<th>Group A</th>
<th>Pre test Mean</th>
<th>Post test Mean</th>
<th>t-test</th>
<th>df</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>26.65</td>
<td>24.06</td>
<td>0.173</td>
<td>28</td>
<td>0.000***</td>
</tr>
<tr>
<td>PCOS Q</td>
<td>21.40</td>
<td>29.06</td>
<td>4.42</td>
<td>28</td>
<td>0.000***</td>
</tr>
</tbody>
</table>
SHBG        | 28.0   | 0.755   | 34.1   | 0.516   | 11.00  | 0.000***
Free testosterone | 4.51   | 0.429   | 2.22   | 0.158   | 25.80  | 0.000***
Homeostatic model assessment insulin resistance | 3.57   | 0.494   | 2.24   | 0.133   | 10.19  | 0.000***
High sensitivity C-reactive protein | 4.30   | 0.620   | 2.33   | 0.228   | 15.87  | 0.000***

Table 8: Comparison of Test Variables within group B between Pre and Post Test values

<table>
<thead>
<tr>
<th>Group B</th>
<th>Pre test</th>
<th>Post test</th>
<th>t-test</th>
<th>Significance</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S. D.</td>
<td>Mean</td>
<td>S. D.</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PCOS Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free testosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homeostatic model assessment insulin resistance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High sensitivity C-reactive protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS
On comparing the mean values of groups A and B on BMI, group A showed better improvement at P ≤ 0.05 (Table 1). On comparing the mean values of groups, A and B on PCOSQ, group A showed better improvement in quality of life at P ≤ 0.001 (Table 2). On comparing the mean values of groups A and B on hormonal levels (SHBG, Free testosterone, HOMA IR and Hs CRP), group A showed better improvement at P ≤ 0.001 (Tables 3-6).

DISCUSSION
The findings of this study reinforce the positive effects of PRE on BMI, quality of life and hormonal levels in women with PCOS. There have been reports on exercise intervention and changes in lifestyle in women with PCOS, but few reports have examined the effects of PRE. It was also hypothesized that PRE can improve menstrual cyclicity in women with PCOS. PRE may counteract the aetiology of PCOS through its effect on body composition (23). This study investigated the combined effects of PRE + Aerobic exercises and diet in the management of PCOS among young women. The effects of Aerobic exercises and diet are well established. The additional benefit observed in group A where PRE was combined with this conventional modality is that PRE improved muscle strength and the muscle’s ability to use insulin. In this study, the subjects had changes in hormonal levels (SHBG, HOMA IR, HsCRP, and free testosterone), BMI and low quality of life. Insulin metabolism is a characteristic of metabolic syndrome in PCOS women (24).

The primary goal of PCOS challenge is to educate, create awareness and diagnose PCOS among very young women at an early stage. As PCOS and obesity are very closely related, the importance of physical activity is to be emphasized among young women. For overweight or obese women with PCOS, a weight loss exercise program results in more regular ovulation and thus the chances of getting pregnant increases. A combined diet and exercise in the form of aerobic activities, resisted exercises or yoga are all effective in women with PCOS.

This study enrolled 24 women with PCOS, 12 each in PRE+ aerobics + diet group and aerobic + diet group respectively. The duration of the study was 24 weeks, having intervention session for six days in a week. The changes experienced in group A in the present study, includes improvement in body mass index and a better quality of life. The group A also demonstrated better symptomatic improvements compared to the group B (25). Previous literature has shown various effects of exercise among PCOS subjects and if the intervention is initiated much earlier, it can actually do wonders both in the symptomatic management as well as in preventing the long-term complications of X syndrome (Hypertension, Diabetes, Hyperlipidaemia and cardiovascular disease).

PRE combined with the conventional aerobic exercises and diet is the modality of choice in subjects with PCOS. This pilot study revealed that 24 weeks of combined exercise intervention with PRE+ aerobic exercises+ diet resulted in improvements on BMI, quality of life and better hormonal profile (Tables 7 and 8). The results can be generalized after performing large-scale clinical trials.

CONCLUSION
A 24 weeks exercise intervention with PRE + aerobic exercises + diet was effective in subjects with PCOS. The combined effect of PRE seems to be more superior to aerobic exercise and diet alone on the BMI parameters, quality of life improvement on PCOSQ and specific hormonal levels.

Limitations and recommendations of the study
The sample size was small and the study included subjects only in the age group between 18-25 years.

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and a BMI range of 25-29. There was no follow up after 24 weeks of intervention. Future studies can be done using large sample size, follow up can be done and effects of different interventions can be studied.

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CONFLICT OF INTEREST: None of the authors have potential conflicting interests declared.

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A study to analyze the effectiveness of exercise program in improving the proprioceptive acuity among patients with chronic neck pain

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ABSTRACT

Introduction and Aim: Neck pain is estimated to affect 10% to 20% of the Indian population every year, with 1/3rd of people developing chronicity, which is predominantly due to impaired proprioception. Clinical texts specifically recommend the assessment and management of proprioceptive dysfunction, there is lack of clear evidence for proprioceptive dysfunction in different subgroups of chronic neck pain.

Materials and Methods: Thirty-two subjects were included and sub-grouped based on their dysfunction. Group A- mechanical neck pain (n= 10), Group B- upper cervical involvement (n= 10) and Group C- cervical radiculopathy (n= 12). The baseline evaluation consisted of Cervico Cephalic Relocation (CCR) test, Deep Cervical Flexor (DCF) endurance, and Neck Disability Index (NDI) questionnaire, which was again taken at the end of two weeks following intervention.

Results: In all three groups, joint position error was observed and following intervention, a significant improvement in the outcomes had occurred, with greater improvement in Group A, followed by B, then C. On comparison between the groups, the right and left rotations of CCR, DCF endurance and NDI (p value< 0.05) were not similar, whereas, the improvement in flexion and extension of CCR (p value> 0.05) were similar.

Conclusion: Proprioception training should form an integral part of rehabilitating patients with chronic neck pain.

Keywords: Neck pain; proprioception; cervical joint position error; Cervico cephalic relocation test.

INTRODUCTION

Neck pain forms a major public health problem, both in terms of personal health and overall well – being, with a prevalence rate of 30 – 50% of adults among general population. Approximately, 50 – 85% of individuals with neck pain do not experience complete resolution of symptoms and may go on to chronic, disabling pain (1). Treatment of chronic neck pain has always been a challenging area for Physiotherapists.

Proprioception involves sensations generated within the body, which contributes to the awareness of relative orientation of body parts, both at rest and in motion. This proprioceptive system depends upon simultaneous activity of different types of mechanoreceptor afferent neurons. They provide information for reflex regulation of muscle tone, awareness of position sense and movement sense, and have been isolated in most spinal tissues (2). Restitution of healthy neuromuscular motor patterns and increased sensory input variation through proprioception training is thought to reduce mechanical stress through improved muscular co-ordination and may prevent recurrence and chronicity of neck pain (3).

Mechanical neck pain is a general term that refers to any type of pain caused by placing abnormal stress and strain on muscles of neck. Typically, mechanical neck pain results from poor postural habits (4). Neck flexion, forward head posture, scapular retraction, forward stoop posture are some of the faulty postural alignments, resulting in neck pain due to increased cervical muscle activity in order to support head in forward position, leading to increased fatigue. Apparently, muscles and other soft tissues tighten up due to excessive overload required to hold the head in position (5). This chronic overload and tightening of soft tissues may result in decreased blood flow and oxygen to the soft tissue, ultimately causing pain. In such cases, a fault in joint position sense becomes apparent due to inappropriate variability in postural control. Due to the disturbance caused to joint position sense, individual is subjected to faulty posture in the continuum. The impaired joint position sense can worsen the alignment of the cervical spine and lead to impaired stability (6).

The presence of high densities of muscle spindles in the slow twitch muscle fibers of small intrinsic deep dorsal and sub occipital muscles play an important role in postural control (7). Therefore, upper cervical...
involvement causes impaired proprioception along with altered afferent input from the facet, in case of facet joint involvement. The impact at many levels of the nervous system can change muscle spindle sensitivity and alter the cortical representation and modulation of cervical afferent input, leading to one of the major physiological basis for proprioception deficit in cervical radiculopathies (8). Moreover, the occurrence of severe degenerative joint disease among individuals deprived of protective proprioceptive sensibility leads to repetitive, longitudinal, impulsive loading, which increases the risk of tissue injury from uncontrolled motion. This highlights the essential role of proprioceptive training in maintaining dynamic cervical spine stability (9).

The proprioceptive training involves Cervico cephalic relocation (CCR) training, Deep cervical flexor (DCF) training and dorsal neck muscle training. CCR training improves the proprioceptive acuity by activating the proprioceptors in different planes of movement (10). DCF training also plays a major role in improving the proprioceptive acuity by activating the relatively high density of muscle spindles present in the deep cervical flexor musculature and decreasing the excessive stress paced on the joints and other structures of the cervical region (11). Fatigue of the dorsal neck muscles deteriorates the ability to reproduce a given joint angle causing a significant proprioception deficit. Hence, training of the dorsal neck muscles plays an important role in improving the proprioceptive acuity (7).

The proprioceptive mechanisms controlling the head on the body can be tested clinically by simple target – matching tasks, to evaluate the ability to relocate the Natural Head Posture (NHP) known as the Cervico Cephalic Relocation test (CCR). In addition, Chattanooga's pressure biofeedback and NDI were used to evaluate endurance of deep cervical flexors and functional outcomes respectively.

It becomes utmost necessity on the part of physical therapist to employ and disturb the vicious cycle of pain, altered proprioception, mal-alignment and pain. Thus, the study was aimed at evaluating the proprioceptive deficit in patients with three categories of neck pain – mechanical neck pain, upper cervical involvement, and cervical radiculopathies and to evaluate the improvement in proprioceptive acuity among the three categories of neck pain patients following intervention.

MATERIALS AND METHODS

The Ethics Committee for students’ projects, Sri Ramachandra Institute of Higher Education and Research, approved this experimental study (REF: CSP/18/SEP/73/263). This study was registered in Clinical Trial Registry- India (CTRI/2019/02/017685).

Method

The Quasi – Experimental study (pre-post design) was conducted in Outpatient Physiotherapy Department, Sri Ramachandra Hospital, Chennai. The recruitment process had started in December 2018 and completed by April 2019 with two weeks follow up for all participants. All the participants received a verbal explanation of the research project and an informed consent was obtained. The sample design was a probability sampling, thirty-five samples were included for the study, out of which there were three drop-outs due to lack of follow-up. Hence, samples included for data analysis were thirty-two.

Study participants

Subjects who met the inclusion criteria were recruited for the study.

Inclusion criteria are both male and female genders, age group: 21-55 years, neck pain with minimum duration of 8-12 weeks, unilateral or bilateral pain in the posterior neck or shoulder region and pain in the cervical region when moving or palpating the cervical spine, upper cervical involvement with or without headache and C1- C2 rotational deficits, patients with cervical radiculopathy.

Exclusion criteria are past trauma to cervical region, past fracture or surgery in cervical spine, deformity of the spine (kyphosis, Scoliosis), inner ear infections, inflammatory rheumatologic disease, vertebro - basilar artery insufficiency, malignancy, neurological evidence leading to balance disturbances (e.g. sensory ataxia).

The patients who had fulfilled inclusion criteria were included for the study. The participants were categorized into three groups depending upon their clinical presentations. Group A – patients with mechanical neck pain (n=10), Group B – upper cervical involvement (n=10), Group C – cervical radiculopathies (n=12). The patients then underwent a pre-test evaluation of CCR test, pressure biofeedback and NDI in order to obtain a baseline measure, followed by intervention for a period of two weeks (three days/ week).

Intervention

1. Cervical joint position sense: relocation with laser feedback:
   - Head relocating to neutral position with eyes opened (vertical/ horizontal/ diagonal) (Figure: 1).
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- Head relocating to predetermined position in range with eyes opened. (vertical/ horizontal/ diagonal)
- Head relocating to neutral position with eyes closed. (vertical/ horizontal/ diagonal)

Fig. 1: Cervical joint position sense training

2. Cervical movement sense: - with laser feedback:
   - Tracing a line (vertical/ horizontal) (figure: 2)
   - Tracing an intricate pattern at slow and fast speed (a figure of eight/ zig – zag/ alphabet) (12).

Fig. 2: Cervical movement sense training

3. Re-education of dcf movement pattern:
   - Gentle and controlled nodding action (Figure: 3a)
   - Repeated and sustained DCF progression from 22 – 30 mmHg with pressure bio – feedback in supine lying, knees bent (Figure: 3b) (12).

Fig. 3a: DCF training

Fig. 3b: DCF training

4. Re-education of dorsal neck muscles:
   - Cranio- cervical extension, cranio cervical rotation (<40°), cervical extension while keeping the cranio-cervical region in neutral done in prone in elbow/ 4 -points kneeling positions. (Figure: 4a)
Co – contraction of deep cervical flexors/extensors (Figure: 4b).

