

**DEVELOPMENT OF CHITOSAN BASED NANOFIBROUS
SCAFFOLDS INCORPORATED WITH BIOACTIVE COMPOUNDS
FOR WOUND HEALING (Project – Report)**

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Technology degree in Biotechnology

By

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BONAFAIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of VISHNU. A. M (38230041) who carried out the project entitled **“DEVELOPMENT OF CHITOSAN BASED NANOFIBROUS SCAFFOLDS INCORPORATED WITH BIOACTIVE COMPOUNDS FOR WOUND HEALING** “under our supervision from October 2019 to March 2020.

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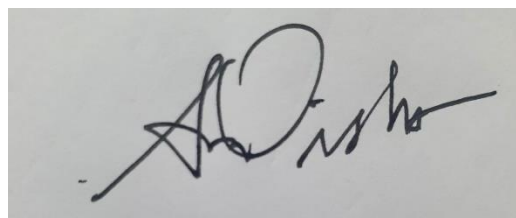
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DECLARATION

I **VISHNU. A. M (38230041)** hereby declare that the Project Report entitled

“DEVELOPMENT OF CHITOSAN BASED NANOFIBROUS SCAFFOLDS INCORPORATED WITH BIOACTIVE COMPOUNDS FOR WOUND HEALING” done by me under the guidance of **Dr. V. Ramesh Kumar** at **Sathyabama institute of Science and Technology, Chennai** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

A handwritten signature in black ink, appearing to read 'Vishnu A. M.', is shown on a light gray background.

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SIGNATURE OF THE CANDIDATE

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ABSTRACT:

Wound healing is a problem faced by surgeons in day-to-day clinical practices. Wounds are prone to bacterial infections which hinders the wound healing process. One other factor affecting the healing of wound is excessive accumulation of ROS. So, increase the wound healing rate and to deal with the prevention of bacterial infections and counteract the excessive accumulation ROS, advanced wound dressings are required. One such type of wound dressing is natural polymer based nanofibrous scaffolds. Natural polymer such as chitosan was combined with a synthetic polymer such as Polyvinyl Alcohol (PVA) for improved mechanical strength. Pomegranate peel extract and gooseberry seed extract were used in combination to provide antibacterial and antioxidant property to the scaffolds. The nanofibrous scaffolds were prepared using a method called electrospinning. The extracts were subjected to GC-MS for identifying the compounds. The scaffolds made from electrospinning was characterized using SEM, FTIR. To test wound healing capacity of the chitosan nanofibrous scaffolds, in-vitro wound healing assay was employed. The in-vitro wound healing assay results showed significant wound healing capacity of the chitosan nanofibers incorporated with bioactive compounds.

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LIST OF ABBREVIATIONS AND SYMBOLS:

gms	-	Grams
ml	-	Millilitre
min	-	Minute
w / v	-	Weight by volume
nm	-	Nanometres
mg	-	Milligrams
mg/ml	-	Milligram per millilitre
mM	-	Millimolar
ug/ml	-	Microgram per millilitre
°C	-	Degree celcius
cm	-	Centimetre
%	-	Percentage
TLC	-	Thin layer chromatography
GC-MS	-	Gas chromatography Mass spectrophotometry

CHAPTER 1: INTRODUCTON

1.1What is a wound?

Wounds are injuries that break the skin or any part of the body. They are caused by many reasons such as cuts, scrapes, punctured skin, blunt force trauma, scratches. Even sutures and stiches during surgeries cause wounds.

1.2 Types of wounds:

Wounds can be classified majorly into two types:

- Open wounds
- Closed wounds

Open wounds:

It involves a break in the skin which leaves the internal tissue exposed. It is usually caused by falls, cuts, blunt force trauma or surgery. Examples of open wounds include:

Abrasions:

An abrasion occurs when the surface of the skin slide or rubs on a rough surface. They produce very little blood.

Lacerations:

Lacerations are the deep opening or tear in the skin. They produce a lot of blood.

Avulsions:

An avulsion involves forcefully tearing away the skin and underlying tissue. It is usually caused during accidents, explosions.

Puncture:

A small hole in the soft tissue is called as a puncture wound. This is caused due to splinters, sharp objects, gunshots.

Closed wounds:

Closed wounds are those that occur when the skin remains intact, but the tissue underneath is not exposed due to blunt force trauma caused when someone falls down. This leads to damaged bones, tissues, and internal organs.

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1.3 STAGES OF WOUND HEALING:

The process of wound healing has been classified into four overlapping stages (Gosain A, DiPietro LA, 2004., S. Guo and DiPietro LA, 2010):

1. Haemostasis
2. Inflammation
3. Proliferation
4. Resolution.

HEMOSTASIS:

Hemostasis begins with fibrin clot formation at the injured endothelial site and platelets adhere (DiPietro LA, et al., 1998). These adhered platelet release cytokines to attract the components of the inflammatory phase.

INFLAMMATION:

The inflammatory phase is initiated by sequential infiltration of Neutrophils, Macrophages and Lymphocytes (Broughton et al., 2006; Campos et al., 2008). The inflammatory cells then help to release some pro-inflammatory cytokines and growth factors such as (TGF)- β , platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), ingesting foreign materials, increasing vascular permeability and promote fibroblast activity (Hunt TK, et al., 1984).

PROLIFERATION:

The proliferative phase usually begins several days after the initial injury. This phase is characterized by capillary growth and granulation tissue formation. Epithelial proliferation and abundant collagen synthesis lead to re-epithelialization and the construction the preliminary dermis. Within the wound bed, fibroblasts produce

collagen as well as glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (ECM).

RESOLUTION:

This is the final phase of wound healing. This phase is also called as remodelling phase. The final phase of wound healing can last for years. For the vascular density of the wound to return to normal regression of the many newly formed capillaries take place. In this phase the remodelling architecture of the ECM approaches that of a normal cell.

1.4 FACTORS AFFECTING WOUND HEALING:

Local factors:

1. Oxygenation
2. Infection
3. Foreign body

Oxygenation:

Oxygen is very important for energy production of the cell and is very critical for all wound healing process. It prevents wounds from infection, induces angiogenesis, increases keratinocyte differentiation, migration, and re-epithelialization, enhances fibroblast proliferation and collagen synthesis, and promotes wound contraction (Bishop, 2008; Rodriguez *et al.*, 2008). Reactive Oxygen Species (ROS) such as Hydrogen peroxide and superoxide act as cellular messengers in key processes such as wound healing. However, increase in ROS transcends the beneficial effect and causes additional damage.

Infection:

When there is an open cut on the skin, the microorganisms present on the surface of the skin gets access to the underlying tissues. The level of infection determines whether the wound is classified as having contamination, colonization, local infection, or invasive infection. *P. aeruginosa* and *Staphylococcus* appear to play an important role in bacterial infection in wounds (., S. Guo and DiPietro LA, 2010).

