DEVELOPMENT AND EVALUATION OF CHITOSAN HONEY HYDROGEL SHEETS AS WOUND DRESSING

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ABSTRACT

Multiplication of antibiotic resistant bacteria complicates the wound healing process nowadays. It has been proved that our ancient medicine honey is a panacea for a lot of diseases and it plays a vital role in wound healing. Hydrogel sheets were developed from chitosan and chitosan-honey in this study to evaluate the required properties for effective wound dressings, such as, folding endurance, degradation, water vapour transmission, swelling ratio, tensile strength, elongation, antimicrobial property and animal study. Based on the Minimum Inhibition Concentration (MIC) value of Manuka honey (*Leptospermum scoparium*), 8% concentration of honey was experimented. The hydrogel sheets were produced from chitosan(C) and chitosan-honey (CH) with lactic acid as solvent. The CH sample showed significant results in physical properties and mechanical properties. It also showed higher zone of inhibition against *Staphylococcus aureus* and *Escherichia coli*, the most wound infecting bacteria and contributed significantly to excision wound healing with 94.4% wound contraction on 12th day. The sheet was also soft, flexible and exhibits its potential to be used as a wound dressing.


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INTRODUCTION

The treatment of wounds has evolved from ancient times. This usually involves preventing infection because the skin, the body’s barrier to infection, is destroyed. Hence, wound healing is a normal biological process that occurs after any injury to the skin. It is achieved through four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling. Healing restores integrity of the injured tissue and prevents organisms from deregulation of homeostasis. An ideal dressing should maintain a moist environment at the wound interface, allow gaseous exchange, act as a barrier to microorganisms and remove excess exudates. It should also be nontoxic, non-allergenic, nonadherent and easily removed without trauma, it should be made from a readily available biomaterial that requires minimal processing, possesses antimicrobial properties and promotes wound healing. An ideal wound healing material should be able to play the role of extracellular matrix (ECM) until host cells can repopulate to form a new natural matrix. During wound healing procedure, epithelium cells are easier to migrate to a moist environment than in a dry environment. Currently, even though there is a variety of wound dressings available ranging from passive adherent/ nonadherent to interactive and bioactive products that contribute to the healing process, bacterial resistance to the antimicrobial agents poses a very serious threat to public health. Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including honey. The use of honey as a traditional remedy for microbial infections dates back to ancient times. It has been reported that honey promotes wound epithelization, reduce inflammation and exudation and accelerates synthesis of collagen and increase DNA content of the granulation tissue. In addition, honey is hygroscopic, which means that it can draw moisture out of the environment and dehydrate bacteria, and its high sugar content and low level pH can also prevent the microbes from growth. The beneficial role of honey is attributed to its antibacterial property with regards to its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) and non-peroxide components, i.e., the presence of phytochemical components like methylglyoxal (MGO). The Leptospermum scoparium, (Manuka honey), the best known of the honeys, has been reported to have an inhibitory effect on around 60 species of bacteria, including aerobes and anaerobes, gram-positives and gram-negatives. The manuka honey is capable of stimulating the monocytes, the precursors of macrophages, to secrete TNF-α. Unlike glucose oxidase, the antibacterial properties from Leptospermum spp. honeys are light and heat stable. Natural honey of other sources can vary as much as 100-fold in the potency of their antibacterial activities, which is due to hydrogen peroxide. It has also been reported that a honey hydrogel dressing and VAP-Chitosan-Honey suspension were developed for enhanced wound healing. Chitosan, a biodegradable, non-antigenic, and biocompatible natural polymer that bears the proxy structure of a glycosaminoglycan (GAG) has also been proved to have desirable wound healing qualities such as hemostasis and bacteriostasis. GAGs are major components of the extra-cellular matrix (ECM). They are known to support cell attachment and proliferation and improve the cell and tissue biocompatibility of biomaterials. Chitosan is slightly crystalline and insoluble when the pH is near or above 7. However, the free amine groups of chitosan can be protonated in an acidic environment, providing additional positive charges that improve its hydrophilicity. Chitosan is easily deformed through external stress, which causes excessive swelling in acidic aqueous environments. Therefore, it is difficult to use chitosan by itself for medical applications, especially as a wound dressing material. Hence, it has been widely used for wound dressings in the form of hydrogel, fiber, membrane, scaffold and sponge. However, chitosan possess ideal wound healing properties by protecting and contracting the
wound in a moist healing environment, when it is in hydrogel form. The demerit of dilution of honey, when it comes to body temperature will also eliminated when it is converted into hydrogel sheet form. In this study, therefore, chitosan and honey were selected to prepare hydrogel sheets as wound dressings. The physical, mechanical and wound healing properties were investigated.