Isometric hold in the range of cervical extension (12)

Outcome measures
Cervico– cephalic relocation test
Blindfolded subjects were seated on a chair paced 90cms from a white wall with the target maze in front. Each subject had a laser pointer attached on the head. Following the procedure, subjects were instructed to memorize the neutral head position to duplicate it after the active movement of maximal amplitude of the head. Once the reference point was achieved, the subject concentrates on this position for a couple of seconds. The subject then performs a maximal flexion/extension and rotation (left and right) for approximately two seconds and tries to find the initial reference point with a maximum of precision without speed instruction. When subject reaches the reference point, he should again concentrate on the reference point for several seconds. 8-10 trials were performed with eyes opened before the actual performance. The actual performance is done with eyes closed and without any feedback (9). The distance between the target point and the relocated point was taken as global cervico- cephalic relocation error.

Cranio cervical flexion test (CCFT)
The CCFT was performed with the patient in supine crook lying with the neck in a neutral position (no pillow) such that the line of the face was horizontal and a line bisecting the neck longitudinally was horizontal to the testing surface. The un-inflated pressure sensor was placed behind the neck so that it abuts the occiput and is inflated to a stable baseline pressure of 20 mm Hg, a standard pressure sufficient to fill the space between the testing surface and the neck but not push the neck into a lordosis. The device provides the feedback and direction to the patient to perform the required five stages of the test. The patient was instructed that the purpose of test was not strength but rather precision. The movement was performed gently and slowly as a head nodding action. The cranio cervical flexion tests the activation and endurance of the deep cervical flexors in progressive inner range positions as the patient attempts to sequentially target five, 2-mm Hg progressive pressure increases from the baseline of 20 mm Hg to a maximum of 30 mm Hg as well as to maintain a isometric contraction at the progressive pressures as an endurance task (13).

Neck disability index (NDI)
It is a self-rated, condition-specific functional status questionnaire with 10 items including pain, personal care, lifting, reading, headaches, concentration, work, driving, sleeping and recreation. The NDI has sufficient support and usefulness to retain its status as the most commonly used self-report measure for neck pain (14).

Following the intervention, the subjects underwent a post- test evaluation. The pre and post -test measures were included for data analysis.

Data analysis
The gathered data was analyzed with IBM SPSS statistics software 23.0 version. For descriptive statistics, frequency and percentage analysis were used for categorical variables whereas, mean and S.D were used for continuous variables. To find the significant difference between the bivariate samples in paired groups the paired sample ‘t’- test was used. For the multivariate analysis, the one-way ANOVA with Turkey’s Post- Hoc test was used. In all the above statistical tools, the probability value 0.05 is considered as significant level.

RESULTS
30 subjects were included in the study and all subjects completed the follow up at 2weeks. On comparing between the groups, post– intervention comparison of CCR, DCF, and NDI shows insignificance in CCR-flexion and extension (p – value 0.19 and 0.07 respectively) and significance in other variables (p –
The analysis was carried out using Turkey’s Post – Hoc test.

**Table 1: Post– intervention comparison of CCR, DCF, and NDI between the groups**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean</th>
<th>SD</th>
<th>f-value</th>
<th>p-value</th>
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</thead>
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<tr>
<td><strong>CCR – flexion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>6.34</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>11.31</td>
<td>6.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>10.30</td>
<td>7.81</td>
<td>1.75</td>
<td>0.191</td>
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<tr>
<td><strong>CCR – extension</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>5.37</td>
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<tr>
<td>Group B</td>
<td>10.71</td>
<td>7.78</td>
<td></td>
<td></td>
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<tr>
<td>Group C</td>
<td>9.6</td>
<td>2.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CCR – Right rotation</strong></td>
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<td>Group A</td>
<td>7.19</td>
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<td>14.74</td>
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<td>Group C</td>
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<tr>
<td><strong>CCR – Left rotation</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group A</td>
<td>7.22</td>
<td>2.76</td>
<td></td>
<td></td>
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<tr>
<td>Group B</td>
<td>12.73</td>
<td>7.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>13.37</td>
<td>6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DCF - Endurance</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>28.4</td>
<td>1.57</td>
<td></td>
<td></td>
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<tr>
<td>Group B</td>
<td>27</td>
<td>1.69</td>
<td></td>
<td></td>
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<tr>
<td>Group C</td>
<td>25.5</td>
<td>1.50</td>
<td></td>
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<tr>
<td><strong>NDI</strong></td>
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<td>Group A</td>
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<td>2.44</td>
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<tr>
<td>Group B</td>
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<tr>
<td>Group C</td>
<td>6.5</td>
<td>3.55</td>
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**Graph 1:** CCR – Mean score of flexion, extension, right and left rotation between the groups

**Graph 2:** Mean score of DCF endurance and NDI between the groups
On analyzing within the groups, it shows that, there occurs a significant difference in all the variables (p value < 0.05) in all the three groups – A (Table 2), B (Table 3) and C (Table 4). This analysis was carried out using paired t test.

Table 2: Analysis of CCR, DCF and NDI in group A within the group

<table>
<thead>
<tr>
<th>Variables</th>
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<td>5.37</td>
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<tr>
<td>Post</td>
<td>13.44</td>
<td>2.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR- Right rotation:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>22.91</td>
<td>7.19</td>
<td>5.59</td>
<td>0.000</td>
</tr>
<tr>
<td>Post</td>
<td>8.17</td>
<td>3.5</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>8.26</td>
<td>2.76</td>
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<td>NDI:</td>
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<tr>
<td>Pre</td>
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<tr>
<td>Post</td>
<td>7.64</td>
<td>2.44</td>
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Table 3: Analysis of CCR, DCF and NDI in group B within the group

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<tr>
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<tr>
<td>pre</td>
<td>27.84</td>
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<td>4.44</td>
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<td>9.73</td>
<td>6.64</td>
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<tr>
<td>CCR – Extension:</td>
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<tr>
<td>Pre</td>
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<td>10.71</td>
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<td>CCR- Right rotation:</td>
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<tr>
<td>Pre</td>
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<td>14.74</td>
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<tr>
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<td>6.46</td>
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<td></td>
</tr>
<tr>
<td>Pre</td>
<td>24.14</td>
<td>12.73</td>
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<td>0.004</td>
</tr>
<tr>
<td>Post</td>
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<td>7.02</td>
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<td>DCF Endurance:</td>
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<td>1.05</td>
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</tr>
<tr>
<td>NDI:</td>
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<td>15.1</td>
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<td>5.57</td>
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<tr>
<td>Post</td>
<td>5.9</td>
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Table 4: Analysis of CCR, DCF and NDI in group C within the group

<table>
<thead>
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<tr>
<td>CCR – flexion:</td>
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<tr>
<td>Post</td>
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Shristhudhi et al: A study to analyze the…..with chronic neck pain

<table>
<thead>
<tr>
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<td></td>
<td>13.37</td>
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<td></td>
<td>10.5</td>
<td>3.55</td>
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DISCUSSION

This experimental study reveals that, there is presence of proprioception deficit not only among chronic mechanical neck pain patients but also in patients with chronic upper cervical involvement and cervical radiculopathy. In past literatures, there are very few evidences for the prevalence of proprioception impairment among patients with upper cervical involvement and cervical radiculopathy. The salient feature of present study is that, it assesses the involvement of proprioceptive deficit into cervical flexion, extension, right and left side rotational movements, as well as the response of each pathology to proprioceptive training. Another salient feature is that, it proposes a treatment protocol aimed at improving the proprioceptive acuity of neck for patients with chronic neck pain, which are specially designed to target the deep sub-occipital muscles and reflex connections, other than gaze stabilization and coordination exercises.

With regard to the postural control system, patients with neck pain demonstrated altered proprioception (tested by cervical joint position sense), balance disturbances, altered head – neck coordination, and altered postural activity of cervical muscles (15). Abnormal Joint Position Error (JPE) also has been detected in patients with chronic neck pain using either tests of ability to relocate the natural head posture after an active movement or to actively relocate a position within a movement plane. These disturbances of postural control have been caused possibly due to altered input from cervical afferents to higher centers (16).

There are abundant receptors in the deep cervical muscles, and multiple cervical central and reflex connections to the vestibular, visual, and postural control systems. In particular, the deep portions of the sub-occipital muscles have the highest cervical receptor density and are known to have a specific role in these reflex and central connections and in postural control (7).

In patients with chronic neck pain, due to overuse, repetitive or serious trauma and muscle weakness, the elasticity of non-contractile tissues becomes enlarged, thereby stabilize the neutral position, and contractile tissues become weak, leading to functional restriction, which in over a period of time leads to proprioception deficit (17).

Abnormal joint position error was assessed with the help of CCR test, which has been an effective outcome measure in evaluating the proprioception deficit. The CCR test when used to test neutral head positioning, executed in its original form (i.e., 10 trials) had a fair to excellent reliability (Intra-Class Correlation Coefficients ranged from 0.52 to 0.81 and from 0.49 to 0.77, for absolute and variable errors, respectively ie., testing the global components of joint position error (18). The findings of this study is more or less in accordance to the present study, and depicted in the tables 2, 3 and 4 which provided a significant improvement in CCR of < 0.0001 within all the three groups.

On the other hand, it has identified that, one of the major causes of postural impairment and proprioception deficit was less endurance of cervical flexor muscle. Following training of deep cervical flexors, the patients had shown substantial improvements. Thus, in addition to the CCR test, DCF endurance test was used as one of the outcome measures in the present study. This has been adopted from a study which stated that, Inter-rater reliability (Intra-Class Correlation Coefficients- ICC) for the CCFT was 0.91 (95% confidence interval; 0.83-0.96), with a reasonable agreement on the Bland-Altman plot.
confirming high reliability of the test (19). In the present study, this training procedure provided a significant improvement in CCR of 0.001, when compared between the subgroups of neck pain in patients with proprioception deficit as documented in the table 1.

The reliability of Neck Disability Index questionnaire was proved by a systemic Review, stating that, the NDI has acceptable reliability, although Intra-Class Correlation Coefficients (ICCs) range from 0.50 to 0.98. In addition, the reported Clinically Important Difference (CID) is inconsistent across different studies ranging from 5/50 to 19/50 (20). The NDI is strongly correlated (>0.70) to a number of similar indices and moderately related to both physical and mental aspects of general health. Henceforth NDI, which was presently used, yielded a significant outcome with a p-value of 0.003(table 1).

The present study protocol intervened cervical proprioception deficit through three types of training programs – proprioception training through target maze, DCF training and Dorsal neck muscle strengthening. According to a study done on proprioceptive training, the results stated that both proprioceptive training and DCF training have a demonstrable benefit on impaired cervical JPE in people with neck pain, with marginally more benefit gained from proprioceptive training (21). The results suggest that improved proprioceptive acuity following intervention with either of the exercise protocol may occur either by an improved quality of cervical afferent input or by addressing input through direct training of relocation sense. Another study (7) stated that, reduced strength of the dorsal neck muscles altered the repositioning acuity in sagittal plane (p-value < 0.0001). Hence, dorsal neck muscle strengthening was included as a part of the treatment regime and proved to be effective in achieving desired outcome.

Clinical implications

The prevalence and management of proprioception deficit among patients with chronic mechanical neck pain and whiplash associated disorders have been proved adequately by the previous studies. It was adopted presently. The subgrouping of neck pain was done in a study and investigated the proprioception deficit among patients with chronic mechanical neck pain (22), whereas, proprioception deficit among subgrouping of neck pain was not attempted in the past studies. In addition, we had intervened the proprioception impairment with training protocols and the outcomes have proven to be clinically significant. Although, statistical analysis in table 1 revealed that CCR test into flexion and extension was not significant with p – values of 0.19 and 0.07 respectively, few outcomes proved statistically significant were CCR test into right and left rotations with p-values of 0.005 and 0.04 respectively. Similarly, the DCF endurance and NDI score proved to be statistically significant with p-values 0.000 and 0.03 respectively.

CONCLUSION

The outcomes of present study revealed the presence of proprioception deficit among chronic mechanical neck pain, chronic upper cervical involvement and cervical radiculopathy patients. Following the intervention, all the three groups responded well, in terms of proprioceptive acuity, DCF endurance and NDI scores. Thus, it may be inferred that, inclusion of such training for chronic neck pain patients may potentially benefit them. In addition, this may be recommended as a part of treatment regimen in out-patient treatment protocol.

FUTURE SCOPE

- Long term effects of the treatment may be evaluated on larger sample size.
- Cervico – cephalic relocation error may be measured in a multi – dimensional plane.

ACKNOWLEDGEMENTS

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REFERENCES

A comparative study on effectiveness of muscle energy technique versus Cyriax’s deep friction technique in adhesive capsulitis

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ABSTRACT

Introduction and Aim: Adhesive capsulitis occurs commonly among middle aged and elderly populations which is typically consist of stiffness in both active and passive range of motions, pain and difficulty in performing normal activities of living. The aim of the study was to compare the efficacy between muscle energy technique and Cyriax technique coupled with mobilisation technique in reduction of pain and in improving the range of motion and functional ability in subjects with adhesive capsulitis.