1.5 NANOFIBROUS SCAFFOLDS FOR WOUND HEALING:

Wound care has improved very much in the past decade from using conventional methods such as ointments, gauze coverages to multifunctional wound dressings, hemostats and tissue engineered dermal substitutes (Robin Augustine et al., 2020). Novel polymeric biodegradable patches were developed for wound coverage. These polymeric biodegradable patches could avoid wound degradation, pathogenic invasion, thus healing the wound faster. However, without any active compounds these patches offer only passive protection (Vig, K., et al, 2017; Clark, R.A., et al, 2007). Active wound dressings can act as alternatives to grafts to protect wound bed, inhibit pathogenic proliferation and foster healing (Vyas, K. and H. Vasconez, 2014). Most of the current wound dressings are made from polymers either natural or synthetic. Some of the naturally used polymers for wound dressings include chitosan, alginate, dextran, cellulose, chitin, elastin, etc (Kenawy, E., et al, 2019). To overcome the limitations of the conventional methods, wound dressings such as hydrogels, cellulose sponges, and nanofibrous scaffolds have been proposed as they protect the wound from microbial invasion and facilitate in the release of bioactive compounds for faster wound healing (Bakhsheshi-Rad et al., 2020; Nguyen et al., 2013; Ojah et al., 2019; Zhang et al., 2019a). Scaffolds made from the above mentioned naturally available polymers usually suffer from poor mechanical properties. This can be easily overcome by crosslinking them with synthetic polymers such as poly (α -esters), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (D,L-glycolic-co-lactic acid) (PLGA), poly (ϵ -caprolactone) (PCL)], poly(vinyl pyrrolidone) (PVP), poly(ethylene oxide) (PEO)/poly (ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(hydroxyethyl methacrylate) (PHEMA), polyurethanes (PUs) (Khamrai, M ., et al, 2019). By crosslinking natural polymers with synthetic polymer, the resulting polymer will have desired mechanical strength. Using the polymers, wound dressings can be made in different forms such as films, sponges, hydrogels, bandages, exosomes, foams, wafers, nanofibers, and nanofibrous materials (including nanofibrous mats, patches, films, membranes, etc.) (Boateng, J.S., et al, 2008). However, the above-mentioned wound dressings have poor antimicrobial property. This can be overcome by incorporating bio-active compounds that offer antimicrobial property to the wound dressing.

1.5.1 CLASSIFICATION OF WOUND DRESSING:

Type of wound dressing	Functions	Limitations	References
Skin substitutes	It replaces damaged skin, reduces scar formation	Host rejection, possible transmission of infection, limited life span	(Ng, S.; Jumaat, N., 2014)
Traditional wound dressing	Protects the wound contamination, provides cushioning, controls bleeding	Frequent changing of wound dressing, causes re-skin damage and pain.	(Hansson, C, 1997)
Interactive wound dressing	Offers moisture at the wound site, good mechanical properties	Limited antimicrobial activity	(Anisha, B.S, 2013)
Bioactive wound dressing	Biocompatible, biodegradable, acts as drug delivery systems	—	(Gupta, K.C., et al, 2014)

As discussed earlier, wound dressings can be made in different forms by using appropriate biopolymers. One such widely used and advantageous form of wound dressing is nanofibrous scaffolds. These nanofibrous scaffolds can be incorporated with bioactive compounds for synergic antimicrobial and antioxidant activity. The most widely used biopolymer for wound dressings is chitosan. It has various advantages such as biocompatibility, biodegradability, cost efficient, etc. By loading suitable antimicrobial and antioxidant source in the nanofibrous scaffolds we can develop cheap and advanced wound with synergic antimicrobial and antioxidant activity, thereby increasing the rate healing.

1.5.2 CHITOSAN NANOFIBROUS SCAFFOLD WITH ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY:

Chitosan is also called as the deacetylated chitin, is a naturally available polycationic linear polysaccharide which is derived by partial deacetylation of Chitin (Cheung, Randy Chi Fai et al, 2015). Chitin is found the exoskeleton of crustaceans, insects, and cell walls of fungi. Chitosan is composed of β -(1-4)-linked d-glucosamine and *N*-acetyl-d-glucosamine randomly distributed within the polymer. Chitosan offers various applications because of its biocompatibility, low allergenicity and biodegradability (Kumar M.N., et al, 2004). Other than that, chitosan also has biological properties such antimicrobial, antitumor and antioxidant (Karagozlu M.Z., Kim S.-K, 2014; Martins A.F., et al, 2014; Ngo D.H., Kim S.K, 2014). Chitosan is a polysaccharide which contains breakable glycosidic bonds and is degraded in lysozyme and several proteases in vivo (Kean, T. and M. Thanou, 2011). As a result of bio-degradation oligosaccharides are released and they are usually non-toxic. By using chitosan nanofibers wound healing is accelerated because the degradation product of the chitosan nanofibers stimulates macrophages and influencing positively on collagen deposition (Singh, R., 2007). The antimicrobial potential of chitosan biopolymer especially against bacteria and fungi is well established and is associated with two principal mechanisms. Firstly, positively charged polymer chains allow them to bind easily with the negatively charged cell walls of microbes and leads to a change in cell permeability (Kong, M., et al, 2010). Bioactive wound dressings can be made from these nanofibers. Bioactive wound dressings are those that are utilized for the delivery of bioactive agents. Examples of Bioactive agents that can be delivered with the help of these dressings include growth factors, nutrients, plant extract, and stem cells. These wound dressings are prepared from natural polymers such as pectin, collagen, alginate, hyaluronic acid, silk fibroin, chitosan, elastin, cellulose, and collagen. Recently reports on bioactive dressings that are patient compliance, biodegradable, and biocompatible. Examples of bioactive wound dressings include Hydrogels, wafers, foams, sponges, transdermal patches, nanofibers. In the current study, we are more concerned about how wound dressings based on nanofibers facilitate faster wound. Let us see how nanofibers are synthesized and how advantageous it is towards wound healing (Colobatiu, L, 2019).

1.5.3 ELECTROSPINNING AND PROPERTIES OF NANOFIBROUS MATERIALS AS WOUND DRESSINGS

Many methods are available for the preparation of nanofibers and nanofibrous scaffolds. But the most commonly used and effective method for preparing nanofibers is electrospinning. This technique uses electrostatic forces to pull the fibres from the droplet formed at the tip of the needle. The formation of nanofibers using electrospinning is influenced by three parameters: solution parameters, ambient parameters, process parameters. The electrospinning technique can produce continuous fibers utilizing a broad range of materials such as polymers and their composites. This method can produce nanofibers/nanofibrous scaffolds with the average diameter ranging from micron to nanosized diameter (Wu, H, 2012). Electrospinning of natural polymers showed poor mechanical strength. However, by combining biopolymers with synthetic polymers, the resulting polymer controls the degradation rate, improve the morphology and mechanical properties of the nanofibrous scaffolds. The parameters used in electrospinning influence the generation of bead-free and smooth electrospun fibers. The electrospun nanofibrous materials have been used to accelerate the wound healing process and to prevent post-surgical infections with controlled and sustained drug release profiles (Liu, M, 2017). By preparing chitosan along with a synthetic polymer such as Polyvinyl alcohol (PVA) improved mechanical strength (Khamrai, M ., et al, 2019) and showed antimicrobial activity which is important for preventing infection at the wound site. As discussed earlier, wound healing is affected by some factors such as bacterial infection, more Reactive Oxygen Species (ROS) produces at the site. In the inflammatory phase, cytokines are produces and immune cells are promoted. Cytokines provoke fibroblast and chondrocyte cells to manufacture reactive oxygen species (ROS) (Volpe, 2018). The uncontrolled level of ROS damages the cell. Since a sustained low-level presence of the ROSs is required for wound healing, the regulation of the amount of the ROSs with an appropriate antioxidant compound is essential to healing the wound (El-Aassar et al., 2020). Another factor affecting wound healing is bacterial infection at the wound site.

