IN VITRO EVALUATION OF ALGINATE FILMS MODIFIED WITH CHITOSAN AND *HEMIGRAPHIS COLORATA* FOR WOUND HEALING APPLICATIONS

DISSERTATION SUBMITTED TO THE UNIVERSITY OF KERALA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN ANALYTICAL CHEMISTRY

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ABSTRACT

Alginate and chitosan are natural biopolymer having immense applications in biomedical field. These materials are extensively used because of their biocompatibility, abundance, non-toxic and low cost nature. In this work, films composed of alginate, chitosan, honey and *hemigraphis colorata* (murikootti) was prepared and characterized. The films were evaluated regarding FT-IR analysis, SEM analysis, in-vitro scratch wound assay, swelling studies, mechanical properties and antibacterial properties. The developed film exhibit faster wound healing property, antibacterial resistance, swelling behavior and mechanical strength. In-vitro studies shows that the film can be effectively developed therapeutically as an effective wound healing agent. Wound healing application of alginate, chitosan, honey and *hemigraphis colorata* are effective for improving wound and skin care system in the current context.

Keywords: Alginate, Chitosan, *Hemigraphis colorata*, wound healing.

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LIST OF ABBREVATIONS

ALG	Alginate
H.colorata	*Hemigraphis colorata*
SEM	Scanning Electron Microscopy
Ca	Calcium
SA	Sodium Alginate
FTIR	Fourier Transform Infrared Spectroscopy
^{0} C	Degree Celsius
Fig	Figure

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CHAPTER 1

INTRODUCTION

Wound healing, as a normal biological process in the human body, is achieved through four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling. These phases and their biophysiological activities must occur in the correct order, at a specified moment, and at an optimal intensity for a specific duration (Table 1; Mathieu et al., 2006).

Phase	Cellular and Bio-physiological Events
Hemostasis	1.vascular constriction 2.platelet aggregation, degranulation, and fibrin formation (thrombus)
Inflammation	1.neutrophil infiltration 2.monocyte infiltration and differentiation to macrophage 3.lymphocyte infiltration
Proliferation	1.re-epithelialization 2.angiogenesis 3.collagen synthesis 4.Extra cellular matrix formation
Remodeling	1.collagen remodeling 2.vascular maturation and regression

Table 1. Normal Wound-healing Process

Several factors affect wound healing such as Ischemic tissues, infection, contamination, wounds with foreign bodies etc.[1] Biopolymers have potential role in wound healing applications and they are abundantly available from natural sources. Cellulose, collagen, alginate, chitosan, hyaluronic acid, etc. are some biopolymers which have bioactive properties such as antimicrobial, immune-

modulatory, cell proliferative and angiogenic of the polymers create a microenvironment favorable for the healing process.[2]

1.1 ALG

1.1.1 STRUCTURE AND PROPERTIES

ALG is a naturally occurring anionic polymer that is primarily derived from brown seaweed. Due to its biocompatibility, low toxicity, affordability, and moderate gelation when divalent cations like Ca^{2+} are added, alginate has been well studied and used for numerous biomedical applications.[3] Brown algae (Phaeophyceae), such as Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum nodosum, and Macrocystis pyrifera, are generally used to produce commercially accessible alginate. Alginate wound dressings keep the wound site's milieu physiologically wet, reduce bacterial infection, and speed up the healing process.

Alginic acid is a linear polymer consisting of D-mannuronic acid and L-guluronic acid residues that are arranged in the polymer chain in blocks. These homogeneous blocks (composed of either acid residue alone) are separated by blocks made of random or alternating units of mannuronic and guluronic acids. Alginates have been observed to undergo proton-catalyzed hydrolysis, which is time, pH, and temperature dependent.[4] Alginates are composed of two acid units, b-(1-4)-linked D-mannuronic acid (M) and a-(1-4)-linked L-guluronic acid (G), which are conjugated in the form of homopolymeric (MM or GG) and heteropolymeric blocks (MG or GM).[5]

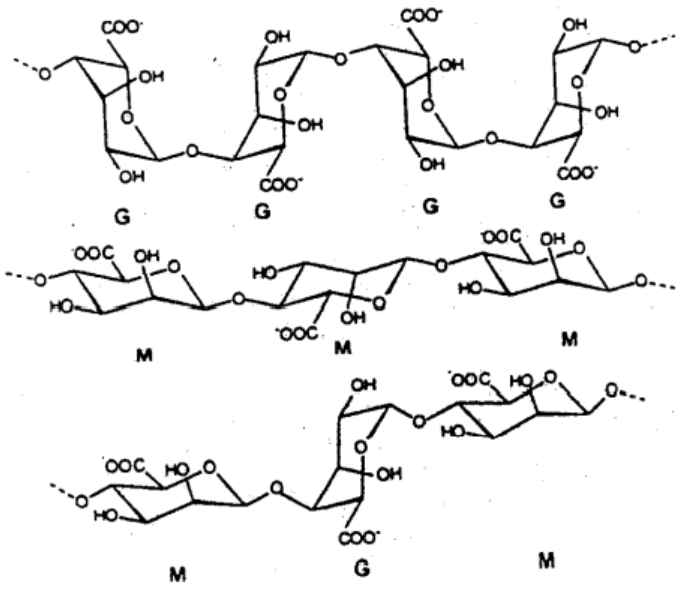


Fig 1: Alginate block types: G = guluronic acid, M = mannuronic acid

Alginates rich in guluronic acid blocks produce gels with significantly higher strength than alginates rich in mannuronate, since the G residues have a larger attraction for divalent ions than the M residues.

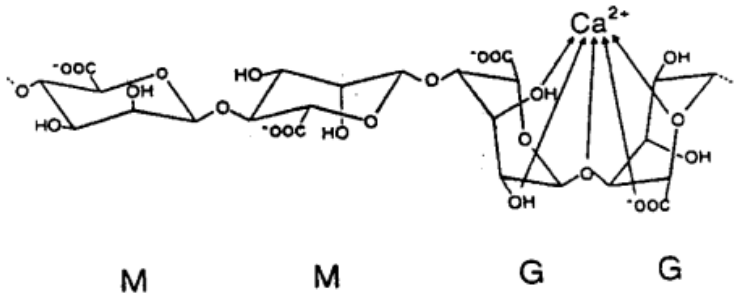


Fig 2: Probable binding mode between the calcium ion and two G residues.

Increasing the length of the G-block and the molecular weight of alginate gels often improves their mechanical qualities.

Alginic acid and its calcium salts are considered non-toxic and biocompatible in general. Alginates have a wide range of applications in the pharmaceutical, protein entrapment, drug delivery system[6-8] scaffolds for tissue engineering, cosmetic, and food industries.[9, 10]

Fig 3: SA powder.