Materials and Methods: 30 subjects were randomly assigned for the experimental study by the selection criteria and separated into two groups: group A –Mobilisation technique with muscle energy technique and group B – Mobilisation technique with Cyriax’s deep friction technique. The period of intervention was 5 sessions per week for three weeks. The therapy progression was evaluated by goniometry and SPADI Scores day prior to treatment and 21 days after treatment.

Results: The analysis showed that the both treatment groups showed improvement comparing pre and post treatment, while group A showed significant difference compared to group B in pain relief, range of motion and functional disability. Statistically comparing the mean values of all the outcome scores of the two treatment groups has showed group A as more efficient than group B at p-value less than 0.001.

Conclusion: The current study concluded that the muscle energy technique with mobilisation technique shows more improvement than the Cyriax’s deep friction technique with mobilisation technique. It shows a better combination therapy for the treatment of Adhesive Capsulitis.

Keywords: Cyriax’s deep friction technique; muscle energy technique; adhesive capsulitis.

INTRODUCTION

Adhesive capsulitis occurs commonly among middle aged and elderly populations which is typically consist of stiffness in both active and passive range of motions, pain and difficulty in performing normal activities of living (1, 2). Adhesive capsulitis is a chronic painful condition with restricted movement in all planes particularly more restrictions on abduction and external rotation of shoulder joint. It affects more commonly middle aged and elderly populations. The condition was explained and differentiated from glenohumeral arthritis in 1872 by Duplay (3, 4). The term “frozen shoulder” was first introduced by Codman in the year 1934. The common symptoms of frozen shoulder are pain and stiffness in the capsular pattern.

The term “adhesive capsulitis” means that this condition sequenced from thickening and eventual contracture of the joint capsule (5). More than 4% of adult population between 40 and 65 years suffered from adhesive capsulitis with more sufferers are women. The exact cause is not known. There had been so many theories proposed, but conclusive and confirmatory results have not been found yet. However, there exists a greater link between adhesive capsulitis, hyperthyroidism, cerebrovascular disease, coronary artery disease, autoimmune disease and diabetes mellitus (6, 7). The main effects of MET can be explained (8) by two distinct physiological processes: Post Isometric Relaxation (PIR) and Reciprocal Inhibition (RI). Deep transverse friction is also known as cross-fiber friction massage, popularized by James Cyriax (9) DTF is a crucial type of soft tissue massage applied especially to the structure such as joint capsules, tendons, muscle bellies, musculotendinous junction, ligaments. The finger directly to the lesion and transverse to the direction of the fibres apply massage.

Deep transverse friction:

Place the thumb or fingertip on the shoulder joint line, supraspinatus, subscapularis and pectoralis major and compress. Moving the client’s skin back and forth over the treated fibers at right angles or perpendicular to the tissue fibers. Pressure should be moderate between a 5 and 7 on a 10 point scale (10, 11).

The aim of the study was to compare the efficacy between muscle energy technique and Cyriax’s technique coupled with mobilisation technique in reduction of pain and in improving the range of motion and functional ability in subjects with adhesive capsulitis.
MATERIALS AND METHODS
Thirty patients were selected based on simple random sampling technique. After getting approval from ethical committee, the patients were selected depending upon the various inclusion and exclusion criteria. All samples included were diagnosed as adhesive capsulitis by orthopedicians and they all showed a capsular pattern of restriction was participated in this study.

Inclusion criteria: Clinically diagnosed adhesive capsulitis (stage II), Age group (40-60 years), Minimum 2 months' duration and marked loss of passive and active ROM.

Exclusion criteria: Age group below 40 years, History of any trauma or surgery, Patients under steroid therapy, un-controlled diabetes, skin infections, polyarthritis, neurological disorder, Medical conditions such as cardiac disease, infection, coagulation disorder were excluded.

Procedure
60 patients with clinically diagnosed adhesive capsulitis reporting to the Physical Therapy Department of ACS Medical College and Hospital. Then tested for the above said inclusion and exclusion criteria and 30 patients who were diagnosed with adhesive capsulitis by orthopaedicians and showed a capsular pattern of restriction included in this study.

After getting approval from ethical committee, aim and technique were explained to the patients and get an informed consent from them. The baseline assessment was recorded. The study was conducted for a period of three weeks. Group A received MET with maitland mobilisation technique and while group B received Cyriax deep friction technique with maitland mobilisation technique. Both groups were given moist heat therapy for 15 minutes before the initiation of treatment and were instructed to do home exercise program. Home exercise program consist of shoulder mobilisation exercises and capsular self-stretching exercises. Post intervention assessment was measured. An experimental design was followed with outcome measures of range of motion and shoulder pain and disability score (SPADI) were collected with pre and post treatment scores.

Prior to the manual therapy, moist heat was given for two groups. Then, maitland graded oscillation technique grade 2 and 3 (for improving range of motion, stretching (3) and to reduce pain (2). shoulder mobilisation exercises consist of pendular exercises, wall ladder exercises, towel exercises, wand exercises and capsular stretching exercises.

Group A
MET for glenohumeral joint restricted flexion
Therapist stood in front of the subject and placed one hand over the top of the patient's involved shoulder at the superior part of the scapula and hold the glenohumeral joint to palpate for motion. The other hand and forearm supported the subject's flexed elbow and flexed the humerus at the glenohumeral joint in the sagittal plane up to the initial point of resistance. The subjects were instructed to extend the elbow against equal opposite force applied by the therapist. Hold it for 3-5 seconds, and then the patients were allowed to relax for 2 seconds, take up the slack and then repeat the procedure for 5 times.

MET for glenohumeral joint restricted abduction
Therapist stood in front of the patient, placed one hand over the top of patient's involved shoulder, cups the glenohumeral joint to palpate for motion and instructed the patients to press the elbow towards their body against equal opposite force applied by the therapist. Hold it for 3-5 seconds, and then the patients were allowed to relax for 2 seconds, take up the slack and then repeat the procedure for 5 times.

MET for glenohumeral joint restricted external rotation
Therapist stood behind the patient and placed his hand superior to the patient's involved glenohumeral joint. Placed his forearm of the other hand medial to the patient's flexed forearm with his hand supporting the patient's hand and the wrist and then instructed the patient to internally rotate the arm by pressing the hand. Against equal opposite force applied by the therapist. Hold it for 3-5 seconds, and then the patients were allowed to relax for 2 seconds, take up the slack and then repeat the procedure for 5 times.

Muscle energy technique was applied for 5 repetitions per set, 5 sets per session, 1 session per day, and 5 days a week for 2 weeks with each repetition maintained for the duration of 7-10 seconds.

Group B
Deep transverse friction was applied to 15 patients in the group. Constant transverse friction were applied around the shoulder joint line, more to be concentrated at the anterior and posterior aspect, at the bicipital groove, serratus anterior, subscapularis and supraspinatus muscle. Deep friction was given 15 minutes per session, five times a week for three week. DTF at bicipital groove for the release of anterior capsule: patient in supine lying, affected side on the edge of the couch with elbow flexed 90° Therapist stands to the side of affected shoulder. Patient’s thenar eminence of one hand on the anterior aspect of the affected shoulder and the other hand should hold the forearm in order to produce internal and external rotation. Therapist apply constant transverse friction force using his weight for pressure application while doing internal rotation of the shoulder by the other hand and pressure is released while doing external rotation. DTF for serratus
anterior muscle: patient is positioned in side lying with affected side facing upwards. Therapist stood at the head end of the table. The patients one hand passive retract the patients affected shoulder to approximate the medial boarder of scapula, creating a space between medial boarder and thoracic cage and with the other hand therapist apply DTF in the space between the scapula and the thoracic cavity. DTF for supraspinatus muscle: patient positioned in sitting with arm fixed in adduction and medial rotation. Therapist stood at the head end of the table and applied DFR using thumb or tip of the index finger reinforced by middle finger.

**Statistical analysis**

All the parameters were assessed using statistical package for social science (SPSS) version 24.0. The outcome scores were measured by using goniometry for range of motion (of external rotation, abduction and flexion) and shoulder pain and disability index (SPADI). Intergroup comparison were analysed using independent ‘t’ test and intragroup comparison were analysed using paired ‘t’ test.

**RESULTS**

Table 1: Comparison of ROM within group A between pre and post-test

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<td>SD</td>
<td>Mean (in degrees)</td>
<td>SD</td>
</tr>
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</tr>
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<td>97.33</td>
<td>18.98</td>
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</table>

Group A: MET GROUP (p<0.001)

Table 1 denotes that there is a highly significant difference between the means of pre-test and post test of ROM values within group A (MET) (p<0.001). There is a marked improvement in the post-test means ROM values. Mean values of abduction range increased markedly compared to flexion and external rotation in group A. Abduction range increased from 44.33° to 86.07°, flexion range increased from 97.33° to 118.21° and external rotation range increased from 12.33° to 33.57°.

Table 2: Comparison of SPADI within group A between pre and post-test

<table>
<thead>
<tr>
<th></th>
<th>Pre-test (%)</th>
<th>Post-test (%)</th>
<th>‘t’ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Total pain score</td>
<td>54.13</td>
<td>10.97</td>
<td>43.71</td>
<td>10.78</td>
</tr>
<tr>
<td>Total disability score</td>
<td>69.73</td>
<td>9.68</td>
<td>58.57</td>
<td>8.35</td>
</tr>
<tr>
<td>Total SPADI score</td>
<td>61.93</td>
<td>8.34</td>
<td>51.14</td>
<td>7.28</td>
</tr>
</tbody>
</table>

Group A: MET technique; P value < 0.001)

SPADI: Shoulder pain and disability index; SD = Standard deviation

**Interpretation:**

The above (Table 2) revealed that there is a highly significant difference between the means of pre-test and post-test of total pain score, total disability score and total SPADI values within group A (MET) (p<0.001). There is a marked improvement in the post-test means of SPADI scores. Total pain score decreased from 54.13 to 43.71, total disability score diminished from 69.73 to 58.57. The overall SPADI value decreased from 61.93 to 51.14.

Table 3: Comparison of pre-test and post-test ROM in group B

<table>
<thead>
<tr>
<th>Range of motion</th>
<th>Pre-test</th>
<th>Post-test</th>
<th>‘t’ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (in degrees)</td>
<td>SD</td>
<td>Mean (in degrees)</td>
<td>SD</td>
</tr>
<tr>
<td>Abduction</td>
<td>50.33</td>
<td>13.16</td>
<td>73.21</td>
<td>14.09</td>
</tr>
<tr>
<td>External rotation</td>
<td>15.67</td>
<td>7.53</td>
<td>23.93</td>
<td>8.59</td>
</tr>
<tr>
<td>Flexion</td>
<td>98.33</td>
<td>19.97</td>
<td>111.79</td>
<td>21.89</td>
</tr>
</tbody>
</table>

SD = Standard deviation

**Interpretation:**

The above (table 3) denotes that there is highly significant difference between the means of pre and post-test range of motion of shoulder abduction, external rotation and flexion in group B (CYRIAX) (p<0.001) there is a marked improvement in the post test abduction ROM values than that of external rotation and flexion values. Abduction range increased from 50.33° to 73.21° degrees, flexion range increased from 98.33° to 111.79° and external rotation range increased from 15.67° to 23.93°.
Table 4: Comparison of pre and post-test SPADI score within group b (Cyriax)

<table>
<thead>
<tr>
<th></th>
<th>Pre-test (%)</th>
<th>Post-test (%)</th>
<th>‘t’ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Total pain score</td>
<td>58.67</td>
<td>11.68</td>
<td>48.71</td>
<td>10.28</td>
</tr>
<tr>
<td>Total disability score</td>
<td>69.60</td>
<td>6.73</td>
<td>65.86</td>
<td>5.95</td>
</tr>
<tr>
<td>Total SPADI score</td>
<td>64.13</td>
<td>7.50</td>
<td>57.64</td>
<td>7.39</td>
</tr>
</tbody>
</table>

SD = Standard deviation

**Interpretation:** The above (Table 4) indicated that there is highly significant improvement in mean values of post-test SPADI scores compared to pre-test SPADI scores within group B (CYRIAX) (p<0.05). The total pain score decreased from 58.67 to 48.71, total disability score reduced from 69.60 to 65.86 and total SPADI score reduced from 64.13 to 57.64.

Table 5: Post-test comparison of mean range of motion in group A and B

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Mean(in degrees)</th>
<th>SD</th>
<th>Mean diff</th>
<th>‘t’ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abduction</td>
<td>A</td>
<td>89.67</td>
<td>20.66</td>
<td>26.10</td>
<td>3.9706</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>63.57</td>
<td>13.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>External rotation</td>
<td>A</td>
<td>33.67</td>
<td>7.90</td>
<td>9.74</td>
<td>3.1811</td>
<td>0.0037</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>23.93</td>
<td>8.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>A</td>
<td>123.67</td>
<td>16.31</td>
<td>14.74</td>
<td>2.1624</td>
<td>0.0396</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>108.93</td>
<td>20.30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD = Standard deviation

**Interpretation:** All the data shown in Table 5 reveals that there was no significant difference in mean, standard deviation (SD), ‘t’-test and p-value of the ROM between group A and group B. but the data proves that there is highly significant difference in mean of post-test’s range of motion of shoulder abduction, external rotation and flexion than pre test scores in group A (MET) than group B (Cyriax; p<0.05).

