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Wound Healing Property of Herbal Dressing Film Using Chitosan and Peel Extracts of *Citrus limetta*

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Abstract

Impaired wound healing is one of the serious problems among the diabetic patients. To prevent complications and damage to the skin tissue and promote fibroblastic growth, many biological dressing materials and skin grafting have been employed. The present study fabricated a novel wound healing gel/film (CH-CL) using chitosan (CH) and methanolic peel extract of Citrus limetta (CL) and investigated the potential towards the wound healing process. During this investigation, the peel extract of CL was subjected to GC-MS to reveal the bioactive compounds responsible for various activities of the extract. Moreover, the CL extract was analyzed for its anti-microbial, anti-oxidant and anti-inflammatory properties. The CH-CL film was also subjected to water absorption capacity and folding endurance. The extract was also investigated for its viability using normal keratinocytes cell lines. The physico chemical characterization of the gel was done to reveal the chemical composition using FTIR, XRD and SEM. The GC-MS analysis results clearly indicated that the gel is biocompatible, possessing anti-microbial, anti-oxidant and anti-inflammatory properties due to its bioactive compounds. Furthermore, the CH-CL film acts as a good water absorbing material with optimum folding endurance which are the key physical properties of a normal wound healing material. Thus, the study concluded that CH-CL gel has been proven as an efficient, cost-economic wound healing gel and can be applied for various types of wounds and other biomedical applications.

Keywords: *Biological Dressing, Biocompatible, Citrus limetta, Health and Well-being, Novel Methods, Novel, Wound Healing Gel.*

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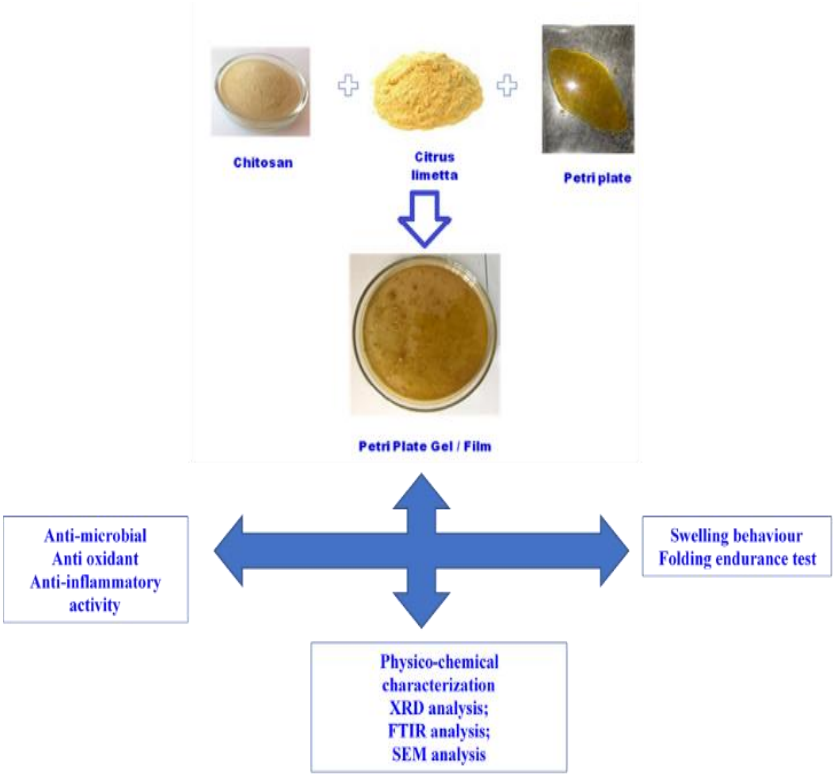

Graphical Abstract

Introduction

Wound healing is an intrinsic capacity of a tissue to regenerating the dermal and epidermal regions of the skin by a natural. It occurs as a substitute reaction to tissue injury and involves processes like inflammation, the proliferation of tissues, formation of fibroplasia and the maturation of scar tissue and is regulated by the deposition of collagen. This process restores the inflamed part to its near normal state [1]. This wound healing is a multifaceted and complex mechanism, but also considered fragile because it is subjected to many disruptions or failures resulting in chronic non-healing wounds. Many systemic factors like diabetes mellitus, infections, arterial and venous diseases and many geriatric metabolic deficiencies contribute to such chronic wounds that do not heal or delay the healing process [2].