1.1.2 ALGINATE IN FILM FORMATION

ALG may produce two kinds of films with distinct properties: water-soluble films and oil-soluble films. Evaporation of an ALG solution or extrusion of an ALG solution into a non-solvent that combines with water, such as acetone or ethanol, can be used to create water-soluble films. These coatings are resistant to grease, fats, and waxes yet allow for the passage of water vapour. When dry, they are brittle, but they can be plasticized using glycerol, sorbitol, or urea. They have strong nonstick qualities and can be used as mould release agents, such as in the production of fibre glass plastics. An extremely low viscosity ALG can be utilised to create a high solids film. A water-soluble film can be converted into a water-insoluble film by treating it with a divalent or trivalent cation or with acid. They can also be created by extruding a soluble ALG solution into a Ca salt

bath. Because ALG hydrogels are analogous to extracellular matrices of live tissues, they may be generated using a variety of cross-linking methods. It has several advantages in the fields of wound healing, medication and protein delivery, cell transplantation, and so on. ALG wound dressing can create a moist microenvironment and reduce bacterial infection at the wound site, allowing for faster wound healing. Depending on the cross linker and cross linking procedures, ALG hydrogels can distribute everything from tiny medicines to macromolecular proteins in a regulated manner.[3]

1.2 CHITOSAN

Chitosan is a linear aminopolysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by β (1–4) glycosidic bonds formed by N-deacetylation of chitin (its parent polymer). Chitin, the second most prevalent biopolymer, is found mostly in crab exoskeletons and fungal cell walls.[11] Chitosan is a polycationic polymer with free acetamide groups and hydroxyl functions coupled to glucopyranose rings that are vulnerable to nucleophilic attack.[12] Chitosan has been used in the production of gels, micro- or nanoparticles, and films. Its physical and biochemical features can be adjusted further to satisfy the requirements of wound healing applications.

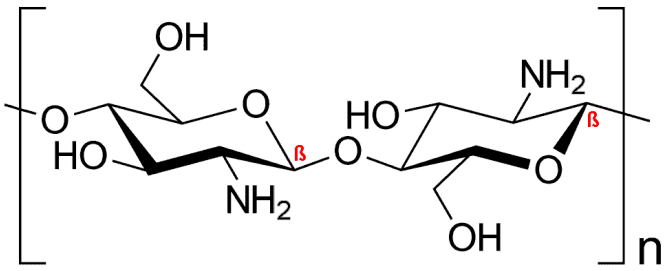


Fig 4: Chitosan

Chitosan is a potent antimicrobial agent and its antimicrobial character is due to presence of its cationic nature. Chitosan's ability to inhibit a wide range of bacteria, fungi, yeasts, and viruses allows it to be used in a broad range of antimicrobial agents in investigations including in vivo and

in vitro interactions in various forms (solutions, films and composites). Studies shows that in the presence of more than 0.025% chitosan, the growth of Excherichia coli, Fusarium, Alternaria and Helminthosporium is inhibited.[13] Chitosan have variety of biomedical applications such as drug delivery, wound healing, antibacterial, fat-binder, homeostatic agent, hypocholesterolemic effect etc. Due to anti-inflammatory effect of chitosan, it is beneficial for the treatment of prolonged inflammation at the wound site.

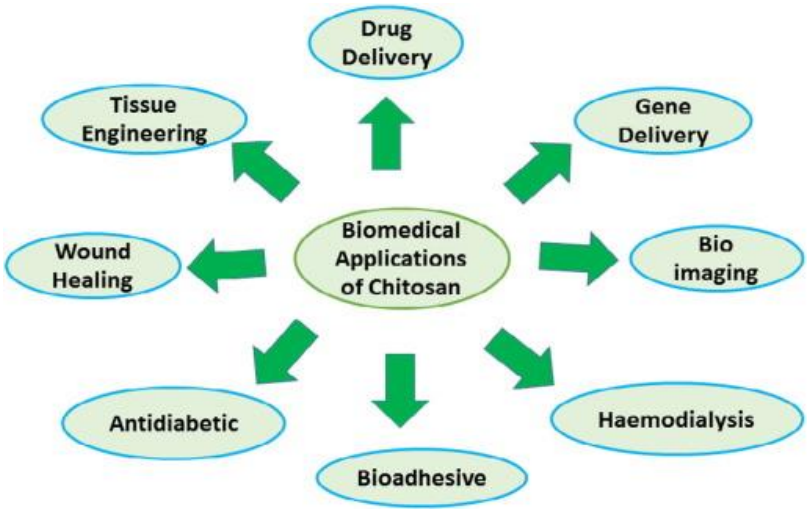


Fig 5: Various biomedical applications of chitosan[14]

Chitosan acts as a non-protein matrix for 3D tissue formation while also activating macrophages for tumoricidal function. It promotes cell proliferation as well as histoarchitectural tissue organisation. Chitosan progressively depolymerizes to produce N-acetyl-b-D-glucosamine, which promotes fibroblast proliferation, aids in organised collagen deposition, and induces an increase in natural hyaluronic acid production at the wound site. It aids in wound healing and scar prevention.

1.3 HONEY

Fig 6: Honey

Honey has long been known to have healing properties. [15] It promotes tissue growth, collagen synthesis, and the formation of new blood vessels in the wound bed.[16, 17] Many studies have reported the use of honey to treat wounds and infections. Honey with antibacterial activity has the potential to be an effective treatment option for wounds infected with, or at risk of infection with, a variety of human pathogens.[18, 19] Honey, when applied to wounds, creates a moist healing environment, quickly clears infection, deodorises, and reduces inflammation, edoema, and exudation. It hastens healing by stimulating angiogenesis, granulation, and epithelialization.[20] The general effects of honey on the healing process are shown in Fig 6.

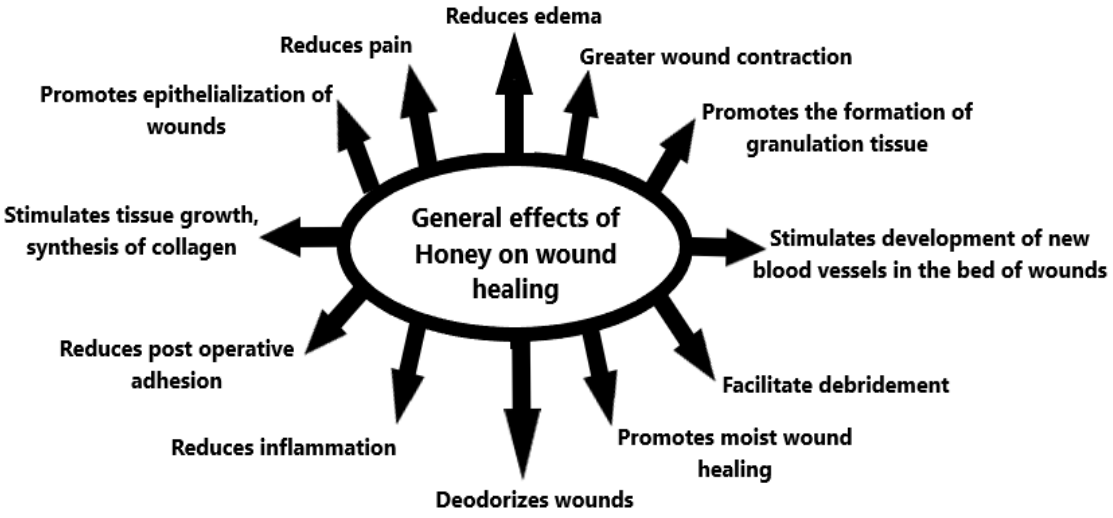


Fig 7: General Effects of Honey on Wound Healing[21]

1.4 HEMIGRAPHIS COLORATA

Hemigraphis colorata (H.colorata), a tropical low-creeping perennial herb that grows to a height of 15 to 30 cm, is an exotic plant adapted to India. When cultivated on the ground, it prostrates and spreads by rooting stems. The upper surface of the leaf has a metallic purple shine, while the ventral side is solid dark purple. The opposite leaves are ovate to cordate, serrate-crenate, 2 to 8 cm long, and 4 to 6 cm broad, with well-defined veins.[22] Because the filament of the outer stamen bears brushes, the word "hemigraphis" means "half writing." The plant has various names, including Aluminium plant, Cemetary plant, Metal leaf, Red flame Ivy, Waffle plant, Java Ivy etc. The plant is known as "murikootti" or "murian pacha" in Kerala because of its incredible ability to heal wounds.[23]