Healing wounds is hampered by infection, a common problem. Wounds on the skin are susceptible to the accumulation of infectious bacteria. Using antibiotics to prevent and treat wound infections has become inefficient due to the spread of antibiotic-resistant bacteria. In addition, it incurs additional costs for individuals and society. Therefore, alternative materials are crucial for fighting pathogens. To incorporate the antimicrobial and antioxidant property to the nanofibers, cheap plant sources with high antioxidant and antimicrobial activity. Let us see the properties of the plant sources in detail.

1.5.4 DESCRIPTION OF SELECTED PLANTS

Punica granatum

TAXONOMICAL CLASSIFICATION

Kingdom –plantae

Subkingdom – Viridiplantae

Class – Mangoliopsida

Order – Myrtales

Family – Lythraceae

Genus – *Punica*

Species – *granatum*

Punica granatum is known as pomegranate. In this study pomegranate peels were taken as a source for incorporating antimicrobial activity. Pomegranate peel is widely recognized for its effectiveness against a broad spectrum of bacterial pathogens. Tabassum & Khan, (2010), studied antimicrobial effect of pomegranate peel extracts against *B. cereus*, *E. coli*, *S. aureus* and *P. aeruginosa*. Ismail, Akhtar, Sestili, Riaz, Ismail & Labbe, (2016) also obtained inhibitory effects of pomegranate peel extracts against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E.coli*, *S.*

typhimurium and *A. niger*. Since pomegranate peels are usually wasted it could be used as a cheap alternate for an antimicrobial source.

Phyllanthus emblica

TAXONOMICAL CLASSIFICATION

Kingdom –plantae

Subkingdom – viridiplantae

Class – mangoliopsida

Order – Malpighiales

Family – Phyllanthaceae

Genus – Phyllanthus

Species – emblica

Phyllanthus emblica is known as Indian gooseberry which is found in the tropical and subtropical regions of Asia. It has been shown that *P. emblica* has vitamin c, β -carotene, calcium, phosphorus, proteins, fats, carbohydrates and phytochemicals for example tannins, saponins, flavonoids, alkaloids, steroid, anthraquinone (Sumalatha D, 2013; Triratnarong D, 2007; Eugeny AP et al, 2009). Most of the time only the flesh of the fruit is used for the production of many products and the seeds are discarded. So, using the seeds as a source for antioxidant activity would make the overall product cheap and feasible for consumers.

CHAPTER 2: REVIEW OF LITERATURE

Various works have been done with different compounds to enhance wound healing. Let us see the works done over time in this review of literature.

Mitra Bagheri et al., worked with chitosan nanofibers containing AgNPs-ZnONPs. By combining Zn/Ag nanoparticles, the nanofibers showed enhanced wound healing potential. It showed a potential of single-device antibiotic combinations with synergic antibacterial and antioxidant properties. The nanofiber mats showed significant inhibition against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli*. The mats proved no cytotoxic effect on fibroblast cells and increased their viability. Nanofibrous scaffolds containing 8.3 % AgNPs-ZnONPs had the highest wound healing potential.

Charernsriwilaiwat. N et al., incorporated *Garcinia mangostana* (GM) extracts into chitosan-based nanofibers for wound healing. The GM extracts with 1, 2 and 3% wt α -mangostin were incorporated into 31 the CS-EDTA/PVA solution and electrospun to obtain nanofibers. These fibre mats showed suitable mechanical and swelling properties. These mats are non-toxic and α -mangostin is rapidly released, which 587 retains the antioxidant and antibacterial activity, and accelerates the wound healing process. Chitosan based nanofibers loaded with GM extracts were synthesized by electrospinning. The mats maintained 90% of their content of α -mangostin for 3 months.

Waghmare SV et al., used starch-based nanofibers as scaffolds for wound healing. Starch based nanofibers were made using electrospinning. These nanofibers had diameter ranging from 110- 330nm. They used Polyvinyl Alcohol (PVA) and Glutaraldehyde for improving the mechanical strength of the nanofibers. Cellular investigations revealed that the nanofibrous mats allowed cell growth and proliferation of dermal cells and have a potential to be employed in biomedical applications like wound healing.

Kharaghani et al. formulated electrospun PVA-chitosan nanofibers loaded with Ag and copper nanoparticles. Their physicochemical properties were confirmed by X-ray diffraction (XRD) analysis and attenuated total reflectance spectroscopy (ATR). The in vitro antimicrobial analysis showed that the nanofibers containing Ag nanoparticles displayed improved antibacterial properties compared to the copper nanoparticles.

Ganesh et al. formulated PVA-chitosan composite electrospun nanofibers co-encapsulated with Ag nanoparticles and sulfanilamide for synergistic wound-healing effect. The successful formulation of Ag nanoparticle-loaded nanofibers physicochemical properties was confirmed by Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) analysis. The scanning electron microscopy (SEM) images of the nanofibers revealed continuous and smooth morphology with an average fiber diameter of 150 nm, which is suitable for the encapsulation of Ag nanoparticles for the treatment of microbial infected wound healing. The swelling analysis displayed that the degree of swelling in PVA–chitosan nanofibers was influenced by Ag nanoparticles/sulphanilamide quantity. The feature mentioned above reveals that Ag nanoparticles/sulphanilamide was crosslinked with the polymer matrix via hydrogen bonding to hinder water uptake and the swelling of the drug-encapsulated nanofibers.

Aadil et al. formulated PVA-lignin electrospun nanofibers loaded with Ag nanoparticles as potential antibacterial wound dressings. The thermal gravimetric analysis (TGA) of nanofibers exhibited a maximum weight loss percentage between 200 and 300 °C, which is due to the decomposition of C-C bonds in lignin. The in vitro antimicrobial analysis of PVA-lignin electrospun nanofibers loaded Ag nanoparticles showed an inhibition zone of 1.1 ± 0.05 and 1.3 ± 0.08 cm against *E. coli* and *B. circulans*, respectively, revealing that Ag nanoparticles encapsulated polymeric nanofibers had good antibacterial efficacy, which is due to its spherical shape, small size, and large surface area.