Despite the efficient inherent natural phenomenon of the skin, complications do arise after an inflammatory process during wound healing. These include sepsis of tissues, disruption of layers of the skin, induction by maggots and chances of spread of infection to adjacent organs as well. Thus, to prevent complications and damages to the skin tissue, development of many biological dressing materials and skin grafting have been employed. As immediate wound coverage happens to be a primary objective in wound therapy and wound dressing plasters involving biomaterials have become popular in wound therapy. Biomaterials derived from animals, microbes, or plants are considered best in designing a wound dressing film because of their mimicking nature of tissues, lower toxicity levels, transport of precise binding sites for specific proteins and transfer of biochemical signals that enhance the process of tissue healing in wounds. Despite of their immunogenicity and states of decomposition at low melting temperatures, they do offer many advantages like biodegradability and they can be designed and developed with ease at molecular levels rather than macroscopic levels [3].

A designed biomaterial should possess a good topography with an efficient architectural assemblage, the best bio-molecular level composition, and good drug chemistry that affords a cell with an effective cell-biomaterial interface for wound healing [4]. Natural polymers like chitin, chitosan, cellulose, and gelatin are biologically proven biomaterial compounds owing to their cytocompatible nature, biodegradability and effective drug carrier delivery at tissue sites. They are employed as implantable materials, scaffold tissue polymers, and regenerative tissues in the field of tissue engineering.

Designing a natural carbohydrate polymeric dressing involving chitosan has been effective in treating femoral wounds. Reports suggest that chitosan offered better wound homeostasis as equivalent to gauze dressings in a mixed breed swine animal model [5,6]. Chitosan accelerates effective wound healing by activating the polymorphonuclear leukocytes at the inflammation site [7]. In combination with PEG, chitosan effectively inhibited infiltration of inflammatory cells and stimulated fibroblast activity at the damaged site, on the other hand, PEG improved the movement of epithelial cells [8].

One approach in designing a wound dressing film is to employ plant extracts with biomaterials to increase the efficacy of biopolymer films in wound therapy [9]. Historically and traditionally in native medicine, many native plants and their products, constituents have been widely for therapeutic management of different types of wounds. Such plant constituents act as effective disinfectants and promote wound debridement, enable a moist environment for the wounds, so that it facilitates an appropriate natural healing climate for the wounds [10].

Sweet lime (*Citrus limettarisso*), commonly called Mosambi in India is a freshly eaten fruit that offers a rich source of Vitamin C and replenishes good energy. Traditionally it is used to treat scurvy, gastrointestinal disturbances, diabetes mellitus, and urinary infections and to boost immunity. Reports reveal presence of multiple constituents in the fruit extracts out of which d-limonene was found to be present in abundance.

As delayed wound healing promotes a serious diabetes-associated complication in humans, a plant product along with the incorporation of a biomaterial happens to be considerably important, Reports suggest animals treated with Limonene depicted a significant diminution of wound size along with downregulation of GITR-expressing cells and decreased in the expression of mRNA for inflammatory mediators, mainly Th1 cytokines [11]. Thus, a prepared novel wound healing gel/film must possess good antioxidant, anti-inflammatory and anti-microbial activity and promote wound healing. The *Citrus limetta* risso peel extract incorporates vitamin C in the wound site stimulates collagen synthesis and chitosan offers effective wound healing at the damaged site. With these properties, the present study planned the fabrication of a novel wound healing film, CH-CL gel to treat all types of wounds.

The plant extract of *Citrus limetta* risso was analyzed for phytochemical analysis by GCMS and *in vitro* pharmacological properties in different concentrations. The prepared material was to for its subjected to physico-chemical parameters of the wound healing gel (CH-CL) by subjecting it to various characterization technique and swelling behavior.