Fig 8: *Hemigraphis colorata*

When applied to a wound, *H. colorata* leaf paste enhanced wound healing in mice, but oral administration was unsuccessful. Wound contraction and epithelialization were faster in mice treated with leaf paste.[24-26] The wound healing activity of *H. colorata* observed enhanced wound healing in the test group that may be due to an increase in collagen concentration per unit area and fibre stability. The Increased wound con-traction and tensile strength may be due to the active constituents present in the extract.[27] The phytochemical elements of *H.colorata* were determined by evaluating crude extracts of its leaves and stem using different solvents, and antibacterial activity against chosen pathogens was evaluated.[28] The ethanolic extract of *H. colorata* showed that it lowers the level of glucose in blood. The steroids and coumarins present in the extract provide anti-diabetes activity.[29]

1.5 SCOPE OF THE STUDY

ALG are one of the most versatile biopolymers and are utilised in a variety of applications. The conventional usage of alginate as an excipient in pharmaceutical products is based on its thickening, gel-forming, and stabilizing qualities. The necessity for longer and more precise medication delivery has increased the need for tailor-made polymers. ALG has been widely studied due to its biocompatibility, low toxicity, relatively low cost and mild gelation with divalent cations. It has extensive applications in wound healing, drug delivery, cell encapsulation etc. Due to the high affinity for chelation with polyvalent metal cations in particular with divalent metal ions, the ALG readily formed the coordination biopolymer cross-linked with metal. To improve the antibacterial activity, the chitosan dissolved in minimum amount of acetic acid is added. The aim of the present study is to develop a wound healing dressing film based on ALG, honey, chitosan and *hemigraphis colorata* by combining the therapeutic properties of chitosan and *hemigraphis colorata* with the hemostatic properties of Ca-ALG.

CHAPTER 2

REVIEW OF LITERATURE

- Ruben Pereira et al synthesized thin hydrogel films were made from alginate and Aloe vera gel in various concentrations (95:5, 85:15 and 75:25, v/v) were synthesized and interpreted. Light transmission behavior, contact angle measurements, and chemical, thermal, and mechanical properties of the films were all investigated. These thin hydrogel films, created through a crosslinking reaction with 5% calcium chloride solution, were also studied in terms of water solubility and swelling behavior. The results showed that Aloe vera increased the transparency and thermal stability of the films. The produced films have appropriate mechanical qualities for skin applications, but solubility experiments revealed that the films are insoluble after 24 hours of immersion in distilled water. The increase in Aloe vera proportion considerably improved the water absorption and swelling behaviour of these films. This study reveals that alginate/Aloe vera films are appropriate for wound healing and drug delivery applications.[30]
- M.D.M. Dantas, D.R.R. Cavalcante et al evaluate the effectiveness of sodium alginate/chitosan-based films and laser therapy in improving burn wound healing. Laser arrays hasten the healing and repair of soft tissue damage. The backs of 60 male rats were burned in six groups: untreated (CTR), dressed with cellulose films (CL), dressed with sodium alginate/chitosan-based films (SC), laser irradiated undressed wounds (LT), laser-irradiated wounds with cellulose (CLLT), and sodium alginate/chitosan-based films (SC) (SCLT). For seven days, laser therapy was used. After 8 and 14 days, the inflammatory response was substantially stronger in the CTR group than in the irradiation groups. With or without dressing films, laser therapy encouraged myofibroblastic differentiation in 8 days. The combination of laser therapy with both dressings improved epithelisation, blood vessel creation, and collagenization, facilitated the quick replacement of type III collagen with type I collagen, and

favoured the better arrangement of newly generated collagen fibres. The use of laser therapy in conjunction with a sodium alginate/chitosan-based bandage promotes burn healing.[31]

- Dong-Keon Kweon et al synthesized water-soluble chitosan (WSC)/heparin (CH) complex Using wound healing WSC and heparin with the ability to attract or bind wound healing growth factors. To assess the wound healing effect, full thickness skin excision on the backs of rats was conducted, and then WSC and water-soluble CH complex ointments were applied to the wounds, respectively. Gross and histologic examinations were done after 15 days. The results of an in vivo test to characterize the effect of WSC and CH complex during the healing process of a full thickness incision revealed that, while the wound was partially regenerated with WSC, the wound treated with water soluble CH complex ointment was nearly completely regenerated with skin appendage structure in the dermis similar to normal skin. According to this study, water-soluble CH complex ointment is the most efficient agent in wound healing.[32]
- Lishan Wang et al evaluated novel chitosan-alginate polyelectrolyte complex (PEC) membranes, cast from aqueous suspensions of chitosan-alginate coacervates with CaCl2, as promising wound-dressing materials. After gamma-irradiation, this coherent PEC membrane had a tensile strength of 10.70 MPa and a strain break of 1.43%. The chitosan-alginate PEC membranes also appeared to aid scar tissue remodeling by enhancing collagen synthesis, compaction of collagen fibers into thicker bundles, and alignment of collagen fibers into parallel bundles. Ease of use, cost effectiveness, biodegradability, ease of formulation, storage stability, and high batch-to-batch reproducibility all contributed to its advantages as a wound dressing.[33]
- Guilherme Ferreira Caetano et al studied the efficacy of a chitosan-alginate membrane to expedite wound healing in experimental skin wounds. In Wistar rats, two wounds (1.5 cm

diameter) were punched and treated with membranes moistened with saline solution (CAM group) or with saline only (SL group). Five rats from each group Histological, flow cytometry, neutrophil infiltration, and hydroxyproline analyses were performed on the wounds/scars.were slain after 2, 7, 14, and 21 days of surgery, and reepithelialization was assessed. The CAM group reepithelialized faster than the SL group on the seventh day, although being equivalent on the previous days. Finally, in rats, chitosan-alginate membrane controlled the inflammatory phase, increased fibroplasia and collagenesis, and accelerated wound healing. In this regard, the chitosan-alginate PEC wound dressing is a promising formulation for use as a new therapeutic option, potentially useful for treating tissue damage and impaired wounds.[34]

- Yingshan Zhou et al prepared composite films of sodium carboxymethylcellulose (CMC), propyl-3-trimethylammonium chitosan chloride (HTCC), and PVA N-(2-hydroxyl) by solvent casting method. The films were evaluated using SEM, FTIR, XRD, and light conduction measurements. The end result revealed that the CMC, PVA, and HTCC in films were somewhat miscible and intermixed via hydrogen bonding. By altering the CMC content, the moisture permeability, tensile properties, swelling properties, and water absorption of PVA/HTCC composite films were investigated. CMC had increased the flexibility and strength of composite films. Adding CMC also increased moisture permeability, swelling ratio, and absorption capacity. Furthermore, composite films with a weight of 40% can be exploited as promising wound dressings with improved antibacterial activity.[35]
- Nirmla Devi and Joydeep Dutta prepared the nanocompsite films of bentonite-chitosan for the application of wound healing. The films' physicochemical features, such as water absorption capacity, folding durability, and thickness, were investigated. FTIR was used to investigate the interactions of chitosan with positive charge and bentonite with negative charge. SEM was

used to determine the surface morphology of films. The hydrophilic characteristic of bentonite improved the water absorption and mechanical strength of films. The produced films shown increased activity against Gram negative and Gram positive bacteria, indicating their potential usage as wound dressing.[36]