Graph 5: Post-test comparison of mean rom between group A and group B

Table 6: Post-test analysis of mean SPADI scores of group A and group B

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>Mean diff</th>
<th>‘t’ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total pain score</td>
<td>A</td>
<td>42.8</td>
<td>10.98</td>
<td>-6.63</td>
<td>1.6442</td>
<td>0.1117</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>49.43</td>
<td>10.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total disability score</td>
<td>A</td>
<td>58.4</td>
<td>8.08</td>
<td>-7.46</td>
<td>2.8137</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>65.86</td>
<td>5.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPADI SCORE</td>
<td>A</td>
<td>50.6</td>
<td>7.33</td>
<td>-7.04</td>
<td>2.5766</td>
<td>0.0158</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>57.64</td>
<td>7.39</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD = Standard deviation

**Interpretation:** Table 6 denotes that there is highly significant difference between the means of post disability score and means of post SPADI scores of group A(MET) and group B(Cyriax) (p<0.05). There is no significant difference exists between mean of post total pain score between two groups.

Graph 6: Post-test comparison of means of SPADI scores between group A and B
From the paired ‘t’ test results, it is observed that p=0.000(P<0.05) thereby significant difference between pre-test and post-test value is seen statistically in both the groups. We observed mean values of abduction, external rotation and flexion of 89.67, 33.67 and 123.67 in group A compared to mean values of 63.57, 23.93 and 103.93 in group B respectively. Mean pain score, disability score and SPADI score of pre and post-test values of group A reduced from 54.13, 69.73, 61.93 to 43.71, 58.57, and 51.14. Mean total disability score has decreased markedly compared to mean total pain score and mean total SPADI score. The comparison of mean values of pain score, disability score and SPADI score between group A and group B shows that MET group achieved a greater improvement in total disability and SPADI score. The total pain score is relatively same between two groups. There is no significant difference in mean pain score between two groups with a p-value of 0.1117 (<0.05).

DISCUSSION

Adhesive capsulitis is a more and more usual disorder of the shoulder joint constantly requiring physiotherapy for its treatment. This study helps to know which very effective adjunct for adhesive capsulitis is. Usman et al., compared the Maitland mobilisation technique with conventional physical therapy methods and shows that Maitland technique is far better than other treatments in treating this condition (12). There are so many researches suggesting the effectiveness Maitland’s mobilisation technique. However, in our current study, we compared which combinations worked well with Maitland’s mobilisation technique.

Uysal et al., prove that Cyriax technique has a remarkable response in pain and stiffness. The results showed that there is a significant difference exists between pre and post-test values of Cyriax group (13). Vaishali et al., have reported application of Cyriax deep friction technique for the treatment of adhesive capsulitis and its effect in relieving adhesion and pain earlier (14). It shows greater improvement than any other physical therapy modalities.

Redda et al., compared the positional release with muscle energy technique and there is a marked improvement with muscle energy technique (15). Physiological effect of MET is by reciprocal inhibition of the antagonist when the agonist contracts, resulting in improving the stretch tolerance of muscle spindle and increasing the range of motion. Therefore, the application of MET is found to be more effective in improving the range of motion of glenohumeral joint when compared to Cyriax technique in adhesive capsulitis. The comparison between mean post-test values between MET and Cyriax group showed that significant difference exist for total disability score and total SPADI score with MET proven to be more beneficial. There is no significant difference exist for total pain scores. Muscle energy technique has also been widely used in the treatment of adhesive capsulitis along with mobilisation technique combination. We can observe that MET can be used to reduce the pain and increase the range of motion and flexibility effectively in combination with Maitland and nulligan mobilisation technique. So far, there is no comparison between METS with Maitland’s mobilisation technique and Cyriax’s DFT with Maitland’s mobilisation technique.

There is a comparison of immediate effects of Cyriax’s DFT to the conventional physical therapy in 2004. Cyriax technique does detach fibrils from their proper formation at the healing breach, and prevent continued adherence at abnormal sites. Thereby it supports the mechanism of relieving capsular adhesions and improving the range of motion of shoulder joints (16). There are also previous studies supporting the result of the study in improving the range of motion as well as pain reduction. Chamberlain reviewed the existing articles related to Cyriax deep friction technique, in which, he referred that traumatic hyperaemia results in enhancement of blood supply to the area. The hyperaemia appears to diminish pain by increasing the speed of destruction of Lewis P substance, probably due to the release of histamine. Lewis p factor is an irritative metabolite, which produces ischaemia when it accumulates (16).

Gehlsen et al., studied the effect of soft tissue mobilisation pressure on fibroblast response and it is found that there is significant increase in healing process and number of fibroblast after soft tissue mobilisation (17).

There are several studies comparing the MET with different techniques. Not a single study compared the efficacy of Cyriax’s DFT to the MET in the combination of mobilisation technique. Both are so effective in combination with other manual therapy treatments. This study compared these two techniques as a means of combination therapy along with Maitland’s technique and found out which works best for adhesive capsulities.

There are so many articles supporting the effects of extensibility of tissues by MET. MET has shown to reduce pain, stiffness and to achieve the greater functional improvements. It was observed that MET relaxes the muscles thereby improves the extensibility of muscles while crossing the restriction barriers (18, 19).

This also coincides with the results of previous study done by Moore et al., who examines the immediate effects of muscle energy technique, which shows a marked improvement in internal rotation and horizontal adduction range of motion (20).
Limitation of the study includes sample size which is small and also further studies are needed to find out the effectiveness of Cyriax’s DFT and METs alone in adhesive capsulitis subjects. The result of this study demonstrated that both MET group and Cyriax group experienced significant improvement in pain and functional status. The study also reveals MET group experienced greater outcomes for all variables in comparison to those receiving Cyriax group.

CONCLUSION

From the result of this study, it is concluded that the MET is more effective than Cyriax deep friction technique in phase II adhesive capsulitis. The result of the study also showed that there is a marked statistical improvement in Muscle Energy Technique as compared to Cyriax technique for adhesive capsulitis patients.

CONFLICT OF INTEREST: None

REFERENCES

15. Redda, K., Mohamed, N. M. Positional release versus muscle energy techniques on functional ability of shoulder in chronic adhesive capsulitis. The 18th international scientific conference Faculty of Physical Therapy Cairo, 2017; 16-17 March.
A study to compare the sagittal posture analysis for single sided and double-sided backpack users

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ABSTRACT

Introduction and Aim: Backpack is a frameless form used to carry manual load/ any sort of equipment that distributes weight across the body and transferring more weight to hips and legs. The student wears the shoulder straps of the backpack on either single or double sides of the shoulder. Wearing backpacks on a single side differs from wearing the backpack on a double side. Methods of usage of backpack bring significant changes in postural alignment and muscle imbalance. Wearing backpack slung or strap over the single shoulder transverse all pressure to the shoulder where the backpack is placed. This results in strain on bones and muscles of the shoulder complex, so in this study is aimed to find the difference in sagittal posture analysis when single and double side backpack is been used.

Materials and Methods: Sagittal posture analysis is a measurement taken from tragus of ear to greater tubercle of the humerus in sagittal posture in students wearing the backpack on a single side and double side. Measurement was taken with load and without load. The study was taken to compare whether there is a difference between wearing bags on a single side and double side by measuring sagittal posture. Measurement shows a considerable change in single-sided and double-sided backpack students and with load and without load.

Results: Study revealed there is a significant change in single-sided backpack students. When the measurement was taken with bags, double-sided backpack students showed a simultaneous decreased measurement on both the right and left side. However, in single-sided backpack students, it showed a decrease in measurement on the side they used to wear the bags and an increased measurement on the opposite side of wearing the bags. Thus, wearing the backpack on a single side may lead to asymmetrical shoulder or postural changes.

Conclusion: Prolonged wearing of bags in a single shoulder will lead to postural changes. When the postural alignment changes, it leads to over straining of shoulder muscles like deltoid, trapezius and back muscles. Finally, this leads to back pain and neck pain. However, wearing backpacks on the double side evenly distributes weight to both the shoulder. Hence, it is advisable to wear bags on the double side rather than a single side.

Keywords: Tragus of the ear; greater tubercle; postural alignment; muscle strain; deltoid; trapezius back muscles.

INTRODUCTION

Carrying a backpack is the most common daily activity among students. The way of wearing influences the student's body alignment. A backpack is a frameless material used by students to carry manual load/ any sort of equipment (1). The modern world changes a student's way of wearing the backpacks. Students started to wear backpacks so with a single sling. Fashion makes students wear bags on the single shoulder, which slowly alters their body alignment (2). The study was taken to compare the sagittal posture analysis in both single-sided and double-sided backpack students. Sagittal posture analysis is done by measuring the distance from the tragus of ear to greater tuberosity. The difference in this measurement will show changes in their shoulder, neck and back alignment (3). This changes the posture of the student. Posture is the attitude assumed by our body when it is stationary or moving. This will be the position to hold the body during sitting or standing. Posture will be the position of all body segments relative to each other and spine. An ideal posture is the one where the body segments are arranged vertically. LOG passes through every joint axis. However, a body segment cannot maintain ideal posture, as LOG falls very close to the joint axis and not through joint axis producing a little stress at joint (4–6).

Good posture will be the position where minimum stress is applied to each joint. It is the position to hold the body straight against gravity. Less strain must be applied to supporting muscles and ligaments. In optimal posture, the spine is stacked on top of pelvis and shoulder must hold the spine in place. Bad posture is the static posture that increases the stress to the joints is a faulty/ bad posture. The improper alignment of shoulder, spine and pelvic will alters ROM. Kyphosis is the extreme forward curvature of the upper spine accompanied by forwarding curvature of lower spine.
Scoliosis is the sideways curvature of the spine when looking from the anterior view, the curvature is easily identified. Lordosis is the increase in posterior concavity which will be common in obesity or pregnancy. Swayback will be related to lordosis. Increase in backward curvature of the spine in the lower region (lumbar) and increase in forwarding curvature of the chest region. In flat-back, the back will be straight with the absence of normal curves of the spine.

Kypholordosis is the combination of kyphosis and posterior lordosis. The posture of the person depends on the arrangement of vertebrae which depends upon intervertebral disc and curves of vertebral column (10). Vertebral column Consist of 33 vertebral bone and 23 IV disk. The spine broadly has 5 regions –cervical, thoracic, lumbar, sacrum and coccyx. The cervical, thoracic and lumbar are movable but sacrum and coccyx are immovable (11). In lateral view, the spine is observed with curves. At birth, it presents a single curve which is posteriorly convex. At later age 4 curves are present in which two are primary and two are secondary. The curve that is posteriorly convex for entire life is the primary or kyphotic curves. The curve that goes for posterior concavity is the secondary/ lordotic curve. Thoracic and sacral are kyphotic and lumbar and cervical are lordotic. The curves are dependent on each other and a part of a closed kinematic chain. Vertebral bodies are connected and separated by IV disc (12-14). The disc comprises of nucleus pulposus and annulus fibrosis and endplates. The endplate contains hyaline cartilage. They undergo calcification with age, annulus fibrosis consists of collagenous fibers and fibrocartilage that surrounds nucleus pulposus contains water, collagen fibers, proteoglycans, and elastin fibers. Type 1 collagen predominates in annulus fibrosis to resist greater tensile forces. Nucleus pulposus is a muco-polysaccharide structure containing 70-90%. Type 2 collagen is greater to resist compressive forces.