Materials and Methods

Preparation of Plant Extract and Phytochemical Analysis

The fresh fruit *Citrus limetta* risso was collected and its peels were removed and stored. They were washed completely with distilled water and made to dry in the shade. It was then made to a fine powder and subjected to solvent extraction Soxhlet apparatus using methanol. The obtained crude methanolic

extract was concentrated and stored at 4°C. GC-MS analysis of the methanolic extract of *Citrus limettarisso* was done in Ultra GC-MS spectrometer QP-2010 (Shimadzu, Japan).

Preparation of Gel

10 % of chitosan was prepared with glycerol and 1 gm of *Citrus limetta* risso peel extract was added to it. This prepared gel was further cast into a petriplate and allowed to set at 50°C for 1 hour and left at 37°C for 48hrs to form the film (Figure 1).

Figure 1. Process and Formation of *Citrus limetta* Gel/Film.

Determination of *in vitro* Pharmacological Properties of CO Extract

The total antioxidant potential (*in vitro*) of *Citrus limetta* risso methanolic extract was assessed by the phospho-molybdenum technique [12]. The anti-inflammatory activity (*in vitro*) was assessed by protein (BSA) denaturation assay and HRBC membrane stabilization studies [13].

Anti-microbial Activity

Sabouraud Dextrose Agar Preparation and Mueller Hinton Agar Preparation

6.5 g medium was added to distilled water (100ml). This was heated and boiled for one minute for complete dissolution. 15.2 g medium was made to add with distilled water (400ml) separately. The mixture was purified in an autoclave machine for fifteen minutes and further cooled. Both the medium is poured onto separate sterile petri dishes of uniform depth. The test microorganisms were made ready. The plates remained swabbed in eight our broth media to be cultured. 10 mm wells were created. 1 mg/ml CO extract (100 µl) was added to wells and made to diffuse in two hours at RT. The control consisted of injections without CO extract. The plates were placed in incubation at a temperature of 37°C for one day with bacteria load and at a temperature of 28°C for 2 days for fungal load. After the prescribed periods, the zone of inhibition was measured [13].

Characterisation of CH-CL Gel

XRD Analysis

A Bruker D8 Advance X Ray diffractometer operating at 40 kV and 30 mA was utilised to record X-ray diffraction (XRD) patterns using a Cu K anode (= 0.1542 nm). The diffraction patterns were recorded at 25°C and at an angular range of 20 to 70°C using a step size of 0.05° per step and a dwell time of 12 sec per increment. The International Centre for Diffraction Data's stoichiometric HAP (JCPDS, card number 01 072 1243) was compared to the resulting pattern [15].

FTIR Spectroscopy

Utilizing FT IR spectroscopy on a Bruker Alpha II device, the functional groups were identified. To create the pellet for FTIR analysis, 2 mg of sample and 200 mg of (potassium bromide) KBr were compressed under hydraulic pressure. Averaging 100 scans, the spectra were recorded between 400 and 4000 cm 1 at a resolution of 2 cm 1 [15].

Scanning Electron Microscopy (SEM)

Samples were fully dried before being ready for SEM analysis. Samples were sputter-coated with platinum for 30 seconds after being placed on stubs using carbon tape. JEOL FE SEM IT800 was used to take SEM pictures. Software in the same instrument was used to record EDS at 20 KV [15].

Swelling Behaviour

The degree of swelling as a marker of water absorptivity of the hydrogel was determined as per the previous method [14]. The CH-CL films were tested for water absorptivity at room temperature. 1 cm^2 Small pieces of CH-CL films were analysed for their weight and then submerged in double distilled water and 0.89% physiological saline solution [14]. After the prescribed interval of time, the water absorbed on the wet swollen gels was dabbed off to remove the water with fine tissue paper and CH-CL dry film specimens were reanalysed for their weights. The experiments were done in triplicates. The gradation of swelling behaviour relates to the absorptive capacity of water by material and is obtained using the equation, noting that Ws and Wd are analysed weights of swollen CH-CL film and dried CH-CL film, respectively.

$$\text{Water absorptivity (\%)} = [W_s - W_d / W_d] \times 100$$

Folding Endurance Test

The testing of folding endurance was analysed to evaluate the flexible nature of the CH-CL film for easy handling and secured application at the wound site. It was analysed by repetitive folding of one CH-CL film at a similar place until the film offered any break or if it folds up to 300 times which was performed manually. The frequent no of times that a film could afford to fold at one similar place, without any significant breakage in the film is considered as the folding endurance value. Thus, the effect of flexibility of the dried CH-CL films was subjected to a folding endurance test [15].