- Xu et al used the hyaluronic acid (HA) and chitosan (CS) to formulate the new wound dressing. Atomic force microscopy (AFM) revealed that as the amount of HA increased, the films got rougher. Increased water-uptake ratio and water contact angle are also considered. However, increasing the HA concentration destabilised favourable properties such as fibroblast adhesion, bovine albumin adsorption, and water vapour permeability (WVP). In vivo animal tests revealed that the CH/HA film can more efficiently speed up wound healing and when the dressing was peeled off again, it minimised the occurrence of re-injury. The findings demonstrated that the CS/HA wound dressing was both affordable and practical.[37]
- T.T. Akhil and Punieethaa Prabhu studied the anti-oxidant, anti-inflammatory and cytotoxic potential of *H.colorata*. The phytochemical contents of several extracts of *Hemigraphis colorata* (Blume) are examined as a result of this research. . The antioxidant capability in vitro was assessed using DPPH and reducing power studies. The antiinflammatory activities of several extracts from the entire plant are investigated using the human RBC membrane stabilisation technique. The findings indicate that ethanolic extracts of *Hemigraphis colorata* have a high antioxidant potential to scavenge different free radicals, which could be attributed to the presence of phytochemicals such as flavonoids, tannins, saponins, and phenolic compounds. The anti-inflammatory activities of *H. colorata* whole plant extracts were investigated using the Human red blood cell membrane stabilization technique.[28]

- Xiaodan Zhang et al carried out Michael addition in Chitosan (CS) and polyethylene glycol diacrylate (PEGDA) in acidic solution. Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), and X-Ray Diffraction were used to examine films with CS/PEGDA weight ratios ranging from 100/0 to 0/100 in 10% increments (XRD). In addition, the mechanical and swelling properties of the films were measured. SEM observation and selected SEM photos of cell seeded surface suggested that the microporous surface structure of the CS/PEGDA films was suitable for cell growth, proliferation, and differentiation. These films have the potential to be employed as wound dressing material.[38]

CHAPTER 3

OBJECTIVES

- Preparation of Ca cross linked ALG film
- Preparation of Ca cross linked ALG, honey, Chitosan film
- Preparation of ALG, honey, Chitosan, *Hemigraphis colorata* mixed films by solvent casting technique for wound healing purpose.
- Examine the tensile strength of the developed films.
- Examine the swelling property of the film.
- To study the antibacterial property of the film.
- Examine the in-vitro scratch wound assay.

CHAPTER 4

MATERIALS AND METHODS

3.1 MATERIALS

- SA
- Honey
- Chitosan
- *Hemigraphis colorata*
- $CaCl_2$
- Glacial acetic acid
- Hydrochloric acid
- NaOH
- Distilled water

Medium viscosity sodium alginate powder (viscosity of 2% solution, 25^0 C ~ 3500 cps) is purchased from Sigma-Aldrich, London. Calcium chloride dihydrate (Merck, Germany) of analytical grade is used as such, without any further purification. Natural honey, used in this study, is purchased from Kerala Agriculture University. The plant *H. colorata* collected locally and was identified by a botanist. Other chemicals used are of analytical grade and used as such, without any further purification.

3.2 APPARATUS AND GLASSWARES USED

- Glass beakers
- Measuring jars
- pH meter
- Magnetic stirrer
- Hot air oven
- Autoclave
- Mechanical stirrer
- Homogeniser
- Sonicator
- Teflon pan

3.3 EXPERIMENTAL METHODS

3.3.1 PREPARATION OF ALG BASED FILMS

Alginate film was prepared using the solvent casting process. Solutions of SA (3%, w/v) was prepared by powder dissolution in distilled water, under mechanical agitation (600 rpm) until the complete dissolution. About honey (5%, w/v), the plasticizer agent was added and stirred well. To this mixture $CaCl_2$ (5 %, w/v) solution was added slowly under constant stirring for about 4 h. After this period of time, solution was exposed to ultrasound sonicator for 15 minutes to remove air trapped bubbles. Then the solution was poured into a teflon pan and dried at 50^0 C for 2 days. The dried film was stripped out and kept in a desiccator.

3.3.2 PREPARATION OF ALG AND CHITOSAN FILM

ALG/ Chitosan film was prepared using the solvent casting method. To obtain ALG/ Chitosan film, chitosan (1 %, w/v) dissolved in minimum amount of acetic acid was added to the solution of ALG (3%, w/v) and honey (5%, w/v). To this mixture $CaCl_2$ (5%, w/v) was added slowly under constant stirring for 4 h. Then the solution was exposed to ultrasound sonicator and dried at 50^0 C for 48 h. The dried film was stripped out and kept in a desiccator.

3.3.3 PREPARATION OF ALG – CHITOSAN – *H. COLORATA* FILM

To obtain ALG/ Chitosan/ *H. colorata* film, *H. colorata* (1%, v/v) was added slowly to the solution contain ALG (3 %, w/v), honey (5 %, w/v) and chitosan (1 %, w/v). To this solution $CaCl_2$ (5 %, w/v) was added slowly under constant stirring for 4 h. The solution was exposed to ultrasound sonicator and dried at 50^0 C for 48 h. The dried film was stripped out and kept in desiccator.

3.3.4 FILM CHARACTERIZATION

3.3.4.1 FT-IR ANALYSIS

The Infrared Spectroscopy technique was used to evaluate the chemical composition of the raw materials (ALG, Chitosan, *H. colorata*) and to study the possible interactions between the compounds. FT-IR spectra were on a PerkinElmer FT-IR Spectrometer (in the range 400- 4000 cm^{-1} with the resolution 4 cm^{-1}). It can identify unknown materials, it can determine the quality or consistency of a sample, and it can determine the amount of components in a mixture. All samples were studied in triplicate.

3.3.4.2 TENSILE STRENGTH

The mechanical properties of the films were evaluated by tensile tests and compared with the skin values. The film test can be performed in dry and wet states. Film samples were cut into specimens 15 mm wide and 50 mm long, following the guidelines of ASTM Standard Method D 882. The initial grip separation was 30 mm and the speed was 1 mm/ s. All samples were run three times. Results of the mechanical tests were expressed as the mean of the measurements +/- SD. Tensile strength (TS) was determined according to the following equation:

$$TS = \frac{Fmax}{A}$$

Where Fmax and A represent, respectively, the maximum force at break and the initial cross-sectional area of the film strip.

3.3.4.3 SWELLING BEHAVIOUR

The swelling properties of the Ca-ALG/chitosan/*H. colorata* films were determined in phosphate buffer for simulated wound fluid (pH 5.5) and in simulated body fluid (pH 7.4). 0.1 g of the film was taken in 100 ml distilled water and 10 ml of other respective solutions. The swollen films were periodically removed and weighed. The weight of the swollen films was determined by blotting them with filter paper to remove moisture adhering to the surface, immediately followed by weighing on an electronic balance. All experiments were done in triplicate. The swelling percentage was estimated by the equation,

$$Swelling\ behaviour = \frac{Ww - Wd}{Wd} \times 100$$

Where Ww represents the wet weight of the film, Wd corresponds to the dry weight of the films. Data were given as the mean + the standard deviation (SD) and based on 3 independent measurements.

3.3.4.4 SEM ANALYSIS

The morphology of the films was examined using JEOL Model JSW 6930LV (Boston, USA) SEM that produce images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contains information about the samples surface topography and composition. The electron beam is generally scanned in a raster scan pattern and the beams position is combined with the detected signal to produce an image. Specimens can be observed in high vacuum, in low vacuum, in wet condition and at a wide range of cryogenic or elevated temperature.

3.3.4.5 IN VITRO SCRATCH WOUND ASSAY

The ALG-chitosan-*H.colorata* film was tested for its wound healing properties using in vitro scratch assay. It is a well-developed method to measure cell migration in vitro. The basic steps involve creating a "scratch" in a cell polymer, capturing images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of cells .In vitro scratch assay is suitable to study on the effects of cell-matrix and cell-cell interactions on cell migration, mimic cell migration during wound healing.

