"COMPARATIVE EVALUATION OF OSTEOGENIC POTENTIAL AND ANTIBACTERIAL EFFICACY OF A NOVEL GUIDED BONE REGENERATION MEMBRANE OF CHITOSAN, *CISSUS QUADRANGULARIS* AND *PUNICA GRANATUM* EXTRACT WITH A CONVENTIONAL MEMBRANE - AN IN VITRO STUDY"

BY

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LIST OF ABBREVIATIONS USED IN THE STUDY

GBR	*Guided Bone Regeneration*
C. quadrangularis	*Cissus quadrangularis*
P. granatum	*Punica granatum*
PEO	Polyethelene oxide
P. gingivalis	*Porphyomonas gingivalis*
ZOI	Zone of inhibition
ECM	Extracellular matrix
CT	Connective tissue
ROS	Reactive oxygen species
MAPK	Mitogen activated protein kinase
SEM	Scanning Electron Microscopy
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared
XRD	X-ray Diffraction
PEG	Polyethene glycol
PCL	Polycaprolactone
Et al	And all
Sr. no.	Serial number
SD	Standard deviation

ANOVA	Analysis of Variance
Ca	Calcium
P	Phosphate
CO_2	Carbon dioxide
PMN	Polymorphonuclear leukocytes
MSC	Mesenchymal stem cells
hrs	Hours
µm	Micrometers
wt	Weight
DMEM	Dulbecco's modified Eagle's medium
OD	Optical Density
MEPGP	Methanolic extract of *Punica granatum* peel
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
ALP	Alkaline Phosphatase
RANKL	Receptor activator of nuclear factor kappa beta (NFkB ligand)

ABSTRACT

STATEMENT OF PROBLEM

Up to 40% of osseointegrated implants may need Guided Bone Regeneration (GBR) as part of the patient's rehabilitation.[4] Having a membrane with antimicrobial activity is essential because, despite the high success rates, microbial infection is the main reason the GBR membrane failures. Conventional commercially available GBR membranes are vulnerable to biomaterial-centered infection, particularly when exposed to infected tissue or an area with high bacterial accessibility, such as the mouth cavity. As a result, there is a need developing GBR membranes with appropriate antibacterial capability to overcome to this problem. The membrane should also be capable of accelerating bone growth can be used to optimize the GBR approach even further.

PURPOSE

To evaluate the osteogenic potential and anti-bacterial activity of a prepared novel membrane from nanofibers of Chitosan and *Cissus quadrangularis* and *Punica granatum* extracts for Guided Bone Regeneration.

METHODS:

The novel GBR membrane was prepared with a base layer of *Cissus quadrangularis, Punica granatum extract* and gelatin. Then electrospun nanofibers of chitosan and Polyethylene oxide (PEO) were deposited on the base layer to form an the novel GBR membrane. The membrane was characterized using SEM analysis to determine the fiber size and morphology of the produced membrane. The novel GBR membrane was subjected to various tests and a commercially available conventional GBR membrane was used as a control group.

The tests included adhesion and proliferation of MG-63 cells to determine the osteogenic potential of the membrane and to compare it to that of the conventional membrane. Cell adhesion was done using trypan blue assay and cell proliferation using MTT assay at 24, 48 and 72 hrs.

The disk-diffusion test was performed to assess the effectiveness of 10% of *Punica granatum* extract incorporated into the novel GBR membrane against *P. gingivalis* after incubation for 48 hours and to compare it with that of a Conventional GBR membrane.

For the hydrolytic degradation studies scaffold's initial weight *(Wi)* before and after 45 days *(Wf)* of incubation in phosphate buffer solution was measured and the percentage weight loss was calculated and was compared with that of the conventional GBR membrane.

RESULT

These results were analyzed and subjected to statistical analysis. Comparison of cell proliferation and cell attachment of the novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours was done using two way ANOVA and Tukeys multiple posthoc procedures. It was found that there is a statistically significant difference ($p= 0.0001$) when the mean cell proliferation and cell attachment scores of novel GBR membrane is compared with the conventional GBR membrane at all the three time points and when comparison was done for each membrane across all the three time points.

Comparison of zone of inhibition of Novel membrane and commercially available membrane was done by Mann-Whitney U test showed that a significantly greater zone

of inhibition (p-value=0.0001) was given by novel GBR membrane as compared to the conventional GBR membrane.

Comparison of Gravimetric weight loss of novel GBR membrane and conventional GBR membrane was done by independent t test and it showed that there was a statistically significant difference in the gravimetric weight loss between novel GBR membrane and conventional GBR membrane. (p=0.0001)

CONCLUSION

It was concluded that the novel GBR membrane showed an increase in MG-63 cell attachment in 48 and 72 hours when compared to the conventional membrane. By MTT assay, the novel GBR membrane was found to be not cytotoxic to MG-63 cells and the viability of cells were maintained.

The novel GBR membrane was found to be an effective antibacterial agent against *Porphyromonas gingivalis* when compared to the conventional GBR membrane which showed no antibacterial property.

The hydrolytic degradation studies showed that the novel GBR membrane showed more gravimetric weight loss as compared to the conventional GBR membrane but it still maintained its gross structure for a period of upto 45 days.

KEYWORDS

Guided Bone Regeneration, Antibacterial activity, *Porphyromonas gingivalis,*

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INTRODUCTION

Dental implant placement to replace lost teeth has increased steadily through the last 30 years. Although it was discovered in the early 1930s, through archaeological excavations that the Mayan civilization used dental implants, there is an ever-increasing demand for novel implant innovations to this day.[1]

The success and long-term prognosis of implant treatment depends primarily on the anchorage of the implant in the jawbone, termed osseointegration. Branemark defined osseointegration as a direct association between a load-bearing endosseous implant and living bone at the microscopic level.[2]

It has been reported that a minimum of 1.8mm of facial bone is required for a successful and satisfactory prolonged implant functioning and aesthetic outcome.[3]

Nonetheless, bone loss or insufficiency, which is a manifestation of a variety of systemic and periodontal disorders, trauma, and malignancies, remains a major obstacle to osseointegration. A substantial volume of bone should exist at the sites of implantation for osseointegrated implants to have a favourable long-term prognosis. To restore the missing bone volume and enable complete implant integration and maintenance during functional loads, various treatments such as bone grafting procedures, alveolar distraction, and Guided Bone Regeneration (GBR) have been used.[4]

GBR is a surgical method for increasing and augmenting alveolar bone volume in locations designated for future implant placement or around previously placed implants. The GBR principle is based on guided tissue regeneration principles.[5]

Because soft gingival tissues grow more rapidly than bone, GBR membranes are positioned between the soft tissue and regenerating bone to prevent gingival tissues from intruding into the alveolar bone site.[6] Thus, bone is directed into the desired position by selectively excluding epithelial and connective tissue using bone grafting and barrier materials.[5]

It is expected that GBR is required as part of the patient's rehabilitation for up to 40% of osseointegrated implants.[4] Numerous investigations have revealed that the longevity of implants inserted in GBR-augmented sites are comparable to those reported for implants positioned in immaculate locations.[7,8,9] The majority of trials reported a success record of far more than 90% after at least one year of function for implants put in augmented locations, with a range of 79% to 100%. [4,10]

The GBR membrane is a vital part of the treatment. It is widely acknowledged that a biomaterial that is structurally and functionally similar to the extracellular matrix (ECM) promotes cell viability and speeds the process of wounded tissue regeneration.[11]

Thus, biocompatibility, cell-occlusion capabilities, host tissues consolidation, clinical manageability, space-making ability, and acceptable mechanical and physical properties are important aspects of the membrane used for GBR therapy. [4]

GBR membranes must be flexible enough to conform to the geometry of the bone deformity and stiff enough to retain the room for bone regeneration.[12]

The barrier membranes used for guided tissue regeneration essentially fall into three subsequent generations: The membranes of the first generation, are non-resorbable membranes which require another surgery to remove them. [13]

To minimize the necessity for surgical removal, the second generation membrane was created to be resorbable. Two bioresorbable membrane types are present which are natural and synthetic membranes. The ability of proteolytic enzymes to break down the scaffolds with natural mechanisms and the display of physiological signals for the establishment and preservation of cell functionality and structure are benefits of scaffolds produced from natural composites. However, there are various difficulties, including early breakdown, downgrowth of the epithelium along the material, and premature material loss. [13]

Synthetic resorbable barrier membranes made of polyesters and their copolymers are biocompatible, but they are not inert since some tissue reactions may be expected during degradation. There is also variability and lack of control over the rate of membrane resorption, which is influenced by factors such as the local pH and material composition. [13]

As the notion of tissue engineering evolved, third-generation membranes emerged to address the shortcomings of initial and succeeding generations of membranes. These membranes not only serve as barriers, but also as delivery systems, releasing specific agents like antibiotics, growth factors, adhesion factors, and so on at the wound site when needed in order to better orchestrate and direct natural wound healing.[13]

Current therapeutically used membranes made of nondegradable woven expanded polytetrafluoroethylene (ePTFE) require a second surgery to remove and have exposure/infection rates ranging from 20 to 44%. The membrane removal surgery may interfere with graft healing and is a burden on both the patient and the physician. Commercially available biodegradable collagen membranes may not need

to be removed, but their disintegration is rapid and unpredictable, and they may not function as a barrier for the 4-6 months required for complete healing and regeneration.[14]

Although conventional commercially available GBR membranes demonstrated promising results, they are vulnerable to biomaterial centered infection, particularly when exposed to infected tissue or an area with high bacterial accessibility, such as the oral cavity. As a result, developing GBR membranes with appropriate antibacterial capability could be a solution to this problem.[11]

Furthermore, a bioactive membrane capable of accelerating bone growth can be used to optimise the GBR approach even further.[15]

This study was carried out keeping in mind the need for a new potential GBR membrane with antibacterial activity and osteogenic potential.

Tissue engineering strategies utilize polymeric biomaterials to fabricate scaffolds which mimic the native extracellular matrix (ECM).[16] The GBR membranes should have high interconnected porosity in the nano-size range to be cell occlusive, have a nanofiber structure to mimic natural nanofiber structures in the extracellular matrix and have adequate mechanical strength. Keeping this in mind the process of electrospinning technique has been used in this study for producing nanofibers.[6]

This technique involves the application of high voltage to a polymer solution or melts resulting in the formation of fibres in the nanometer to micrometer range. The capability to fabricate scaffolds with well-defined architecture including controlled pore size, fibre diameter and topography which favours the cell growth and

the resemblance to the *in vivo* like the architecture of ECM makes electrospinning a unique technique.[16]

Chitosan (1-4,2-amino-2-deoxy-D-glucan) is a polysaccharide with a linear structure that is largely present in arthropod exoskeletons and is the deacetylated version of chitin. [17] Due to the material's porosity pattern, simplicity of chemical complexation, biodegradability, biocompatibility, antibacterial characteristics, and highly favourable attraction to in vivo molecules, chitosan has a longstanding background of use in tissue engineering applications. The electrospun chitosan nanofiber membranes generally offer acceptable adequate oral bioavailability and proteolysis rates over a clinically significant substantial amount of time. In comparison to other commercial membranes, the mechanical properties and the ability to handle them are subpar to support growing bones.[6]

Because of its stiff chemical structure, polycationic nature in solution, and unique intra- and intermolecular interactions, chitosan's electrospinnability is primarily limited. The formation of strong hydrogen bonds prevents the free movement of polymeric chain segments exposed to the electrical field, leading to jet break-up during the process. Moreover, the repulsive force acting between ionic groups on the polymer backbone is expected to hinder the formation of sufficient chain entanglements to allow continuous fibre formation, generally resulting in nanobeads instead of nanofibers.[20]

Combining chitosan with a second natural or artificial polymeric phase is the most effective and straightforward way to increase its electrospinnability. The eventual applications of chitosan nanofibers won't be restricted by this co-spinning agent, which is often an easily electrospinnable polymer like Polyethylene oxide

(PEO) which is a hydrophilic, non-toxic and biodegradable polymer having FDA approval for its use in various pharmaceutical formulations.[16]

Cissus quadrangularis (Vitaceae), is a sprawling shrub with a fleshy quadrangular thick stem. Due to its capacity to reconnect bones, the plant known as "Bone Setter" is also called as "Asthisamdhani" in Sanskrit and "Hadjod" in Hindi.[21] It is hypothesized that the primary component of *C. quadrangularis* is an isolated phytogenic steroid. The pharmacological properties of *C. quadrangularis* include excellent antioxidant properties, free radical scavenging potential, antibacterial activity, anti-osteoporotic activity, antitumor activity, analgesic activity, antipyretic activity, and bone fracture healing activity.[22,23] This unidentified anabolic steroid may operate on the bone's estrogenic receptors, according to studies on the healing of fractures. It has been observed that *C. quadrangularis* acts by stimulating metabolism and increasing mineral absorption of calcium, sulphur, and strontium by the osteoblasts in fracture healing. The effectiveness of *C. quadrangularis* on early ossification and bone remodelling has been reported.[24,25] Studies report the use of *C. quadrangularis* in reducing post-operative inflammation, pain and swelling, as well as fracture mobility and reported an accelerated healing of fractured jaw bones. Studies have concluded that it helps in reducing the period of immobilization and early rehabilitation.[26] *C. quadrangularis* increases osteogenesis, and there is evidence from prior literature to support its usage as a natural alternative or preventive therapy for bone disorders including osteoporosis.[27] According to research, *C. quadrangularis* extract may control osteoblastic activity by promoting the mineralization process and alkaline phosphatase activity.[28]

The pomegranate (*Punica granatum*) peel also contains flavonoid polyphenols, anthocyanins and tannins including ellagitannins, ellagic acid and punicalgin all of which have anti-bacterial functions.[29] Extracts of *P. granatum* peel produce optimal polyphenols in triggering osteoblastic proliferation by inducing osteocalcin, BMP-2 and collagen type I. This way it plays a role in bone remodelling and can contribute to bone repair.[30]

Commercially available GBR membranes are prone to biomaterial-centered infection, especially when exposed to infected tissue or an environment where bacteria are easily accessible, such as the oral cavity. Therefore, creating GBR membranes with adequate antibacterial properties may be a way to address this issue.[11] The GBR technique can also be improved even more by using a bioactive membrane that can quicken bone formation. [15]

The requirement for a new prospective GBR membrane with antibacterial activity and osteogenic potential guided the conduct of this work.

This study aimed to fabricate a novel GBR membrane with osteogenic and antibacterial properties with Chitosan as a scaffold[14], *Cissus quadrangularis* for its osteogenic potential[26] and *Punica granatum* peel extract for its anti-bacterial property.[29]

NEED FOR THE STUDY

- A sufficient volume of bone should be present at the sites of implantation for osseointegrated implants in order to obtain a satisfactory long-term prognosis.
- Different strategies, such as bone-grafting techniques and Guided Bone Regeneration (GBR), have been applied to restore the lost bone and allow implant placement even in cases with inadequate bone.[4]
- The GBR technique uses a membrane as a physical barrier to isolate the area around the bone defects. By positioning the membrane between the soft tissue and regenerating bone, the membrane prevents the invasion of fibrous connective tissue into the defect space, allowing the bone to be "guided" into the desired position.[11]
- Up to 40% of osseointegrated implants may need GBR as part of the patient's rehabilitation, according to estimates.[4] According to reports, the GBR method has a 95.5% implant survival rate.[31]
- Having a membrane with antimicrobial activity is essential because, despite the high success rates, microbial infection is the main reason the GBR membrane fails.[32-33]
- According to studies, *Porphyromonas gingivalis* makes up 37.5% of the bacteria linked to peri-implant disorders.[34]
- Conventional commercially available membranes lack the synergistic effect of being antibacterial as well as osteogenic in nature.
- Taking into consideration the need of the hour, this study aims to develop a novel GBR membrane with osteogenic and antibacterial properties with

Chitosan as a scaffold[14], *Cissus quadrangularis* for its osteogenic potential[26] and pomegranate (*Punica granatum)* peel extract for its anti-bacterial property.[29]

- Chitosan possesses important material properties, including biocompatibility, low immunogenicity, and controllable degradation and non-toxic degradation products.[14]
- *Cissus quadrangularis* has been shown to be effective in promoting early ossification and bone remodeling. It also stimulates metabolism and increases osteoblasts' uptake of the minerals calcium, sulphur, and strontium during the repair of fractures. [26]
- The pomegranate (*Punica granatum*) peel also contains flavonoid polyphenols, anthocyanins and tannins including ellagitannins, ellagic acid and punicalgin all of which have anti-bacterial functions.[29]
- Extracts of pomegranate (*Punica granatum*) peel produces optimal polyphenols in triggering osteoblastic proliferation by inducing osteocalcin, BMP-2 and collagen type-I. This way it plays a role in bone remodeling and can contribute to bone repair.[30]

HYPOTHESIS

NULL HYPOTHESIS

There is no significant difference in the osteogenic potential and antimicrobial properties of the novel guided bone regeneration membrane formed of Chitosan and *Cissus quadrangularis* and *Punica granatum* extracts when compared to a conventional resorbable membrane.

ALTERNATIVE HYPOTHESIS

There is a significant difference in the osteogenic potential and antimicrobial properties of the novel guided bone regeneration membrane formed of Chitosan and *Cissus quadrangularis* and *Punica granatum* extracts compared to a conventional resorbable membrane.

AIM AND OBJECTIVES

AIM OF THE STUDY

To evaluate the osteogenic potential and anti-bacterial activity of a prepared novel membrane of Chitosan and *Cissus quadrangularis* and *Punica granatum* extracts for Guided Bone Regeneration.

OBJECTIVES

1) To evaluate the osteogenic nature of the novel GBR membrane using MTT Assay as a measure of cell adhesion and proliferation.

2) To evaluate antibacterial property of the novel GBR membrane against *Porphyromonas gingivalis* using disk diffusion method.

3) To compare the osteogenic nature and antibacterial properties of the novel GBR membrane with a conventional resorbable GBR membrane.