Results

Gas Chromotography-Mass Spectrometer (GC-MS)

The GC-MS report of methanolic *Citrus limetta* risso revealed compounds like coumarins, anthraquinone, flavonoids, saponins, carotenes, polyphones, vitamin C, terpenes, Limonoids and linalool (Figure 2).

HRBC Membrane Stabilization Method

The effect of methanolic extract of *Citrus limetta* risso peel extract on hypotonic saline prompted lysis of erythrocytes is a known indication of a good anti-inflammatory property in a plant. *Citrus limetta* risso peel extract showed a dose-related higher inhibition percentage indicating membrane stabilizing potential with conc. from 50 µg/ml to 1000 µg/ml and the obtained results were compared with diclofenac sodium considered as standard (200 µg/ml) (Table 1).

Protein Denaturation Method

Citrus limetta risso peel extract showed a dose-related higher inhibition in the denaturation of bovine serum albumin in conc. from 50 µg/ml -1000 µg/ml and the obtained results were compared with diclofenac sodium taken as standard in a conc. 200 µg/ml (Table 2.

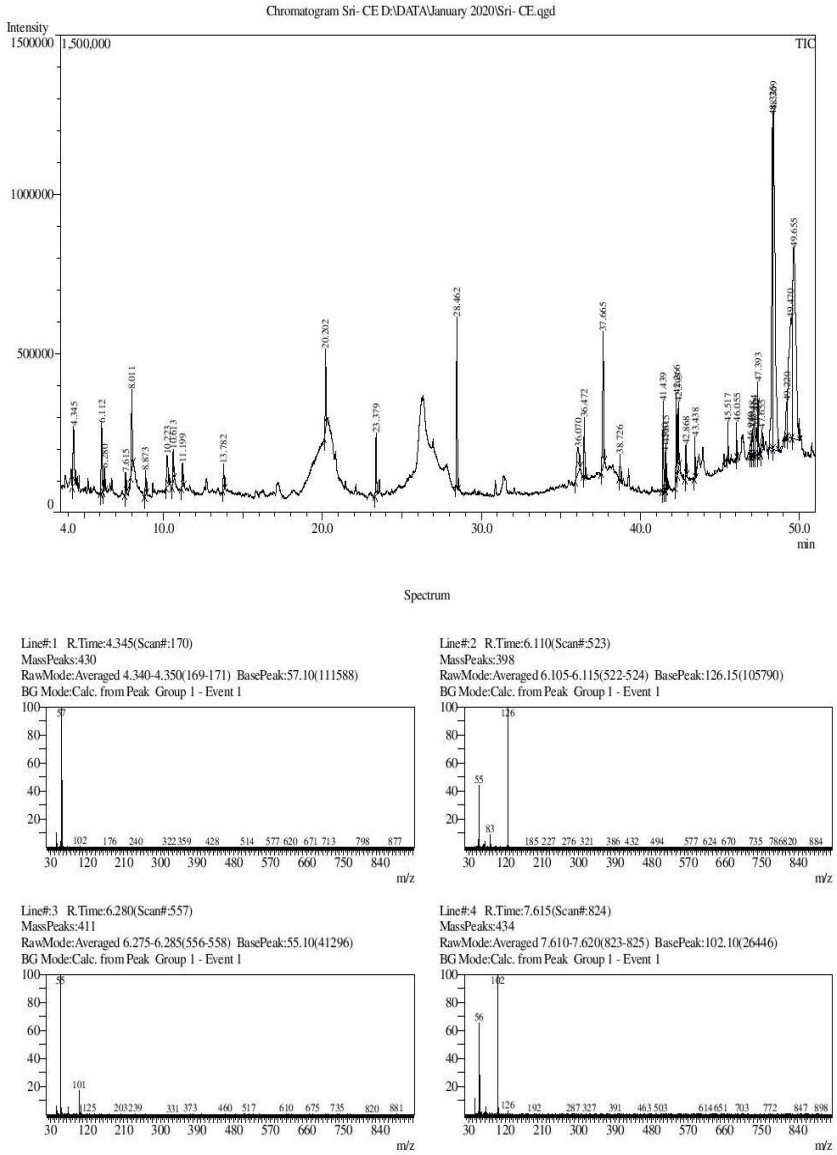

Figure 2. GCMS Report of *Citrus limetta* risso Peel Extract.

Table 1. HRBC Membrane Stabilization Study of *CL* Extract.

S. No	Conc. (µg/ml)	Methanolic CL extract Haemolysis inhibition (%)	Diclofenac sodium Haemolysis inhibition (%)
1	50	21.24	
2	100	37.65	
3	200	49.81	87.6
4	300	59.14	
5	400	64.88	