Cell line used for the study: RAW cell line

Cell lines and maintenance

RAW cell line (monocyte /macrophage-like cells) was procured from the National Centre for Cell Sciences (NCCS), Pune, India.

Cell culture media and maintenance

The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-Himedia), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 1% antibiotic cocktail containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). The cell containing TC flasks ($25cm^2$) were incubated at 37^OC at 5% CO_2 environment with humidity in a cell culture incubator (Galaxy® 170 Eppendorf, Germany).

Preliminary procedure:

RAW cells (1 million cells/well) were seeded on 6 well plates and allowed to acclimatize to the culture conditions such as 37 $^{\circ}C$ and 5% CO_2 environment in the incubator for 24 h. The test samples were prepared in cell culture grade DMSO (10 mg/mL) and filter sterilized using 0.2 µm

Millipore syringe filter. The samples were further diluted in DMEM media and added to the wells containing cultured cells of at least 80% confluency at final concentrations of 25, 50, 100 µg/mL respectively. Untreated wells were kept as control.

Scratch wound assay procedure

(i) Scrape the cell monolayer in a straight line to create a ‘‘scratch’’ with a 200 µL pipette tip. Remove the debris and smoothen the edge of the scratch by washing the cells once with 1 ml of the growth medium and then replace with 5 ml of fresh medium

CRITICAL STEP: It is important to create scratches of approximately similar size in the assessed cells and control cells to minimize any possible variation caused by the difference in the width of the scratches.

(ii) To obtain the same field during the image acquisition, create markings to be used as reference points close to the scratch. The reference points can be made by etching the well plate lightly with a razor blade on the outer bottom of the dish or with an ultrafine tip marker. After the reference points are made, place the dish under a phase-contrast microscope, and leave the reference mark outside the capture image field but within the eye-piece field of view. Acquire the first image of the scratch.

(iii) Place the well plate in a tissue culture incubator at 37 ^{0}C. Photomicrographs were taken for varying durations (0 hour, 12 hours, 24 hours and 36 hours). The time frame for incubation should be determined empirically for the particular cell type used. The well plates can be taken out of the incubator to be examined periodically and then returned to resume incubation.

CRITICAL STEP: Choose a time frame of incubation that allows the cells under the fastest migrating condition to just achieve the complete closure of the scratch.

(iv) After the incubation, place the dish under a phase-contrast microscope, match the reference point, align the photographed region acquired in Step 6 and acquire a second image. Likewise images should be taken till the complete closure of the wound.

3.3.4.6 ANTIBACTERIAL STUDY

Antibacterial assay by agar well diffusion method

Procedure: Agar well diffusion method is widely used to evaluate the antimicrobial activity of the test sample. Mueller-Hinton agar (15-20 mL) was poured on glass petriplates of same size and allowed to solidify. Standardized inoculum of the test organism was uniformly spread on the surface of the plates using sterile cotton swab. Four wells with a diameter of 8 mm (20 mm apart from one another) were punched aseptically with a sterile cork borer in each plate. The test sample (50 and 100 µL) was added into the wells T1 and T2 directly from the sample. Gentamycin (40µl from 4 mg/ml stock) and the solvent used for sample dilution were added as positive and negative control respectively. The plates were incubated for 24 h at 36ºC ± 1ºC, under aerobic conditions. After incubation, the plates were observed and the zone of bacterial growth inhibition around the wells was measured in mm.

Culture media Details:

Muller Hinton Agar medium (HIMEDIA- M173) is used for determination of susceptibility of microorganisms to antimicrobial agents. Suspend 38 grams in 1000 ml distilled water. Heat until it boils to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile petri plates.

CHAPTER 5

RESULTS AND DISCUSSION

ALG and Chitosan are the natural polymers which have inherent properties that make them appealing for use in wound healing treatment. The application of alginate and chitosan for wound healing is due to these polymers having favorable and distinctive attributes. These include having a high absorption capacity, biocompatible, non-toxic, non-immunogenicity, and low cost. Incorporating *H. colorata* shows significant wound healing as well as anti-inflammatory activities. ALG-chitosan-*H.colorata* film was prepared by the dissolution of ALG, chitosan, *H.colorata*, honey in suitable proportion and dropwise adding $CaCl_2$ on a magnetic stirrer for 4 h. Then the solution was poured into a teflon pan and dried at 50^0 C for 2 days. The therapeutical and mechanical properties of chitosan like biodegradability, non-toxicity, anti-bacterial effect and biocompatibility enhances the wound healing property of ALG. Honey in the film promotes autolytic debridement, wound tissue development, and anti-inflammatory actions, all of which speed up the wound healing process. Furthermore, honey promotes wound healing outcomes by lowering the incidence and excessive scar formation.

Fig 9: Preparation of ALG-chitosan-*H.colorata* polymer by solvent casting process.

Fig 10: ALG-honey-$CaCl_2$ film.

Fig 11: ALG-honey-Chitosan- $CaCl_2$ film.

Fig 12: ALG-honey-chitosan-*H.colorata*-$CaCl_2$ film.

4.1 SEM ANALYSIS

The surface morphology of ALG/ Chitosan/ *H.colorata* film was studied using Scanning Electron Microscopical images. Fig 13 show the surface morphology of ALG/ Chitosan/ *H.colorata* which seen as small depression without visible pores. The surface morphology of SA films is smooth and homogenous, but films with chitosan and *H.colorata* have a rougher surface. This matrix's composition aids in the absorption of wound fluids, keeping the wound dry.