REVIEW OF LITERATURE

1. **J M Dietzel et al in 2001** published a paper outlining the electrospinning of polyethylene oxide fibres, where they show that it is possible to control the deposition of sub-micron polymer fibres on a substrate and decrease the instability of the chaotic oscillation of the electrospinning jet by using an electrostatic lens element and collection target of opposite polarity. It is feasible to reduce or completely get rid of the bending instability by adding a secondary external field to the jet that has the same polarity as the surface charge. Utilizing wide angle x-ray diffraction, optical microscopy, and environmental SEM, fibre mats of electrospun PEO have been studied. [35]
2. **Ambarish Sanyal et al in 2005** carried out a study in which they examined the extract of the stem of *C. quadrangularis* in terms of its probable ability to enhance mineral growth. The stem extract is high in calcium ions (about 4% by weight) and phosphorus, both of which are required for bone fracture healing. Furthermore, they demonstrated that unique morphology calcite crystals can be generated by simply reacting the calcium ions present in the extract with CO2 bubbled directly through the extract. Furthermore, they demonstrated the creation of true biogenic CaCO3 crystals with exquisite morphology simply by bubbling CO2 into an aqueous stem extract of an indigenous medicinal plant. The presence of phosphorus in the plant can also be used to synthesize hydroxyapatite, utilizing conventional understanding of bone-fracture repair with advanced material synthesis techniques.[36]
3. **Ying Yang et al in 2006** used SEM and jet current measurements to examine the effects of critical variables on fibre formation during electrospinning of a polyethylene oxide solution (PEO). Particularly at humidity levels over 70%, the

ambient humidity has a significant impact on the development of fibres from PEO water solutions. Lowering the humidity or using a solvent other than water can lessen the affection. Both negative and positive voltages can be used in the process, however, the polarity affects the stability and fibre sizes. It was discovered that fluctuations in the current and fibre sizes correlated. Positive voltages result in a smaller average fibre diameter than negative voltages. The number of different sized diameter fibres was lower at needle to target distances of 30 cm than it was at 20 cm and 25 cm. It was discovered that short target distances prevented fibre development whereas long target distances led to a wider variation of fibre diameters.[37]

4. **T. C. Santos et al. in 2007** examined the impact on the activation of human polymorphonuclear neutrophils (PMNs) on chitosan-based membranes. Isolated human PMNs were cultured on freshly produced chitosan or chitosan/soy membranes. Chitosan's effect on the activation of PMNs was evaluated using measures of lysozyme and reactive oxygen species (ROS). The results established that osteopontin was generated by human PMNs treated with chitosan. Osteopontin is a glycosylated phosphoprotein that promotes cell adhesion or cell proliferation in specific cell types. Additionally, osteopontin may be involved in granulomatous inflammation. The in vitro results showed that PMNs triggered by granulocyte colony stimulating factor (G-CSF) and chitosan accumulated osteopontin mRNA and released osteopontin into their culture. The outcomes demonstrated a novel role for chitosan in regulating the early stages of wound healing, demonstrating that osteopontin is produced by migrating PMNs. Instead of the glucosamine residue, the N-acetyl-glucosamine unit of the chitosan molecule was thought to be responsible for the macrophage NO secretion.[38]

5. **Shu-Hua Teng et al in 2008** designed a three-Layered Membranes of Collagen/Hydroxyapatite and Chitosan for GBR. The central layer of the sandwich-shaped membrane was constructed of chitosan, and the top and bottom layers were made of collagen containing 20 weight percent hydroxyapatite (HA). Through a layer-by-layer filtration process, the aforementioned three layers were created into an integrated membrane from their separate slurries. Fourier-transform infrared (FTIR), x-ray diffraction (XRD) tests verified the membrane's phase and composition. The SEM analysis of the membrane's morphology revealed that it had a porous structure and structural integrity. The inclusion of the collagen/HA composite layers gave the membrane remarkable flexibility and bioactivity, while the chitosan layer guaranteed its high tensile strength and elastic modulus. These findings imply that the integrated membrane developed in this study could be used as a GBR material.[15]
6. **Duenpim Parisuthiman et al in 2008** directed a study in which the effects of ethanol extract of *C. quadrangularis* (CQ-E) on osteoblast differentiation and function were analyzed using murine osteoblastic cells. Alkaline phosphatase (ALP)activity and the extent of mineralized nodules were significantly increased in treated cells compared with controls. ALP activity was dramatically reduced by the addition of an extracellular regulated kinase inhibitor and an ap38 mitogen-activated protein kinase (MAPK) inhibitor. The increased ALP activity effect of CQ-E is probably mediated by MAPK dependent pathway, according to these data, which revealed that CQ-E may regulate osteoblastic activity via raising ALP activity and mineralization process.[28]
7. **Bhagath Kumar Potu et al in 2009** led a study where the effects of petroleum ether extract of *C. quadrangularis* on bone marrow mesenchymal stem cell

proliferation and osteogenic differentiation were evaluated. After being cultured for 4 weeks in conditions including or omitting *C. quadrangularis*, mesenchymal cells (MSCs) were stained for alkaline phosphatase. Von Kossa staining demonstrated the calcification of the extracellular matrix. A cell proliferation experiment (MTT Assay) was also performed on cultures of marrow mesenchymal stem cells in normal media and osteogenic media supplemented with this extract. The extracellular matrix calcification and differentiation of marrow mesenchymal stem cells into ALP-positive osteoblasts were both aided by treatment with this extract. The proliferation, differentiation, and calcification rates of cells cultured in osteogenic medium containing *C. quadrangularis* were higher than those of control cells. MSCs are converted into osteoblasts by *C. quadrangularis* extract. As early as 15 days following the start of the treatment, MSCs cultured in basal medium and treated with *C. quadrangularis* extract demonstrated calcium accumulation in the extracellular matrix.[27]

8. **Hua Hong et al in 2009** conducted a study with the objectives of improving the embrittlement of the chitosan membrane by addition of polyethylene glycol (PEG) to chitosan and to quicken the rate of deterioration by combining gelatin with the binary chitosan-PEG mixture. In the case of the chitosan-PEG-gelatin (CPG) membrane, the tensile compliant increased. According to in vitro degradation results, however it disintegrated more quickly and much more than chitosan membrane. Comparing the CPG membrane to the chitosan membrane, it demonstrated a considerable improvement in both flexibility and degradation. With the help of C2C12 cells, cell adhesion, vitality, and proliferation onto the surface of the CPG membrane were assessed in vitro and measured using a methyl thiazolyl tetrazolium (MTT) reduction test. [39]

9. **Sh. Abdollahzadeh et al in 2010** conducted a study to determine the effectiveness of *P. granatum* peel methanolic extract (MEPGP) against *Candida albicans, Streptococcus mutans, Staphylococcus aureus, Streptococcus salivarius, Streptococcus sanguinis, and Staphylococcus epidermidis*. Paper discs containing MEPGP at doses of 4 mg/ml, 8 mg/ml, and 12 mg/ml were subsequently put on media after the aforementioned oral organisms were cultivated in blood agar and Mueller Hinton media. The agar disc diffusion method was used to assess the antibacterial activity. MEPGP demonstrated antibacterial activity against *S. aureus* and *S. epidermidis* at all concentrations. The only concentrations of MEPGP that were effective against *L. acidophilus, S. mutans*, and *S. salivarius* were 8 mg/ml and 12 mg/ml. Additionally, MEPGP did not suppress *A. viscosus* or *C. albicans* at all concentrations.[40]
10. **Mehdi Pakravan et al in 2011** conducted a study of chitosan/PEO electrospinning. A highly deacetylated chitosan in 50% acetic acid was electrospun at moderate temperatures in the presence of a low content of polyethylene oxide (10wt% PEO). A strong hydrogen bonding between chitosan and PEO chains is formed, making their blends electro spinnable. Increasing chitosan/PEO ratio led to a fiber diameter reduction. Producing defect free nanofibrous mats from the electrospinning process and with high chitosan content is particularly promising for antibacterial film packaging and filtration applications.[20]
11. **H. Hülya Orak et al in 2011** conducted a study which assessed three different *P. granatum* genotypes to determine their antibacterial and antifungal properties. Three bacteria strains, *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Salmonella Enteritidis* (ATCC 13076) and two fungi

Aspergillus parasiticus (NRRL 2999) and *Aspergillus parasiticus* (NRRL 465), were used to investigate the antibacterial and antifungal activity of peel extracts. Against all of the studied bacterial and fungal strains, all extracts demonstrated outstanding antibacterial and antifungal activity. The peel extracts from genotype 19-66 had the highest total phenolic content among the extracts, but their antibacterial activity was the lowest. Additionally, its acidity was found to be noticeably lower than that of other *P. granatum* peels. This finding could help to explain why the presence of organic acids could enhance the antibacterial effects of phenolic compounds.[41]

12. **Valcinir Vulcani et al 2012** conducted a study sought to create nanostructured membranes based on mixtures of chitosan and polyoxyethylene (PEO), assess their thermal and morphological characteristics, and determine their in vitro biocompatibility through agar diffusion cytotoxicity tests for three different cell lines. The result was a nanostructured fibrous membrane with fibers of around 200 nm in diameter, a rough surface, and a thickness of one to two millimeters. The cytotoxicity test results demonstrated the non-toxicity of the chitosan/PEO membranes to the cells investigated in this work. Additionally, the electrospinning method was successful in producing nanostructured chitosan/PEO membranes that, in preliminary in vitro experiments utilising cell lines, demonstrated biocompatibility.[42]
13. **Arawatti Siddaram et al in 2012** led a study in which clinical evaluation was done to check the efficacy of the Asthishrankhala (*C. quadrangularis Linn*) for early mobilization in the management of Colle’s fracture. Colle's fracture is a fracture at the distal end of the radius, at its cortico-cancellous junction with typical displacement. 30 registered, clinically diagnosed and confirmed patients

of Colle's fracture were selected for the present clinical trial. They were randomly divided in following three groups of 10 patients each, Group A treated with only external application, Group B-treated with only internal application and Group C-treated with both external and internal application of *C. quadrangularis*. At the end of the study, it was found that results were highly significant in group B & C (combined therapy) and can be concluded that Asthishrankhala is effective in the management of Colle's fracture as it is safe, cost effective and free from any side effects.[43]

14. **Nimisha Singh et al. in 2013** investigated the osteogenic potential of *C. quadrangularis*. In their study, 60 individuals with mandible fractures were separated into two groups. Group 1 patients were given *C. quadrangularis* capsules, and fracture repair was measured by osteopontin expression during treatment. Group 2 served as the control group. In the study, clinical and radiological data suggested that group 1 had better fracture healing. All of the group 1 samples evaluated for osteopontin expression utilising western blot analysis and flow cytometry revealed considerable amounts of osteopontin protein expression and CD4+ T cells expressing osteopontin, respectively. They determined that *C. quadrangularis* speeds up fracture healing and causes early callus remodelling.[44]
15. **M. Kharaziha et al in 2013** developed novel aligned nanofibrous composite membranes for GBR. The membranes were developed through electrospinning of PCL-forsterite nanopowder. The membranes were characterized with regard to structural and mechanical properties, degradation, bioactivity and cellular interactive responses. Results showed that optimized nanofibrous composite membrane with significantly improved tensile strength and elastic modules was

achieved through addition of 10 wt.% forsterite nanopowder into PCL membrane. Aligned fibrous membranes revealed smaller fibre sizes and significantly enhanced and anisotropic mechanical properties. Furthermore, composite nanofibrous membranes possessed significantly improved cellular responses in terms of attachment, proliferation and mineralization of pre-osteoblasts compared to PCL membrane.[12]

16. **J. Janani et al in 2013** tested the antibacterial efficacy of pomegranate rind, seed, and pith extracts against a number of oral pathogens, including *Staphylococcus aureus, Staphylococcus epidermis, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Candida albicans*. The oral pathogens were cultivated in Mueller-Hinton medium before extracts were added to the medium on paper discs at concentrations of 4 g/ml, 8 g/ml, 12 g/ml, and 100 g/ml. The disc inhibition method was used to assess the antibacterial activity. Relatively to diluted extracts, 100 mg/ml concentrations of Methanolic Rind Extract (MRE) and Aqueous Rind Extract (ARE) were found to be efficient against the chosen oral bacteria. According to the study, pomegranate rind extract can effectively treat oral diseases.[45]

17. **Shiqing Ma et al in 2014** developed a novel asymmetric chitosan GBR membrane that consists of a loose layer that can enhance cell adhesion and stabilize blood clots and a dense layer that isolates the bone defect from the invasion of surrounding connective fibrous tissue. The chitosan membrane was cross-linked by sodium tripolyphosphate (TPP) which increased the membrane's tensile strength. By using a scanning electron microscope (SEM), FT-IR, XRD tests, and a tensile test machine, the physical characteristics of an asymmetric chitosan membrane were evaluated. The membrane's biocompatibility was

assessed using the MTT assay and Live/Dead cell staining for MC3T3-E1 osteoblasts cultivated on the membrane. An in vitro investigation revealed the membrane's high biocompatibility. The asymmetric chitosan membrane is a promising GBR membrane for bone regeneration.[46]

18. **Hemal R. Brahmkshatriya et al in 2015** conducted a study the purpose of which was to evaluate the effect of *C. quadrangularis* in healing process of maxillofacial fracture. All of the patients received postoperative care, antibiotics, and analgesics along with the open reduction internal fixation technique. There were two groups of patients. For six weeks, Group 1 received one 500 mg capsule of *C. quadrangularis* three times per day, while Group 2 (the control group) received no additional medication. Pre- and post-operative assessments of pain, edema, fragment mobility, serum calcium, and serum phosphorus were made on days 1, 21, and 45. Compared to Group 2, Pain, Swelling, and Fragment Mobility were less in Group 1. The results showed that *C. quadrangularis* aids in lowering discomfort, edema, and fracture mobility and speeds up the healing of fractured jaw bones. Serum calcium and phosphorus levels were likewise high, and Group 1's bone healing was clearly visible on day 21 as compared to the control group It does not produce any toxic effects when used orally and due to its various inherent pharmacognostic properties.[26]
19. **Hengjie Su et al in 2016** conducted in vitro and in vivo evaluations of a novel post-electrospinning treatment to improve the fibrous structure of chitosan membranes for GBR. A novel process was investigated for acidic salt removal from chitosan electrospun in 70% trifluoroacetic acid (TFA) by treating with triethylamine (TEA)/acetone and di-tert-butyl dicarbonate (tBOC) instead of the common Na2CO3 treatment. Membrane degradation after 4 weeks in PBS with

100 μg ml−1 lysozyme and osteoblastic proliferation were similar between TEA/tBOC-treated and Na2CO3-treated membranes. Rat 8 mm critical-sized calvarial defects covered with TEA/ tBOC-treated chitosan membranes prevented soft tissue infiltration and supported new bone growth similar to a commercial collagen membrane at 12 weeks based on microCT analyses. Hence our novel TEA/tBOC process significantly improved nanofiber structure and mechanical strengths of electrospun chitosan membranes as compared to Na2CO3 treated membranes, without affecting *in vitro* degradation or cytocompatibility, improved membrane mechanical properties to be greater than a commercial PLA membrane and to be in range of commercial collagen membranes and supported calvarial bone defect healing similar to collagen.[19]

20. **Livia A. P Gomes et al in 2016** conducted a study aimed to evaluate the antimicrobial activity of pomegranate glycolic extract (PGE) against the periodontal pathogen *P. gingivalis* by using *Galleria mellonella* as *in vivo* model. For each group, fifteen larvae were employed. The right proleg received PGE injections, while the left last proleg received an inoculation with *P. gingivalis* suspension. After that, the larvae were kept in the dark at 37°C. Any dose of PGE injection statistically increased the survival rates of the larvae. The analysis of the data revealed that enhanced larval survival rates were evident at all PGE concentrations. Using a *G. mellonella* in vivo model, the PGE demonstrated antibacterial activity against *P. gingivalis.*[47]
21. **H. E Schneider et al in 2016** revealed the results of electrospinning poly (ethylene oxide), a material that may be used as scaffolding in tissue engineering. The applied electric field, solution flow rate, separation between the syringe tip and the collector, and collector geometry are process variables that can be

changed to alter the diameter of the fibre. The PEO nanofibers were produced using polymer solutions with concentrations ranging from 14% to 22%. (by weight). The samples were then examined under a scanning electron microscope. The diameter of the fibres increases alongside concentration in this regime of fibre production.[48]

22. **Sedef Tamburaci et al in 2017** fabricated novel composites membranes composed of chitosan matrix and polyhedral oligomeric silsesquioxanes (POSS) by solvent casting method. The surface roughness, protein adsorption, and swelling capacity of membranes were all improved by the introduction of Octa-TMA POSS nanofiller into the chitosan matrix, according to the results. Plasma protein adsorption on the surface was enhanced by the addition of POSS filler to the polymer matrix. With chitosan/POSS composites, high cell viability values were attained. Additionally, results from tests on cell growth and ALP activity revealed that POSS incorporation greatly raised the ALP activity of Saos-2 cells grown on chitosan membranes. This could be thought of as a potential candidate for GBR applications.[49]
23. **Ibrahim Elgali et al in 2017** evaluated the components and biological processes involved in GBR. According to experimental findings, various alterations of the mechanical and physicochemical characteristics of membranes may encourage bone regeneration. New experimental results further imply that the membrane compartment itself, rather of just acting as a passive barrier, actively promotes the healing processes in the underlying defect during GBR. An important approach in this area of research is the systematic optimization of barrier and bioactive characteristics in membrane materials.[4]

24. **Shue Jin et al in 2018** tested electrospun silver ion-loaded calcium phosphate/chitosan antibacterial composite fibrous membranes for GBR in an in vitro investigation. By incorporating silver ion-loaded CaP via a one-step electrospinning process and then crosslinking with vanillin, a novel type of biomimetic and bioactive silver ion-loaded calcium phosphate/chitosan (Ag-CaP/chitosan) membrane with antibacterial capacity was effectively generated. The Ag-CaP/CS membranes demonstrated a sustained release of Ag+, which inhibited staphylococcus mutans adherence and growth. Cell adhesion and MTT assay findings demonstrated that the Ag-CaP/CS membranes were compatible with bone marrow stromal cells. Thus, these electrospinning Ag-CaP/CS nanofiber membranes have a high potential for use in GBR.[11]
25. **Remya KR et al in 2018** produced hybrid polycaprolactone/polyethylene oxide scaffolds with variable fibre surface shape, enhanced hydrophilicity, and biodegradability for bone tissue engineering applications in an in vitro investigation. On blended scaffolds, improved biological response with good cell adhesion, viability, and proliferation of human osteoblast sarcoma (hos) cell lines was demonstrated. The study found that incorporating PEO on PCL scaffolds improved the properties of PCL and facilitated the fabrication of scaffolds with improved hydrophilicity, mechanical properties, and tunable degradation profiles, as well as better cell viability, making it an ideal candidate for bone tissue engineering applications.[16]
26. **Tulasi Nayak et al in 2019** led a study to determine whether *C. quadrangularis* promotes quantifiably early healing and the benefits of including it into a routine mandibular fracture treatment plan. In this study, two groups of patients with mandibular fractures who had received equivalent care were compared: one

group underwent surgery along with oral *C. quadrangularis*, and the other group underwent simply surgery. 30 consecutive consenting patients with isolated traumatic mandibular fractures participated in the study. An Ayurvedic practitioner gave 15 patients in the *C. quadrangularis* group two capsules of it (250 mg each) b. d. for 42 days following trauma. The Ayurvedic medicine was not administered to the control group. In this trial, biochemical results suggested that patients who had eaten *C. quadrangularis* formed calluses more quickly, but there was no discernible radiological or clinical improvement when compared to the control group.[50]