6	500	69.71	
7	1000	81.32	

Table 2. Protein Denaturation Study of CL Extract.

S. No	Conc. (µg/ml)	CL Extract Protein denaturation Inhibition (%)	Diclofenac Sodium Protein denaturation Iinhibition (%)
1	50	25.79	
2	100	38.21	
3	200	56.42	87.61
4	300	59.62	
5	400	64.83	
6	500	69.14	
7	1000	79.92	

Determination of Total Anti-oxidant Property

Table 3 and Figure 3 show the antioxidant values of CL extract. The findings of the study showed that there was a dose-dependent increase in the percentage of total anti-oxidant activity for both *Citrus limetta* Risso peel extract and ascorbic acid standard ranging from 100 to 500 microgram per ml. IC_{50} for standard is 284 and IC_{50} for CL extract is 279.

Table 3. Total Antioxidant Capacity

Conc. (µg/ml)	Ascorbic acid % Inhibition	Os % Inhibition
50	18.47±1.08	18.13±1.13
100	30.64±1.62	30.16±1.87
200	45.12±1.94	44.92±2.24
300	46.73±1.37	45.87±1.69
400	53.75±1.82	53.22±0.79
500	60.72±1.09	58.79±0.05
IC $_{50}$ (µg/ml)	284	279

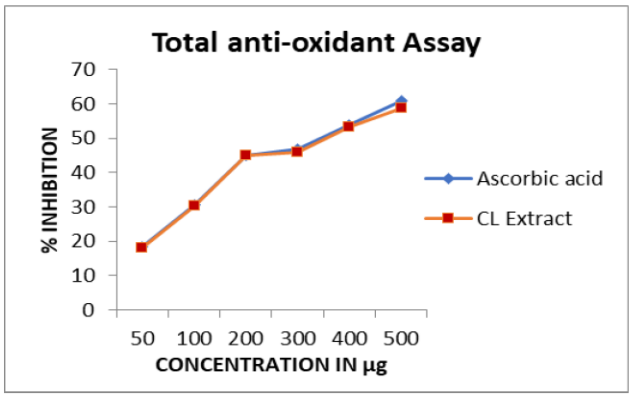

Figure 3. Shows the Anti-oxidant Property of the CL Extract.

Anti Microbial Activity

In the present investigation, showed that methanolic peel extract of CL was active against locally isolated human pathogens like *Pseudomonas aeruginosa*, and *Candida albicans* as shown in Table 4 and figure 4 and among all these, the CL extract was highly resistant towards *Pseudomonas aeruginosa*.