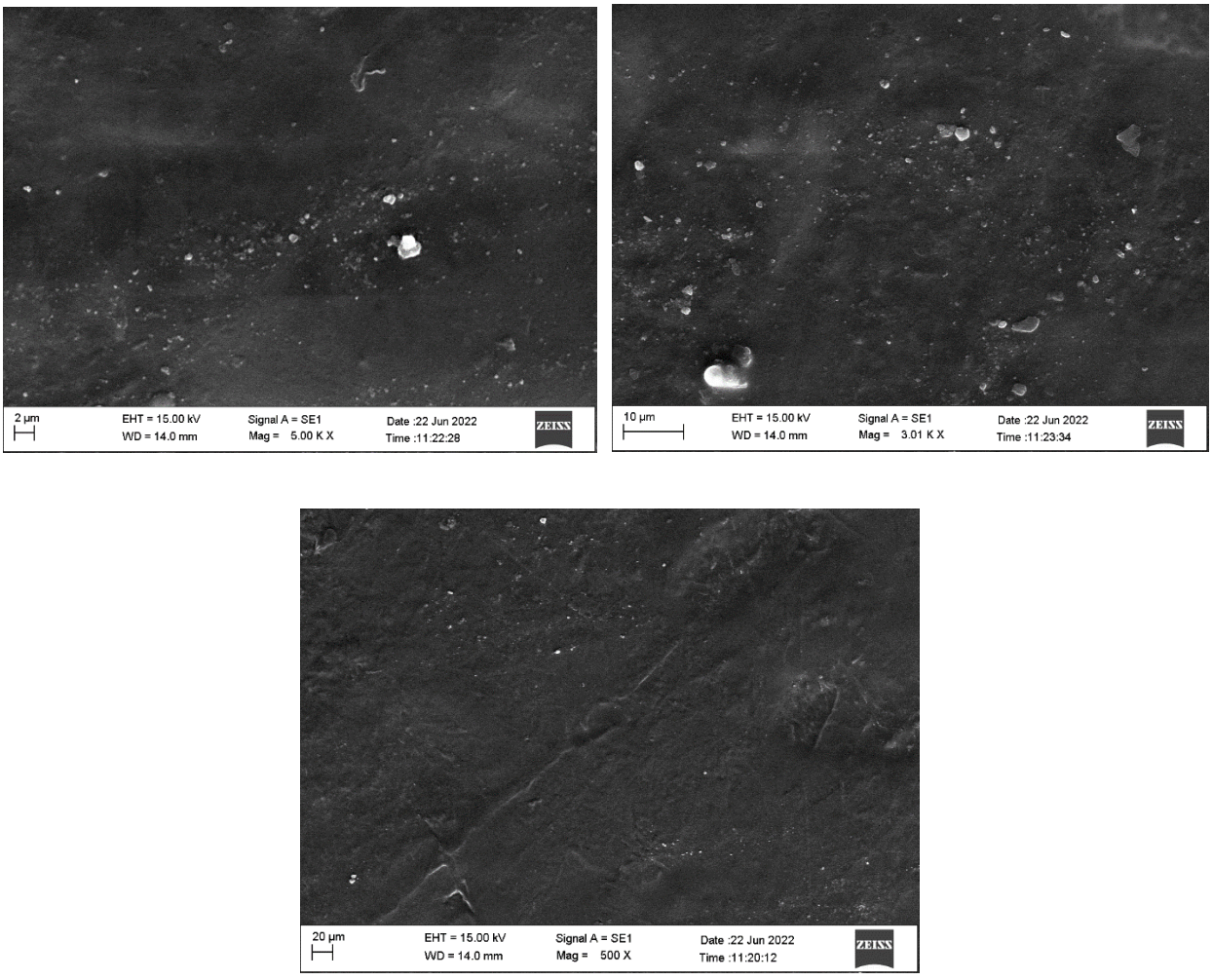


Fig 13: SEM images of ALG-Chitosan- *H.colorata* film

4.2 FTIR ANALYSIS

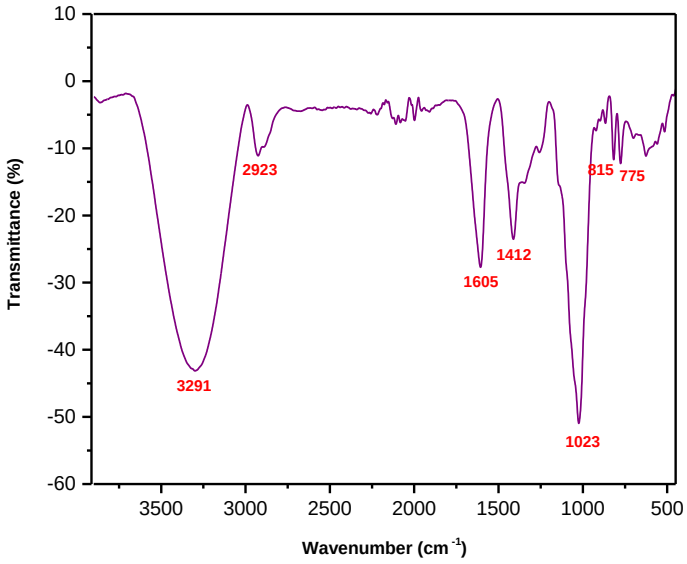


Fig 14: FTIR spectra of ALG

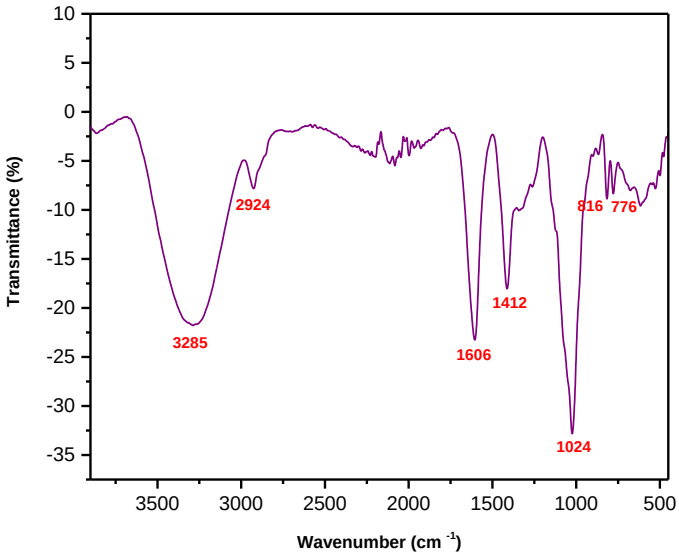


Fig 15: FTIR spectra of ALG- Chitosan

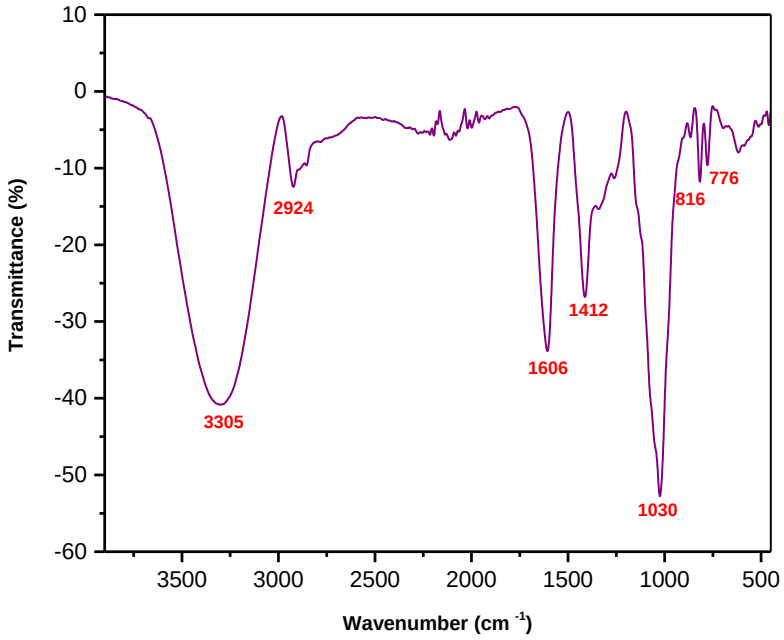


Fig 16: FTIR spectra of ALG-Chitosan-*H.colorata*

The interaction between alginate, chitosan and *H.colorata* was studied using FTIR spectroscopy. In the case of ALG polymer Fig 14, the broad absorption band at 3291 cm^{-1} is due to the O-H stretching vibration of alginate. The peak located at 2923cm^{-1} is indexed to C-H stretching vibration. The strong asymmetric stretching absorption band at 1605 cm^{-1} and weaker symmetric stretching band near 1412 cm^{-1} are due to the presence of carboxylate anions. The band at 1023 cm^{-1} are the characteristic of the C-O-C linkage.

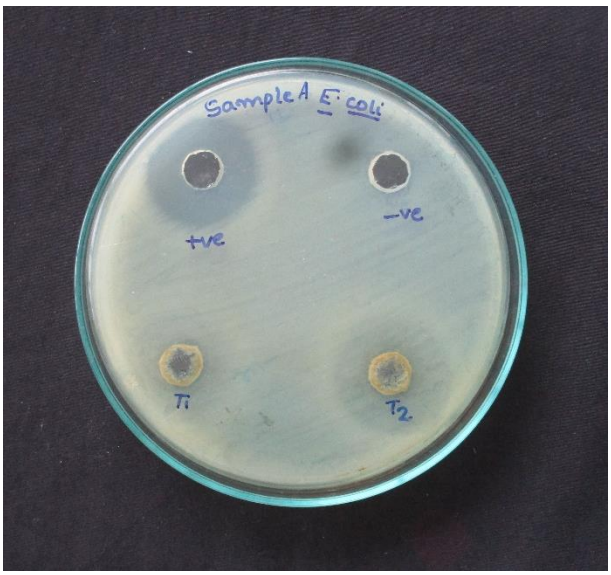
In the case of ALG-Chitosan, Fig 15, the absorption band at 3285 cm^{-1} is due to O-H group. The peak at 2924 cm^{-1} represent the C-H stretching vibration. The peaks at 1606 cm^{-1} and 1412 cm^{-1} is due to the asymmetric and symmetric stretching of carboxylate anions respectively. The strong asymmetric stretching absorption band at 1650 cm^{-1} and weaker symmetric stretching band near 1430 cm^{-1} are due to the presence of carboxylate anions.