27. **B. A Dikici et al in 2019, c**reated a bilayer polycaprolactone (PCL) membrane by combining the processes of electrospinning and emulsion templating. First, an air plasma treatment was applied to a thick polymerized high internal phase emulsion (polyHIPE) made of photocurable PCL, which was reported to enhance cellular penetration. The best solvent mix for electrospinning a nanofibrous PCL barrier layer atop PCL polyHIPE was subsequently identified. The biocompatibility and barrier properties of the electrospun layer were demonstrated over a period of four weeks in a test tube using histological staining. After in vitro evaluations of cell viability and PCL polyHIPE's propensity to support blood vessel ingrowth, a chick chorioallantoic membrane experiment was employed to further analyze cell infiltration. The results showed that the PCL polyHIPE was able to sustain cell infiltration, calcium and mineral deposition in bone cells, and blood vessel development through pores for at least four weeks.[51]
28. **Meifei Lian et al in 2019**, fabricated a unique bi-layered membrane for GBR applications that has osteogenic and antibacterial properties. The membrane's

loose layer (LL) was made of conjugated electrospun poly (lactic-coglycolic acid) (PLGA)/gelatin nanofibers incorporating dexamethasone-loaded mesoporous silica nanoparticles, while the dense layer (DL) was made of conventionally electrospun PLGA nanofibers loaded with the broad-spectrum antibiotic doxycycline hyclate. In contrast to the DL membrane, which remained dense enough to act as a barrier, the LL membrane, according to morphological studies, had a porous and loosely packed structure that was beneficial for cell attachment and infiltration. The composite membrane also displayed increased osteoinductive characteristics for rat bone marrow stem cells, according to in vitro studies on osteogenesis. Alkaline phosphatase activity, calcium deposition, and osteocalcin expression improvements all corroborated this. Through in vitro antimicrobial experiments, the effective antibacterial efficiency of the DCH/PLGA membrane was identified. As a result, its osteogenic and antibacterial properties were combined.[52]

29. **Sri Hernawati et al in 2020** investigated the inhibitory effects of red pomegranate (*P. granatum*) extract on the growth of *Fusobacterium nucleatum* and *P. gingivalis* in vitro. Using a disc-diffusion approach, this study sought to determine the inhibitory efficacy of pomegranate peel extract on the development of *F. nucleatum* and *P. gingivalis*. The discs were soaked in various quantities of red pomegranate extract and in petri dishes containing an agar medium and inoculated with *P. gingivalis* bacteria, 0.2% chlorhexidine (positive control) and sterile distilled water (negative control) were inserted. Growth of *F. nucleatum* and *P. gingivalis* are inhibited by pomegranate extract. [29]

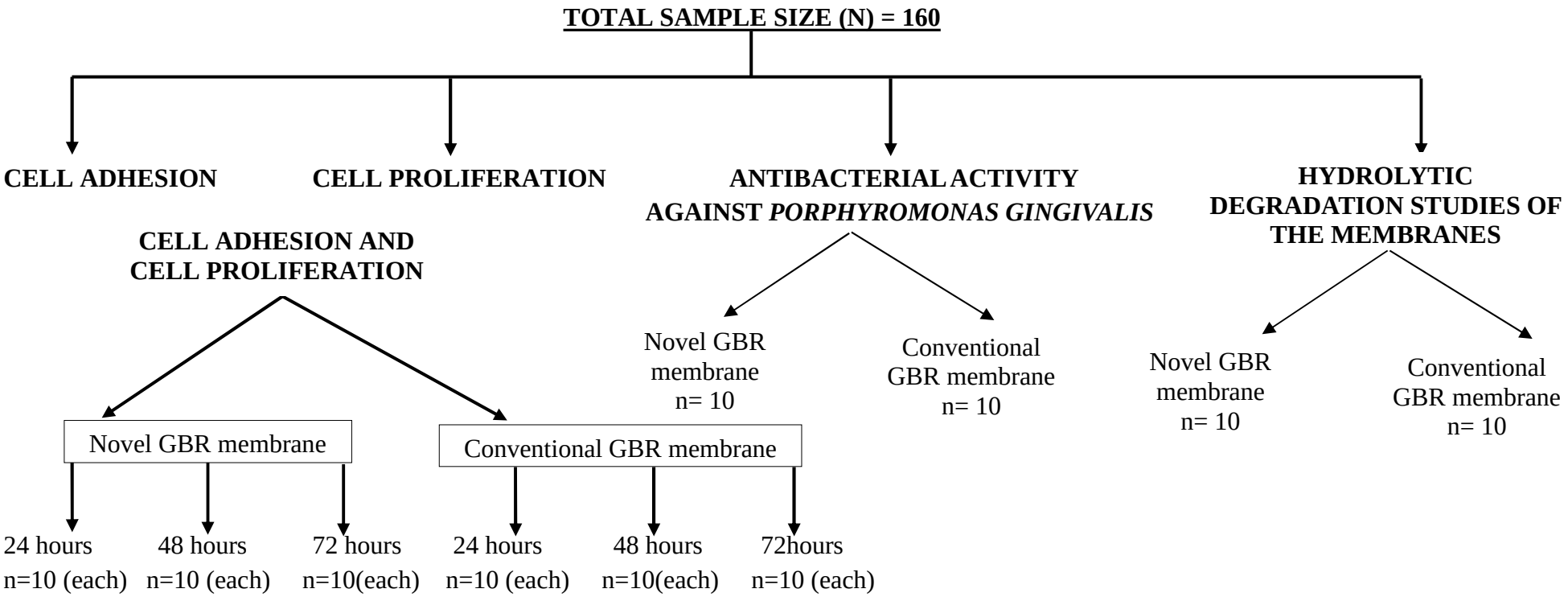
MATERIAL AND METHODOLOGY

SOURCE OF DATA:

1. KLE Academy of Higher Education (KAHER'S)

- The Department of Prosthodontics, Crown and Bridge, KLE Vishwanath Katti institute of Dental Sciences, Belagavi.
- Dr. Prabhakar Kore's Basic Science Research Centre, Belagavi.
- Department of Pharmaceutics, KLE college of Pharmacy, Belagavi.

2. Department of Mechanical Engineering, Indian Institute of Technology (IIT)-Goa.


TOTAL SAMPLE SIZE (N) = 160
CELL ADHESION
CELL PROLIFERATION
ANTIBACTERIAL ACTIVITY AGAINST *PORPHYROMONAS GINGIVALIS*
HYDROLYTIC DEGRADATION STUDIES OF THE MEMBRANES
CELL ADHESION AND CELL PROLIFERATION
Novel GBR membrane
Conventional GBR membrane
24 hours n=10 (each)
48 hours n=10 (each)
72 hours n=10(each)
24 hours n=10 (each)
48 hours n=10(each)
72hours n=10 (each)
Novel GBR membrane n= 10
Conventional GBR membrane n= 10
Novel GBR membrane n= 10
Conventional GBR membrane n= 10

MATERIALS AND ARMAMENTARIUM

MATERIALS:

- Chitosan
- *Cissus quadrangularis extract*
- *Punica granatum* (Pomegranate) peel extract
- Poly ethelene oxide (PEO)
- Acetic acid
- Gelatin
- Commercially available standard resorbable GBR membrane
- MG 63 osteoblast like cell lines
- Dulbecco's Modified Eagles Medium (DMEM)
- 20% foetal bovine serum
- MTT reagent (tetrazolium bromide)
- Dimethyl sulfoxide (DMSO)
- Trypan blue stain
- Phosphate buffer solution (PBS)
- Deionized water
- *P. gingivalis* bacterial strains (ATCC No.33277)

Table 1: Materials used in the study.

MATERIALS	DESCRIPTION	MANUFACTURER
Chitosan	Code no: 02697	LOBA Chemie Pvt. Ltd, Mumbai
Cissus quadrangularis extract	Batch: KBAL-0919-009	Kshipra Biotech PVT. LTD, Indore
***Punica granatum* (Pomegranate) peel extract**	Batch no: KBPL/PG/01042	Kshipra Biotech PVT. LTD, Indore
Poly ethelene oxide (PEO)	Batch No.: MKCN4119	Sigma Aldrich
Gelatin	Product code:1314-13	SBF, Pharma Pvt. Ltd, Ahmedabad, Gujarat
Acetic acid	Product code:11040	Molychem, Mumbai
Commercially available standard resorbable GBR membrane	Bioresorbable collagen membrane Batch No. HG.055	Healiguide
MG 63 cell lines	-	NCCS, Pune
Dulbecco's Modified Eagles Medium (DMEM)	Ref:AL007S LOT No: 0000515574	Hi-media Laboratories Pvt Ltd
MTT reagent (tetrazolium bromide)	LOT No.: - 0000173715	Hi-media Laboratories Pvt Ltd
20% foetal bovine serum	LOT No.: 42G5176K	Gibco by life technologies
Antibiotic- antimycotic solution	LOT No.:000D482174	Hi-media Laboratories Pvt Ltd
Trypan blue stain	LOT No.: - 2024334	Hi-media Laboratories Pvt Ltd
Phosphate buffer solution (PBS)	LOT No.: 0000457707	Hi-media Laboratories Pvt Ltd
Trpsin- EDTA solution	LOT No.: 0000297540	Hi-media Laboratories Pvt Ltd
Distilled water	Batch No:-007M15	Rankem Chemicals, Avantor, India
Mueller Hinton agar	Culture media	Hi-media
Blood agar	Culture media	Hi-media

ARMAMENTARIUM:

- Electrospinning device
- Sputter coater
- Scanning Electron Microscope
- Microscope
- Digital analytical balance
- CO_2 Incubator
- Spectrophotometer
- Laminar air flow
- Haemocytometer
- Tissue culture plate
- Anaerobic jar
- Vacuum oven

Table 2: Armamentarium used in the study.

MATERIALS	MANUFACTURER
Electrospinning device	E-SPIN Nanotech Pvt. Ltd - Super ES-1
Scanning Electron Microscope	Thermofischer (FEI) Quanta FEG 200
Sputter coater	Leica EM ACE 200
Microscope	3-D microscope HRX-01, HIROX Europe
Digital analytical balance	Wensar, an ISO 9001:2015 certified
Laminar air flow	Quesst International, Bangalore
Haemocytometer	KLEU/BSRC
Micro Titre Plate Reader	KLEU/BSRC/HF/2012-13
CO_2 Incubator	New Brunswick- an Eppendorf company
Tissue culture plate	Tarsons company, Korea
Electric Loop sterilization	Hi-Media, Mumbai
Anaerobic jar	Hi-Media, Mumbai

METHODOLOGY:

A) PREPARATION OF THE MEMBRANE-

1) Preparation for base layer of membrane
    a) MIC and MBC of *Punica granatum* extract

2) Solution preparation for Electrospun membrane

3) Electrospinning for nanofibre production

B) Characterisation of the fabricated membrane

C) Osteoblastic adhesion and proliferation on membranes.

1.MTT assay

2.Cell attachment assay

D) Antibacterial activity against *Porphyomonas gingivalis*- DISK-DIFFUSION TEST

E) Hydrolytic degradation studies

1. Preparation for base layer of membrane:

Pure dry *Cissus quadrangularis* stem extract (Fig 1) was procured Kshipra Biotech PVT.LTD:KBPL/PI156/20-21

Punica granatum peel extract (Fig 1) was procured from Kshipra Biotech PVT.LTD: KBPL/PG/01042

a) MIC and MBC of Punica granatum extract

Punica Granatum peel extract (Fig 1) was procured (Kshipra Biotech PVT.LTD:KBPL/PI156/20-21). The assessment of antimicrobial properties of the *Punica granatum* extract was carried out using standard strain of *P. gingivalis* (ATCC 33277). These strains were maintained on nutrient agar slope and thioglycolate medium respectively at 4^0 C. These strains were sub cultured onto agar plates at 37^0 C for typical colony morphology and used for the study. Anti-microbial activity was tested as per CLSI guidelines (Clinical Laboratory Standard Institute Guidelines) by using Mueller Hinton Agar on 90mm diameter petri dishes.

The sample of *Punica granatum* extract was vortexed in the centrifuge tube for 30 seconds. Following which 96 well culture plates were taken and 10 wells were allotted for each extract in triplicates. A volume of 100 µl broth was added in all the wells and 100 µl of each extract was added in first well, double dilution scheme was implied for the extract to requisite concentrations up to the 10th well. Therefore, concentration in subsequent wells reduced by 50 percent. A volume of 10µl of inoculum was added to all 10 well and kept for incubation in McIntosh and Fildes anaerobic jar for 2 days. After 48 hours, resazurin dye was added and observed for colour change from blue/violet to slight pink/magenta except positive control. The concentration at which resazurin is just reduced to resafurin by colour change from

blue to pink taken as MIC value.[53] Results recorded in triplicate outcomes and mean value was taken.

The antimicrobial activity assessed using Resazurin dye method was confirmed with disc diffusion method. The disc diffusion method was carried out using Mueller Hinton Agar.

The values of MIC, MBC were recorded at 10 % for *Punica granatum* extract**.**

The values were utilized as this extract was incorporated into the formation of the membrane.

After the concentrations for antibacterial efficacy were established the base layer of the membrane was prepared. 3 wt% of *Cissus quadrangularis* extract [54] and 10 wt % of *Punica granatum* extract was mixed thoroughly using the magnetic stirrer.

2 wt % of Gelatin (SBF, Pharma Pvt. Ltd, Ahmedabad, Gujarat) in an aqueous solution was incorporated into the mixture while continuous stirring to form a homogenous mixture of *C. quadrangularis* extract, *P. granatum* extract and gelatin.

A 20 cm diameter teflon tray was used to cast the mixture, and it is gently spread to create an even liquid coating. The foundation layer of the membrane was created by vaporizing the layer in an oven at 50°C for 8 hours

2) Solution preparation for Electrospun membrane:

Separate solutions of chitosan (LOBA Chemie Pvt. Ltd, Mumbai, Code no: 02697) and PEO (Sigma Aldrich Batch No.: MKCN4119) were made at a concentration of 6 wt% in 50 wt% aqueous acetic acid. To guarantee complete solute dissolution and produce homogenous liquid, the mixing of the two liquids was carried out at room temperature using a laboratory magnetic stirrer for 18 to 24 hours. (Fig 3) The prepared solutions were maintained in a sealed container at ambient

temperature for 4 hours to allow for degassing. Then, chitosan/PEO solution was created by combining the two liquids in ratios of 4:1 and 1:1, respectively.

3) Electrospinning for nanofibre production

- Electrospinning was carried out utilizing a lateral setup that included a configurable micro-syringe piston and an adjustable high DC voltage source. (E-SPIN Nanotech Pvt. Ltd - Super ES-1) (Fig 5)
- The solutions were injected using a 20-gauge flat-tip needle and a 10 mL syringe with a Luer-Lock attachment with a 12 mm syringe width. The syringe was attached to the micro-syringe compressor with a grip and connected with an automotive clip.
- Fiber mats were gathered on tinfoil that was fastened to a barrel collector, onto which base layer of the membrane prepared earlier is attached.
- Flow rates of 1 mL/hour was used and voltages between 17 to 22 kV were the operating variables.
- All tests were performed with humidity levels of 46% and atmospheric pressure, the temperature was maintained at 24 degrees Celsius.
- The fibre presence was then confirmed using the digital microscope at 100µm. (Fig 7 and 8)

B) **Characterisation of the fabricated membrane -** The determination of fiber size and morphology of the produced membrane will then be done by Field Emission Scanning Electron Microscope (FESEM) analysis (Thermofischer, FEI). (Fig 11) Sputter coating of 4 nm was done with gold palladium alloy using sputter coater (LEICA EM ACE 200). FESEM was operated at 20 kV. (Fig 9 and 10)

C) Osteoblastic adhesion and proliferation on membranes.

Maintenance of cell lines: Cell lines of MG- 63 were procured from National Center for Cell Sciences (NCCS) Pune. The data sheet with sixteen short tandem repeat (STR) loci proved to be 100% matching with ATCC STR profile. After procuring the cell lines, maintenance and subculturing of the cells was done by preparing 100ml of complete media comprising of DMEM (89ml) (Hi-media, LOT No: 0000515574) Foetal bovine serum (10ml), (Gibco by life technologies LOT No.: 42G5176K) and antibiotics (1ml) (Hi-media, LOT No.:000D482174**).** The cells were maintained in 5% CO_2 incubator. All the procedure of cell culture was performed in Class II cabinet by considering all the aseptic conditions. On cells reaching the 85% confluency, trypsinization was performed using trypsin and subculturing was done and cells were observed under inverted microscope. (Fig 16)

MTT Assay (Fig 17): During the cells in log phase of growth MTT assay was performed. MTT assay was performed for 24 hours, 48 hours and 72 hours in 92 well plates.

Initially on 96 well plates markings were done by considering negative control (without adding membrane), positive control (Conventional GBR membrane) and testing membrane (Novel GBR membrane) in triplicates wells. The novel GBR membranes and conventional GBR membranes were placed inside the wells of 96

well plates. (Fig 18) Trypan blue was performed and number of viable cells were counted and calculated and seeded 5000 cells per well. Later complete media was added to make the volume of 150 microliter. After 24 hours 20ml of MTT dye, the plate was wrapped in silver foil as MTT is photosensitive and incubated for four hours. The supernatant was slowly removed and discarded without disturbing the formazan crystals, 100ml of DMSO was added to dissolve the crystals and by using Spectrophometer at around 570nm reading was obtained and calculated the proliferative index by dividing the Optical Density (OD) of test with OD of control multiplied by 100. Similar procedure was performed for 48hours and 72 hours. The percentage of proliferative index obtained in an excel spread sheet for further data analysis.

Cell Attachment Assay: After 24 hours and 48 hours and 72 hours, for the negative control wells trypsin was added and the wells containing membrane cells around the membranes was carefully removed and discarded. The membranes were transferred in small Eppendorf bulb and trypsin was added to detach the cells on the membrane, using trypan blue assay (Fig 19) cell obtained were counted and entered in an excel spread sheet for data analysis.

D) **Antibacterial activity against *Porphyomonas gingivalis* - DISK-DIFFUSION TEST-**

- The disk-diffusion test was performed to assess the effectiveness of 10% of *Punica granatum* extract incorporated into the novel GBR membrane against *P. gingivalis* standard bacterial strains and to compare it with that of a conventional GBR membrane.
- The blood agar plate was streaked with standard bacterial strains.

- After the inoculum dries, 6mm diameter and 3mm depth well were punched with a sterile cork borer.
- The sterile commercially available membrane and a novel GBR membrane were punched of diameter 5 mm and a width of 2 mm (ASTM F67). (Fig 20)
- Each plate contained two membranes:

1. *Experimental group-* The novel GBR membrane

2. *Control group-* The conventional GBR membrane.

- The plates were then be incubated for 48 hours at 37°C. (Fig 21)
- There was absence of bacterial colonies around the antibacterial agent forming a zone of inhibition. When the antibacterial activity is present, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is called the zone of inhibition which was measured in millimeters.

E) HYDROLYTIC DEGRADATION STUDIES[16]

- Hydrolytic degradation studies were done in Phosphate buffer solution (PBS) at 37°C.
- 20mm-diameter circular specimens were maintained at 37 °C in a beaker containing 30mL of pH 7.4 phosphate buffer solution. (Fig 23)
- The scaffold's initial weight *(Wi)* (Fig 22) before and after 45 days *(Wf)* of incubation in PBS was measured (Fig 24) and the percentage weight loss was calculated.