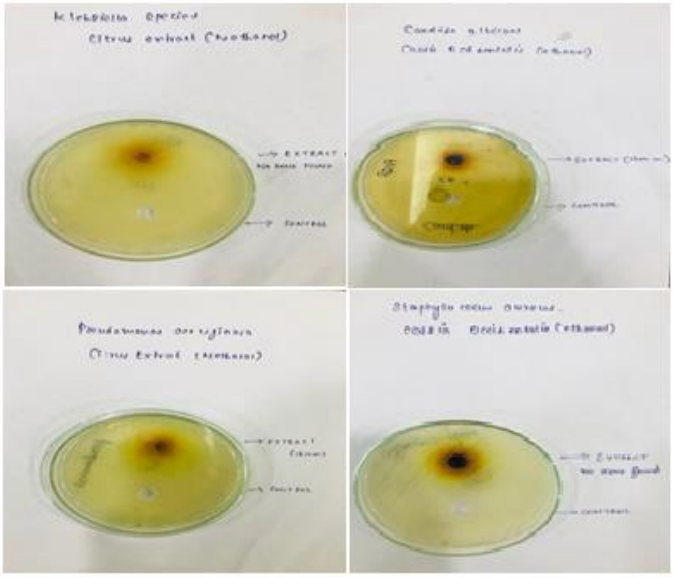

Figure 4. Shows the Anti-microbial Activity of *Citrus limetta* Extract.

Table 4. Antimicrobial Activity of the *Citrus limetta* Extract.

S. No	Test pathogens	Zone of inhibition	Positive Control
1.	*Staphylococcus aureus*	No zone found	Linezolid 30 mm
2	*Candida albicans*	13mm	Flucoconazole 28mm
3	*Klebsiella species*	No zone found	Cephalosporin 18mm
4	*Pseudomonas aeruginosa*	18mm	Piperacillin/tazobactam 30 mm

Characterization of Wound Dressing Film

Fourier Transformer Infrared Spectroscopy (FTIR)

In figure 5, the resutls showed the infrared spectrum of chitosan with absorption bands viewed around 2933 and 2879 cm^{-1} and it can be indicative of C-H symmetric and asymmetric stretching. These bands represent typical characteristics of polysaccharides like xylan [15] glucans [16] and carrageenan's [17]. A band that appears at 1589 cm^{-1} is indicative of N-H bending of a primary amine. The bending of CH_2 with symmetrical deformation was indicative and confirmative with the presence of bands at around 1411 cm^{-1}. The bands in 1032 cm^{-1} were responsive to C-O band stretching. Reports suggest that all these bands were found in the spectra of samples containing chitosan [18].

X-Ray Diffraction

The XRD 2θ values indicate the peaks at 36.67^0 and 42.87 which correspond to the reflection from 112 and 130 showing the occurrence of chitosan. The 2θ values 632.20^0 and 76.36^0 correspond to the reflection from 315 and 416 showing the occurrence of the presence of *Citrus limetta* in the gel (Figure 6).

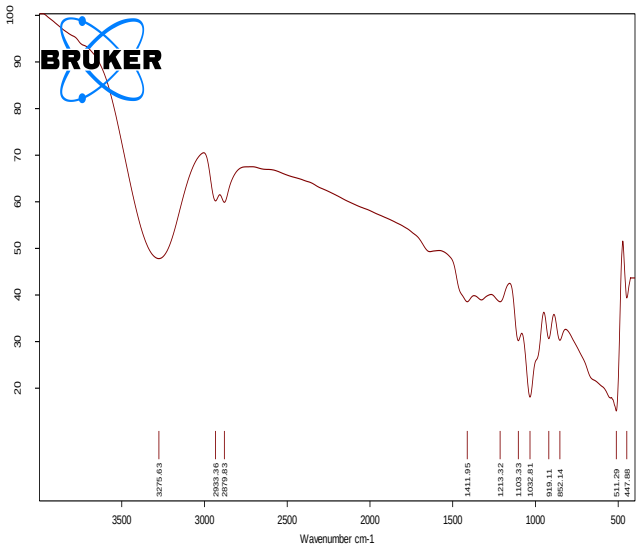

Figure 5. FTIR Analysis of the *Citrus limetta* Gel.