Panagiota Moutsatsou et al., produced nanofibers with polyaniline–chitosan (PANI/CS) using electrospinning. Increase in PANI content resulted in membranes with higher hydrophobicity and higher electrical conductivity. It was found that none of the membranes showed any toxic effects on osteoblasts and fibroblasts, and that they all supported cell attachment and growth, even to a greater extent than tissue culture plastic. The membrane with the PANI/CS ratio 1:3 supports better cell attachment and proliferation for both cell lines due to a synergistic effect of hydrophilicity retention due to the high chitosan content and the conductivity that PANI introduced to the membrane.

Yang et al. designed Janus nanofiber wound dressings from PVP and ethyl cellulose via a side-by-side electrospinning technique. The nanofibers were loaded with Ag nanoparticles and ciprofloxacin. The successful preparation of the dual drug-loaded Janus nanofibers was confirmed by FTIR and XRD. The in vitro drug release revealed a controlled release pattern of the drug from the nanofibers. The in vitro antibacterial analysis demonstrated the antibacterial activity of Janus nanofibers against both *S. aureus* and *E. coli*, indicating a synergistic action of both the Ag nanoparticles and ciprofloxacin.

Lee et al. formulated electrospun chitosan-based nanofibers encapsulated with Ag nanoparticles for bacteria-infected wound care. The SEM images displayed homogeneous nanofibrous structures with uniform morphology and mean fiber diameters of 460 ± 80 nm, 126 ± 28 nm for plain nanofibers, and nanoparticle-loaded nanofibers, respectively. The in vitro antimicrobial evaluation of nanofibers demonstrated a high zone of inhibition against *P. aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (MRSA), suggesting that the chitosan-based nanofibers encapsulated with Ag nanoparticles are potential therapeutics for the treatment of infected wounds

CHAPTER 3: AIM AND OBJECTIVE

3.1 AIM:

To develop Chitosan-based nanofibrous scaffolds incorporated with bioactive compounds from *Punica granatum* and *Phyllanthus emblica* for wound healing.

3.2 OBJECTIVES:

1. To evaluate the phytochemical compounds.
2. To estimate the antioxidant activity by DPPH assay.
3. To estimate the antidiabetic activity by α -amylase inhibition assay.
4. To estimate the anti-inflammatory activity by Membrane stabilization assay.
5. To evaluate the biochemical compounds by GCMS.
6. To characterize nanofibers using SEM.
7. To perform in-vitro wound healing assay

CHAPER 4: MATERIALS AND METHODS

4.1 MATERIALS

1. Conical flask
2. Test tubes
3. Weighing balance
4. Shaker incubator
5. Millipore filter
6. Centrifuge
7. Eppendorf
8. Petri plates
9. Measuring cylinder
10. UV-spectrophotometer

4.2 METHODOLOGY

4.2.1 *Sample collection:*

The samples were collected from nearby fruit shop, and they were thoroughly washed with tap water and then shade dried.

4.2.2 *Sample preparation:*

Collected pomegranates were peeled and the peels were crushed, and shade dried. Gooseberries are seeded and the seeds were shade dried and crushed.

4.2.3 *Solvent extraction:*

The powdered samples were extracted by soaking them in methanol separately. The extracts were separated from the residues by filtering through Whatman No.1 filter paper. The residues were extracted twice with the fresh solvent and extracts were combined. The extracts were stored in a refrigerator ($5\pm 2^{\circ}\text{C}$) until used for further analysis (Verma, M., et al., 2018). Next, 10% of gooseberry extract and 10%

of pomegranate peels extract was mixed together in the ratio of 1:1 for further analysis.

4.2.4 Phytochemical screening:

Phytochemical examinations were carried out for all the extracts as per the standard methods.

4.2.4.1 Detection of alkaloids:

4.2.4.1.1 Mayer's Test:

Take few ml of extract, add a drop Mayer's reagent. Appearance of white creamy precipitate indicates the presence of alkaloids.

4.2.4.2 Detection of carbohydrates:

Benedict Test:

Take 0.5 ml of sample and add 1ml of Benedict's reagent. The mixture is heated on a boiling water bath for 2 minutes. Appearance of precipitate indicates the presence of sugar.

4.2.4.3 Detection of Glycoside:

Take few ml of sample, add concentrated HCl, add 2ml of glacial acetic acid and now add 1ml of H₂SO₄. Appearance of violet ring below the brown ring and green ring confirms the presence of glycosides.

4.2.4.4 Detection of saponins:

1ml of sample is added with 2ml of distilled water and mix well. Development of stable foam suggests the presence of saponins.

4.2.4.5 Detection of phenol

To the extract, 200µl of neutral ferric chloride solution was added and shaken well. Dark green or Violet or Purple or dark brown color indicates the presence of phenol.

4.2.4.6 Detection of flavonoids

To the extract, few drops of 1% NaOH solution, were added. A yellow colour formation within short period is positive for flavonoids.

4.2.4.7 Detection of tannins

To the extract, few drops of lead acetate solution was added and observed for brownish green or a blue-black coloration.

4.2.4.7 Detection of Terpenoids:

To a 2ml of extract, few drops of saturated solution of trichloro acetic acid are used formation of coloured precipitate confirms the presence of terpenoids.

4.3 ESTIMATION OF ANTIOXIDANT ACTIVITY BY DPPH METHOD

The antioxidant activity of the mixture of the two extracts was measured by DPPH (1, 1- diphenyl 2-picrylhydrazyl) free radical scavenging activity (Blois, 1958). One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (20-120 µg/mL) extract mixture. The mixture was then allowed to stand for 30 min incubation in dark. One mL of DPPH solution mixed with 1 mL of methanol was used as the control. The decrease in absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The percentage of inhibition was calculated using the following formula:

$$\% \text{ Of DPPH}^{\bullet} \text{ radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

4.4 ESTIMATION OF ANTIDIABETIC ACTIVITY BY α -AMYLASE INHIBITION ASSAY:

α - amylase enzyme inhibition assay was carried out based on the starch-iodine test (Xiao *et al.*, 2006). The total assay mixture was composed of various concentration (20-120 µg/mL) ofextract, 10 µL of alpha amylase enzyme prepared in 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) and was incubated at 37°C for 10 min. Then soluble starch (1%, w/v) was added to each reaction set and incubated at 37°C for 60 min. One hundred µL of 1 M HCl was added to stop the enzymatic reaction and followed by 200 µL of iodine reagent (5 mM I 2- and 5-mM KI) was added. The colour change was noted and the absorbance was read at 595 nm. The control reaction representing 100% enzyme activity did not contain any plant extract. A dark blue colour indicates the presence of starch; a yellow colour indicates the absence of starch, while a brownish colour indicates

partially degraded starch in the reaction mixture. In the presence of inhibitors from the extracts, the starch added to the enzyme assay mixture is not degraded and gives a dark blue colour complex, whereas no colour complex is developed in the absence of the inhibitor indicating that starch is completely hydrolyzed by α -amylase.

$$\% \text{ Of } \alpha\text{-amylase enzyme inhibition} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