- The recovered scaffoldings were air-dried, rinsed with deionized water, and their weight was measured.
- The following equation was used to estimate the percentage of weight loss:

Gravimetric weight loss (%) = $(Wi\text{-}Wf) \div Wi \times 100$

Fig 1. Chitosan, *Punica granatum* and *Cissus quadrangularis* extracts

Fig 2: Polyethelene oxide

Fig 3: Chitosan and PEO solutions in 50 wt% aqueous acetic acid

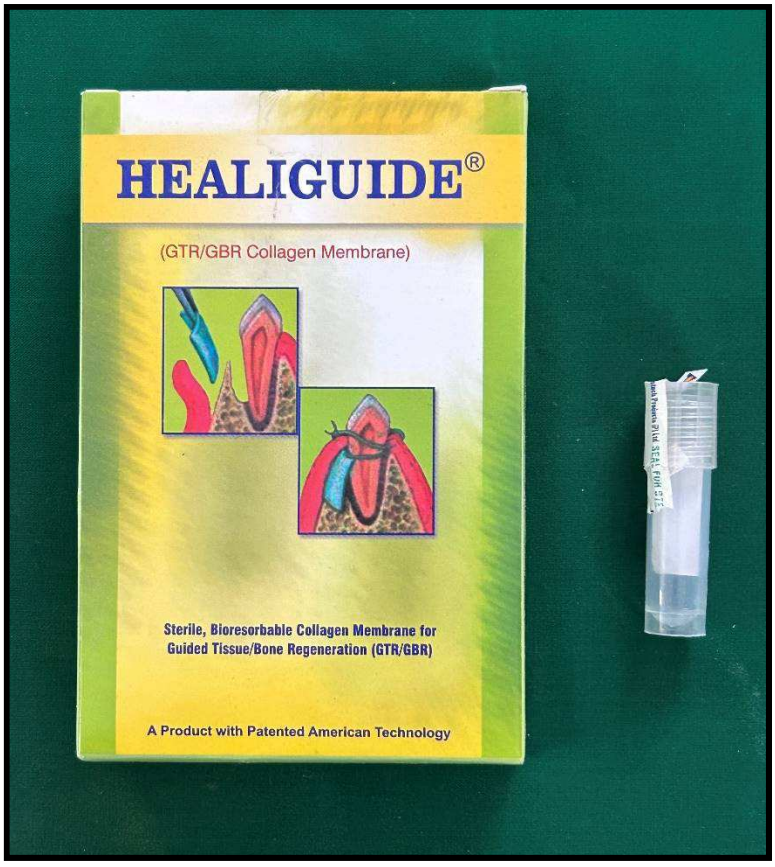


Fig 4: Conventional (commercially available) GBR membrane

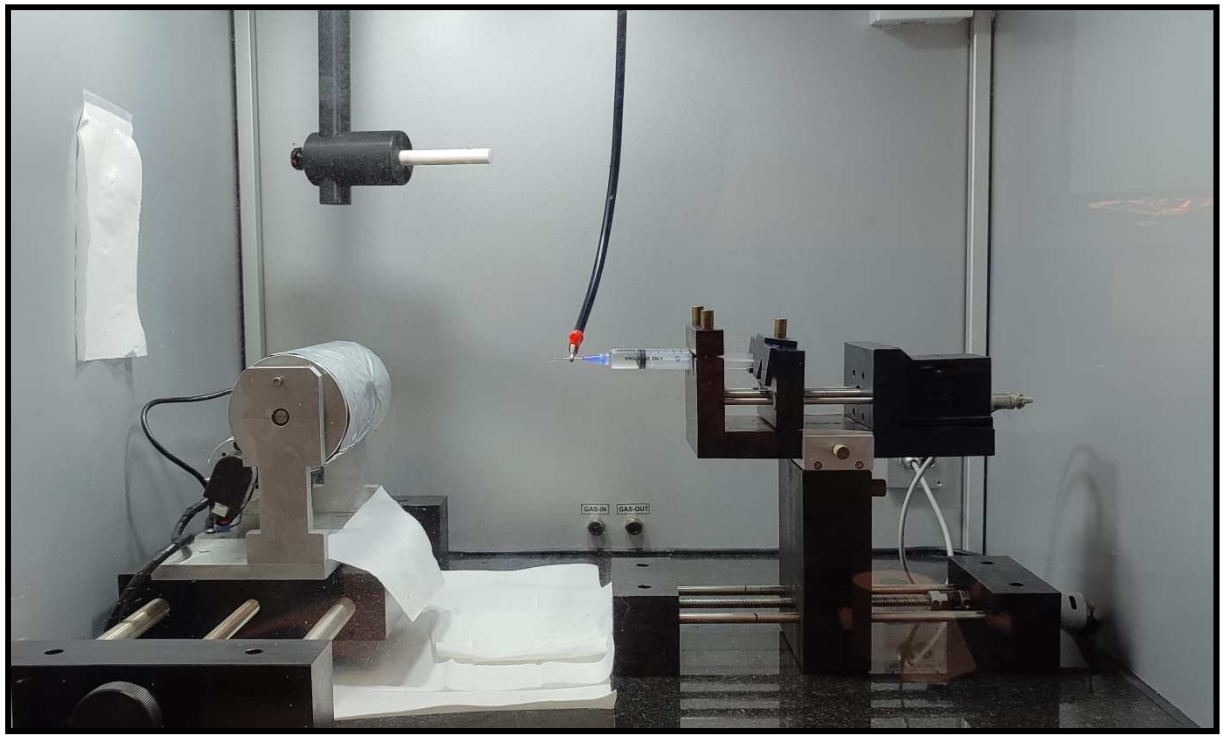


Fig5: Electrospinning machine

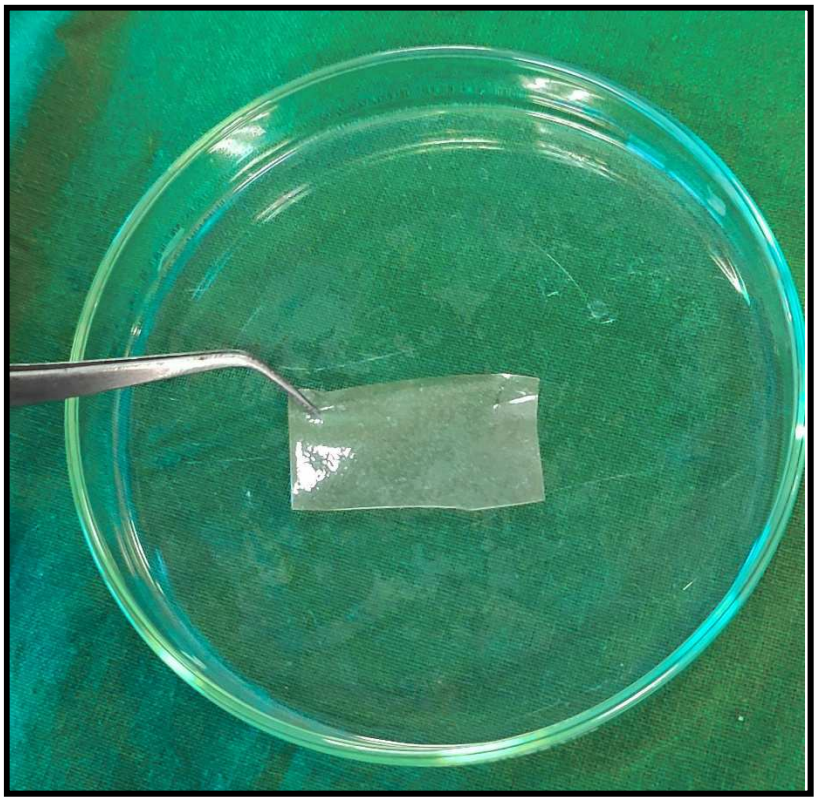


Fig 6: Novel GBR membrane

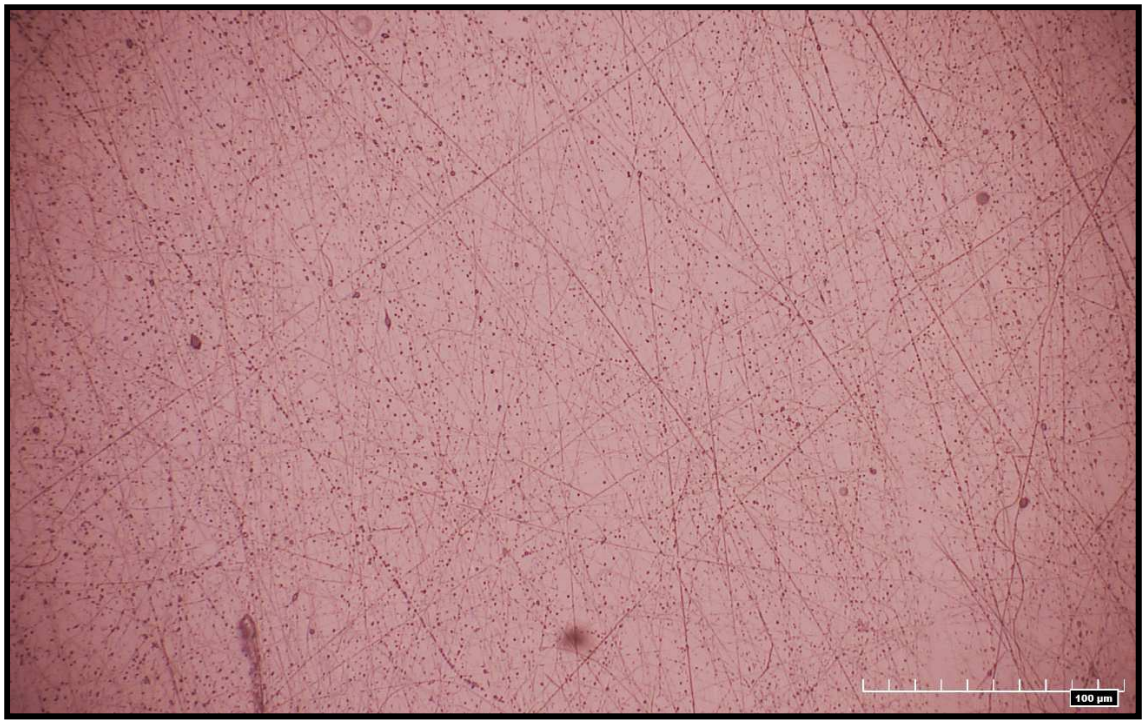


Fig 7:Microscopic image of nanofibres deposited on membrane at 100 µm magnification (1)

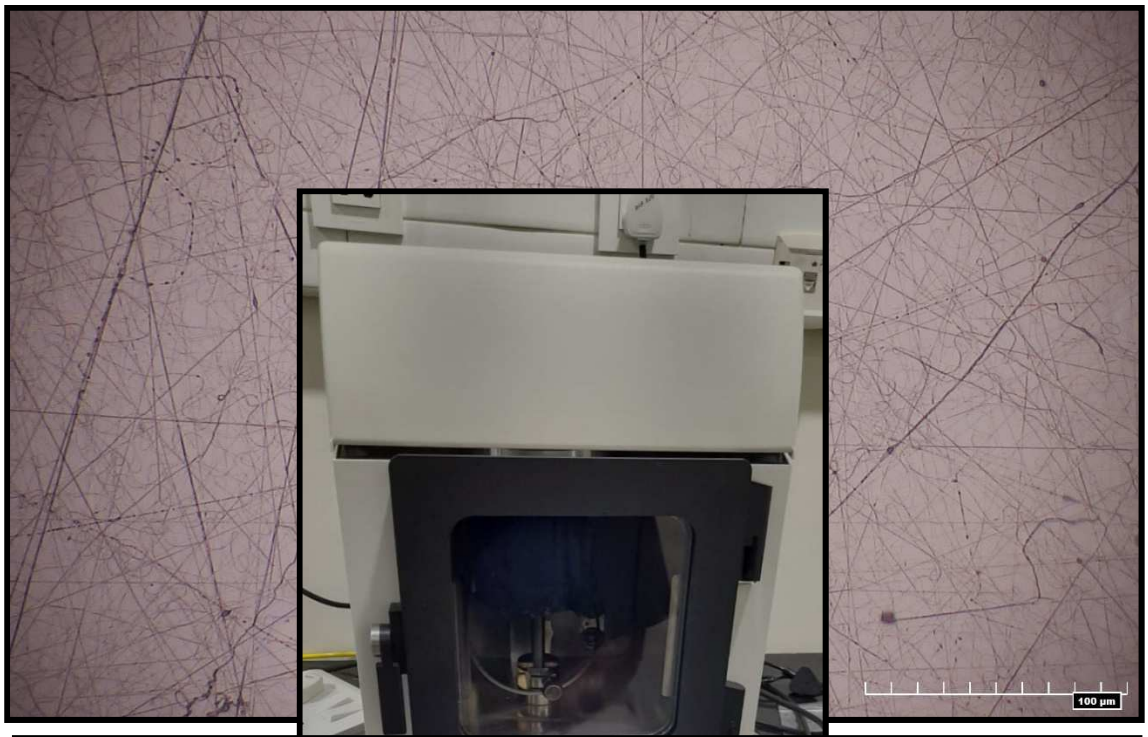


Fig 8:Microscopic image of nanofibres deposited on membrane at 100 µm magnification (2)

Fig 9: Sputter coater

Fig 10: Sputter coating before FESEM

Fig 11: Field Emission Scanning Electron Microscope

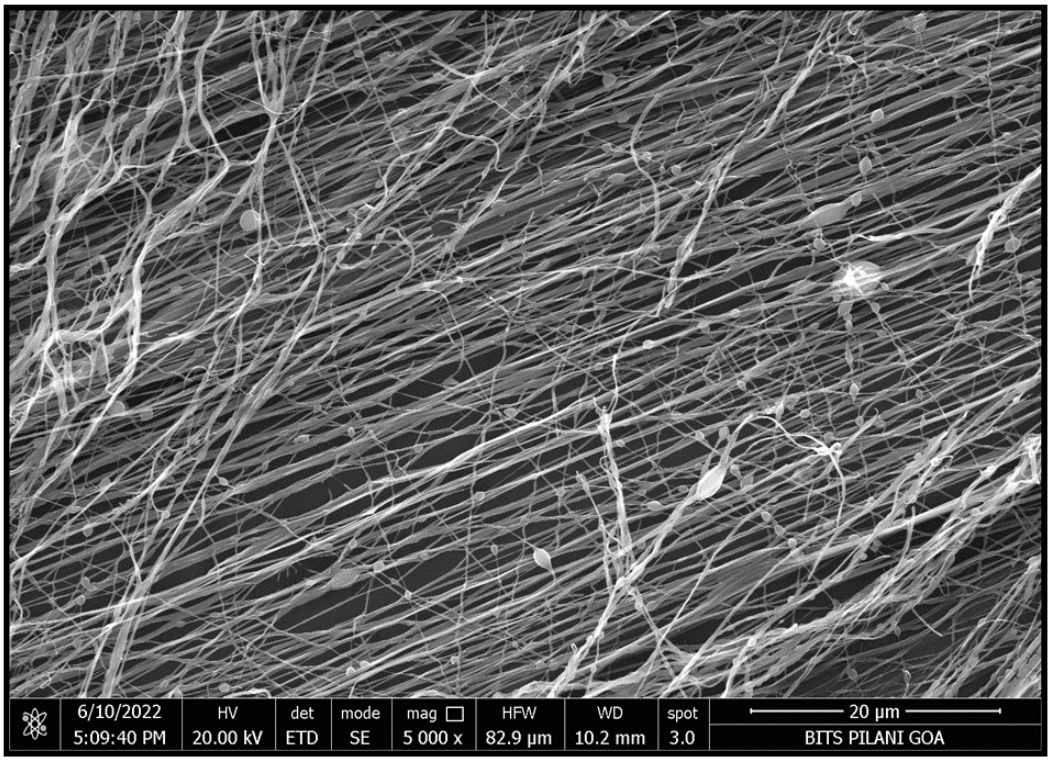


Fig 12: FESEM images of nanofibres deposited on membrane at 20µm magnification (1)

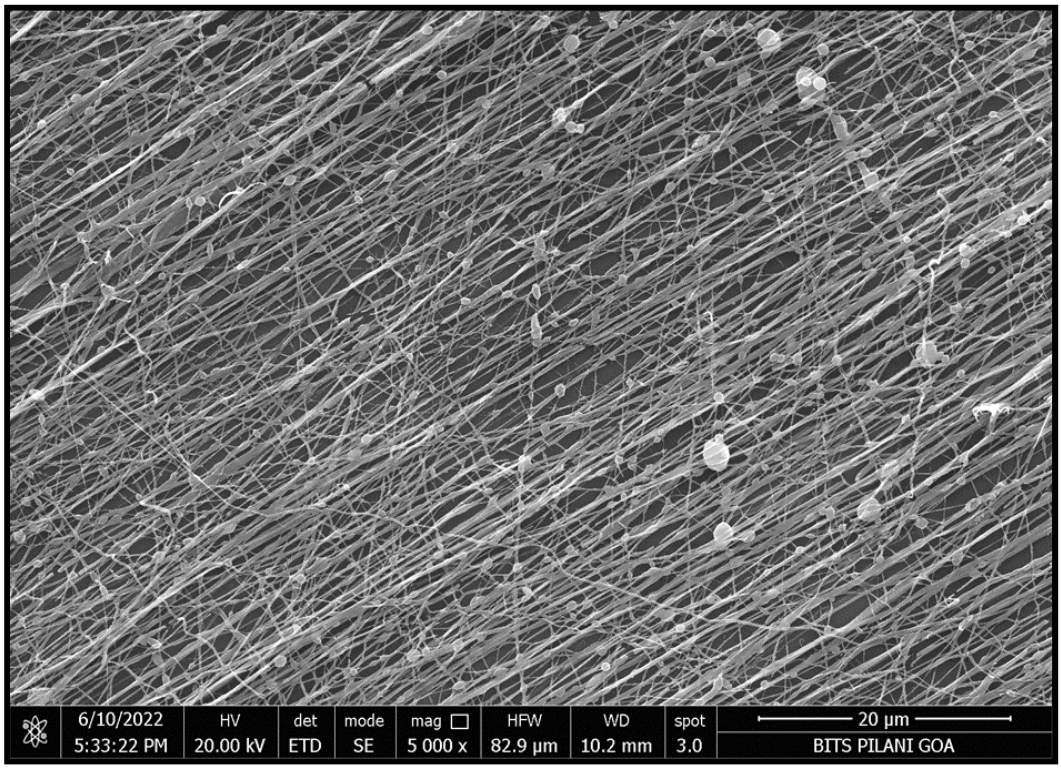


Fig13: FESEM images of nanofibres deposited on membrane at 20µm magnification (2)