MATERIALS AND METHODS

Materials

Chitosan was purchased from Sigma Aldrich Chemicals, Ltd (Bangalore, India). Manuka honey was obtained from MediHoney, Canada, Derma Sciences Inc. and Lactic Acid was purchased from HI-PURE chem industries, (Chennai, India).

Prepared hydrogel sheets

![Appearance of hydrogel sheets peeled off and placed in a petri dish](image)

Table 1

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sample</th>
<th>Sample Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>2% Chitosan</td>
</tr>
<tr>
<td>2</td>
<td>CH</td>
<td>2% Chitosan – 8% Honey</td>
</tr>
</tbody>
</table>

Evaluation of films (i). Physical Properties

Thickness of the film was measured using a calibrated micrometer which can measure the thickness with least count of 0.001 mm. To determine weight uniformity of the each film, five specimens of size 2.0 cm x 2.0 cm of all films were weighed on electronic balance and mean weight was calculated. Folding endurance was determined by repeatedly folding one film at the same place till it breaks or folded upto 300 times manually to find the flexibility of film. Degradation test was carried out by immersing the samples in solvents for different time periods using the area/volume ratio = 0.1 cm⁻¹. The degradation index (Di) was calculated based on the mass loss using the equation, \( \frac{[Wo-Wf]}{Wf} \times 100 \), Wo- Initial Weight and Wf- Final Weight.
(ii). Transmission Properties

Water Vapour Transmission (ASTM E 96-95)
To measure the water vapour penetration, desiccant method was used. The films were cut and placed on top open bottles containing 5 gms. of silica gel and held in place with a screw lid (test area: 4.9 cm$^2$). The bottles were conditioned in desiccators containing silica gel for 12 hours. The bottles were then placed in desiccators containing NaCl at 30° C (75% relative humidity). The equilibrium vapour penetration was determined by weighing the bottles at 6, 12 and 24 hours, respectively. The water vapour transmission (WVT) was calculated as follows:

\[ \text{WVT in g./hr.m}^2 = \frac{G}{t \times A} \]

Where, 
- G- Change in weight of Silica gel(gms)
- t- Time during which G occurred
- A- Test Area (m$^2$)

Swelling ratio

The water uptake was assessed gravimetrically. The weights of the completely dried films were determined with an analytical balance. Strips of chitosan and chitosan-honey films (2x 2 cm$^2$) were immersed in deionized water at 37° C in an incubator for 24 hours. The resultant swollen film was gently blotted with filter paper to remove excess surface water and weighed again. The water uptake of the film is the increase in weight, expressed as a percentage. The water uptake of different samples was calculated using the following method:

\[ \text{Water uptake (\%)} = \frac{100 \times (W_2 - W_1)}{W_1} \]

Where, 
- $W_1$ is the weight of completely dried sample; 
- $W_2$ is the weight of the swelled sample at 37° C for 24 hrs.