4.5 ESTIMATION OF ANTI-HEMOLYTIC ACTIVITY BY MEMBRANE STABILIZATION ASSAY.

4.5.1 Preparation of Red Blood cells (RBCs) suspension

The Blood was collected from healthy human volunteer who has not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tube. The tubes were centrifuged at 3000 rpm for 10 min and discard the pale-yellow plasma. Then, about 10 mL of PBS was poured into centrifuge tube, centrifuged at 3000 rpm for 5 min, discard and discard the supernatant and repeat the washing process once again. The volume of the dissolved red blood pellets obtained was measured and reconstituted as a 40% v/v suspension with isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4). The buffer solution contained 0.2 g of NaH_2PO_4 , 1.15 g of Na_2HPO_4 and 9 g of NaCl in 1 litre of distilled water. The reconstituted red blood cells (resuspended supernatant) were used as such normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

4.5.2 Heat induced hemolysis

The reaction mixture (2 mL) consisted of 1 mL of different concentrations of plant extracts (20-120 $\mu\text{g/mL}$) extract and 1 mL of 10% RBCs suspension, instead of test sample only saline was added to the control test tube (Shinde et al., 1989). All the centrifuge tubes containing reaction mixture were incubated in water bath at 56°C for 30 min. At the end of the incubation the tubes were cooled under running tap

water. The reaction mixture was centrifuged at 3000 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. Aspirin was used as a standard drug.

The percentage inhibition of hemolysis was calculated as follows:

$$\text{Percentage of hemolysis inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

4.6 ANTIBACTERIAL ACTIVITY OF THE PLANT EXTRACTS:

The microorganisms of Gram-positive bacteria such as *Staphylococcus aureus*, and Gram-negative bacteria *Escherichia coli* and *Pseudomonas argenoisa* were used for the evaluation of the antibacterial activity.

4.6.1 Agar well diffusion method

Nutrient broth agar medium was prepared according to the standard methods (peptone-5 g, yeast extract-3 g, NaCl-5 g, distilled water- 1000 mL, agar-20 g). The required amount of ingredients was weighed and suspended in the required volume of 100 mL of distilled water and then autoclaved at 15 lbs and 121°C for 15 min. The hot medium was poured in sterile petri plates which were kept in the aseptic Laminar chamber. The medium was allowed to solidify for 15 min.

Antibacterial activity of plant extracts was carried out using agar well diffusion method (Kubo et al., 2002). The solidified nutrient agar in the petri plates was inoculated by dispensing the inoculum using sterilized cotton swabs which are previously immersed in the inoculum containing test tube and spread evenly onto the solidified agar medium. Five wells were created in each plate with the help of a sterile well-borer of 8 mm diameter. The plant extracts were then poured into each well containing 250, 375, and 500 µg/mL concentrations. All the plates with extract loaded wells were incubated at 37°C for 24 h and the antibacterial activity was assessed by measuring the diameter of the inhibition zone formed around the well. Tetracycline (25 µg) was used as the positive control. zones >6 mm were considered as inhibitions resulting from significant antibacterial activity.

4.7 PREPARATION OF CHITOSAN, PVA AND PLANT EXTRACT FILM FORMING MIXTURE:

Method used by Mirzaei., et al 2016 was carried out with a slight modification. PVA used in the procedure was replaced with PVA. Chitosan (3.0 Wt. %) and PVA (3.0 Wt. %) solutions were prepared separately by dissolving chitosan and PVA powder in aqueous acetic acid (90 V/V %) under magnetic stirring at 37 °C for 24 h. The obtained solutions were then mixed together in weight ratio of CS/PVA, 90:10, as the required polymer solution for electrospinning with 3.0 Wt. % of total solid. Plant extract dissolved in 90% (v/v) aqueous acetic acid for 20% (w/w) concentration and then add to Chitosan/PVA blend solution (polymer solution) to obtain polymer/extract solution with polymer to extract weight ratio of 90: 10.

4.7.1 PREPARATION NANOFIBROUS SCAFFOLDS WITH BIOACTIVE COMPOUNDS USING ELECTROSPINNING:

The electrospinning processes were carried out using Espin Nano. To produce extract loaded CS/PVA nanofibrous mats, the polymer solutions with different amounts of the extract was placed into a 5 ml plastic syringe with a blunt-ended 18-G stainless steel needle. An aluminium foil was wrapped on the rotating drum as collector and was located at the distance of 17 cm from the needle. A syringe pump fed the solution to the needle tip at the injection rate of 0.5 ml/h. The wire of a DC positive high voltage was connected to the metallic needle and the collector to the ground. The applied voltage, and drum speed were fixed at 20 kV and 200 rpm, respectively (Mirzaei., et al, 2016).

4.8 ANTIBACTERIAL ACTIVITY OF CHITOSAN NANOFIBROUS SCAFFOLDS:

The antimicrobial activity of nanocomposite scaffolds was evaluated by the disk diffusion method against a gram-negative bacterium (*Escherichia coli*) and a gram-positive bacterium (*Staphylococcus aureus*). The microbial culture medium was prepared according to the manufacturer's protocol and poured into 10 cm plates, followed by separate preparation of a suspension of 0.5 McFarland bacteria. Sterilized cotton swabs from the suspension prepared on plate surface containing Mueller Hinton agar were cultured uniformly. The scaffolds were prepared by incubating the specimens in a 37 °C incubator for 24 h, and then the inhibitory areas around the scaffolds were measured (Bagheri, M., et al, 2021).

4.9 GC-MS ANALYSIS OF EXTRACT:

Gas Chromatography:

A Shimadzu GC-2010 Plus gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20°C per minute to 450°C and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode).

Mass spectrum:

A Direct connection with capillary column metal quadrupole mass filter period mass spectrometer operating in electron ionization (EI) mode with software ver. 2.6 was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 1000 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra

were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 1000 at 1 second per scan.

Mass spectrometry library search:

Identification of the components of the compound was matching their recorded spectra with the data bank mass spectra of NIST library V11 provided by the instrument's software. GCMS metabolomics Database was used for the similarity search with retention index.

4.10 FOURIER TRANSFORM INFRARED SPECTROSCOPY:

Fourier transform infrared spectroscopy (FTIR) analysis to evaluate the functional groups involved in the formation of nanoparticles [Anu et al.,2017]. Fourier transform infrared spectroscopy (FTIR) uses the mathematical process (Fourier transform) to translate the raw data (interferogram) into the actual spectrum. FTIR method is used to obtain the infrared spectrum of transmission or absorption of a fuel sample. FTIR identifies the presence of organic and inorganic compounds in the sample. Depending on the infrared absorption frequency range $600\text{--}4000\text{ cm}^{-1}$, the specific molecular groups prevailing in the sample will be determined through spectrum data in the automated software of spectroscopy (P. Mohamed Shameer & P. Mohamed Nishath, 2019)

4.11 SCANNING ELECTRON MICROSCOPY:

The morphology of the chitosan nanofibrous scaffolds were studied using a Scanning electron microscope.