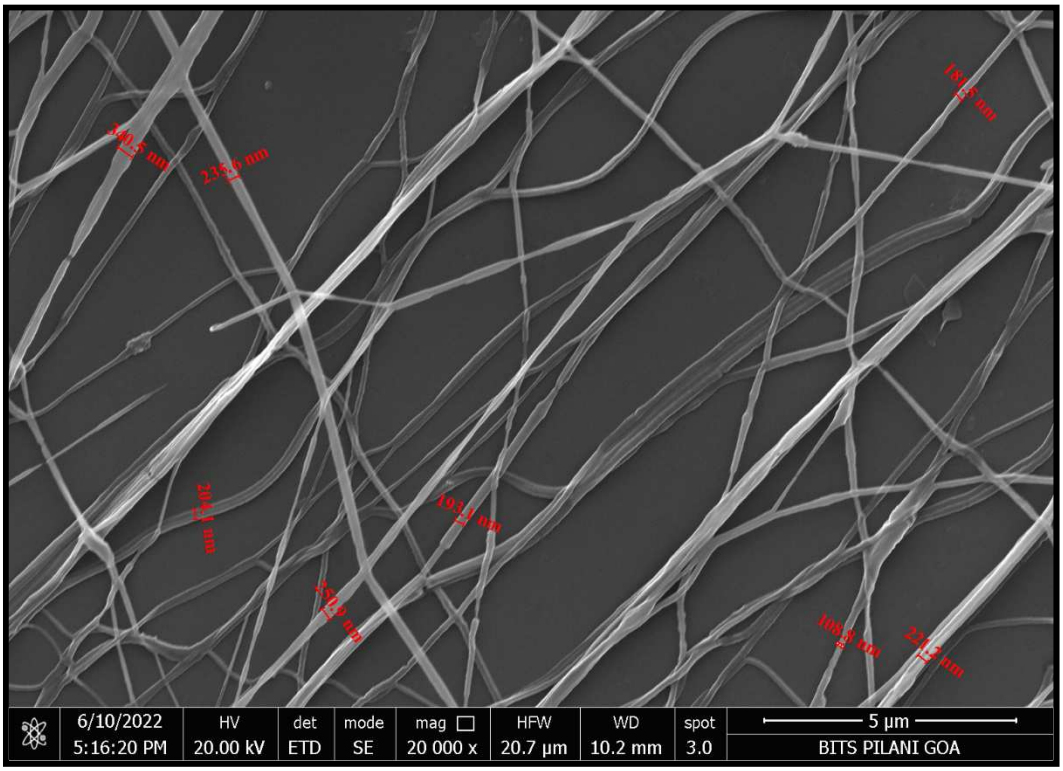


Fig14: FESEM images of nanofibres deposited on membrane at 5 µm magnification with fibre diameter measurements

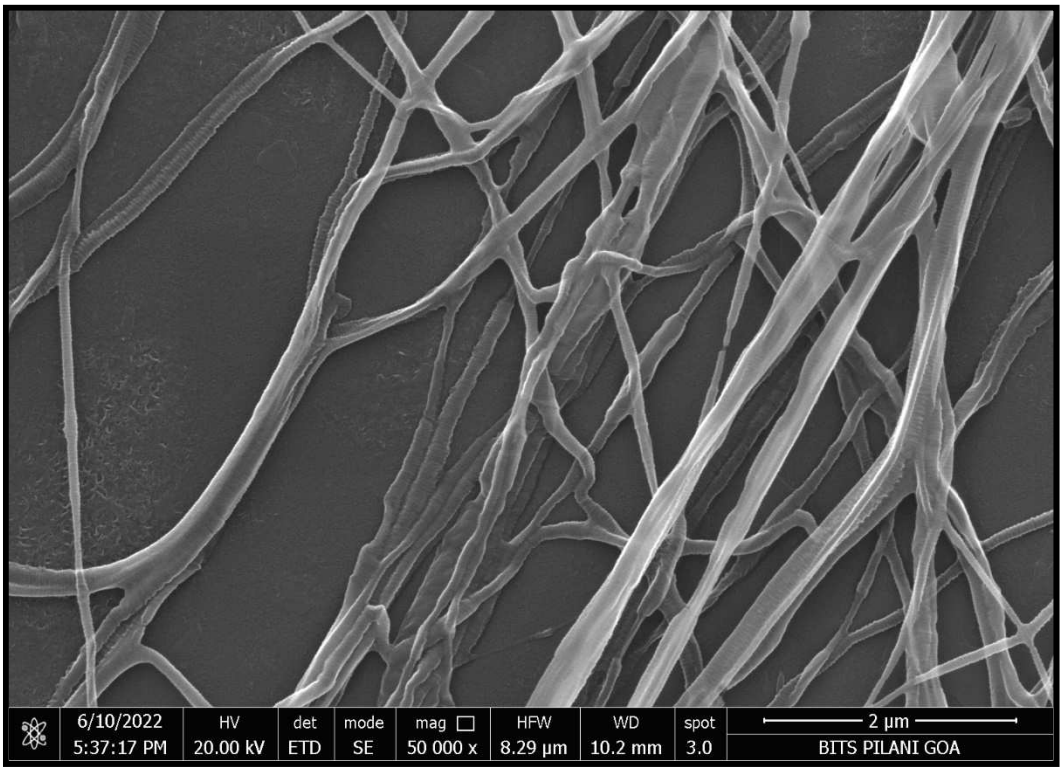


Fig 15: FESEM images of nanofibres deposited on membrane at 2 µm magnification

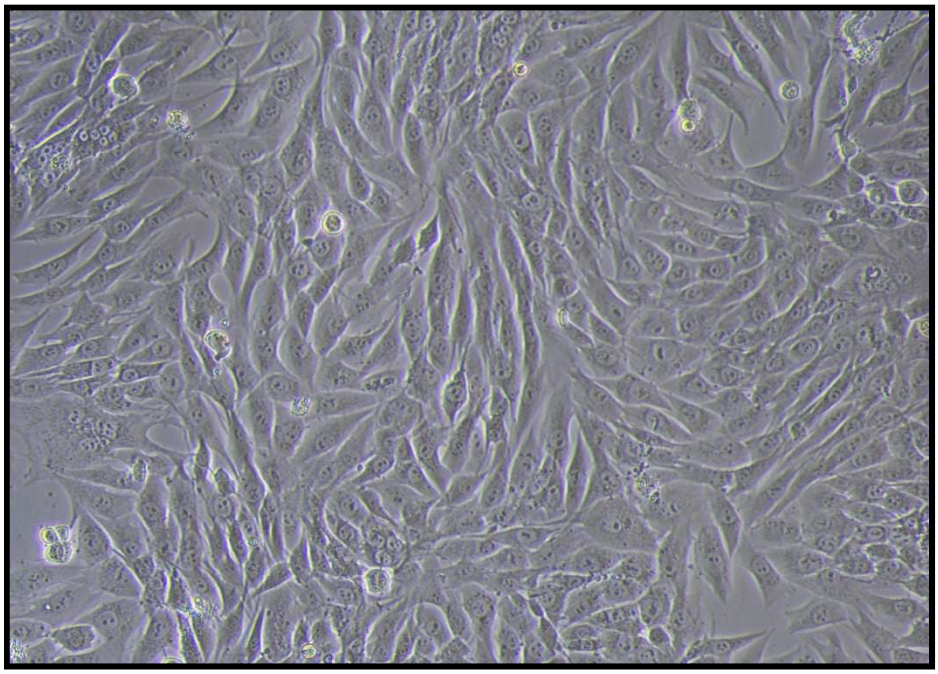


Fig16 : MG-63 cells under the microscope

Fig17 : Materials used for MTT assay

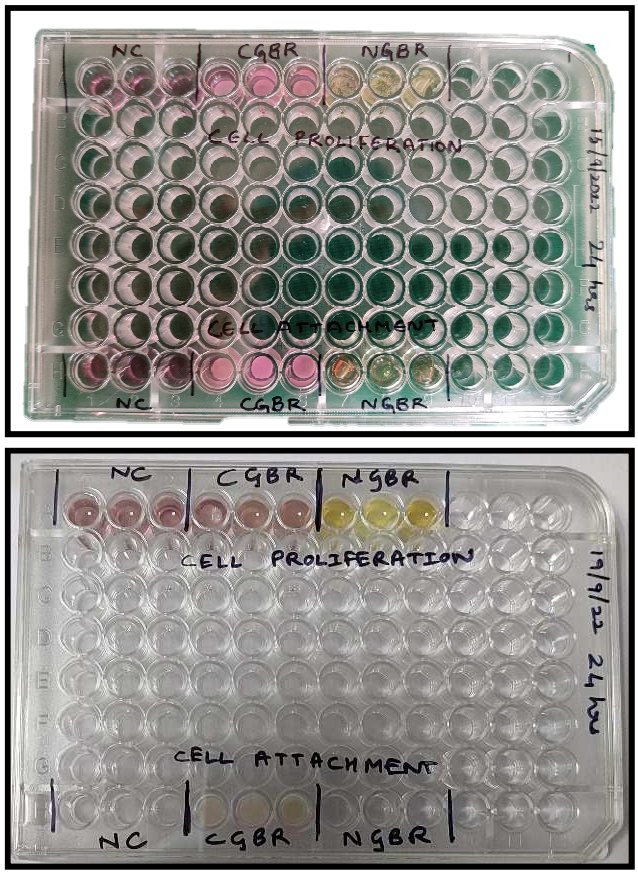


Fig18 : MTT assay

Fig 19: Haemocytometer with Trypan blue dye for cell attachment by Trypan blue assay

DISC DIFFUSION METHOD

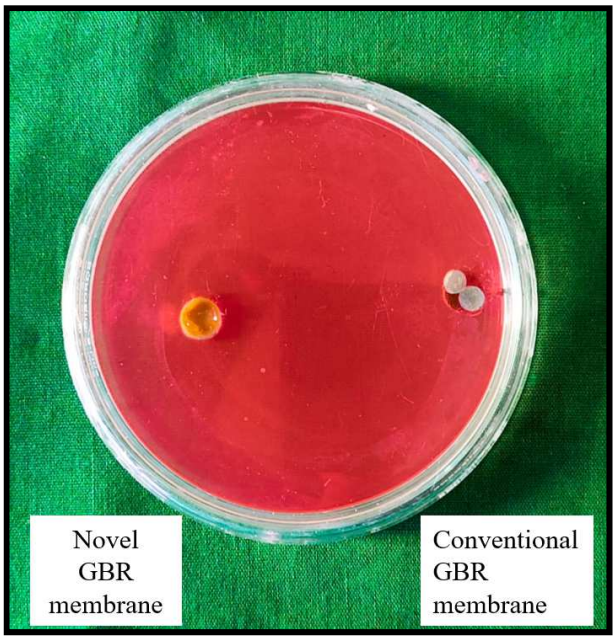


Fig20: Novel GBR membrane and conventional membrane placed in blood agar streaked with *P. gingivalis*

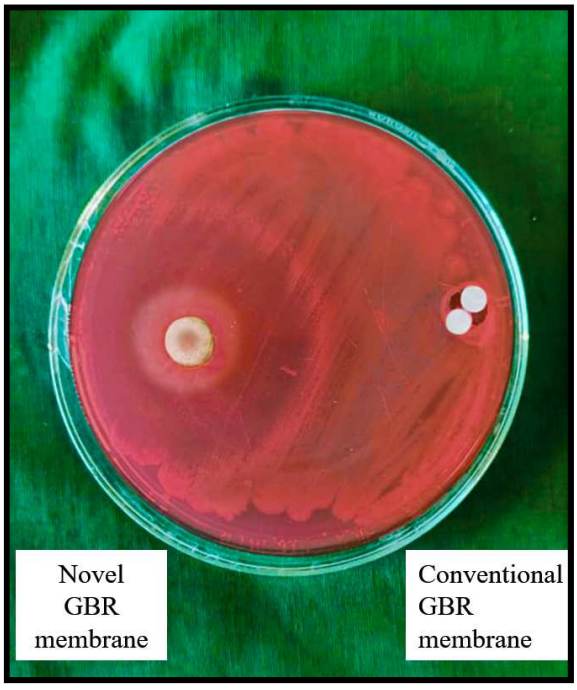


Fig 21: Zone of inhibition around novel GBR membrane placed in blood agar streaked with *P. gingivalis* after 48 hours

Hydrolytic degradation studies

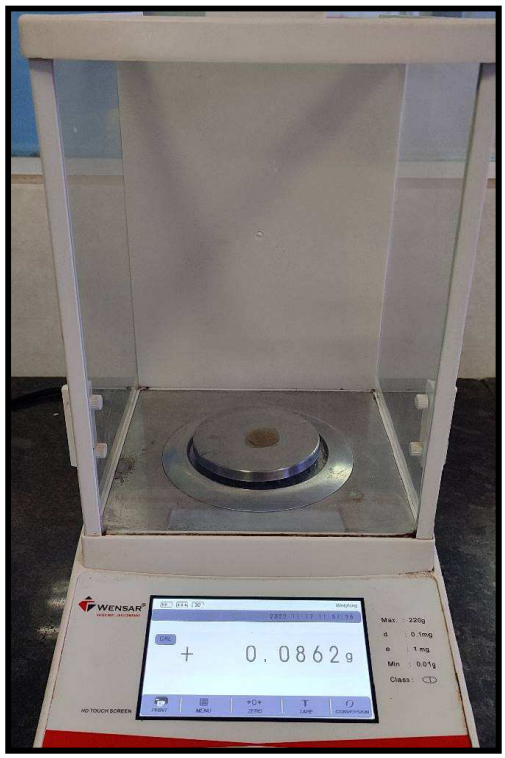


Fig 22: Initial weight of the novel GBR membrane

Fig 23: Novel GBR membrane in phosphate buffer solution

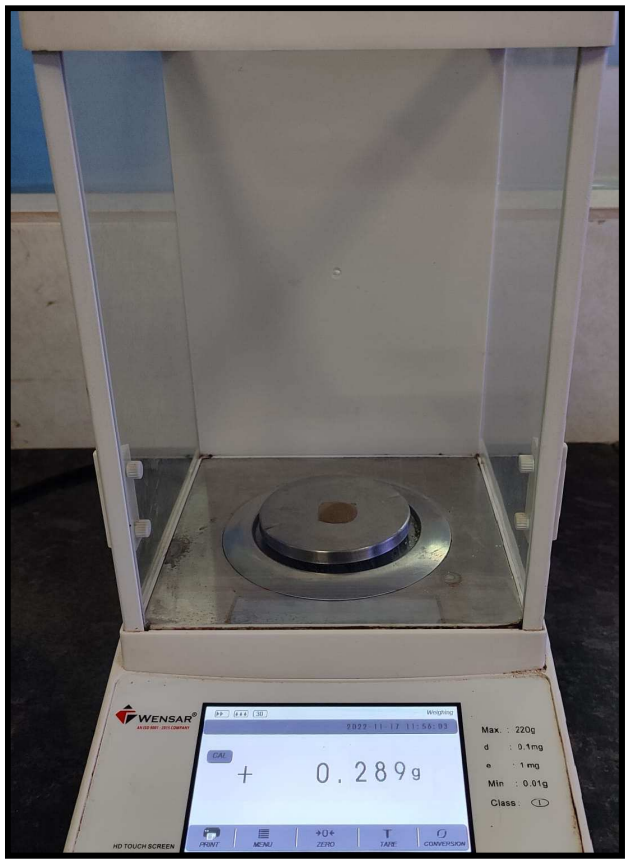


Fig 24: Weight of the novel GBR membrane at 45 days

RESULTS

The results of the cell proliferation, cell attachment, zone of inhibition, and the gravimetric weight loss percentage comparing the novel GBR membrane and conventional GBR membrane were tabulated.

These results were analyzed and subjected to statistical analysis using SPSS software version 20. Descriptive statistical measures such as Mean, Standard deviation were calculated for cell proliferation (Table 3), cell attachment (Table 6), Zone of inhibition (Table 9), gravimetric weight loss (Table 10).

Table 3: Summary of cell proliferation in novel GBR membrane and conventional GBR membrane and three time points, 24 hours, 48 hours and 72 hours

Factors	Levels of factor	n	Mean	SD	SE	95% CI for mean	
						Lower	Upper
Group	Novel membrane	30	81.23	6.02	1.10	78.99	83.48
	Conventional membrane	30	204.40	8.43	1.54	201.25	207.55
Time	24 hours	20	137.70	66.08	14.78	106.77	168.63
	48 hours	20	141.50	55.62	12.44	115.47	167.53
	72 hours	20	149.25	67.87	15.18	117.48	181.02
Group* Time	Novel membrane with 24hrs	10	73.30	0.82	0.26	72.71	73.89
	Novel membrane with 48hrs	10	87.30	0.82	0.26	86.71	87.89
	Novel membrane with 72hrs	10	83.10	0.74	0.23	82.57	83.63
	Conventional membrane with 24hrs	10	202.10	1.37	0.43	201.12	203.08
	Conventional membrane with 48hrs	10	195.70	1.16	0.37	194.87	196.53
	Conventional membrane with 72hrs	10	215.40	1.07	0.34	214.63	216.17

As seen in Table 3 and Graph 1, 2 across time intervals it was observed that novel GBR membrane had 81.23±6.02 cell proliferation whereas conventional GBR membrane had 204.40±8.43 cell proliferation. The conventional GBR membrane has shown increase in proliferation of cells from 24 hours (202.10±1.37) to 72 hours (215.40 ± 1.07). The novel GBR membrane has shown increase in proliferation of cells from 24 hours (73±0.82) to 72 hrs. (83.10±0.74). Conventional GBR membrane

and novel GBR membranes has shown around 10% of increase in proliferation of cells from 24 hours to 72 hours.

Table 4: Comparison of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours with mean cell proliferation scores by two way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value
Main effects					
Group	227550.42	1	227550.42	217481.814	0.0001*
Time	1386.03	2	693.02	662.3522	0.0001*
2-way interaction effects					
Group*Time	1666.03	2	833.02	796.1575	0.0001*
Error	56.50	54	1.05		
Total	230658.98	59			

*$p<0.05$ indicates statistically significant

Two-way ANOVA was performed to compare the mean cell proliferation scores of novel GBR membrane and conventional GBR membrane at three time points that is 24 hours, 48 hours and 72 hours as seen in Table 4 and Graph 2. The test revealed that there was statistically significant difference between the groups when only the groups were compared, and also when the groups were compared across time ($p=0.0001$) as seen in Table 4.