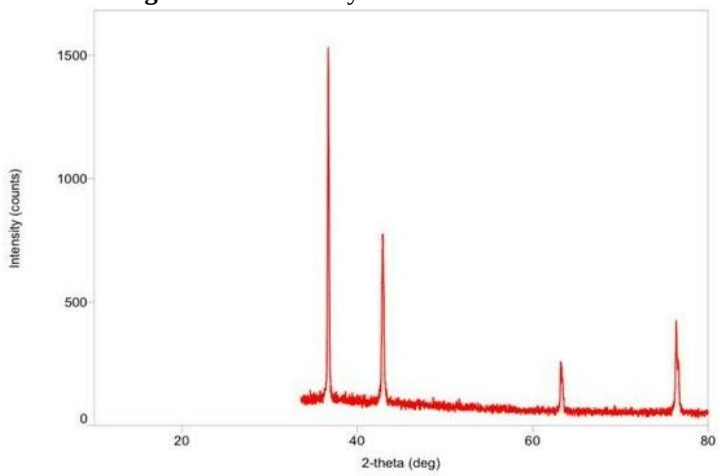

Figure 6. XRD Analysis of the *Citrus limetta* Gel.

Scanning Electron Microscope Analysis (SEM)

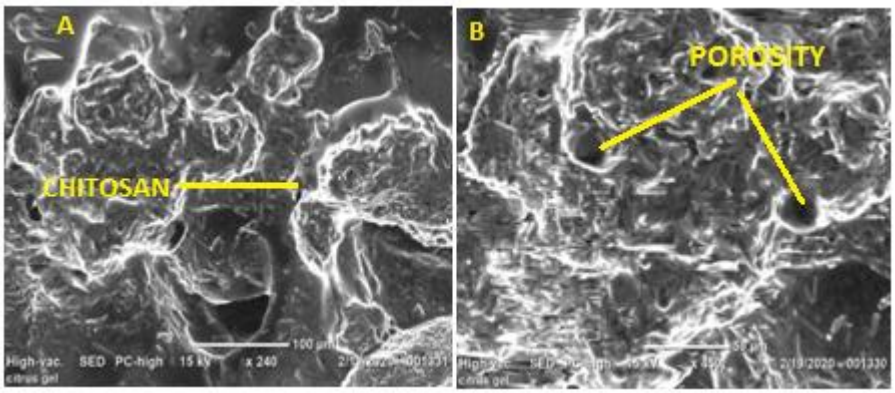

Figure 7. Showing SEM imageCH-CL gel (A) shows the chitosan network (B) shows the porosity of the gel.

Measurement of Water Absorptivity of CH-CL Gel

The water resorbing capability of CH-CL films was markedly increased with the inclusion of CL extract into the chitosan gel, which may be due to hydrophilic properties of *Citrus limetta* extract [19] and the results were shown in table 5.

Folding Endurance

Folding endurance of CH-CL film was significantly increased with the inclusion of CL extract into the chitosan gel when compared with chitosan film which was shown in table 5 [20].

Table 5. Water Absorption Capacity and Folding Endurance of the Film.

S.NO	Composition	Water absorption capacity (%)	Folding endurance
1	**Chitosan film**	1101.87±9.08	202±5.50
2	**CH-CL film**	1134.45±11.13	238±6.55

Discussion

The CL peel extract was evaluated for phytochemical analysis by GCMS and the wound dressing film CH-CL was evaluated for its physico-chemical characteristics. GCMS analysis revealed the presence of coumarins, anthraquinone, flavonoids, saponins, carotenes, polyphones, vitamin C, terpenes, Limonoids and linalool that inducted antimicrobial activity and wound healing activity for the film. In addition, the phytochemicals impart anti-oxidant and anti-inflammatory properties to the wound healing gel which helps in healing the wound quickly even in diabetic patients.

CL methanolic extract at a known conc. 50 µg/ml was capable of inhibiting haemolysis by 21.24%. This percentage of inhibition gradually increased with increasing concentration of CL extract and at conc. of 1000 µg/ml. The CL extract inhibited the haemolysis at 81.32% which was on par with the values of diclofenac sodium at conc. of 200 µg/ml. The anti-inflammatory property of CL extract may be because of the existence of bioactive compounds and compounds like vitamin C in the CL extract [21]. When this extract was incorporated with chitosan to form a gel or film, it was able to show an anti-inflammatory activity at the implant site which eventually rapidly assisted the wound healing.