4.12 IN-VITRO WOUND HEALING ASSAY:

Materials and Requirements:

- Dulbecco's Modified Eagle's medium with supplements (serum, antibiotics)
- Phosphate buffered saline

- Bovine serum albumin
- Poly-L-lysine stock
- Tissue culture dishes with 60mm
- Razor or fine sharpie marker
- p200 pipette tips
- Hemocytometer
- Phase-contrast microscope
- Stage incubator
- CO₂ incubator
- Video camera and
- Image analysis software

PROCEDURE

Coating of cell culture dishes

Take 60-mm culture dish and coat with ECM substrates and incubate the dish overnight for 2 hours at 37°C without shaking. Then remove unbound ECM substrate and coat with 3 ml of bovine serum albumin at 37°C for 1 hour. Wash it with PBS and add media into the dish before plating the cells

Cell culture

Re suspend the sub confluent growing cells by washing with PBS twice in culture dish. Add trypsin and mix the cell with serum containing medium. Disperse the cell equally by pipetting the solution and rock the dish gently. Using hemocytometer, cell counts of aliquot from cell suspension are determined. Add cells to 60mm culture dish to form confluent monolayer. Incubate them at 37°C for 6 hours to adhere to substrate and develop into monolayer.

In Virto Scratch assay

Scrape monolayer of the cell horizontally using p200 pipette tip and debris were removed by washing with PBS. Wash the cell again with 1ml of growth medium and replace it with 5 ml of medium. Create a reference mark on the culture plate by using a razor blade or with fine tipped marker. Then the cells were treated with extract at 40 μ g/ml . cells without treatment is considered as control and positive control will be cell treated with EGF (2ng/ml). Place the culture dish on the phase contrast microscope and record the first image of scratch at 0 hour. Keep the culture dish in CO2 incubator, observe them at the interval of 24h using the microscope and record the second and third set of Image. Migration rate of the cells are analysed by using the software "ImageJ".

CHAPTER 5: RESULTS AND DISCUSSION

5.1 SAMPLE EXTRACTION:

Gooseberry seeds and pomegranate peels were soaked in methanol and crude extract was filtered using Whatman filter paper. The filtrate was centrifuged, and the supernatant was refrigerated.

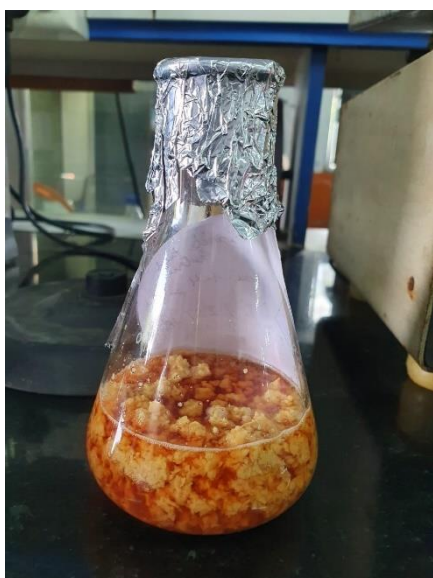


Fig.1 pomegranate peel extract

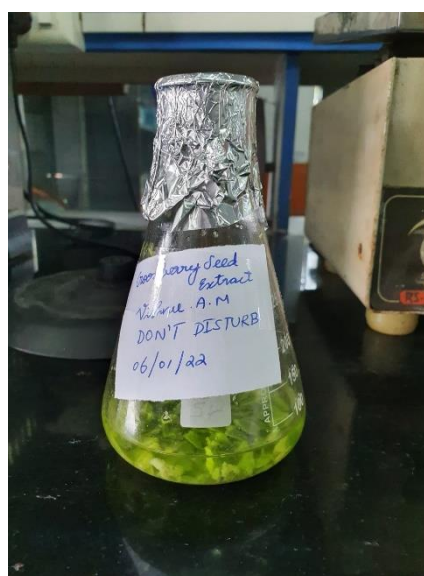


Fig. 2 Gooseberry seed extract

5.2 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS FROM MIXTURE OF PLANT EXTRACT:

The phytochemical screening of the extracts is listed below. “+” sign indicates the presence of the phytochemical. Whereas “-“ indicates the absence of the phytochemical

S. No	Phytochemicals		Methanol
1	Carbohydrates		+
2	Glycosides		+
3	Phenol		+
4	Terpenoids		+
5	Saponins		-
6	Steroids		-
7	Tannins		+
8	Alkaloids		+

5.3 ESTIMATION OF ANTIOXIDANT ACTIVITY BY DPPH ASSAY:

The extract was tested for its antioxidant activity. The extract was subjected to DPPH assay. The plant extract was allowed to react with violet colored DPPH solution, which was reduced to yellow color product, diphenyl picryl hydrazine. The rate of conversion indicates the presence of antioxidant potential in the plant sample and the reduction reaction in plant sample occurs in the concentration dependent manner. The scavenging capacity of the sample were compared with the standard, Gallic acid which is a stronger DPPH scavenger.

Table 1. Estimation of antioxidant activity:

S.no	Concentration	Absorbance	% Of Inhibition
1	Control	0.241	
2	20	0.165	

3	40	0.123	
4	60	0.143	
5	80	0.105	
6	100	0.098	
7	120	0.092	

5.4 ESTIMATION OF ANTI-DIABETIC ACTIVITY BY α -AMYLASE INHIBITION ASSAY:

To know whether this lichen species have antidiabetic or not, α -amylase activity is done using both the extracts. The activity was found to be similar for both aqueous and methanol extracts. The percentage of inhibition of methanol extract at 100 μ /g concentration was 58.4%, whereas for aqueous extract it was found out to be 51.1%.

Table 2. Estimation of antidiabetic activity:

S.no	Concentration	Absorbance	% Of Inhibition
1	Control	0.126	
2	20	0.713	
3	40	0.685	
4	60	0.631	
5	80	0.480	
6	100	0.475	
7	120	0.467	

5.5 ESTIMATION OF ANTI- HEMOLYTIC ACTIVITY BY MEMBRANE STABILIZATION METHOD.

To analyse if the sample has the ability to inhibit the hemolysis of red blood cells. HRBC method was selected for the *in vitro* evaluation of anti-inflammatory property because the erythrocyte membrane is analogous to the lysosomal membrane (Shenoy *et al.*, 2010) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release. The results indicated that the plant extracts showed significant inhibition at increasing concentrations. The highest % inhibition was 93.5% at 120 µg/ml and the lowest was at 48.45 % at 20 µg/ml. The IC₅₀ value of the extracts were found to be 61.91%

Table 3. Estimation anti-hemolytic activity:

S.no	Concentration	Absorbance	% Of Inhibition
1	Control	1.841	
2	20	0.949	48.45%
3	40	0.661	64.09%
4	60	0.417	77.34%
5	80	0.270	85.33%
6	100	0.254	86.20%
7	120	0.119	93.5%

5.6 ANTIBACTERIAL ACTIVITY OF THE PLANT EXTRACTS:

The combination of the plant extracts was tested for its antibacterial activity against four microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Micrococcus luteus*. The plant extracts were tested in three different concentrations (i.e) 250 µg/ml, 375 µg/ml, 500 µg/ml. The zone of inhibition is measured in mm.