Table 5: Pair wise comparison of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours with mean cell proliferation scores by Tukeys multiple posthoc procedures

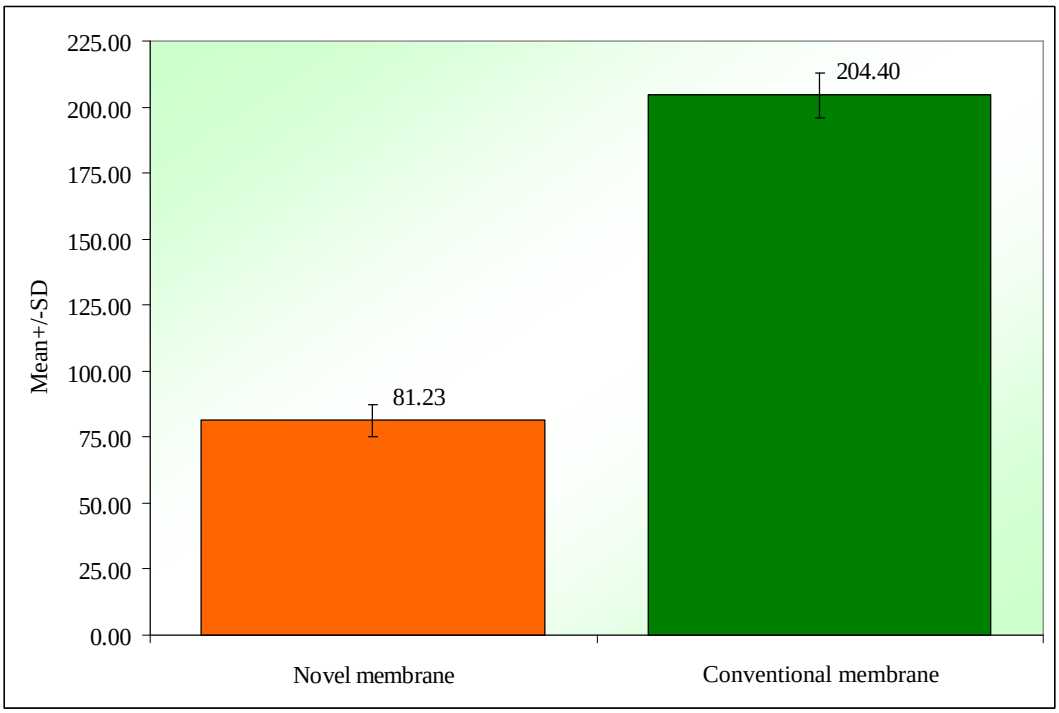
Interactions	Novel membrane with 24hrs	Novel membrane with 48hrs	Novel membrane with 72hrs	Conventional membrane with 24hrs	Conventional membrane with 48hrs	Conventional membrane with 72hrs
Mean	73.30	87.30	83.10	202.10	195.70	215.40
SD	0.82	0.82	0.74	1.37	1.16	1.07
Novel membrane with 24hrs	-					
Novel membrane with 48hrs	p=0.0001*	-				
Novel membrane with 72hrs	p=0.0001*	p=0.0001*	-			
Conventional membrane with 24hrs	p=0.0001*	p=0.0001*	p=0.0001*	-		
Conventional membrane with 48hrs	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-	
Conventional membrane with 72hrs	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-

*p<0.05 indicates statistically significant

Pair-wise comparison of novel GBR membrane and conventional GBR membrane at three time points that is 24 hours, 48 hours and 72 hours to compare the mean cell proliferation scores by Tukeys multiple posthoc procedure. It was found that there is a

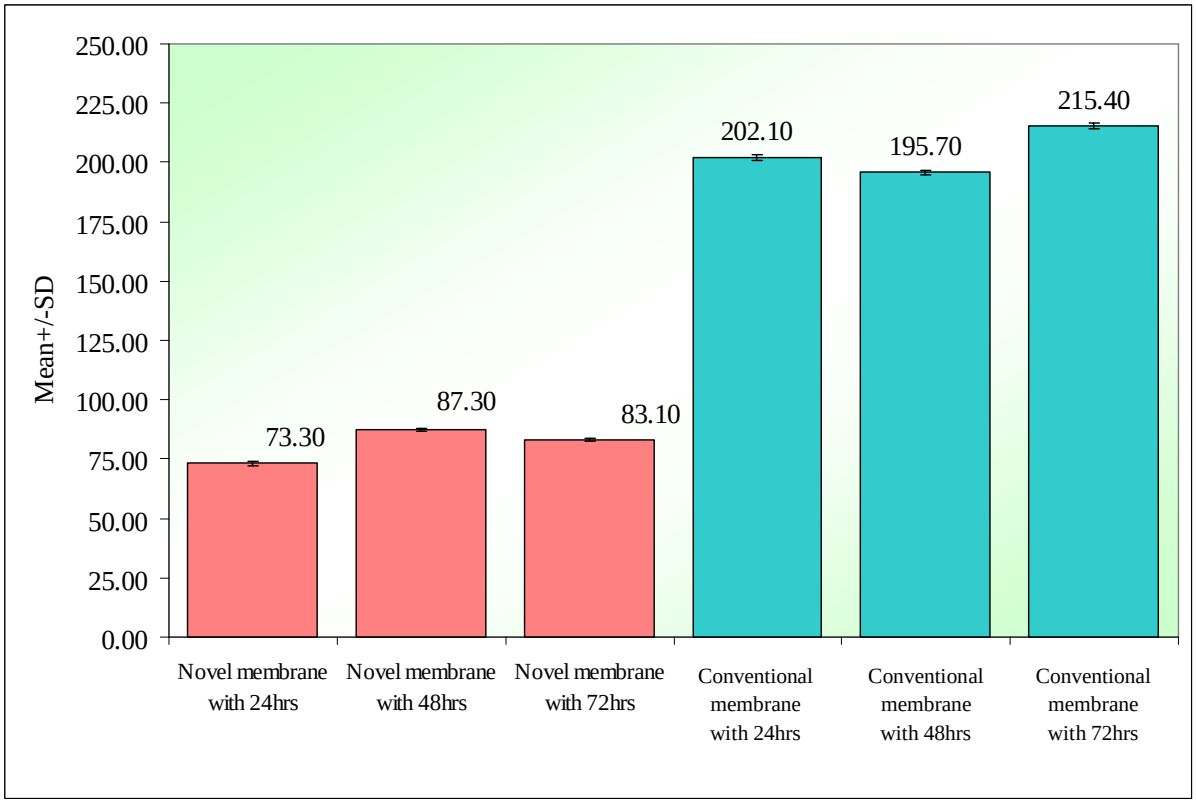
statistically significant difference (p= 0.0001) when the mean cell proliferation of novel GBR membrane is compared with the conventional GBR membrane at all the three time points and when comparison was done for each membrane across all the three time points as seen in Table 5.

Graph 1: Comparison of novel GBR membrane and conventional GBR membrane with mean cell proliferation scores



Comparison of mean cell proliferation scores time intervals of the novel GBR membrane and conventional GBR membrane it was observed that novel membrane had 81.23±6.02 cell proliferation whereas commercially available membrane had 204.40±8.43 cell proliferation as seen in Graph 1.

Graph 2: Comparison of the mean cell proliferation scores of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours.



Comparison of the mean cell proliferation scores of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours. It is revealed that there was difference between the groups when compared across time 24 hours, 48hours and 72 hours. The conventional GBR membrane showed an increased mean cell proliferation when compared to the novel GBR membrane across all the three times that are compared (24hours, 48 hours and 72 hours)

Table 6: Summary of cell attachment ($x10^3$) in novel GBR membrane and conventional GBR membrane and three time points, 24 hours, 48 hours and 72 hours

Factors	Levels of actor	n	Mean	SD	SE	95% CI for mean	
						Lower	Upper
Group	Novel membrane	30	797.60	406.80	74.27	645.70	949.50
	Conventional membrane	30	561.13	440.77	80.47	396.55	725.72
Time	24 hours	20	1113.55	57.49	12.86	1086.64	1140.46
	48 hours	20	712.50	400.04	89.45	525.28	899.72
	72 hours	20	212.05	21.30	4.76	202.08	222.02
Group* Time	Novel membrane with 24hrs	10	1057.80	8.16	2.58	1051.96	1063.64
	Novel membrane with 48hrs	10	1102.40	3.44	1.09	1099.94	1104.86
	Novel membrane with 72hrs	10	232.60	3.86	1.22	229.84	235.36
	Conventional membrane with 24hrs	10	1169.30	2.11	0.67	1167.79	1170.81
	Conventional membrane with 48hrs	10	322.60	2.41	0.76	320.87	324.33
	Conventional membrane with 72hrs	10	191.50	2.01	0.64	190.06	192.94

As seen in Table 6 and Graph 3 across the three time periods the mean cell attachment of novel GBR membrane was797.60± 406.80 and for conventional GBR membrane it was 561.13±440.77.

In 24 hours, the conventional GBR membrane showed increase in number of cells attached per ml of cell suspension with 1169.30 ± 2.11 cells attached as compared to novel GBR membrane at 24 hours with 1057.80± 8.16 cells attached.

Whereas in 48 hours and 72 hours novel GBR membrane showed increase in number of cells attached per ml cell suspension with cell attachment values of 1102 ± 3.44 and 232.60 ± 3.86 respectively.

Table 7: Comparison of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours with mean cell attachment ($x10^3$) scores by two way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value

Main effects					
Group	838747.27	1	838747.27	46722.0470	0.0001*
Time	8159957.03	2	4079978.52	227273.406	0.0001*
2-way interaction effects					
Group*Time	2272300.23	2	1136150.12	63288.7418	0.0001*
Error	969.40	54	17.95		
Total	11271973.93	59			

*p<0.05 indicates statistically significant

Two-way ANOVA was performed to compare the mean cell attachment scores ($x10^3$) of novel GBR membrane and conventional GBR membrane at three time points that is 24 hours, 48 hours and 72 hours as seen in Table 7 and Graph 4. The test revealed that there was statistically significant difference between the groups when only the groups were compared, and also when the groups were compared across time (p= 0.0001) as seen in Table 7.

Table 8: Pair wise comparison of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours with mean cell attachment ($x10^3$) scores by Tukeys multiple posthoc procedures

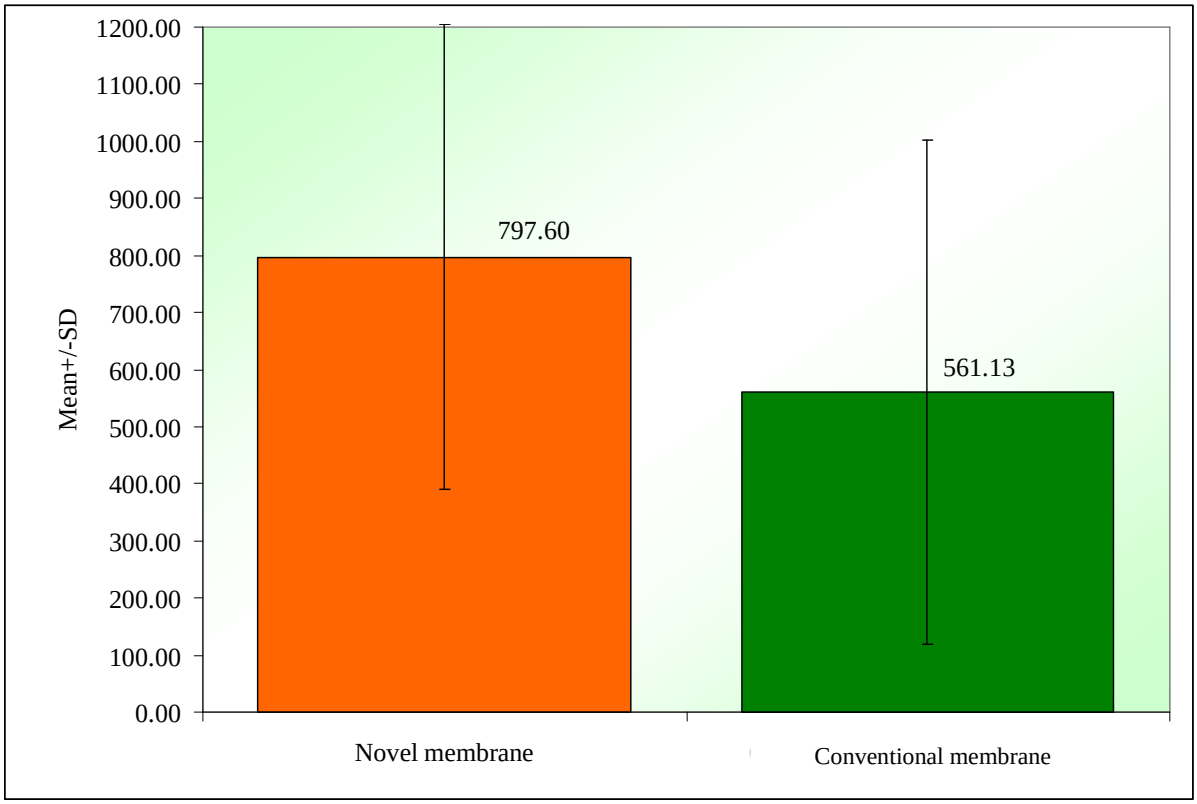
Interactions	Novel	Novel	Novel	Convention	Convention	Convention

	membrane with 24hrs	membrane with 48hrs	membrane with 72hrs	al membrane with 24hrs	al membrane with 48hrs	al membrane with 72hrs
Mean	1057.80	1102.40	232.60	1169.30	322.60	191.50
SD	8.16	3.44	3.86	2.11	2.41	2.01
Novel membrane with 24hrs	-					
Novel membrane with 48hrs	p=0.0001*	-				
Novel membrane with 72hrs	p=0.0001*	p=0.0001*	-			
Conventional membrane with 24hrs	p=0.0001*	p=0.0001*	p=0.0001*	-		
Conventional membrane with 48hrs	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-	
Conventional membrane with 72hrs	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-

*p<0.05 indicates statistically significant

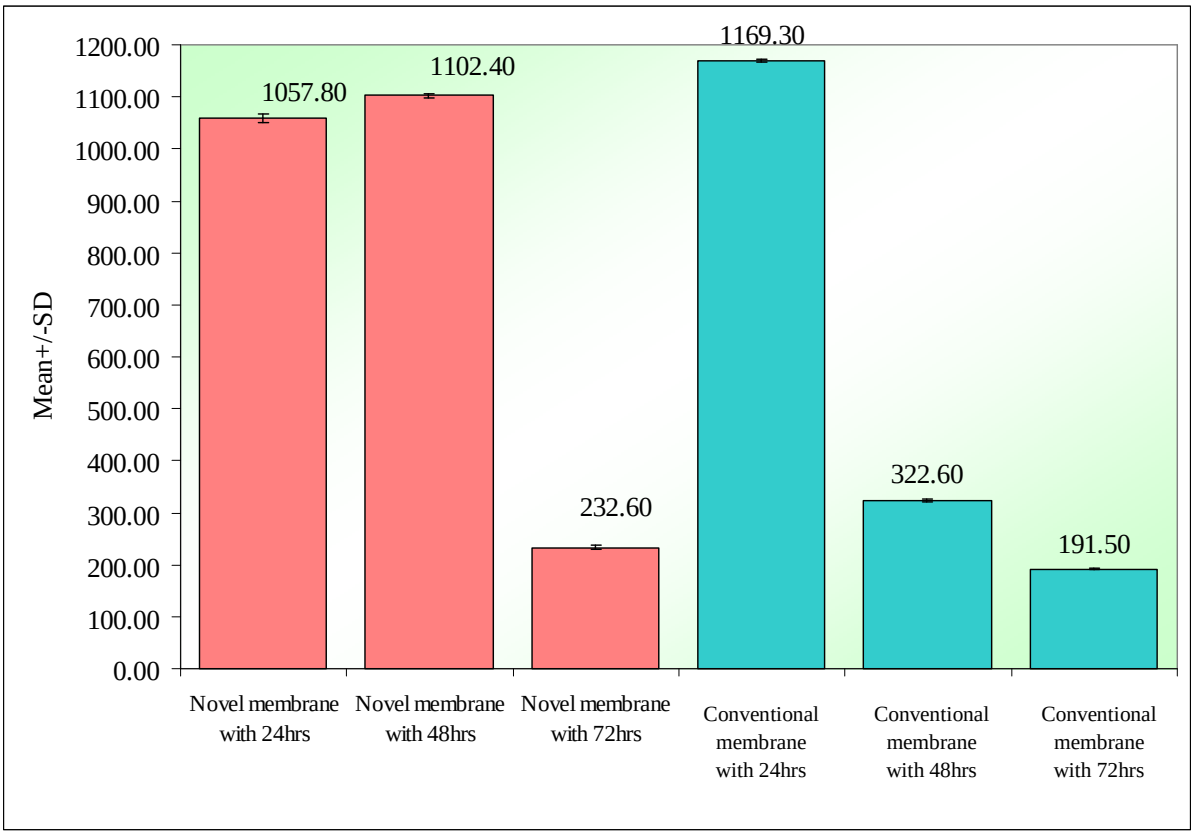
Pair-wise comparison of novel GBR membrane and conventional GBR membrane at three time points that is 24 hours, 48 hours and 72 hours to compare the mean cell attachment scores ($x10^3$) by Tukeys multiple posthoc procedure. It was found that there is a statistically significant difference (p= 0.0001) when the mean cell attachment of novel GBR membrane is compared with the conventional GBR membrane at all the three time points and when comparison was done for each membrane across all the three time points as seen in Table 8.

Graph 3: Comparison of novel GBR membrane and conventional GBR membrane with mean cell attachment ($x10^3$) scores



Comparison of novel GBR membrane and conventional GBR membrane with mean cell attachment ($x10^3$) scores at three time periods the mean cell attachment of novel GBR membrane was 797.60± 406.80 and for conventional GBR membrane it was 561.13±440.77.

Graph 4: Comparison of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours with mean cell attachment ($x10^3$) scores



Comparison of the mean cell attachment (10^3) scores of novel GBR membrane and conventional GBR membrane at 24 hours, 48 hours and 72 hours. It is revealed that there was a difference between the groups when the two groups were compared and also within each group when compared across time at 24 hours, 48 hours and 72 hours. In 24 hours, conventional GBR membrane showed increase in number of cells attached per ml of cell suspension, whereas in 48 hours and 72 hours novel GBR membrane showed increase in number of cells attached per ml cell suspension.