(iii). Mechanical Properties

Tensile Strength and % Elongation (ASTM D 882-12)
Tensile strength was evaluated using an Instron Universal Testing instrument (Model 4206, Instron Ltd., Japan) with a 2 kg load cell. Film of the required dimension without any air bubbles or physical imperfections were held between two clamps positioned at a distance of 3 cm. During the measurement, the top clamp was pulled at a rate of 100 mm/minutes and the force and elongation were measured upon breaking the films. The results from a film sample that broke down between the clamps were used. Measurements were run in triplicate for each film. Tensile strength and percent elongation were calculated by applying the following equations:

\[ \text{Tensile strength} = \frac{\text{Force at break (N)}}{\text{Initial cross sectional area of the sample (m}^2\text{)}} \]

\[ \text{Elongation \%} = \frac{\text{Increase in length at breaking point mm}}{\text{Initial length mm}} \times 100 \% \]

(iv) Antimicrobial property -- Agar diffusion test (SN 195920: 1992)
Plate Count Agar (PCA) plate was prepared and 100 µl of the selected dilutions of respective bacterial cultures were spread plated in duplicate. The film with the diameter of 2cm ± 0.1cm was taken for the analysis. Both the sides of samples were pre sterilized under ultra violet radiation for 15 minutes. Sterile bacteriostasis agar was dispensed in sterile petridishes. Broth cultures (24 hours) of the test organisms were used as inoculum. Using sterile cotton swab, the test organisms (Escherichia coli & Staphylococcus aureus) were swabbed over the surface of the agar plate. Pre sterilized samples were placed over the swabbed agar surface by using sterile spatula and forceps. After placing the samples, all the plates were incubated at 37ºC for 18 to 24 hours. After incubation the plates were examined for the zone of bacterial inhibition around the sample. The size of the clear zone was used to evaluate the inhibitory effect of the film.

(v) Animal Study: Excision wounds
Excision of wounds was made as described by Morton and Malone. Animals, wister rats weighing 100-150gms, were anaesthetized with anaesthetic ether and shaved on the back with electric clipper by placing them on operation table. A full thickness 1.5cm x1.5cm excision wound was created on the back of all animals. The animals were divided into 4 groups of 5 animals in each. Animals of Group I was treated as control; Group II were applied with standard cipladin ointment; Group III animals were treated with sample C and Group IV animals were applied with sample CH. The wound was treated with respective treatment till
epithelialization and the wound contraction was studied by tracing the raw wound area on day 4, 8 and 12 on graph paper. The size reduction and percentage of wound closure was recorded. The experimental protocol was approved by the Institutional Animal Ethics Committee, PSG Institute of Medical Sciences & Research.

**RESULTS AND DISCUSSIONS**

**Physical Properties**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Thickness (mm) (Mean±SD)</th>
<th>Weight (gms.) (Mean±SD)</th>
<th>Folding Endurance (Mean±SD)</th>
<th>Degradation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.280±0.14</td>
<td>0.201±0.05</td>
<td>143±4.3</td>
<td>41±9.2</td>
</tr>
<tr>
<td>CH</td>
<td>0.312±0.03</td>
<td>0.243±0.18</td>
<td>289±5.5</td>
<td>48±30.1</td>
</tr>
</tbody>
</table>

Statistical analysis

Five readings were taken in all measurements and expressed as means ± standard deviations. Single factor analysis of variance (ANOVA) was employed to evaluate the statistical significance of the results. Statistical significance was associated with a probability \( P<0.05 \).

Table 2. shows that thickness of prepared films, ranged between 0.280-0.312 mm. The deviation in thickness was minimized by pouring equal volume of solutions in a same diameter petridish. When compared, the addition of honey increased the thickness, weight and folding endurance. To observe the degradation of films all the films were soaked in solvent for 1 hour and observed for mass loss. It was very rapid in the initial 30 mins and then minimum reduction was observed. It is observed that the degradation % increases with the addition of honey, which may be because of reduction in degree of crosslinking.