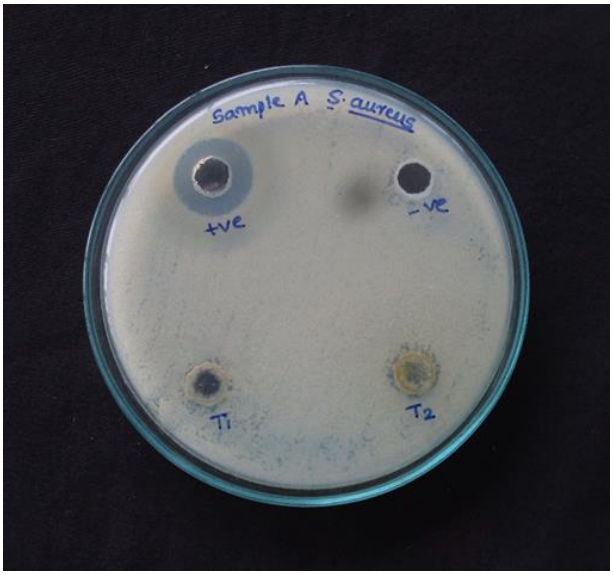
In the case of ALG-Chitosan-*H.colorata* Fig 16, characteristic absorption band was found at 3305 cm-1, which represent the O-H group with increase in intensity. The intense peaks at 1606 cm^{-1} and 1412 cm^{-1} is due to the asymmetric and symmetric stretching of carboxylate anions respectively. The peak at 2924 cm^{-1} represent the C-H stretching vibration. The strong intense band found at 1023 cm^{-1} are the characteristic of the C-O-C linkage.

The functional groups in the prepared films are shown in Fig 14, 15&16 with their most significant vibration modes. There appear to be no substantial differences between pure alginate films (AG) and alginate films with chitosan and H.colorata. The vibration bands from the COO, CH, C O, OH, and C O C groups may be observed in all spectra.

4.3 ANTIBACTERIAL STUDIES

The antibacterial properties of ALG films are being investigated against indicator organisms like *E-coli* (gram negative bacteria), *S.aureus* (gram positive bacteria) and *streptococcus* by plate assay and the formation of a clear zone was obtained as shown in the Fig 17. Gram negative bacteria were more resistant to the antibacterial action than gram - positive. It is also obvious that antibacterial activity is stronger in ALG- Chitosan-*H.colorata* film. It is clear from the results that the chitosan and *H.colorata* in the ALG matrix can considerably reduce microbial development during the wound healing process. The developed film is more effective in combating *Streptococcus* and *E.coli* than *S. aureus.*





(1) (2)

(3)

Fig 17: Antibacterial activity of ALG-Chitosan- *H.colorata* film against (1) *E.coli* (2) *S.aureus* (3) *Streptococcus.*

4.4 TENSILE STRENGTH

The mechanical properties of a wound dressing are significant to its wound-protective function. Biofilms used as a wound dressing should have sufficient elasticity and mechanical qualities, including suitable bendability and stretchability, to fit to the softness of the skin.[39] Tensile strength is a mechanical resistance for the film that may be related to the cohesiveness between the matrix of the filmogenic polymer chains, whereas elongation is a measure of the film's flexibility, or its ability to stretch before rupture.

	Thickness (nm)	Tensile Strength (MPa)	Maximum Load (N)	Load at Break (N)	Yield Strength (MPa)	Young's Modulus (MPa)	Elongation at Break (%)
1	0.28	3.16	30.9917	-0.34	-	22.53	59.11
2	0.29	3.10	31.4871	-0.07	-	20.19	46.98
3	0.26	2.45	22.2871	-1.00	-	30.92	38.38
4	0.24	3.39	28.4605	-0.07	-	23.49	52.16
Mean	0.27	3.03	28.3066	-0.37	-	24.28	49.16
Std.deviation	0.02	0.40	4.23	0.44	-	4.63	8.74
Coeff. of variation	8.29	13.34	14.93	-118.48	-	19.08	17.77

Table 2: Standard Test Method for Tensile Properties of ALG-chitosan- *H.colorata* film.

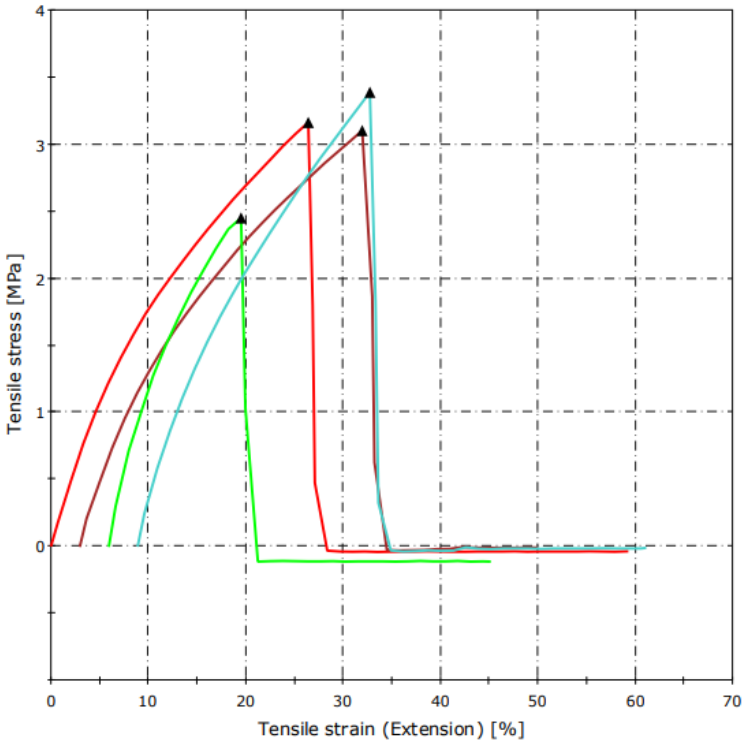
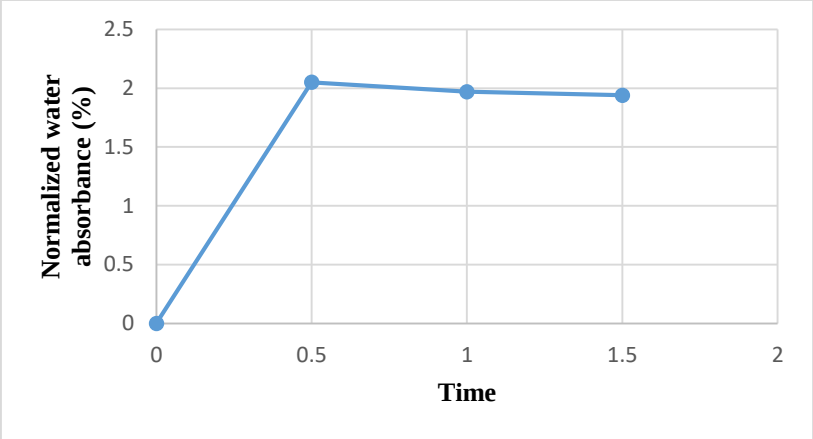


Fig 18: Tensile strength of ALG-Chitosan-*H.colorata* film.