Table 4a. Activity against *S. aureus*

Sample concentration	Zone of inhibition
250µg/ml	21mm

375 µg/ml	23mm
500 µg/ml	25mm

Table 4b. Activity against *E. coli*

Sample concentration	Zone of inhibition
250µg/ml	17mm
375 µg/ml	19mm
500 µg/ml	22mm

Table 4c. Activity against *M. luteus*

Sample concentration	Zone of inhibition
250µg/ml	20mm
375 µg/ml	22mm
500 µg/ml	23mm

Table 4d. Activity against *B. subtilis*

Sample concentration	Zone of inhibition
250µg/ml	27mm
375 µg/ml	28mm
500 µg/ml	32mm



Fig 1a. *M. luteus*



Fig 1b. *E. coli*



Fig 1c. *B. subtilis*



Fig 1d. *S. aureus*

5.7 PREPARATION OF NANOFIBROUS SCAFFOLDS USING ELECTROSPINNING:

The polymer solution was made into nanofibers by using electrospinning. The resulting nanofibers was found to have a thickness of 20-30 μm .



Fig 2. Chitosan nanofibers

5.8 ANTIBACTERIAL ACTIVITY OF CHITOSAN NANOFIBROUS SCAFFOLDS:

The prepared scaffolds were tested for antibacterial activity against one gram-positive bacterium (*S. aureus*) and one gram-negative bacterium (*E. coli*). The results showed that the scaffolds had significant antibacterial activity. This could be advantageous for preventing wound infections.

Table 5. Zone of inhibition.

Bacterium	Zone of inhibition
<i>S. aureus</i>	24mm
<i>E. coli</i>	18mm



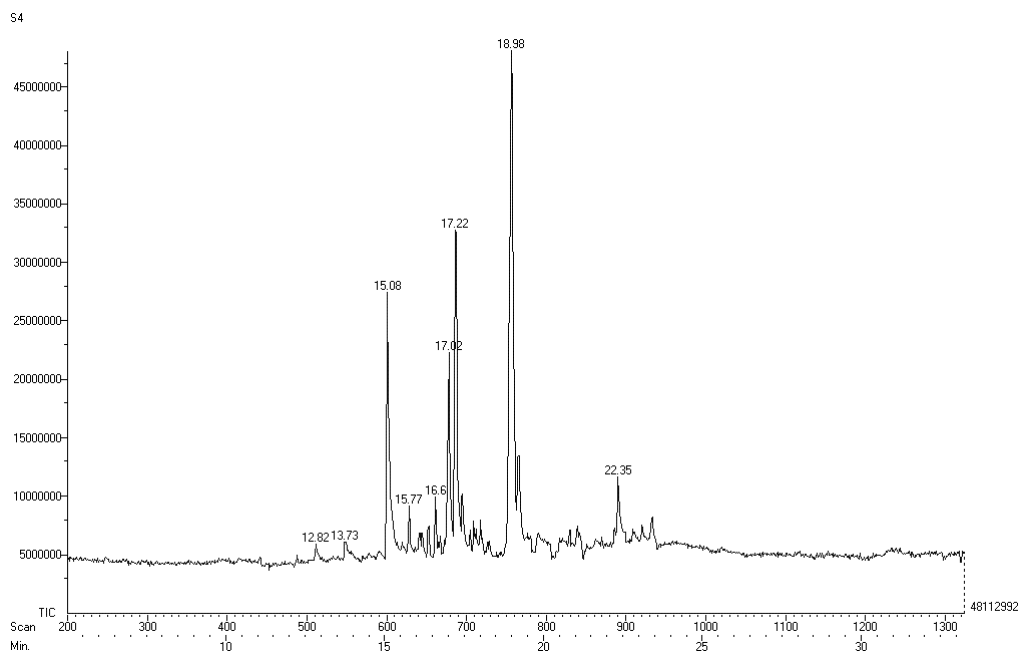
Fig 2a. *S. aureus*



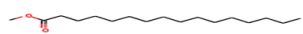
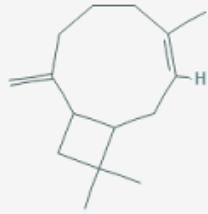
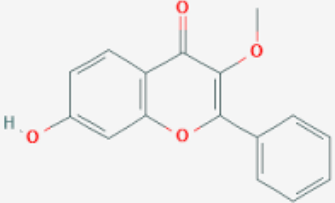
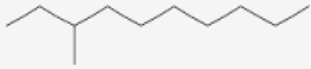
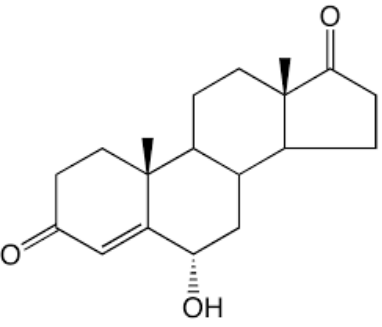
Fig 2b. *E. coli*

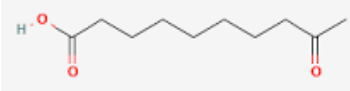
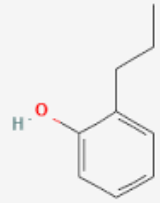
5.9 GC-MS ANALYSIS OF EXTRACT:

Fig 3. GC-MS graph



S.no	Compounds	Structure
1	Phytol	<chem>CCCC(C)CCCC(C)CCCC(C)CCCC(C)O</chem>
2	Pentadecane	<chem>CCCCCCCCCCCCCCC</chem>

3	Hexadecanoic acid, methyl ester	
4	Humulen-(V1)	
5	4H-1- Benzopyran-4-one, 7-hydroxy-2-phenyl-	
6	Decane-3-methyl	
7	4- Androsten-6a- ol-3, 17-dione	

8	Dodecanoic acid	
9	Phenol-2-propyl	

5.10 SCANNING ELECTRON MICROSCOPY:

The morphology of nanofibers such diameter, texture, and dimensions, were studied using the Scanning Electron Microscope (VEGA3 TESCAN). From the images it can be seen that the nanofibers are formed without any beads. The size of the nanofibers ranged from 179.42 nm to 242.42 nm. Mirzaei et al., 2016 showed the nanofibers loaded with 30% plant extract had diameters ranging from 275 nm to 375 nm.