The motion segment comprises two adjacent vertebrae along with IV disc, the ligament that connects them. The spine mobility is the sum of movement of each motion segment. The coupling will be the association of one motion about an axis with another motion of different axis. IV disc adds tremendous ROM y allowing tilting of vertebrae on each side. Flexion and extension occur as the result of tilting and gliding of superior vertebra over the inferior vertebra. The spinal column undergoes for axial compression, bending, torsion, and shear force, which depends on the type, duration, and rate of loading. The force acts through the long axis of the spine perpendicular to the disc. This compressive load will be transmitted from the superior endplate to inferior endplate through trabecular bone and another cortical shell. The nucleus pulposus gets deformed by the compressive force. When this force act, the nucleus will exhibit a swelling pressure. Stress is created in annulus fibrosis when swelling pressure is distributed in all directions. The force of the nucleus pulposus is an interaction pair (15-17). The pressure is transmitted to vertebral bodies. Thus, disc and trabecular bone undergo deformation. The endplate will fail first when there is a high load. The IV disc also exhibit creep that produces changes in disc compression and function. Both compression and tension are created during bending. During forward flexion, the anterior disc, ligament, and muscle will go compression and the posterior structure go for tension. In the lateral bending ipsilateral side of the disc is compressed and the contralateral side is stretched. Torsional forces are created during rotation. The outer layer of vertebral bodies, disc, and orientation of facet provide torsional stiffness. Translation occurs when a shear force acts on the midplane of the disc. Zygapophyseal joint resists shear forces. When there is sustained load; disc exhibits creep and the zygapophyseal joint resists the entire shear force. Hence, adaptation of wearing bags on the single shoulder will lead to such kinds of postural deformities. Change in wearing the backpack on the single side also affects the shoulder complex. Overloading on the single side may affect the dynamic stability of the shoulder joint. Shoulder complex composed of scapula, humerus, and clavicle and is a combination of three joints that links upper extremity to the thorax. It includes sternoclavicular joint, acromioclavicular joint, Acapulco thoracic joint and glenohumeral joint. Static position, humeral headrest on fossa, the gravity acts parallel to shaft in the downward direction. It needs vertical force to restore/maintain equilibrium; this force is supplied by the deltoid, supraspinatus, long head of biceps and triceps (18). The mechanism of joint stability is passive. The translator component of rotator cuff muscles prevents shear between the humeral head and glenoid fossa. Deltoid along with infraspinatus, teres minor and subscapular create a pure rotation.

Dynamic stability is described as muscles and soft tissues used to control the movement and other structures like rotator cuff muscles. The deltoid is an anterior stabilizer of the GH joint in abduction and external rotation. Rotator cuff muscle provides dynamic stability during GH mobility. It is the main source of dynamic stability (19). The instability of the glenohumeral joint is a common disorder. Trauma, repetitive motion or frequent dislocation of the shoulder joint lead to this condition. Symptoms occur as pain during activities such as overhead activity,
carrying heavy items, swimming. Wearing backpack for a prolonged time will lead to degenerative changes at shoulder complex specifically at AC joint and GH joint. A heavy load will compress the spine leading to scoliosis or can lead to any incorrect postural adaptation.

MATERIALS AND METHODS

The trial was conducted in 100 students from Saveetha College of Physiotherapy. Subjects included in the study were under the age group of 19-21 yrs. Both male and female subjects were included. Each subject was asked about their usage of bag either single side or double side. Based on their usage 100 subjects were divided into two groups. One group with students wearing their bags on a single side and another group wearing bags on a double side. Every subject was provided with information about the study and asked for their participation. After their full agreement; subjects were included in the study. Subjects with previous shoulder pain or recently undergone any surgery in the shoulder are contraindicated to the study.

Group A included 50 subjects who wear their bags on double sides of the shoulder with both males and females. Group B included 50 subjects who wear their bags on a single side of shoulder with both males and females. Each subject's postural alignment was observed in the lateral view. Subjects were made to lean against a straight wall with the head, scapula and gluteal region touching the wall. Greater tubercle was palpated. The midpoint of greater tuberosity was marked with a skin marker. Measurement was taken from the tragus of the ear to greater tuberosity using inch tape. The inch tape must behold straight for accurate measurement. Subjects were measured without wearing bags and wearing bags on both sides of the shoulder. The load of the backpack differs in each subject. The subject was measured with their daily carrying load.

Inclusion criteria: Healthy male and female college students were included in the study. Subjects with the age group of 19 to 21 were included. The students taken for the study didn't have any recent injury or surgery in the neck, shoulder or back.

Exclusion criteria: Subjects with congenital deformity are excluded. Students who already had fracture or dislocation at the shoulder joint is not included. Subjects with any changes in postural alignment due to other pathological causes are excluded.

RESULTS

On average almost 70% of double-sided backpack students showed decreased measurement on both sides on wearing bags when compared to the measurement taken without bags. They showed a decreased measurement of bilaterally. However, in single-sided backpack students, when the measurement was taken with the bag, almost 90% of students showed decreased measurement on the side where they used to wear their bags and increased measurement on the opposite side of wearing bags. This shows the unilateral variations in single-sided backpack students when they wear their bags. Hence there is a possibility for the occurrence of the asymmetrical shoulder or postural changes in single-sided backpack students.

Table 1: Comparison between the values of group A without a bag for right and left

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A without a bag (Right)</td>
<td>50</td>
<td>21.029</td>
<td>1.950</td>
</tr>
<tr>
<td>Group A without a bag (Left)</td>
<td>50</td>
<td>21.037</td>
<td>1.965</td>
</tr>
</tbody>
</table>

Table 2: Comparison between the values of group A with a bag for right and left

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A with the bag (Right)</td>
<td>50</td>
<td>20.709</td>
<td>1.950</td>
</tr>
<tr>
<td>Group A with a bag (Left)</td>
<td>50</td>
<td>20.771</td>
<td>1.967</td>
</tr>
</tbody>
</table>

Table 3: Comparison between the values of group B without a bag for right and left

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group B without a bag (Right)</td>
<td>50</td>
<td>21.258</td>
<td>2.200</td>
</tr>
<tr>
<td>Group B without a bag (Left)</td>
<td>50</td>
<td>21.244</td>
<td>2.207</td>
</tr>
</tbody>
</table>

Table 4: Comparison between the values of group B with a bag for right and left

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group B with a bag (Right)</td>
<td>50</td>
<td>21.258</td>
<td>2.193</td>
</tr>
<tr>
<td>Group B with a bag (Left)</td>
<td>50</td>
<td>21.282</td>
<td>2.257</td>
</tr>
</tbody>
</table>

DISCUSSION

The study revealed that there is a difference in measurement in single-sided and double-sided backpack students. In students wearing their backpacks on a single side, it was found that values decreased on the side in which the students wear the strap of the bag. Several authors revealed about the effects of using
backpacks with heavy loads. Merati et al., states the effects of a backpack with 10% of body weight of subjects on cervical and shoulder posture in school children. They found a reduced craniovertebral angle, increased sagittal shoulder posture in loaded conditions and concluded, a load of a backpack with 10% body weight will be too heavy for school going children’s (20).

Few types of research found the effects of backpack weight on schoolgirls with vertebral communities. Taimela et al., showed the effects of backpack weight on high school girl students with a spinal deformity like scapula asymmetry, vertebral column deviation, and unsymmetrical shoulder. Their study included students with variable backpack loads like 10%, 12.5%, 15% and 75% of body weight. The result proved only 10% of body weight as backpack load did not show any effect on dropped shoulder and suggested students should not carry a backpack heavier than 10% of body weight (21). Gent et al., study revealed the effects of load placement in posture and spinal curvature. The results proved changes in trunk forward flexing and craniovertebral angle and proved there is decreased effect when the load was placed at the lower spine and to minimize postural adaptations (22). Vicas-Kunse found that effects of backpack shoulder straps length and found changes in upper trapezius pain threshold and craniovertebral angle (23). Viry et al., concluded backpack with shorter straps is more beneficial than a backpack with longer straps stated the effects of single side and double side strap backpack on lung function, which included forced vital capacity, forced expiratory volume in 1 second and peak expiratory flow (24). Wedderkopp et al., concluded that decrease in forced vital capacity more in single side backpack students than double side backpack students and no significant changes in forced expiratory volume and peak expiratory flow in both types of wearing backpacks (25).

Those previous studies resulted in the effects of using backpacks with heavy loads and incorrect usage of bags. This study will be a comparative one independent of a load of the backpack. The backpack load will be a variable one according to the subject’s usage. The study compares the measurement from the tragus of the ear to the midpoint of greater tubercle in the sagittal plane in both single-sided and double-sided backpack. The measurement was taken both sides with bags and without bags. Of 100 students almost 15 double-sided backpack wearing students and 5 single side backpack wearing students did not show any considerable changes in measurements, hence neglected. In single side backpack wearing students, the measurement was reduced on the side on which students wear their bags. The study results there might be postural changes and shoulder alignment in single-sided backpack students.

CONCLUSION

In single-sided backpack students, the measurement showed a difference between both sides of the shoulder. This shows the chance for the occurrence of the asymmetrical shoulder. Prolonged loading on a single side of the shoulder; may compress the IV disc, changes the orientation of the body of vertebra leading to alteration of spinal curves. Changes in the spinal curve results in postural deformities like scoliosis or kyphosis. Overloading on a single side of the shoulder also results in muscle weakness due to over contraction. This finally results in neck pain, shoulder pain and back pain. Hence, it is advisable to wear backpacks on both sides. This distributes the weight evenly on both sides. Students must be aware of the disadvantage of wearing bags on a single side and even the load of the bag affects the body position. Hence loading the bag over for about 10% of your body weight is considerable.

CONFLICT OF INTEREST: There is no conflict of interest from other authors.

REFERENCES


Assessment of autonomic dysfunctions in patients with non-cirrhotic portal fibrosis
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ABSTRACT

Introduction and Aim: Autonomic dysfunction has been observed in both alcoholic and nonalcoholic chronic liver diseases. Autonomic (both parasympathetic and sympathetic) functions are affected in these diseases. However, there are few studies on autonomic dysfunctions in non-cirrhotic portal fibrosis (NCPF) compared with cirrhosis. Therefore, in the present study we have assessed autonomic functions in patients NCPF and cirrhosis.

Materials and Methods: Autonomic function such as heart rate variability (HRV) and conventional autonomic function tests (AFTs) were assessed in 3 groups of patients. Groups 1 included patients with NCPF, Group 2 were those with compensated cirrhosis (Child A) and Group 3 included age, sex and BMI-matched healthy volunteers. Patients with diabetes, cardiac or renal insufficiency, neurological disorders, on diuretics / beta blockers for at least 2 weeks prior to AFTs were excluded.

Results: Total Power (TP) of the HRV spectrum was reduced significantly in NCPF (p<0.001) and cirrhosis participants (p<0.001) when compared to the controls. LF-HF and VLF was significantly reduced in both NCPF and cirrhosis when compared to controls. LF-HF ratio was reduced in NCPF and increased in cirrhosis and the difference between them was statistically significant. There was no difference in the Mean RR of the three groups. When compared to the controls, RMSSD, SDNN, NN50 was significantly reduced in NCPF and Cirrhosis. The difference in pNN50 was significantly reduced in Cirrhosis (p<0.001) when compared to controls. Among Conventional AFTs, heart rate response to standing expressed as 30:15 ratio was reduced in NCPF and Cirrhosis. ΔDBP IHG was similar in all the three groups.

Conclusion: The present study reports the presence of autonomic imbalance in patients suffering from NCPF and hepatic cirrhosis, which is more prominent in cirrhosis. Sympathovagal imbalance, decreased HRV, decreased vagal and increased sympathetic modulations of cardiac functions in these patients predispose them to higher CV risks and CV morbidities.

Keywords: Non-cirrhotic portal fibrosis; Cirrhosis; Autonomic function tests; Heart rate variability; Sympathovagal balance.

INTRODUCTION

Chronic liver disease is associated with various cardiovascular abnormalities, the most common characteristic of which is a hyperdynamic circulatory state (1, 2). Autonomic dysfunction is seen in both alcoholic and nonalcoholic chronic liver diseases (3-6). Both parasympathetic and sympathetic functions are affected. The prevalence and severity of autonomic dysfunction is related to the severity of hepatic dysfunction and is independent of etiology (7). However, there are only few studies on autonomic dysfunction in Non cirrhotic portal fibrosis (NCPF) and Extra-hepatic Portal Vein Obstruction (EHPVO) in which the liver functions are normal (8, 9). The etiopathogenesis of autonomic dysfunction in NCPF is yet unexplained.

Recent studies have reported that patients with chronic liver disease have poor outcome and bad clinical presentations, if autonomic dysfunctions are present. The prevalence and severity of autonomic dysfunction appears to be related to the severity of liver disease and association of autonomic dysfunctions is linked with an increase in morbidity and mortality (5, 6). Both sympathetic and parasympathetic (vagal) functions, which can be assessed by noninvasive methods, are affected in patients with chronic liver disease (4, 5). Autonomic dysfunction in chronic liver disease may be secondary to the deranged liver function or can occur as a consequence of portal hypertension per se (5). Other factors such as impaired vascular hypo-responsiveness that are possibly related to a circulating vasodilator or due to presence of false neurotransmitters or a true neuropathy may also contribute. Extrahepatic portal venous obstruction (EHPVO) and non-cirrhotic portal fibrosis (NCPF) are two other diseases in which portal hypertension occurs. Previous studies have shown that a hyperkinetic circulatory state exists in patients with noncirrhotic portal hypertension, which could be
caused by expanded plasma volume secondary to enlargement of portal bed (8). To best of our knowledge the contributions of the autonomic system in modifying the systemic and portal circulation in these patients are still not known.

Recently, analysis of heart rate variability (HRV) has been reported to be a noninvasive sensitive marker in assessment of autonomic dysfunctions. Therefore, in the present study we have compared the autonomic functions in 3 groups of patients: NCPF, Child A cirrhosis and controls.