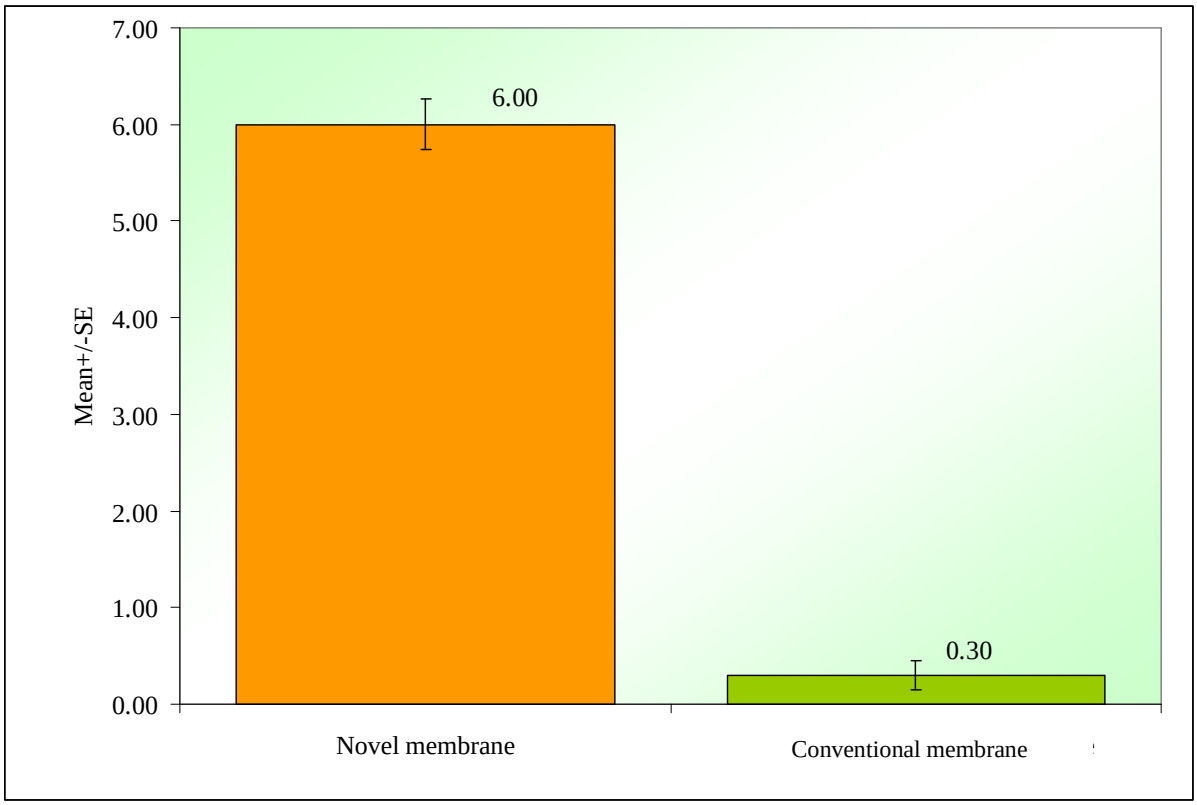
Table 9: Comparison of Novel membrane and Conventional membrane with Zone of inhibition (in mm) by Mann-Whitney U test

Groups	*n*	Mean	SD	Mean rank	Z-value	*p*-value
Novel membrane	10	6.00	0.82	15.50	3.7418	0.0001*
Conventional membrane	10	0.30	0.48	5.50		

*p<0.05 indicates statistically significant

Comparison of the zones of inhibition of novel GBR membrane and conventional GBR membrane was done by Mann-Whitney U test. As seen in Table 9 and Graph 5 the novel GBR membrane showed a zone of inhibition of 6.00 ± 0.82 whereas the conventional GBR membrane showed a zone of inhibition of 0.30±0.48. The comparison between the two groups showed that a significantly greater zone of inhibition (p-value=0.0001) was given by novel GBR membrane as compared to the conventional GBR membrane.

Graph 5: Comparison of the zone of inhibition (in mm) of Novel GBR membrane and conventional GBR membrane



Comparison of the zones of inhibition of novel GBR membrane and conventional GBR membrane was done by Mann-Whitney U test. The novel GBR membrane showed a zone of inhibition of 6.00 ± 0.82 whereas the conventional GBR membrane showed a zone of inhibition of 0.30±0.48.

Table 10: Comparison of novel GBR membrane and conventional GBR membrane with Gravimetric weight loss by independent t test

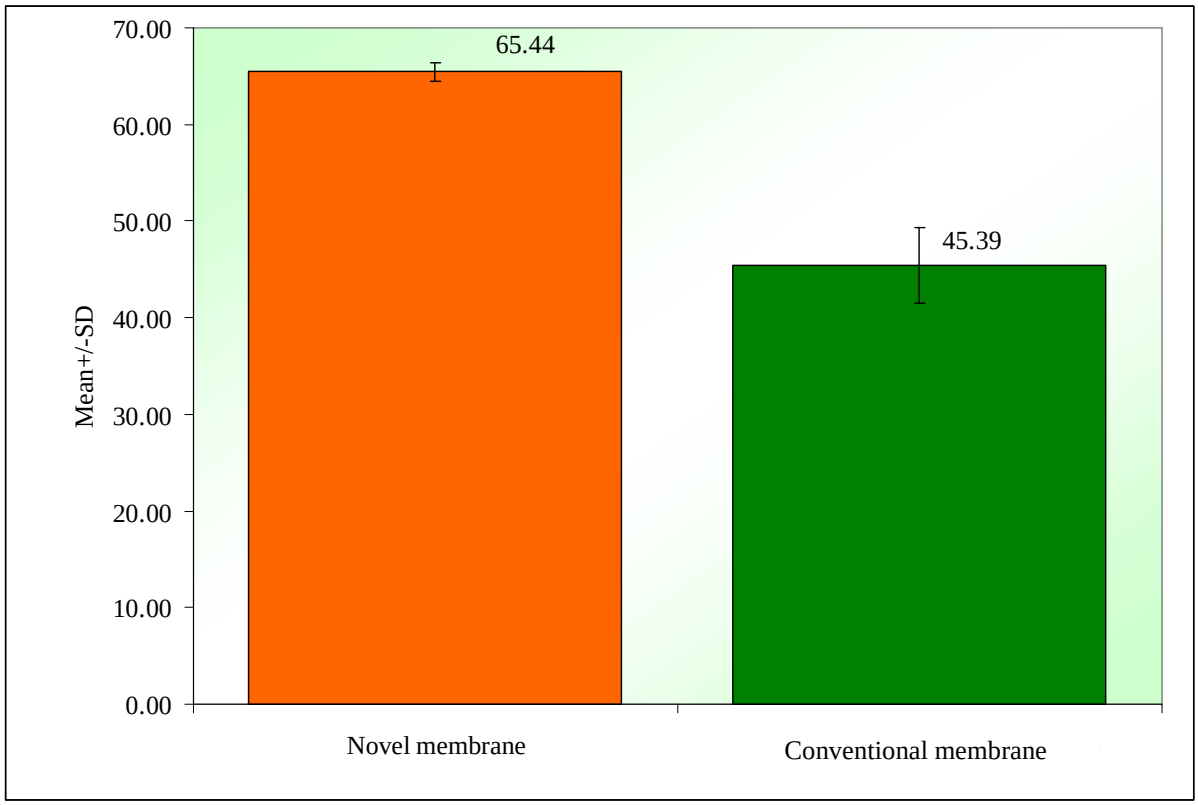
Groups	*n*	Mean	SD	SE	t-value	*p*-value
Novel GBR membrane	10	65.44	0.97	0.31	15.8308	0.0001*
Conventional GBR membrane	10	45.39	3.89	1.23		

*p<0.05 indicates statistically significant

As seen in Table 8 and Graph 6 comparison of the gravimetric weight loss of novel GBR membrane and conventional GBR membrane was done by independent t test. The novel GBR membrane showed a gravimetric weight loss of 65.44±0.97 whereas the conventional GBR membrane showed a Gravimetric weight loss of 45.39±3.89.

The comparison between the two groups showed that there was a statistically significant difference in the gravimetric weight loss between novel GBR membrane and conventional GBR membrane. (p=0.0001)

Graph 6: Comparison of novel GBR membrane and conventional GBR membrane with Gravimetric weight loss



Comparison of the Gravimetric weight loss of novel GBR membrane and conventional GBR membrane was done by independent t test. The novel membrane showed a Gravimetric weight loss of 65.44±0.97 whereas the conventional membrane showed a Gravimetric weight loss of 45.39±3.89.

DISCUSSION

Guided bone regeneration is a surgical method for increasing and augmenting alveolar bone volume in locations designated for future implant placement or around previously placed implants. The GBR principle is based on guided tissue regeneration principles.[5] Because soft gingival tissues grow more rapidly than bone, to stop gingival tissues from penetrating the alveolar bone location, GBR membranes are positioned between the soft tissue and growing bone.[6] Thus, bone is directed into the desired position by selectively excluding epithelial and connective tissue using bone grafting and barrier materials.[5]

The GBR membranes should have high interconnected porosity in the nano size range to be cell occlusive, have a nanofiber structure to mimic natural nanofiber structures in the extracellular matrix and have an adequate mechanical strength. Keeping this in mind the process of electrospinning technique has been used in this study for producing nanofibers.[6]

Researchers have extensively investigated the electrospinning technology to produce scaffolds of tissue engineering that replicate the fibro porous structure of natural ECM. Electrospinning stands out as a different method due to its potential to create scaffolds with clearly defined architecture, including controlled size of porosity, fiber diameter, and surface that fosters cell development. It is also distinguished by its similarity to the in vivo structure of ECM.[16]

Electrospinning is an electrohydrodynamic process in which a liquid droplet is subjected to a high electrostatic force generated by the applied electric field to form a jet, followed by stretching and elongation to generate fibre (s).[48]

The processing parameters—most importantly, the solution concentration—play a significant role in the creation of electrospun fibres and the control of their diameters. Other parameters include the voltage that is applied, the liquid's flow rate and the distance between the syringe tip and the collector.[48]

One of the most critical process parameters at work throughout the electrospinning process is solution concentration, which influences polymer viscosity. The solution becomes more viscous as the concentration of the solution increases. The electrostatic force on the solution will not be strong enough to launch a jet from the tip of the syringe at exceptionally high viscosities (due to a very high solute concentration). In this limit, the applied electric field required to initiate a jet would be of the same order of magnitude as the polymer’s electric breakdown voltage. When the solution viscosity is reduced (but kept relatively high) by decreasing the solution concentration, big helix-shaped fibres form, and further reduction in the solution viscosity results in smooth, continuous fibres.[48]

Chitosan has been commonly used in tissue engineering practices because of its porosity structure, simplicity of chemical modification, biodegradability, biocompatibility, antimicrobial properties, and significant affinity to in vivo macromolecules.[6] However, chitosan's electrospinnability is principally limited due to its stiff chemical structure, polycationic nature in solution, and particular intra- and intermolecular interactions. Strong hydrogen bonding prohibits polymeric chain segments subjected to an electrical field from moving freely, which causes a jet to break apart in the process. Additionally, the production of adequate chain entanglements to permit continuous fiber formation is predicted to be hampered by

the repulsive force operating across ionic groups on the polymer backbone, typically leading to nanobeads rather than nanofibers.[20]

The most effective and simple technique to boost chitosan's electrospinnability is to combine it with a second natural or artificial polymeric phase. This co-spinning agent, which is frequently an easily electrospinnable polymer like Polyethylene oxide (PEO), is a hydrophilic, biocompatible, and biodegradable polymer with FDA approval for use in various pharmaceutical formulations.[16]

The success of chitosan PEO-assisted electrospinning is believed to be the consequence of strong hydrogen bonds formed between ether groups in PEO and hydroxyl and amino groups in chitosan, as shown by FTIR in the study by Pakravan et al.[20]

It is speculated that PEO may act as a "carrier" of chitosan in the electrospinning process via those physical bonds.[20]

As proven by the above studies in this study electrospinning was performed with 6 wt% each of chitosan and PEO solutions by combining them in 1:1, 4:1 chitosan/PEO ratios.

Similar result was found in the study by Pakravan et al wherein increasing chitosan content in the chitosan-PEO blend solutions led to a significant reduction in nanofiber diameters (from 123 to 63 nm for 1:1 and 4:1 chitosan/PEO blends, respectively, at room temperature). This is likely related to a reduction in viscosity and increased conductivity when increasing the chitosan content from 50 to 90%.[20]

In the study by Vulcani et al 4wt% chitosan solution and one part of a 3wt% PEO solution was subjected to electrospinning resulting in a final concentration ratio

of 4:1 resulting in the formation of membranes in the diameters ranging between the submicron and nanoscale.[42]

In the study by Remya et al the electrospinning of Polycaprolactone (PCL) polymer by blending it with a water-soluble polymer Polyethylene oxide (PEO) having a molecular weight of Mv~1,00,000 resulted in superior mechanical properties and an increased fiber diameter.[16]

Osteogenesis, induced by osteoblastic cells, is characterized by a sequence of events, involving cell attachment, cell proliferation and followed by the expression of osteoblast phenotype.

CELL ATTACHMENT

Cells placed on a substrate, as is widely known, will first undergo adhesion and spreading before proliferating. During this process, cells may detect a wide range of chemical and physical signals from the environment and react to them by integrating, evaluating, and responding to them. With this ability, cells can change their shape, dynamics, function, and even outcome.[55]

Cells are inherently sensitive to the physical characteristics of the materials with which they interact. One of the most studied characteristics of materials is the three-dimensional morphology of the substrate. As per early observations, it was indicated that cells interacted with their substrate through ‘ruffled membranes’, focal adhesions, adhesion plaques, or in the recent past, focal contacts. The focal contacts can be described as specialized microstructures anchored within the cell to cytoskeletal microfilaments which underlie the cytoplasmic membrane. The components of the microstructure traverse the cytoplasmic membrane, thus permitting

the cell to bind to its extracellular matrix via proteins called integrins. As a consequence of changes in the interaction of integrins with both extracellular and intracellular proteins, the signals from the extracellular environment may be transferred to the cell resulting in new gene transcription as well as new protein synthesis. As per the original hypothesis, the focal contacts should be of a certain length, in order to promote adhesion.[55,56]

Cells migrate and orient themselves using the substrate's topography. MSCs (mesenchymal stem cells) can proliferate best on surfaces with a slight degree of surface roughness.[56]

Studies by Luo et al and Geiger et al have found that surface topography can influence cellular behaviors such as adhesion, morphology, migration, and orientation. They have shown that when cells attach to the substrate, they first contact with the convex region. Thus, the moderate surface roughness is the most suitable for MSCs growth.[55, 57]

Comparison of the study groups with respect to Cell Attachment at 24 hours,48 hours and 72 hours by Tukey's multiple posthoc procedures were done, and it was found to be cell attachment was found to be significant (p-value-≤0.001) between the Novel GBR membrane and conventional GBR membranes at all the time intervals as seen in Table 8.

Across the three time periods the mean cell attachment of novel GBR membrane when compared with conventional GBR membrane was found to be higher with a statistically significant difference (p-value-≤0.001) between the two groups. (Table 6 and Graph3)

In 24 hours, conventional GBR membrane showed increase in number of cells attached per ml of cell suspension, whereas in 48 hours and 72 hours novel GBR membrane showed increase in number of cells attached per ml cell suspension. (Table 6 and Graph 4)

A deep cellular infiltration into the nanofibrous novel membrane is expected as they allow for larger number of attached cells per area.[58]

Fibre diameters and pore size of bio-scaffolds plays an important role in cell growth, attachment, infiltration, and differentiation by changing the scaffolds characteristics such as scaffold pore size, mechanical property and surface tomography.[59] Decuzzi et al addressed that the existence of microstructures or nanostructures on substrates increases the surface contact angle which in turn increases the surface energy leading to maximal cell adhesion.[60]

According to the study by Mhatre et al wherein they have characterized the surface topography of the same commercially available membrane and discovered that it has a smooth surface and is non-porous.[61]

This could be the reason for the higher mean cell attachment of novel GBR membrane when compared with conventional GBR membrane

Study by Remya et al revealed that Polycaprolactone (PCL)-Polyethylene oxide (PEO) blend scaffolds also supported cell attachment of human osteosarcoma (hOS) cells as depicted by the elongated spindle morphology of hOS cells on all the scaffolds when observed under fluorescent microscope.[16]

In the study conducted by Jin et al a novel type of biomimetic and bioactive silver ion-loaded calcium phosphate/chitosan (Ag-CaP/CS) membrane with

antibacterial ability was successfully developed. The Ag-CaP/CS composite membranes were cytocompatible with bone marrow stromal cells. With increasing culture time, the proliferation of cells on the nanofiber membranes significantly increased. The incorporation of CaP facilitated cell adhesion on the surface of membranes, thereby significantly promoting the proliferation of BMSCs. All of the membranes facilitated good attachment and growth of cells except for pure Chitosan membrane.[11]

This is in accordance with the studies conducted by Lopez-Perez et al and Silva et al which showed that unmodified Chitosan membranes did not perform sufficiently to mediate cell adhesion.[62,63]

CELL PROLIFERATION

Cell proliferation was evaluated by the MTT assay. Cells were seeded onto the membranes and evaluated at intervals of 24 hours, 48 hours, and 72 hours. It was found that both conventional GBR membrane and Novel GBR membrane are not cytotoxic to MG- 63 cells and the viability of cells were maintained from 24 hours to 48 hours. Both the compared membranes have shown around 10% of increase in proliferation of cells from 24 hours to 72 hours.

However, across time intervals it was observed that more cell proliferation occurred on conventional when compared with the novel membrane with a statistically significant difference with a p-value <0.001. (Table 3 and Graph 1)

The results are in accordance with the studies done previously by Vulcani et al on nanostructured membranes of blends of chitosan and PEO using HEp-2 (human larynx carcinoma), VERO (African green monkey kidney cells) and McCoy (mouse

fibroblasts) cell lines and they were also found to be not cytotoxic to fibroblasts and epithelial cells.[42]

Study by Remya et al revealed that Polycaprolactone (PCL)-Polyethylene oxide (PEO) blend scaffolds also supported cell proliferation of hOS cells using MTT assay, as all the scaffolds exhibited more than 80% metabolic activity.[16]

The increased cell proliferation with the novel membrane can be attributed to the *C. quadrangularis* extract in the membrane. It has been shown by Sanyal et al that the stem extract of *C. quadrangularis* contains a high percentage of calcium ions (ca. 4% by weight) and phosphorus both essential for bone-fracture healing.[36]

In the study conducted by Parisuthiman et al effects of ethanolic extract of *C. quadrangularis* on osteoblast differentiation and function were analyzed using murine osteoblastic cells. They concluded that at the concentrations of 100 μg/ml or less, the proliferation was unaffected indicating that *C. quadrangularis* had no cytotoxic effect on cells.[28]

According to Potu et al. 2009, treatment of the mesenchymal cells grown in osteogenic media with *C. quadrangularis* plant extract resulted in a 3-fold increase in the cell proliferation rate in comparison with cells grown in basal media alone. The exact molecular mechanism involved in *C. quadrangularis* -promoted osteogenesis remains to be explored. However, some evidence suggests that Wingless-related integration site signaling (Wnt signaling) may be involved. This pathway has been shown to play a significant role in the control of osteoblast production and bone formation.[27]

ANTIMICROBIAL ACTIVITY AGAINST *P. GINGIVALIS*

Comparison of the zones of inhibition of Novel GBR membrane and conventional GBR membrane was done by Mann-Whitney U test. The comparison between the two groups showed that a significantly greater zone of inhibition (p-value ≤0.001) was given by novel GBR membrane as compared to the commercially available membrane as seen in Table 9 and Graph 5.