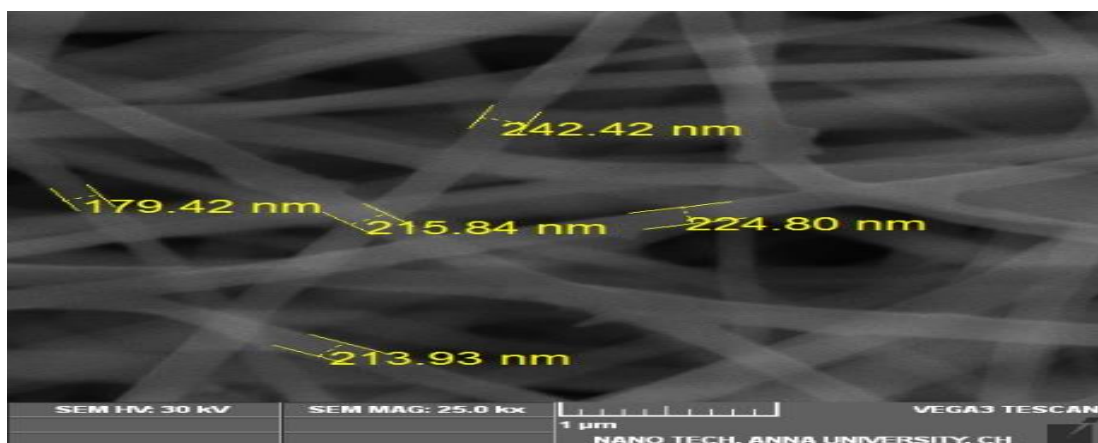


Fig 4. Chitosan nanofibers with diameter

5.11 FOURIER TRANSFORM INFRARED SPECTROSCOPY:

The functional groups in the nanofibers were determined using Fourier Transform infrared spectroscopy, to understand the polymer interaction and confirm any unfavourable interaction between the polymers during processing. The sharp peak at 1746.44 cm^{-1} was assigned to C=O stretching vibration. Another sharp peak at 2113.54 cm^{-1} was related to the stretching vibration of CC (CC refers to C, C triple bonds). One significant peak was noticed at 1233.25 cm^{-1} corresponds to the C-H deformation vibration. Another peak can be found at 1371.14 cm^{-1} which is assigned to the symmetrical deformation vibration of C-H bond. A peak at 958.44 shows C-C skeleton vibration.

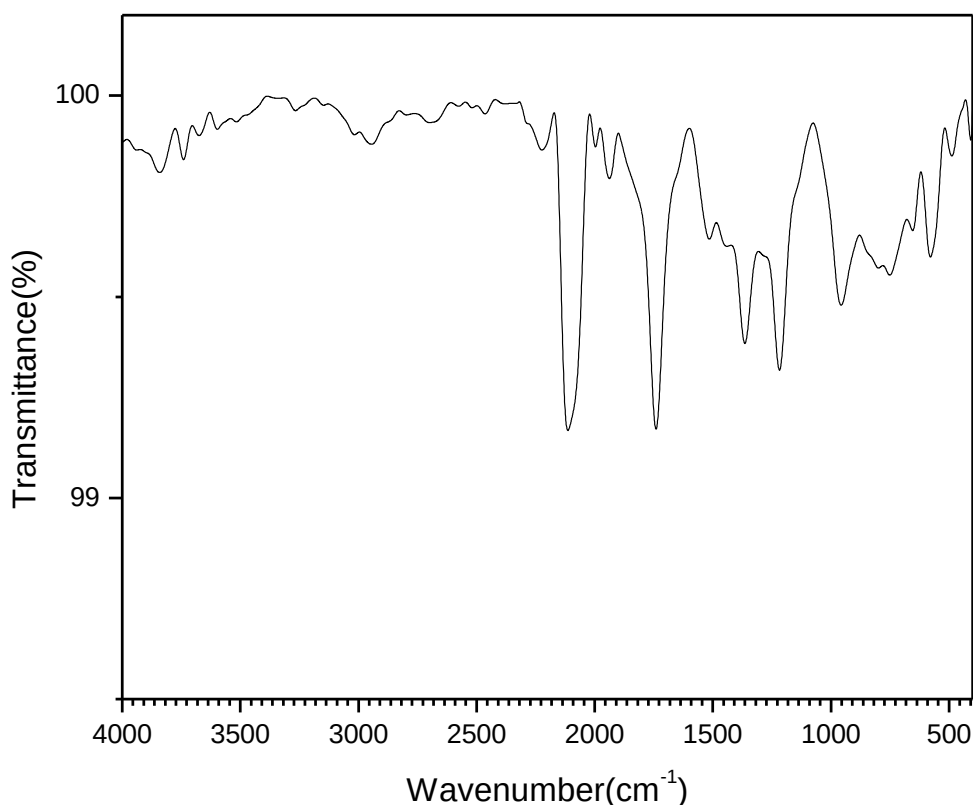


Fig 5. FTIR graph

5.12 IN-VITRO WOUND HEALING ASSAY:

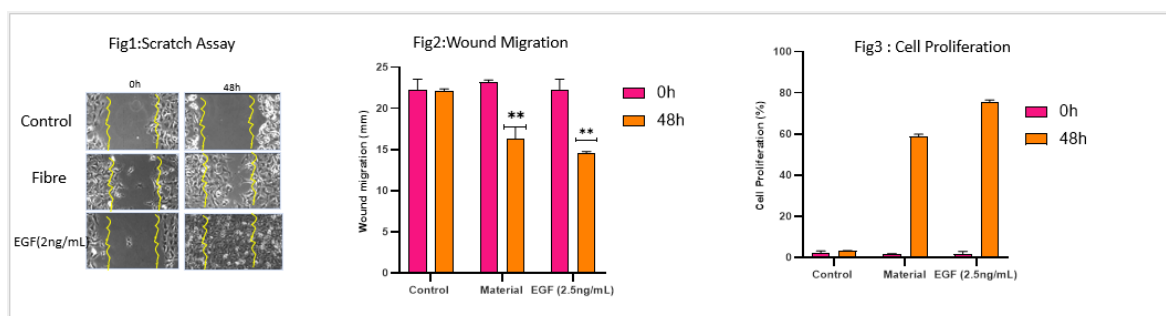


Fig 6. Wound healing assay results.

The in-vitro wound healing assay was employed to test the wound healing potential of the chitosan nanofibers loaded with plant extracts. The image above shows the results of the wound healing assay. A normal scratch in the middle of the fibroblast (L929) acted as a wound. Scratch without any growth factors acted as a negative control and another scratch with Epidermal Growth Factor (EGF) acted as a positive control. After 48 hours, it can be seen that wound on which the fibre was placed migrated about 6mm. The positive control containing EGF showed wound migration about 8 mm and the negative control showed no migration at all. The nanofiber used as a wound dressing increased the cell proliferation by 60%. The positive control containing EGF showed 80% cell proliferation. The negative showed no cell proliferation. Chitosan nanofibers loaded with bioactive compounds shows accelerated wound healing potential.

6.SUMMARY AND CONCLUSION:

In this study, nanofibrous incorporated with bioactive compounds from pomegranate peel extract and gooseberry seed extract were successfully developed. The extracts showed significant antioxidant, antibacterial, anti-hemolytic properties. The scaffolds were characterized by FTIR, SEM. The FTIR results showed no negative interactions. The size of the nanofibers ranged from 170nm to 250nm. GC-MS results listed the various compounds present in the extract that were responsible for various properties. The in-vitro wound healing assay results showed that the scaffolds containing 90:10 weight ratio of Chitosan/PVA: Plant extract showed 60%

cell proliferation and the wound migrated by 6mm in 48 hours. This shows significant wound healing potential of the scaffolds. Since the raw materials used for making these are cheap and they are extracted from waste materials. These scaffolds can be a cheap alternative for commercially available wound dressings. However, these scaffolds need further in-vivo study to commercialize them.

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