MATERIALS AND METHODS

We included all patients (>13years) who presented to Medicine and Gastroenterology units of our hospital with clinical features of NCPF and cirrhosis, over a two year and four months period (March 2011-August 2013). The research protocol was approved by our institutional ethics committee and all participants had given written informed consent.

Autonomic function was assessed in 3 groups of patients. Groups 1 included patients with NCPF, Group 2 were those with compensated cirrhosis (Child A) and Group 3 included age, sex and BMI matched healthy volunteers. Patients with diabetes, cardiac or renal insufficiency, neurological disorders, on diuretics / beta blockers for at least 2 weeks prior to Autonomic function test (AFT) were excluded.

The diagnosis of Non cirrhotic portal fibrosis in Group 1 patients was based on the presence of portal hypertension as evidenced by two out of three of the following –a) Esophageal or gastric varices on endoscopy b) USG or CT evidence of dilated portal vein (≥ 1.3 cm) c) Splenomegaly (Spleen ≥ 13 cm on USG) in the absence of liver involvement (liver biopsy s/o NCPF or no cirrhosis)

Cirrhosis (Group 2) was diagnosed based on clinical features of shrunken liver (<8cm on USG) or surface nodularity on USG, USG features (dilated portal vein >1.3cm), biochemical parameters (low albumin and prolonged prothrombin time) and/or endoscopic evidence of oesophageal/gastric varices.

Parameters assessed for AFT included Conventional AFT (30:15 Ratio, E: I Ratio, Δ DBP IHG (Diastolic BP change with Isometric handgrip), Frequency Domain Analysis (FDI) and Time Domain Analysis (TDI) of HRV.

Statistical analysis of data

Table 1: Age, anthropometric and basal cardiovascular parameters of controls, NCPF and cirrhosis patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=30)</th>
<th>NCPF (n=35)</th>
<th>Cirrhosis (n=30)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.6±8.3</td>
<td>36.8±9.2</td>
<td>39.43±10.9</td>
<td>0.15</td>
</tr>
<tr>
<td>BMI</td>
<td>19.9±2.3</td>
<td>19.4±2.81*</td>
<td>21.2±2.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>107.9±10.2</td>
<td>107.3±12.1</td>
<td>109.1±13.4</td>
<td>0.83</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>66.4±7.8</td>
<td>66.7±9.6</td>
<td>66.0±9.6</td>
<td>0.96</td>
</tr>
<tr>
<td>Heart Rate/min</td>
<td>71.7±9.2</td>
<td>71.1±12.7</td>
<td>72.7±13.5</td>
<td>0.86</td>
</tr>
</tbody>
</table>
Wyawahare et al: Assessment of autonomic …… non-cirrhotic portal fibrosis

Data expressed are mean± SD. The star mark (*) depicts comparison with control and hash mark (#) depicts comparison between NCPF and Cirrhosis

Table 2: Frequency domain parameters of Controls, NCPF and Cirrhosis patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=30)</th>
<th>NCPF (n=35)</th>
<th>Cirrhosis (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (ms²)</td>
<td>806.1±531.8</td>
<td>250.4±195.4***</td>
<td>140.57±89.7***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VLF (ms²)</td>
<td>182.7±149.5</td>
<td>55.3±46.1***</td>
<td>39.9±26.8***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LF (ms²)</td>
<td>287.4±203.3</td>
<td>79.4±70.1***</td>
<td>58.73±46.2***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HF (ms²)</td>
<td>335.9±289.7</td>
<td>115.7±100.9***</td>
<td>41.9±32.5***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LFnu</td>
<td>52.8±15.9</td>
<td>44.4±18.0</td>
<td>54.9±20.8</td>
<td>0.053</td>
</tr>
<tr>
<td>HFnu</td>
<td>47.2±15.9</td>
<td>55.6±18.0</td>
<td>45.1±20.8</td>
<td>0.053</td>
</tr>
<tr>
<td>LF-HF ratio</td>
<td>1.47±1.15</td>
<td>1.05±0.9*</td>
<td>1.7±1.1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.05

Data expressed are mean ± SD. The star mark (*) depicts comparison with control, hash mark (#) depicts comparison between NCPF and Cirrhosis

Table 3: Time domain parameters of controls, NCPF and cirrhosis patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=30)</th>
<th>NCPF (n=35)</th>
<th>Cirrhosis (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean RR(s)</td>
<td>83±11.66</td>
<td>87.97±13.92</td>
<td>84.67±12.61</td>
<td>0.2842</td>
</tr>
<tr>
<td>RMSSD(ms)</td>
<td>46.29±30.04</td>
<td>28.9±15.76**</td>
<td>19.88±10.89***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SDNN</td>
<td>42.1±19.36</td>
<td>26.17±11.81***</td>
<td>22.51±15.64***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NN50</td>
<td>68.77±65.99</td>
<td>37.00±44.59*</td>
<td>9.3±17.21***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pNN50</td>
<td>18.57±18.51</td>
<td>14.37±20.12#</td>
<td>2.65±4.74***</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

* p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.05

Data expressed are mean ± SD. The star mark (*) depicts comparison with control and hash mark (#) depicts comparison between NCPF and Cirrhosis

Table 4: Classical autonomic function testing parameters of controls, NCPF and cirrhosis patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n=30)</th>
<th>NCPF (n=35)</th>
<th>Cirrhosis (n=30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30:15 ratio</td>
<td>1.41±0.18</td>
<td>1.30±0.18**</td>
<td>1.25±0.16**</td>
<td>0.002</td>
</tr>
<tr>
<td>E: I ratio</td>
<td>1.33±0.2</td>
<td>1.27±0.13</td>
<td>1.21±0.12**</td>
<td>0.0123</td>
</tr>
<tr>
<td>Δ DBP Hg</td>
<td>14.8±6.86</td>
<td>13.86±4.94</td>
<td>15.3±5.93</td>
<td>0.6072</td>
</tr>
</tbody>
</table>

* p < 0.05; **p < 0.01

Data expressed are mean ± SD. The star mark (*) depicts comparison with control

DISCUSSION

The total power (TP) of heart rate variability (HRV) was significantly less in both NCPF and cirrhosis groups suggesting that in NCPF and cirrhosis patients the vagal modulation of cardiac functions is considerably reduced as TP of HRV in general represents the magnitude of cardiac parasympathetic activity (10, 11). TP represents the enormity of heart rate variability, which is primarily a vagal function, and decreased TP is the index of decreased HRV (10, 11). It has been reported that decreased HRV is associated with increased cardiovascular (CV) risks, and all cause morbidity and mortality (12-14). Thus, decreased TP in NCPF and cirrhosis groups indicates increased CV risks in these patients. Among NCPF and cirrhosis groups, the TP in cirrhosis group was about 45% less compared to NCPF group, suggesting that cirrhosis patients are more vulnerable to CV risks, morbidities and mortality. Further, HFnu and time-domain indices (RMSSD, SDNN, NN50, and pNN50) of HRV were significantly less in cirrhosis patients compared to NCPF patients. These findings establish the greater decrease in vagal potency in cirrhotic patients compared to NCPF patients, as HFnu and time-domain indices are measures of parasympathetic drive of cardiac control (10, 11). Suggesting that cirrhotic patients more susceptible to CV morbidities.

In addition, The LF-HF ratio was more in cirrhosis group compared to NCPF group, indicating a greater sympathovagal imbalance in cirrhotic patients, as LF-HF ratio is a marker of sympathovagal imbalance and increased sympathetic activity (10, 11). This was further evidenced by increased LFnu in cirrhosis group compared to NCPF group, as LFnu is a marker of sympathetic drive to the heart (10, 11). Thus, these findings indicate that cirrhotic patients had increased sympathetic activity compared to NCPF patients. Findings of the present study also indicate that sympathovagal imbalance is more in hepatic cirrhosis compared to NCPF, which is due to both decreased vagal activity and increased sympathetic activity.

On analysis of conventional autonomic function tests (CAFT), it was found that 30:15 ratio and E: I ratio were decreased in NCPF and cirrhosis group.
compared to control group, and the decrease was more pronounced in cirrhosis group. As, both 30:15 ratio and E:I ratio are measures of parasympathetic reactivity (15), these findings indicate decreased vagal reactivity in NCPF and cirrhosis, which is more in cirrhotic patients compared to NCPF patients. However, there was no significant alteration in sympathetic reactivity in these patients, as there was no significant difference in ΔDBPⅩHG, the marker of sympathetic reactivity (15), among the groups.

Findings of the present study indicate the presence of sympathovagal imbalance in NCPF and cirrhosis group, which is more intense in cirrhotic patients. The sympathovagal imbalance is contributed by both decreased parasympathetic activity and increased sympathetic activity. Though there is decreased parasympathetic reactivity, sympathetic activity remains unaltered. Thus, these findings indicate that the autonomic imbalance in NCPF and hepatic cirrhosis patients are mostly due to the reduction in vagal component of autonomic modulation, though increased sympathetic activity contribute to some extent. Though the exact cause of greater degree of sympathovagal imbalance in cirrhotic patients cannot be ascertained from this study, increased body mass index (BMI) might be a potential contributor to the autonomic imbalance, which was significantly high in cirrhosis group compared to NCPF group, as increase in BMI has been reported to decrease parasympathetic and increase sympathetic activity. However, the cause-effect relationship between BMI and autonomic imbalance cannot be established from the findings of the present study.

The present study reports the presence of autonomic imbalance in patients suffering from NCPF and hepatic cirrhosis, which is more prominent in cirrhosis. Sympathovagal imbalance, decreased HRV, decreased vagal and increased sympathetic modulations of cardiac functions in these patients predispose them to higher CV risks and CV morbidities. An earlier study reported autonomic dysfunction in 12 (67%) patients with EHPVO, 3 (25%) patients with NCPF and 12 (80%) patients with cirrhosis (7). However, only 3 patients had NCPF in their study population and frequency domain parameters were not studied. Another study has shown greater likelihood of variceal bleed in NCPF cases with autonomic dysfunction (8). Autonomic dysfunction was reported in patients with extra-hepatic portal vein thrombosis, however there was no correlation with hemodynamic abnormalities (9).

Non-cirrhotic portal fibrosis (NCPF) is one of the important causes of upper gastrointestinal hemorrhage in a patient with normal liver function in tropical countries. It causes presinusoidal portal hypertension and hypersplenism (16, 17). NCPF patients with dysautonomia are prone to frequent falls, gastrointestinal symptoms which hamper their quality of life. In this study, dysautonomia was found in all 24 NCPF patients, of whom 10 were symptomatic. Portal hypertension is the underlying pathology in both cirrhotic and non cirrhotics. In cirrhosis, endocannabinoids and excess nitric oxide production has been implicated as a causative factor (18, 19). The exact etiology is not known in NCPF. Prognosis is worse in cirrhosis with autonomic dysfunction (20, 21). In earlier studies, cirrhosis patients were found to have objective evidence of improvement in cardiovascular parameters post-transplant (22, 23). Future research can elucidate the role of endotoxins in causation of dysautonomia in patients of NCPF and correlate its prognostic significance.

Study limitations are that it’s a cross sectional study. More longitudinal studies are needed to establish causality between NCPF and dysautonomia. Also, we did not study the quality of life indicators in these patients. Cirrhosis patients were diagnosed based on ultrasonography and laboratory parameters and biopsy was not undertaken. We have not estimated the biochemical markers of CV risks in these patients and the sample size is less in each group. Due to less sample size, the multiple regression analysis could not be done to assess the link of BMI to sympathovagal imbalance in these patients. Future studies on larger sample size are warranted to assess if CV risks in NCPF and hepatic cirrhosis are linked to autonomic imbalance, and the interventions required to reduce the CV risks in patients suffering from these disorders.

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Effectiveness of student-oriented counselling on behavior, attitude and academic performance of first year professional undergraduate students

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ABSTRACT

Introduction and Aim: Student oriented counselling is given for the purpose of improving well-being, alleviating distress and enhancing coping skills. It focuses to facilitate student achievement, improve behaviour, attitude, attendance, academic performance and to take decisions for their future. The present study is done to assess and compare classroom behavior, attitude towards college and academics and academic performance of first year professional undergraduate students before and after counselling intervention.

Materials and Methods: An experimental pre-test, post-test, control group design was framed for present study. As per simple random sampling 150 students were taken for study group with counselling intervention and 150 students for control group without counselling intervention to investigate the effect of Student Counselling on student class room behaviour, attitude towards college & academics and academic performance of first year professional under graduate students. A structured questionnaire was administered for both the groups after one month of college entry, internal marks were collected and one to one personal counselling was given to study group for eight months whereas the control group was withheld. After eight months, post-test was given for both the groups and internal marks were collected.

Results: The collected data was analysed using SPSS software version ±23.0. The independent ‘t’ test results revealed that, in study group there was statistically significant difference after counselling i.e., post-test, in mean score of class room behaviour (t = 4.684 at p = 0.0001), attitude towards college and academics (t = 7.266 at p = 0.0001), student problems affecting academics (t = 10.097 at p = 0.0001) and academic performance (t = 11.013 at p = 0.0001) among students comparable to pre-test as compared to control group.