The antimicrobial effect of the membrane can be attributed to the *Punica granatum* extract within the membrane. The pomegranate (*P. granatum*) peel contains flavonoid polyphenols, anthocyanins and tannins including ellagitannins, ellagic acid and punicalgin all of which have anti-bacterial functions.[9]

In the research by Orak et al they found that antibacterial effect of *P. granatum* extract could be due to the low phenolic compounds and more acidic nature due to increasing presence of organic acids.[41]

Gomes et al through their study found that this extract consists of gallic tannins and alkaloids which accounted for the significant antimicrobial action against *P. gingivalis*, a Gram-negative bacterium.[47]

In the study conducted by Hernawati et al they found that red pomegranate extract had a zone of inhibition similar to what was found in this study between the concentrations of 6.25% and12.5%.[29]

In another study conducted by Abdollahzadeh et al it was found that the methanolic extract of *P. granatum* peel (MEPGP) had antibacterial activity against *S. aureus* and *S. Epidermidis, L. acidophilus, S. mutans* and *S. salivarius* at concentrations of 8mg/ml and 12mg/ml.[40]

In a study conducted by Janani et al they concluded that Methanolic Rind Extract of P. granatum were found to be effective against oral microorganisms like *Staphylococcus aureus, Staphylococcus epidermis, Klebsiella pneumonia, Pseudomonas aeuriginosa* and *Candida albicans.*[45]

HYDROLYTIC DEGRADATION STUDIES

The novel membrane showed more gravimetric weight loss as compared to the conventional membrane and it was statistically significant. (p-value 0.0001) (Table 10 and Graph 6). This could be due to the presence of PEO nanofibers in the membrane. It has been shown by studies by Remya et al by using SEM micrographs that the presence of PEO affected the degradation by marked fibre rupture and thinning as well as formation of pores on fibre surface. They conducted gravimetric weight loss studies of the PCL-PEO blend scaffolds at 1 month and 3 months and they found that weight loss increased with increasing PEO content. They concluded that weight loss is due to the elution of PEO from the scaffolds. They also analysed the degradation behaviour qualitatively by observing the changes in the fibre architecture.[16]

In another study conducted by Norowski et al they tested the in-vitro degradation of cross linked and uncrosslinked chitosan. This study was performed in PBS containing 100 mg/ml lysozyme, a level much higher than physiological conditions. A high level of lysozyme was used in this study to accelerate degradation and accentuate any possible differences in biodegradation caused by crosslinking of the chitosan polymer. They concluded that the most mass loss was exhibited by the uncrosslinked chitosan mats by a statistically significant amount.[14]

In a similar study Su et al tested the treated electrospun chitosan membrane for in-vitro degradation and found that there was no significant difference between degradation of the different treated membranes as measured by percent change in mass. Membrane mass was reduced significantly over time.[6]

During our review of the literature, we found that no study has been done combining these unique biomaterials and techniques for fabrication of a GBR membrane. Therefore, in this regard, more studies, with detailed qualitative and quantitative analysis for the functioning of this membrane in all the aspects can be carried out in the future.

SCOPE OF THE STUDY

The present study evaluated and compared the osteogenic nature of the novel GBR membrane of chitosan, *Cissus quadrangularis* and *Punica granatum* extract with a conventional membrane using MTT Assay as a measure of cell adhesion and proliferation. It also evaluated the antibacterial property of the novel GBR membrane against *Porphyromonas gingivalis* using disk diffusion method and compared it with the conventional membrane.

Further research to indicate the osteogenic nature of the membrane could be to assess the differentiation of cells with parameters like ALP, RANKL, Alizarin Red stain, deposition of calcium deposits with Von Kossa Staining.

This study should be expanded by the use of various other cells like Human bone marrow cells obtained from a healthy donor.

Establishing the novel GBR membrane's antibacterial properties on various strains of microorganism that may cause peri implant infections is important.

Since microbial growth may vary over various periods of time for various bacteria, additional research may be done to incorporate the antimicrobial assessment over a longer time period.

This study could serve as a springboard for future investigations into the antimicrobial effectiveness of various combinations and permutations of phytopharmaceutical extracts from various plant parts, as well as pharmaceutical composition and the gradual optimization of desired antimicrobial properties.

As the study was conducted in vitro, further in vivo parameters should be included with variable clinical conditions and long term follow up.

LIMITATIONS OF THE STUDY

1. The novel GBR membrane's impact on various microbial strains causing peri-implantitis was not tested.
2. While quantitative studies have been done, qualitative assessments should also be done and must be associated with quantitative information.
3. Osteoblast like cells MG-63 were used in the study. The immunocytochemical profile of extracellular matrix proteins obtained from MG-63 cells differ from those of normal human osteoblasts. Thus, there could be an inconsistency in its cell differentiation abilities when compared to a normal human osteoblast.

CLINICAL IMPLICATIONS

- The novel GBR membrane have electrospun nanofibers with high interconnected porosity in the nano size range and have a nanofiber structure to mimic natural nanofiber structures in the extracellular matrix. This has attributed to the increase in proliferation and attachment of the cells.
- Since commercially available membranes lack the beneficial properties of being both osteogenic and antibacterial, microbial infection is the primary cause of GBR membrane failure. Overcoming obstacles will be made easier by the use of novel GBR membrane. The clinical use of this novel GBR membrane has a great future prospective even as a preventive measure for periimplant infections.
- With these advantages, tissue engineering can be an excellent method for repairing large segmental bone loss caused by tumours, trauma, or fractures or caused by metabolic illnesses like diabetes and osteoporosis.
- Biopolymers and biomimetic biomaterials are readily available, which has created the ideal environment for tissue engineering to become a common procedure in implant surgery.

CONCLUSION

Within the limitations of the present in-vitro study, the following conclusions can be drawn:

- The novel GBR membrane showed an increase in MG-63 cell attachment in 48 and 72 hours when compared to the conventional membrane. By MTT assay, the novel GBR membrane was found to be not cytotoxic to MG-63 cells and the viability of cells were maintained. An increase in proliferation in was seen from 24 to 72 hours in both the novel GBR membrane and conventional GBR membrane.

- The novel GBR membrane was found to be an effective antibacterial agent against *Porphyromonas gingivalis* when compared to the conventional GBR membrane which showed no antibacterial property.

- The hydrolytic degradation studies showed that the novel GBR membrane showed more gravimetric weight loss as compared to the conventional GBR membrane but it still maintained its gross structure for a period of upto 45 days.

- Thus, the novel guided bone regeneration membrane of chitosan, *Cissus quadrangularis* and *Punica granatum* extract has osteogenic potential and antimicrobial properties against *Porphyromonas gingivalis* when compared to a conventional resorbable membrane. Thus, proving our research hypothesis.

- This makes room for additional studies in this area. Moreover, the natural biopolymers available for tissue engineering have a vital role to play in enhancing the potential for bone regeneration.

SUMMARY

The study evaluated the osteogenic potential and anti-bacterial activity against *Porphyromonas gingivalis* of a novel guided bone regeneration membrane of chitosan, *Cissus quadrangularis* and *Punica granatum* extract and compared it with a conventional membrane.

The novel GBR membrane had a base layer of *Cissus quadrangularis, Punica granatum extract* and gelatin. Then electrospun nanofibers of chitosan and PEO were deposited on the base layer to form an the novel GBR membrane. The membrane was characterized using SEM analysis to determine the fibre size and morphology of the produced membrane. The novel membrane was subjected to various tests and a commercially available conventional membrane was used as a control group.

The tests included adhesion and proliferation of MG-63 cells to determine the osteogenic potential of the membrane and to compare it to that of the commercially available membrane. Cell adhesion was done using trypan blue assay and cell proliferation using MTT assay at 24, 48 and 72 hrs.

The disk-diffusion test was performed to assess the effectiveness of 10% of Punica granatum extract incorporated into the novel GBR membrane against *P. gingivalis* after incubation for 48 hours and to compare it with that of a conventional GBR membrane.

For the hydrolytic degradation studies scaffold's initial weight *(Wi)* before and after 45 days *(Wf)* of incubation in phosphate buffer solution was measured and the percentage weight loss was calculated and was compared with that of the conventional GBR membrane.

The data obtained from all of the above tests was statistically analyzed to draw a conclusion from experimental data.

It was concluded that the novel GBR membrane showed an increase in MG-63 cell attachment in 48 and 72 hours when compared to the conventional membrane. By MTT assay, the novel GBR membrane was found to be not cytotoxic to MG-63 cells and the viability of cells were maintained.

The novel GBR membrane was found to be an effective antibacterial agent against *Porphyromonas gingivalis* when compared to the conventional GBR membrane which showed no antibacterial property.

The hydrolytic degradation studies showed that the novel GBR membrane showed more gravimetric weight loss as compared to the conventional GBR membrane but it still maintained its gross structure for a period of up to 45 days.

This makes room for additional studies in this area. Moreover, the natural biopolymers available for tissue engineering have a vital role to play in enhancing bone's potential for regeneration.

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ANNEXURE – I

ETHICAL CLEARANCE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
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Sl. No. : 1459

CERTIFICATE

This is to Certify that the synopsis titled

Comparative evaluation of osteogenic potential & antibacterial efficacy of a novel guided bone regeneration membrane of chitosan, cissus quadrangularis and punica granatum extract with a conventional membrane an invitro study *Submitted by*

Dr. _ P. G. Student /

Staff, Guided by Dr. Santosh. Y. Nelogi *from Department of* Prosthodontics & crown & Bridge *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE-II-

CERTIFICATE OF ANALYSIS FOR POMEGRANATE EXTRACT

KSHIPRA BIOTECH PVT. LTD.
(An ISO 9001:2015 | WHO – GMP | FSSAI | Organic Certified Company)
CIN. : U24100MP2015PTC038928 | GSTIN: 23AAFCK7461H1ZI | PAN: AAFCK7461H |

CERTIFICATE OF ANALYSIS

PRODUCT NAME:	**POMEGRANATE EXTRACT**	**BOTANICAL NAME:**	**PUNICA GRANATUM LINN**
BATCH NO. :	**KBPL/PG/010420**	**PARTS USED:**	**PEEL**
HER :	**10:1**	**EXTRACTED BY :**	**ETHANOL & WATER**
MFG. M. :	**MARCH, 2020**	**EXP. M. :**	**MARCH, 2022**

ASSAY DATA			
ELLAGIC ACID	**40.0% MIN.**	**HPLC**	**40.18%**
QUALITY DATA			
IDENTIFICATION	**STANDARDIZATION**	**TEST METHOD**	**RESULT**
ASPECT	BROWN YELLOW FINE POWDER	VISUAL	COMPLIED
ODOR & TASTE	CHARACTERISTIC	ORGANOLEPTIC	COMPLIED
PARTICLE SIZE	NLT 95% THROUGH 80 MESHES	USP<786>	COMPLIED
LOSS ON DRYING	5.0% MAX.	USP<731>	3.52%
ASH	5.0% MAX.	USP<281>	2.90%
HEAVY METALS	10PPM MAX.	USP<231>II	COMPLIED
PB	≤3PPM	USP<251>	COMPLIED
AS	≤2PPM	USP<211>II	COMPLIED
MICROBIOLOGICAL DATA			
TOTAL AEROBIC COUNT	NOT MORE THAN 10000CFU/GRAM	USP<61>	220CFU/GRAM
YEAST & MOLD COUNT	NOT MORE THAN 100CFU/GRAM	USP<61>	20CFU/GRAM
E. COLI	NONE DETECTED	USP<62>	COMPLIED
SALMONELLA	NONE DETECTED	USP<62>	COMPLIED
S. AUREUS	NONE DETECTED	USP<62>	COMPLIED

STORAGE STORE IN A COOL DRY PLACE, AVOIDING SUNLIGHT DIRECTLY
SHELF LIFE TWO YEARS WHEN SEALED WELL

Corporate Office: 221, 2nd Floor, Phadnis complex, 89/1, M.G. Road, Near Kothari Market, Indore- 452007 (M.P) INDIA,
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Mfg. Facility : Jamna Nagar, Maxi Road, Dewas 455001 (M.P) INDIA,
Email: info@kshiprabiotech.com, biotechkshipra@gmail.com ,
Skype: kshipra.biotech Web : www.kshiprabiotech.com

006-CB-QMS

ANNEXURE-III-

CERTIFICATE OF ANALYSIS FOR CISSUS QUADRANGULARIS EXTRACT

KSHIPRA BIOTECH

KSHIPRA BIOTECH PVT. LTD.

(An ISO 9001:2015 | WHO – GMP | FSSAI | Organic Certified Company)

CIN. : U91100MP2015PTC035005 | GSTIN: 23AAFCK7501H1Z1 | PAN: AAFCK7501H |

CERTIFICATE OF ANALYSIS

PRODUCT: CISSUS QUADRANGULARIS (CISSUS) D.E.
ACTIVES: TOTAL KETOSTERONES> 10%
PART USED: STEM
SOLVENTS USED: METHANOL, WATER

BATCH: KBAI-0919-009
DATE: SEPTEMBER' 2019
SHELF LIFE: 3 YRS
COUNTRY OF ORIGIN: INDIA

TEST	SPECIFICATIONS	RESULTS	PROTOCOL
PHYSICO-CHEMICAL			
DESCRIPTION	BROWINSH COLOUR POWDER WITH SLIGHTLY BITTER TASTE	COMPLIES WITH DESCRIPTION.	VISUAL/ ORGANOLEPTIC
IDENTIFICATION	BY TLC	POSITIVE	BY TLC
SOLUBILITY : WATER	MINIMUM 50%	COMPLIES	USP <561>
PH 1% WATER SOLUTION	5-7	7	USP <791>
BULK DENSITY(UNTAPPED) (TAPPED)	0.4-0.8 G/ML 0.5-0.9 G/ML	0.58 G/ML 0.74 G/ML	USP <616>
TOTAL ASH	MAXIMUM 20%	3.57%	USP <561>
LOSS ON DRYING	MAXIMUM 5%	3.93%	USP <731>
MESH SIZE	100% PASSES THROUGH #40	COMPLIES	USP <786>
ASSAY			
TOTAL KETOSTERONES	**MINIMUM 10%**	**11.98%**	**GRAVIMETRIC**
MICROBIAL PROFILE			
TOTAL PLATE COUNT	NMT 10000 CFU/G	COMPLIES	USP <61>
YEAST & MOULDS	NMT 100 CFU/G	COMPLIES	USP <61>
E-COLI	ABSENT	COMPLIES	USP <62>
SALMONELLA	ABSENT	COMPLIES	USP <62>
S.AUREUS	ABSENT	COMPLIES	USP <62>
PSEUDOMONAS	ABSENT	COMPLIES	USP <62>
HEAVY METALS PROFILE			
HEAVY METALS CONTENT	NOT MORE THAN 10 PPM	COMPLIES	BY ICPMS/ AAS

- STORAGE: STORE IN COOL, DRY PLACE IN CLOSED CONTAINERS, AWAY FROM DIRECT SUNLIGHT
- NO-IRRADIATED. NON-GMO

HERBAL EXTRACTS, BEING A NATURAL PRODUCT ARE SUBJECT TO MINOR VARIATIONS IN COLOUR, TEXTURE AND SMELL OVER A PERIOD OF TIME AND FROM BATCH TO BATCH.

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Mfg. Facility : Jamna Nagar, Maxi Road, Dewas-455001 (M.P) INDIA.
Email: info@kshiprabiotech.com, biotechkshipra@gmail.com . Web : www.kshiprabiotech.com

ANNEXURE-IV-

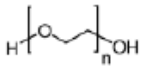
CERTIFICATE OF ANALYSIS FOR POLYETHENE OXIDE

Sigma-Aldrich.

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Poly(ethylene oxide) - average Mv 600,000, powder



Product Number:	**182028**
Batch Number:	**MKCN4119**
Brand:	ALDRICH
CAS Number:	25322-68-3
MDL Number:	MFCD00081839
Formula:	(C2H4O)nH2O
Quality Release Date:	29 OCT 2020

Test	Specification	Result
Appearance (Color)	White	White
Appearance (Form)	Powder	Powder
Infrared Spectrum	Conforms to Structure	Conforms
Loss on Drying 105 Degrees Celsius, 45 Minutes	≤ 1.0 %	0.4 %
Viscosity C= 5%, H2O at 25 Degrees Celsius	4500 - 8800 cps	6100 cps
Assay Alkalies and Other Metals as CaO	≤ 2.0 %	0.2 %

Michael Grady, Manager
Quality Control
Milwaukee, WI US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

ANNEXURE-V-

CERTIFICATE OF ANALYSIS FOR CHITOSAN

LOBA Chemie
LABORATORY REAGENTS & FINE CHEMICALS
ISO 9001-2015 CERTIFIED

CERTIFICATE OF ANALYSIS

Product Name	:	CHITOSAN - FROM SHRIMP SHELLS 75% (deacetylated)			
Lot No.	:	Sample COA	Analyzed On	:	09-May-2021
Mol. Formula	:		Mol. Weight	:	
Code No.	:	02697	CAS No.	:	9012-76-4
Mfg. Date	:	May-2021	Exp. Date	:	Apr-2025
HAZ. / P.G.	:	-	UN No.	:	-

Sr.	Tests	Specifications	Results
1	Appearance	White to beige powder or flakes	Beige flakes
2	Deacetylation	Min 75%	75.01%
3	Ash content	Max 1.0%	1.0%
4	Moisture content	Max 10.0%	8.19%
5	Viscosity (1% solution)	Min 200 cps	535.1 cps

CONCLUSION -This above product complies as per the specifications of **LOBA CHEMIE PVT. LTD.**

Varsha
Chemist

Shashikant Gaikwad
QC Manager

LOBA CHEMIE PVT. LTD.

Works : Plot No. D-22, MIDC, Tarapur Industrial Area, Tarapur, Boisar, Taluka- Palghar, Dist. Palghar, Pin-401506
Tel: 91.02525-663630/39/34
Regd Office : 107 Wode House Road, Jehanghir Villa, Colaba, Mumbai-400005
Tel: 91.22.6663 6663, Fax: 91.22.22151099
info@lobachemie.com | www.lobachemie.com

ANNEXURE-VI-

CELL PROLIFERATION

Sr. no	Novel GBR membrane			Conventional GBR membrane		
	24 hours	48hours	72 hours	24 hours	48 hours	72hours
1	73%	87%	83%	202%	196%	215%
2	75%	88%	83%	200%	195%	214%
3	74%	87%	84%	202%	197%	216%
4	73%	89%	83%	204%	194%	215%
5	73%	87%	83%	203%	196%	217%
6	73%	87%	84%	202%	195%	214%
7	74%	86%	82%	200%	197%	215%
8	72%	87%	83%	202%	196%	215%
9	73%	87%	84%	204%	194%	216%
10	73%	88%	82%	202%	197%	217%

ANNEXURE-VII-

CELL ATTACHMENT ($\times 10^3$)

Sr.no	Novel GBR membrane			Conventional GBR membrane		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
1	1060	1100	230	1170	320	190
2	1061	1101	240	1165	325	189
3	1049	1100	230	1167	324	192
4	1038	1111	230	1171	319	194
5	1065	1103	230	1170	326	195
6	1060	1101	239	1168	323	190
7	1060	1100	230	1172	320	189
8	1061	1104	232	1171	324	192
9	1060	1104	234	1169	324	192
10	1064	1100	231	1170	321	192

ANNEXURE-VIII-

ZONE OF INHIBITION (in millimeters)

Sr. no.	Novel GBR membrane	Conventional GBR membrane
1	7	0
2	5	0
3	6	1
4	7	0
5	6	0
6	6	0
7	7	1
8	5	1
9	5	0
10	6	0

ANNEXURE- IX-

GRAVIMETRIC WEIGHT LOSS PERCENTAGE

Sr. no	Novel GBR membrane	Conventional GBR membrane
1	66.47	48
2	65.71	42.15
3	65.93	38.59
4	65.94	49.16
5	65.08	48.57
6	64.60	39.53
7	67.19	45.87
8	64.75	46.14
9	64.09	47.79
10	64.64	48.07