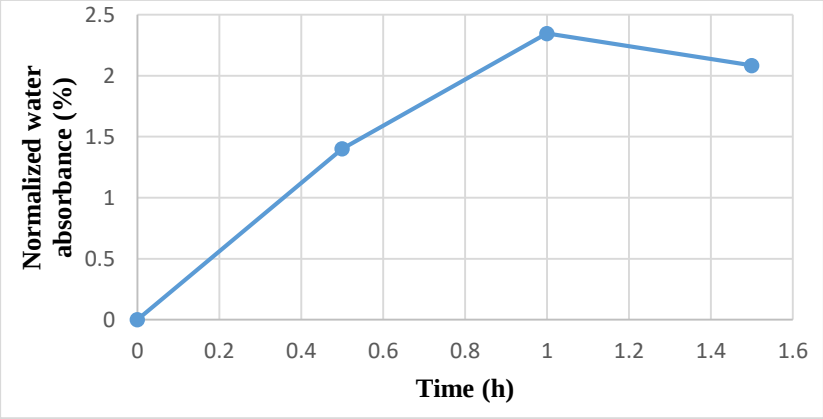
The tensile strength of the developed film is shown in Fig 18. From Table 2: the mean elongation at break value of ALG-Chitosan-*H.colorata* film was 49% which was high when compared to the ALG-Chitosan film (23%).[40] Skin tensile strength values are typically in the 2.5-16 MPa range. When these values are compared to the mechanical characteristics of the produced films, all films show appropriate qualities for skin application.

4.5 SWELLING BEHAVIOUR

At room temperature, the swelling behavior of the biofilms was investigated for their potential utility in wound healing. Because of the hydrophilic nature and physical cross linking of polymer, when they are soaked in various media, their bonds began to break off and the composites began to disintegrate.[41]



(1)



(2)

Fig 19: Swelling behavior of ALG-Chitosan-H.colorata film in phosphate buffer at (1) pH 5.5 (2) pH 7

The pH sensitive swelling behavior of the film was investigated at phosphate buffer solution of pH 5.1(SWF), pH 7(SBF) respectively are provided in the Fig 19. The swelling percentage increases when pH increases. At pH 5.5, the swelling increased up to 1.94% whereas in pH 7, 2.34% swelling was found. The degree of swelling rises with increasing pH due to the presence of

the carboxylate anion. That is, the carboxylate anion in ALG increases electrostatic repulsion and facilitates swelling.[42]

4.6 INVITRO SCRATCH WOUND ASSAY

In vitro scratch wound assay was carried out to observe the effect of ALG-Chitosan-*H.colorata* on the healing process. The scratch substance, diluted with DMEM, was introduced to the culture cells to detect RAW 264.7 cell movement, and images were recorded as given in the Fig 20. The wound assay is a simple and affordable in vitro method that provides reliable data on the wound healing ability. It assess fibroblast migration, wound proliferation, and wound re-epithelialization potential. The wound healing potential of the film is vividly shown in Fig 20.

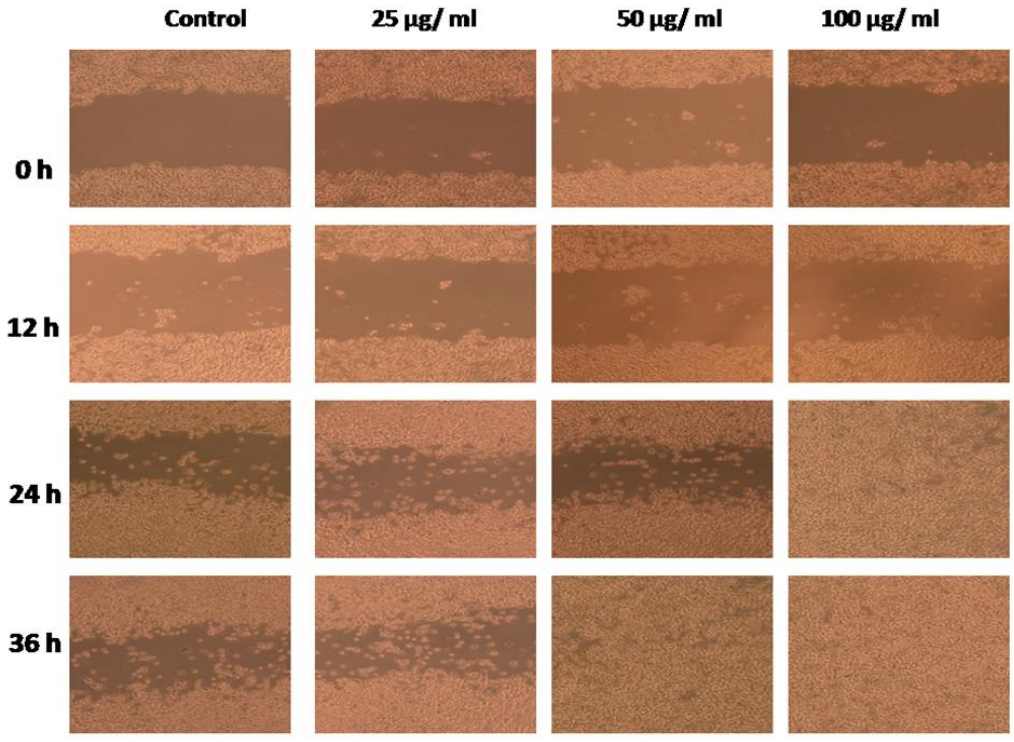


Fig 20: Scratch wound healing assay of ALG-Chitosan-*H.colorata* film.

Even after 24 hours of incubation, the wound remained comparable in the control experiment. When treated with the sample, wound was fully healed at the time duration of 36 hours. The wound healing efficiency was found out to be in a concentration and time dependent manner. The maximum efficacy was elicited by the concentration **100 µg/ ml** at the time duration of **36 hours**. This indicates that the ALG-Chitosan-*H.colorata* film has the potential to be developed therapeutically as an effective wound healing agent. The scratch assay findings show that the biocompatible, non-toxic chitosan, *H.colorata* used in ALG film have excellent wound healing qualities, as well as antibacterial, anti-inflammatory, and antiseptic characteristics. This is a preferable alternative to the long-term usage of current drugs, which have several adverse effects.

CHAPTER 6

CONCLUSIONS

Effective wound healing using natural materials is acquiring immense attention in the current status. In this study, a simple and fast procedure was propsed for creating innovative Alginate/Chitosan/*H.colorata* thin films using the solvent-casting process. SEM analysis results gave the rough matrix's composition which aids in the absorption of wound fluids. The physicochemical characterisation of the produced films revealed that chitosan adds to the mechanical characteristics of alginate films. The water absorption and swelling behaviour of the produced films are affected by both the chitosan/*H.colorata* content of the film and the pH of the solution. The chitosan and *H.colorata* employed in the film have increased antibacterial resistance for an effective wound healing process. According to the invito assay result, it can be considered elite candidate for the management of wound. The combined results from various studies provided for a better understanding of how chitosan and *H.colorata* impact the growth of alginate film characteristics, as well as its potential application in domains like as the biomedical and pharmaceutical industries. This work also suggest that alginate/chitosan/*H.colorata* films is a preferentiable alternative to the long term usage of current drugs since it have suitable properties for wound healing.

CHAPTER 7

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