Conclusion: The student oriented counselling showed improved positive classroom behaviour, attitude towards college and academics and improved academic performance in scoring good marks in examinations.

Keywords: Student counselling; behavior; attitude; academic performance; professional undergraduate students.

INTRODUCTION

There are many issues commonly experienced by students in college that can some time pose major challenges to study, play, socializing and living i.e., anxiety, stress, home sickness, difficulty in adjustment to new college environment, ragging from fellow seniors, language difficulty etc. Transition from school/college to university can be extremely challenging, for both the student and academic staff involved in teaching the new cohort. This transition has been identified as a major cause of anxiety amongst first-year university students (1). Failure to successfully manage such transition may result in significant distress, poor academic performance, and increased drop-out rates (2). It is notable that the transition to university may be particularly difficult for mature students with families, for students who are the first generation to go to university, and for students who come from ethnic minorities that are underrepresented in a student population (3). Student misbehaviours such as disruptive talking, chronic avoidance of work, clowning, interfering with teaching activities, harassing classmates, verbal insults, rudeness to teacher, defiance, and hostility (4), ranging from infrequent to frequent, mild to severe, is a thorny issue in everyday classroom. Teachers usually reported that these disturbing behaviours in the classroom are intolerable (5) and stress-provoking (6), and they had to spend a great deal of time and energy to manage the classroom (7, 8). Stress is interpreted as a more general term that describes the effects of psychosocial and environmental factors on physical or mental well-being (9). Mental stress in psychiatric disease and in daily life contributes to oxidative stress in the body (10). The adjustment difficulties of college students have been an emerging issue. Many studies have proved that the adjustment difficulties like appetite disturbance, concentration problems and depression are most evident in freshmen (11). Medical education
is inherently stressful and demanding. Overwhelming burden of information leaves a minimal opportunity for the student to relax and recreate. Stress and depression have been consistently linked to mental and physical health effects (12). An optimal level of stress enhances learning while excess of stress can cause health problems. This results in reduction of students' self-esteem and affects their academic achievement. A high level of stress may have negative effect on cognitive functioning and learning of students in medical school (13). The young student population is vulnerable to stress of higher professional education due to competitive environment. Comparing stress between medical and non-medical students shows that medical students perceive higher stress (14, 15). If left unattended, any level of stress can lead to sleeping disorders, burnout, drop out etc. The objectives of this study were to determine the effectiveness of student oriented counselling on student classroom behaviour, attitude towards college and academics and academic performance of first year professional undergraduate students namely Allied Health Science, B.Sc. (Nursing) and MBBS.

**METHODOLOGY**

The design for the study was experimental pre-test, post-test control group design framed to investigate the effect of student counselling on student class room behaviour, attitude towards college and academics and academic performance. The sample of the study (n=300) consisted of (n=150) 1st year MBBS, BSc nursing, BSc allied science students in study group and (n=150) first year MBBS, BSc Nursing, BSc Allied Science students in control group from Chennai. The tool consisted of part -1 demographic data, part -2 structured questionnaire to assess the student classroom behavior, attitude towards college and academics and student problems affecting academics. Content validity of the questionnaire was obtained from experts. Reliability of the questionnaire was tested by using test-retest reliability method (r=0.7).

**Counselling Session**

The fresher students in both study group and control group were assessed with a structured questionnaire after one month of college entry. Four sessions of counselling were given to students in study group. The counselling sessions were based on their personal problems and the academic difficulties in their studies so that their situations could be improved. The students were allowed to express their problems and full confidentiality was assured to them. We offered encouragement and solutions to their problems and promised to support them whenever they wanted. Depending upon the problems, the students were continuously supported, trained for their academic success. Students in control group were withheld from counselling. Finally, after 8 months post-test was given to both the groups.

**RESULTS**

**Table 1:** Demographic variables among first year professional undergraduate students (n=300)

<table>
<thead>
<tr>
<th>Demographic variables</th>
<th>Control group (n=150)</th>
<th>Study group (n=150)</th>
<th>X2</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-18 years</td>
<td>73</td>
<td>48.6</td>
<td>98</td>
<td>65.4</td>
</tr>
<tr>
<td>19-21 years</td>
<td>56</td>
<td>37.4</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td>22-24 years</td>
<td>21</td>
<td>14</td>
<td>19</td>
<td>12.6</td>
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<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>66</td>
<td>40</td>
<td>56</td>
<td>37.3</td>
</tr>
<tr>
<td>Female</td>
<td>90</td>
<td>60</td>
<td>94</td>
<td>62.7</td>
</tr>
<tr>
<td><strong>Native</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamil Nadu</td>
<td>109</td>
<td>72.7</td>
<td>98</td>
<td>65.3</td>
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<td>Other state</td>
<td>41</td>
<td>27.3</td>
<td>52</td>
<td>34.7</td>
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<td><strong>Family income per month</strong></td>
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<tr>
<td>10000-20000 (Low)</td>
<td>31</td>
<td>20.7</td>
<td>28</td>
<td>18.7</td>
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<tr>
<td>20001-30000 (Middle)</td>
<td>85</td>
<td>56.7</td>
<td>79</td>
<td>52.7</td>
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<tr>
<td>&gt;30000 (High)</td>
<td>34</td>
<td>22.7</td>
<td>43</td>
<td>28.7</td>
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<td><strong>Medium of study in school</strong></td>
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<tr>
<td>English</td>
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<td>58</td>
<td>94</td>
<td>62.7</td>
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<tr>
<td>Tamil</td>
<td>42</td>
<td>28</td>
<td>34</td>
<td>22.7</td>
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<tr>
<td>Others</td>
<td>21</td>
<td>14</td>
<td>22</td>
<td>14.7</td>
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<tr>
<td><strong>Education background of the family</strong></td>
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<tr>
<td>Educated</td>
<td>95</td>
<td>63.3</td>
<td>96</td>
<td>64</td>
</tr>
<tr>
<td>Uneducated</td>
<td>55</td>
<td>36.7</td>
<td>54</td>
<td>36</td>
</tr>
<tr>
<td><strong>Place of education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rural</td>
<td>65</td>
<td>43.3</td>
<td>74</td>
<td>49.3</td>
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<tr>
<td>Urban</td>
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<td>50.7</td>
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<td><strong>Type of family</strong></td>
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</tr>
<tr>
<td>Joint</td>
<td>43</td>
<td>28.7</td>
<td>40</td>
<td>26.7</td>
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<tr>
<td>Nuclear</td>
<td>91</td>
<td>60.7</td>
<td>90</td>
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<td>Single parent</td>
<td>16</td>
<td>10.7</td>
<td>20</td>
<td>13.3</td>
</tr>
</tbody>
</table>

NS- Not significant; *p<0.05; **p<0.01; ***p<0.001
Table 1: Among 300 students selected for study i.e., 150 in study group and 150 in control group, 98 (65.4%) were among the age category of 16-18 years in study group and 73 (48.6%) in control group. With regard to sex, the majority were females 94 (62.7%) in study group and 90 (60%) in control category. 109 (72.2%) were from Tamil Nadu in control group and 98 (65.3%) from Tamil Nadu in study category. With regard to family income 85 (56.7%) fall in middle class in control group and 79 (52.7%) middle class in study group. Concerning to medium of study 94 (62.7%) were from English medium in study group and 87 (58%) from English medium in control category. Regarding families’ educational background 96 (64%) in study group and 95 (63.3%) from control group were educated. 85 (56.7%) in control group were from urban educational background. With regard to type of family 91 (60.7%) in control group and 90 (60.0%) from study group were from nuclear family.

Table 2: Comparison of mean score of student classroom behavior among first year professional undergraduate students

<table>
<thead>
<tr>
<th>Test</th>
<th>Study group</th>
<th>Control group</th>
<th>Mean difference</th>
<th>'t' value / p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Pre test</td>
<td>6.76</td>
<td>3.42</td>
<td>6.52</td>
<td>3.06</td>
</tr>
<tr>
<td>Post test</td>
<td>9.95</td>
<td>2.71</td>
<td>8.39</td>
<td>3.05</td>
</tr>
</tbody>
</table>

NS- Not significant; *p<0.05; **p<0.01; ***p<0.001

Table 2 demonstrates the comparison of mean score of classroom behaviour among first year professional undergraduate students between the study and the control group during pre-test and post-test. The pre-test mean score of behaviour was 6.76 with SD 3.42 in the study group and 6.52 with SD 3.06 in the control group with mean difference of 0.220. The results of the independent ‘t’ test revealed that there was no statistically significant difference in mean score of behaviour as shown by independent ‘t’ value 0.640 at p = 0.523. The post-test mean score of behaviour was 9.95 with SD 2.71 in the study group and 8.39 with SD 3.05 in the control group with mean difference of 0.220. The independent ‘t’ test results revealed that there is statistically significant difference in mean score of behaviour as shown by independent ‘t’ value 4.684 at p = 0.0001.

Table 3: Comparison of mean score of attitudes towards college and academics among first year professional undergraduate students

<table>
<thead>
<tr>
<th>Test</th>
<th>Study Group</th>
<th>Control Group</th>
<th>Mean Difference</th>
<th>'t' value / p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Pre Test</td>
<td>28.09</td>
<td>11.163</td>
<td>26.79</td>
<td>10.287</td>
</tr>
<tr>
<td>Post Test</td>
<td>36.63</td>
<td>10.240</td>
<td>27.99</td>
<td>10.370</td>
</tr>
</tbody>
</table>

NS- Not significant; *p<0.05; **p<0.01; ***p<0.001

Table 3 demonstrates the comparison of mean score of attitudes towards college and academics among the first year professional undergraduate students between the study and the control group during pre-test and post-test. The pre-test mean score of attitude was 28.09 with SD 11.163 in the study group and 26.79 with SD 10.287 in the control group with mean difference of 1.293. The independent t test results revealed that there was no statistically significant difference in mean score of attitude as shown by independent t value 1.043 at p = 0.298. The post-test mean score of attitude was 36.63 with SD 10.240 in the study group and 27.99 with SD 10.370 in the control group with mean difference of 8.647. The independent ‘t’ test results revealed that there is statistically significant difference in mean score of attitude as shown by independent ‘t’ value 7.266 at p = 0.0001.

Table 4: Comparison of mean score of student problems affecting academics among first year professional undergraduate students

<table>
<thead>
<tr>
<th>Test</th>
<th>Study Group</th>
<th>Control Group</th>
<th>Mean Difference</th>
<th>'t' value / p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Pre Test</td>
<td>95.18</td>
<td>29.418</td>
<td>97.33</td>
<td>30.682</td>
</tr>
<tr>
<td>Post Test</td>
<td>59.10</td>
<td>27.703</td>
<td>94.45</td>
<td>32.728</td>
</tr>
</tbody>
</table>

NS- Not significant; *p<0.05; **p<0.01; ***p<0.001

Table 4 demonstrates the comparison of mean score of student problems affecting academics among the first year professional undergraduate students between the study and the control group during pre-test and post-test. The pre-test mean score of student problems was 95.18 with SD 29.418 in the study group and 97.33 with SD 30.682 in the control group with mean difference of 2.410. The independent t test results revealed that there was no statistically significant difference in mean score of student problems as shown by independent t value 0.617 at p = 0.538. The post-test mean score of student problems was 59.10 with SD 27.703 in the study group and 94.45 with SD 32.728 in the control group with mean difference of 35.347. The independent ‘t’ test results revealed that there is statistically significant difference in mean score of student problems as shown by independent ‘t’ value 10.097 at p = 0.0001.
Koundinya et al: Effectiveness of student-oriented ….. undergraduate students

problems was 59.10 with SD 27.70 in the study group and 94.45 with SD 32.728 in the control group with mean difference of 35.347. The independent t test results revealed that there is statistically significant difference in mean score of student problems as shown by independent ‘t’ value 10.097 at p = 0.0001.

Table 5: Comparison of mean score of academic performance among first year professional undergraduate students

<table>
<thead>
<tr>
<th>Test</th>
<th>Study Group</th>
<th>Control Group</th>
<th>Mean Difference</th>
<th>'t' value / p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Pre Test</td>
<td>51.52</td>
<td>9.85</td>
<td>52.11</td>
<td>10.15</td>
</tr>
<tr>
<td>Post Test</td>
<td>59.47</td>
<td>7.82</td>
<td>63.65</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 5 demonstrates the comparison of mean score of academic performance among the first year professional undergraduate students between the study and the control group during pre-test and post-test. The pre-test mean score of academic performance was 51.52 with SD 9.85 in the study group and 52.11 with SD 10.15 in the control group with mean difference of 7.96. The independent t test results revealed that there was statistically significant difference in mean score of academic performance as showed by independent ‘t’ value 7.753 at p = 0.0001. The post-test mean score of academic performance was 59.47 with SD 7.82 in the study group and 63.65 with SD 7.8 in the control group with mean difference of 11.54. The independent ‘t’ test results revealed that there was statistically significant difference in mean score of academic performance as shown by independent ‘t’ value 11.013 at p = 0.0001.