Denaturation of proteins is a marked feature of inflammation. The release of autoantigens into the joints leads to arthritic diseases which is one of the autoimmune disorders [22]. The secondary and tertiary structures of the tissue proteins are lost due to the invasion of autoantigens. The phytochemicals present in the CL extract exhibit anti-inflammatory property and promote control over the assembly of autoantigens and eventually prevent protein denaturation.

CL extract at a concentration of 50 µg/ml exhibited 25.79% inhibition against denaturation of proteins. The percentage inhibition was directly proportional to rise in conc of the CL extract. CL extract at conc. 1000 µg/ml exhibited denaturation of proteins at 79.92% which was on par with diclofenac sodium at a conc.200 µg/ml. Thus, it clearly showed that compounds like alkaloids, phenols, and flavonoids contribute to control the denaturation of protein when compared with the standard drug diclofenac sodium.

Preclinical models that simulate acute or compromised wounds, such as those associated to diabetes and nutrition, can be created in a variety of ways, including in mice, rabbits, and pigs. There are numerous ways to generate these, the most popular being

excision or incision. Once a viable model has been identified for a study, researchers must choose suitable and repeatable procedures that will enable tracking the evolution of the wound over time. Wound tracing, photographic recording (including image analysis), biophysical approaches, and/or invasive protocols requiring wound biopsies are among the non-invasive methods that can be used to complete the assessment [23].

Bioactive phytocompounds in plants like phenolic acids, and tannins. flavonoids, quinones, proanthocyanins and anthocyanins, lignans, coumarins, and catechins possess redox properties that impart antioxidant properties to plant products and hence delay or prevent the onset of degenerative diseases. Those redox properties of plants allow them to act as effective hydrogen donors, reducing mediators, and scavengers of free radicals. The methanolic extract of CL revealed the presence of Vitamin C, flavonoids, anthraquinone, terpenoids, carotenoids and saponins. High concentration of polyphenols, Vitamin C and emodins in the extract could be the possible reason for CL to exhibit the anti-oxidant property [24]. Methanolic peel extract of CL was active against locally isolated human pathogens like Pseudomonas aeruginosa, and Candida albicans and among all these the extract was highly resistant towards *Pseudomonas aeruginosa*. The antimicrobial property supports the CH-CL in preventing any infection in the wound site with rapid healing.

FTIR results revealed the presence of chitosan in the gel with all the peak values when compared to the literature and hence the activity of chitosan can be achieved when it is mixed with citrus peel extract. The results of XRD confirm the findings of FTIR and reveal the presence of chitosan and citrus extract with the 2θ values. The SEM image of CH-CL gel reveals the presence of chitosan forming a fibrous network with lot of pores which allows the bioactive compounds of CL extract to reach the wound site and hence quick wound healing will be accomplished.

The water absorption capacity of CH-CL film showed an increase in the value compared to pure chitosan film which may be due to the hydrophilic properties of *Citrus limetta* extract along with the hydrophilic property of chitosan. There was a marked difference in folding endurance between the chitosan film and CH-CL film and this supports the CH-CL film in ease of handling while placed in the wound site. The flexibility of the film also helps to cover the wound site irrespective of its size and shape

Conclusion

Compromised wound healing is considered a serious threat faced by patients suffering from diabetes mellitus and burn injuries. In line with that, the present study fabricated and evaluated a natural and novel wound healing gel/film using chitosan (CH) and methanolic peel extract of *Citrus limetta* (CL in its therapeutic efficiency for wound health. The GC-MS analysis results indicated that the gel is biocompatible, possessing anti-microbial, anti-oxidant and anti-inflammatory properties due to its bioactive compounds. Furthermore, the CH-CL film acts as a good water-absorbing material with optimum folding endurance which are the key physical properties of a normal wound healing material. Thus, the CH-CL gel has been proven as an efficient, cost-economic wound-healing gel, and can be used for treating diabetic wounds and burns rapidly after further in vivo experiments.

Conflict of Interest

The author hereby declares that there is no conflict of interest.

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