DISCUSSION

The student oriented counselling support students directly in their academic life to foster, promote, and increase interpersonal competencies and academic achievement. The Counselling believes that sound education involves the overall development of the student. This includes the social, emotional, intellectual and physical aspects of students’ lives.

In the present study comparison of mean score of students’ classroom behavior between study and control group during pretest, had no statistically significant difference p=0.523 level and homogeneity between the groups was maintained. In post-test the mean score of study group was 9.95 with a mean difference of 2.667 from control group 8.39 which was statistically significant with P=0.001 compared to control group which proved that student counselling had a positive impact in improving classroom behaviour. It is also supported by an earlier study done at Kenya that showed effectiveness of guidance and counselling in managing student behaviour established that there was a correlation coefficient of r=0.503 between guidance and counselling and the management of student behaviour (16). The findings agree with an earlier study that individual attention is useful addressing student behaviour (17). Additionally, another study also confirms that guidance and counselling services are instrumental in academic performance, self-understanding and career choice (18).

The comparison of mean score of student attitude towards college and academics study and control group during pre-test had no statistically significant difference p=0.298 level and homogeneity between the groups was maintained. However post-test confirmed the statistically significant difference in the level of attitude towards college and academics between the study group and control group during post-test (P=0.0001). This shows the change of attitude among students towards college and academics who underwent counselling. The current study results were accordance with an earlier study, which revealed that guidance and counselling enhances self-esteem among the learners. The learners acquire evaluative aspect of their self-concept, which makes them value themselves. Guidance and counselling develops positive self-image and makes the learners feel that their teachers accommodate them and listen to them. Moreover, the use of guidance and counselling to mould learners has made them develop positive attitude towards that particular alternative corrective measure (19).

The comparison of mean score of students’ problems affecting academics among study and control group during pre-test results revealed that in pretest the groups had no statistically significant difference at p=0.538 hence homogeneity in between groups was maintained. However, post-test confirmed that the problems faced by students in study group was gradually decreased compared to control group at (p =0.0001). The study revealed that counselling helped students in understanding themselves and adjusting to the new transition in their lives. Our study correlates with a study which states that students who completed a course of counselling show significantly greater improvement from depression and anxiety than those students who drop out or have an unplanned therapy ending (effect size 1.03, 0.85, respectively). That study stated that the stage at which students drop out the therapy is important than those students dropping out of therapy before the third stage are the most vulnerable to the above problems (20). This was according to practitioner pre- and post-therapy severity ratings of depression and anxiety.
Findings of our study states that there was a good improvement in academic performance of students who underwent counselling sessions compared to students who did not attend counselling at (P= 0.0001). The current study results were in accordance with another study, which revealed that the stakeholders felt that guidance and counselling programme was effective in varying degrees in boosting secondary school students’ personal, career and academic competencies (21).

CONCLUSION
The individual counselling had a positive influence on the academic performances. The results of our study showed that the student oriented counselling improved positive classroom behaviour, good attitude towards college and academics and academic performance by scoring good marks in examinations.

REFERENCES
Screening of pathogenic strains of *Pseudomonas aeruginosa* in wound infections using a mushroom myco-model as a host - a novel approach

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(Received: September 2019    Revised: October 2019    Accepted: December 2019)

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ABSTRACT

**Introduction and Aim:** The detection of pathogenic strains of *Pseudomonas aeruginosa* is usually carried out by antimicrobial susceptibility testing, molecular characterisation, selective isolation, 16S rRNA sequencing, immunological assays and through other methods. This study aimed to use mushroom as a host model for detecting pathogenic strains of *P. aeruginosa* in wound infection.

**Materials and Methods:** The strains of *P. aeruginosa* from wound infection were isolated. The pathogenic, non-pathogenic and ATCC strains of *P. aeruginosa* as well as *Escherichia coli* were injected respectively into the cap region of the mushroom and incubated for the production of lesion. The collagen was extracted from mushroom by salt precipitation method and pure collagen was purchased commercially. The collagen degradation by all the above organisms was detected by performing UV spectrophotometric analysis. The organisms were allowed to grow on medium containing extracted collagen and pure collagen.

**Results:** The lesions were found at the site of the injected region on mushroom cap when injected with pathogenic strain of *P. aeruginosa* while the rest of the strains failed to produce lesion. The collagen was degraded when infected with the pathogenic strain and the rest of the strains failed to degrade collagen. This is in accordance with the OD value. The medium containing collagen also showed growth only for pathogenic strain of *P. aeruginosa*.

**Conclusion:** Thus, the study proved that mushroom can be used as a host model to differentiate the pathogenic and non-pathogenic strains of *P. aeruginosa* from wound infections.

**Keywords:** Collagen; wound infection; mushroom; myco-model; *Pseudomonas aeruginosa*.

**INTRODUCTION**

A large number of detection methods have been developed utilizing the optical, electrochemical, biochemical and physical properties of pathogenic bacteria. The predominant techniques currently used to identify microbial pathogens rely upon conventional clinical microbiology monitoring techniques. Usually, immunological and molecular methods are followed for screening of pathogenic strains. It is reported that animals, plants, insects can be used as host models. Hence, in this present study, an innovative approach is tried out using mushroom as a host model to screen the pathogenic strains of *Pseudomonas aeruginosa* in wound infections by injection method and UV spectrophotometric analysis.

Collagen is the major protein component of human skin. Collagenase is an enzyme produced by *P. aeruginosa*, which penetrates human skin by utilizing collagen (1). It breaks down the peptide bond and liberates hydroxyproline from collagen (2). Collagen acts as a source for the pathogenic bacteria to grow and survive in a wound. Since mushroom contains collagen, it can be used as a source to identify pathogenic *P. aeruginosa*.

Hence, in the present study, mushroom is used as model to determine the pathogenic strains of *P. aeruginosa*. Collagenase acts as the key factor in detecting the pathogenic strains of *P. aeruginosa* by collagen degradation. Based on degradation of collagen due to injection of pathogenic isolate of *P. aeruginosa* lesions are produced by the organism on the mushroom, which can be used as an identification system. Lesions are directly related to the pathogenicity of the strain (3). It has been found that the size of lesions is directly related to the measure of pathogenicity (4).

**MATERIALS AND METHODS**

The samples were collected from wound infections at the Government Hospital of Tambaram Sanatorium, Chennai and the strains of *P. aeruginosa* were isolated by standard biochemical procedures.

**Injection method**

The button mushrooms (*Agaricus bisporus*) were purchased commercially and thoroughly checked to be lesion free with fresh cap regions. Mushrooms were injected at the cap region respectively with 75µl of pathogenic, non-pathogenic and ATCC strains of *P. aeruginosa* (ATCC-27853) as well as *Escherichia coli*. A mushroom injected with sterile saline was used as negative control. The injection procedure was carried out using microinjection needle by saline soaking method and moist chamber method.
Shiva Priya and Sathish Kumar: Screening of pathogenic…..a novel approach

The injected mushrooms were soaked in a glass beaker with 10 ml of sterile saline (Saline soaking method) and the injected mushrooms were placed in a beaker provided with a moist chamber (Moist chamber method). The beakers were covered tightly and incubated at 37°C for 24 hours. The method of injection was carried out at an angle of 30° as depicted in the fig. 1.

![Fig 1: Injection method](image)

**Collagen extraction from mushroom**

The collagen protein was extracted from mushroom by salt precipitation method with the following steps like drying, grinding, extraction and purification followed by ninhydrin test.

**Drying the mushroom**

The mushrooms were dried to remove their moisture content by spreading it on a tray. The tray was placed in hot air oven for 4 hours at 80°C for further drying then the mushrooms were sun dried for 2-3 days.

**Grinding the mushroom**

The dried mushrooms were powdered finely using a mixer. The mushroom powder was used for the collagen extraction. The collagen extraction was carried out using salt precipitation. The extraction was carried out in neutral salt solutions by the gradual addition of sodium chloride.

**Extraction and purification of collagen**

The sodium chloride solution (0.45M) was prepared with a pH of 7.5 with stirring for 24 hours. The test tubes were filled with 3ml sample and the salt solution was added gradually along with regular stirring. After 24 hours the precipitated protein was subjected for purification. In the process of purification, all the added salts must be removed from the collagen. The precipitate was mixed with a buffer containing SDS, Tris-HCl and phenol for 3minutes. The precipitate that comes out of it will contain salt less concentrated collagen.

**Ninhydrin test**

A small quantity of extracted compound was allowed to react with 2% Ninhydrin. Ninhydrin solution was prepared by dissolving 0.8g of Ninhydrin in 10ml of acetone or ethanol. Ninhydrin solution was allowed to react with the extracted product. After 5 minutes the tubes were checked for any colour change. The negative control maintained was non collagen protein while positive control was pure collagen. Pure collagen was commercially purchased.

**UV spectrophotometric analysis**

Both extracted and pure collagen was used separately as sources for UV spectrophotometric analysis. The test tubes were provided with minimal media of collagen and sodium chloride. The different tubes were inoculated with pathogenic, non-pathogenic and ATCC strains of *P. aeruginosa* as well as *E. coli*. The test tubes were incubated at 37°C and the samples were collected for every hour until 5 hours. The readings were noted under UV spectrophotometry at a wavelength of 280nm. The trials were carried out in triplicates.

**Medium containing collagen**

Pure collagen medium and extracted collagen medium was prepared. Pure collagen medium constituted pure collagen powder, sodium chloride and agar powder while extracted collagen medium constituted with extracted collagen powder, sodium chloride and agar powder. The pathogenic, non-pathogenic and ATCC strains of *Pseudomonas aeruginosa* as well as *Escherichia coli* were streaked and incubated at 37° C for 24 hours.

**Statistical analysis**

One-way ANOVA was carried out for the triplicate values of UV spectrophotometric screening procedure using MS-Excel.
RESULTS

Fig. 2A & 2B: Positive reaction: Lesion was observed on mushroom at the site of injected area when injected with pathogenic strains of *P. aeruginosa*.

Fig. 3A & 3B: Negative reaction: No lesion was observed on mushroom at the site of injection when injected with non-pathogenic strains of *P. aeruginosa*.

**Injection method**

The strains isolated were found to be *Pseudomonas aeruginosa*. The injection method resulted in lesions at the site of injection only when injected with pathogenic strains of *Pseudomonas aeruginosa* (Fig. 2A & 2B) while the rest of the strains showed no lesion (Fig. 3A & 3B).

**Collagen extraction from mushroom**

The extracted collagen was confirmed by ninhydrin test showing positive result due to presence of hydroxyproline.

**UV Spectrophotometric analysis**

The OD values showed rapid decrease in case of pathogenic strains of *P. aeruginosa* while for the rest of the strains they remain unchanged in both the extracted and pure collagen (Fig. 4).

**Medium containing collagen**

Green coloured colonies were observed on the medium with collagen when inoculated with pathogenic strains while the rest of the strains failed to grow on the medium containing collagen. The results were obtained due to the production of collagenase enzyme by the pathogenic strains of *P. aeruginosa* present in the wound infection which degrades the collagen of the mushroom while the non-pathogenic strains fail to produce collagenase enzyme.

**Statistical analysis**

In accordance with the one-way ANOVA, there was significant difference at \( \alpha=0.05 \) between the OD
values of pathogenic isolates of \textit{P. aeruginosa} and ATCC strain of \textit{P. aeruginosa}. The null hypothesis was accepted since the critical value was smaller than that of F value.

**DISCUSSION**

The present study focussed on using mushroom as a host model to screen pathogenic isolates of \textit{P. aeruginosa} in wound infection. It is very difficult to handle laboratory animals for pathogenic identification hence mushroom can be used as a host model. Presence of collagen in mushroom has been reported (5). This method would be cost effective than other screening procedures.

In the past few years, a number of different invertebrate host model systems have been described. Experiments with plants, insects, protozoa, nematodes and slime moulds have recently come to the forefront in the study of pathogenic microorganisms (6). Among the alternative experimental models, plants were economic screening tool to identify putative virulence determinants (7).

A recent study suggested the use of plant (\textit{Lactuca sativa}) as a model for screening the pathogenic isolates of \textit{P. aeruginosa} from clinical samples in which the pathogenicity level varies from strain to strain (4). A similar work was carried out in the present study-using mushroom as a host model.

Inoculation procedure was performed using mushrooms in which 15 mushrooms were inoculated by dispensing a drop of appropriate \textit{Staphylococcus aureus} cell suspension on the cap for enterotoxin production (8). With the above reference, the inoculation procedure was carried out in this study on the cap region of mushroom.

Thus, the present study showed that mushroom can be used as a host model to screen pathogenic isolates of \textit{P. aeruginosa} present in wound infections. Future studies are warranted to screen other wound infecting pathogens capable of producing collagenase enzyme.

**ACKNOWLEDGEMENT**

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