**Transmission Properties**

**Water vapour Transmission**

Graph 1

*Water Vapour Transmission (g/m²/day) of the samples*

Water vapor transmission was measured under steady-state conditions by considering the contribution of the moisture absorbed by the film as negligible. As stated in the literature\(^\text{67}\), water vapour transmission varies inversely with thickness due to the mass difference. The water vapour transmission for chitosan honey films ranges between 6000-12000 g/m²/day, which is comparable to the commercial wound dressing. It can also be seen that the rate of vapour penetration increases at a faster rate between 6-12 hrs. than between 12-24 hrs.
**Water Vapour Transmission Test**

**Figure 2**
*shows the water vapour transmission test method*

**Swelling ratio**

**Graph 2**
*Swelling ratio of prepared samples*

![Graph showing swelling ratio of prepared samples]

**Swollen sample**

**Figure 3**
*Appearance of the hydrogel sheet in swollen condition*

The result shows that the average equilibrium water uptake of chitosan and chitosan-honey films after 24 hrs., which ranged between 605 and 650. With the addition of honey, equilibrium swelling in water(ESW) decreases, which indicates reduced the water retention ability of the samples. This can be explained by the hydrophilic nature of chitosan polymer.
**Mechanical Properties**

**Tensile test and elongation %**
The films suitable for wound dressing should preferably strong but flexible. Since in both the samples, lactic acid was expected to act as both solvent and plasticizer, the films are more elastic. It is known that there is an inverse relationship between tensile strength and elongation of biopolymer film. The addition of honey in the film reduces the strength and increases elongation at break\(^6\).

**Tensile Strength Tester**

**Figure 4**

*Position of hydrogel sheet during tensile strength testing*

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tensile strength (N/mm(^2)) and Elongation % at break of the samples</strong></td>
</tr>
<tr>
<td>Samples</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>CH</td>
</tr>
</tbody>
</table>

**Bioevaluation properties**

**Antimicrobial test- Agar Diffusion Test**
The zone of inhibition with various films against *S.aureus* and *E.coli* was different in agar disc diffusion technique as shown in Table 4. Blank chitosan films shows minimum bacterial inhibition compared to chitosan honey film, which can be explained by the inherent antimicrobial property of chitosan and honey. The bacterial inhibition is higher in all the cases of *S.aureus* than *E.coli*. Hence, it is clear that both Chitosan and Honey contributes to antibacterial property and the addition of honey increases the zone of inhibition. Since honey is hygroscopic in nature, it draws moisture out of the environment and dehydrate bacteria, and its high sugar content and low level pH can also prevent the microbes from growth\(^4\).

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zone of Inhibition of the samples against S.aureus and E.coli</strong></td>
</tr>
<tr>
<td>Samples</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>CH</td>
</tr>
</tbody>
</table>

**Statistical Analysis**
To identify the effect of addition of honey ANOVA were performed between the samples for all the characterization studies in specific reference to wound dressing. Even though the test results shows variation numerically between the sample, the statistically there is no significant difference between the tested samples (p>0.05). There was a significance difference between blank chitosan and chitosan-honey samples in almost all characterization studies(p<0.05).
Animal Study
The percentage of wound contraction in treated and control groups were analyzed in the excision wound sites and the results are shown in Table 5 and Figure 5.

Macroscopic observation of excision wounds
Wound (0th Day)

Wound Healing (consecutive days)

<table>
<thead>
<tr>
<th></th>
<th>4th Day</th>
<th>8th Day</th>
<th>12th Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Standard</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Sample C</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>Sample CH</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 5
Visual appearance of wound during the animal study
Table 5
Percentage wound contraction

<table>
<thead>
<tr>
<th>Sample</th>
<th>4th day</th>
<th>% C</th>
<th>8th day</th>
<th>% C</th>
<th>12th day</th>
<th>% C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200.8</td>
<td>10.7</td>
<td>139.6</td>
<td>37.9</td>
<td>81.0</td>
<td>64.0</td>
</tr>
<tr>
<td>Std. Ointment</td>
<td>189.4</td>
<td>15.8</td>
<td>92.7</td>
<td>58.8</td>
<td>34.5</td>
<td>85.5</td>
</tr>
<tr>
<td>Sample C</td>
<td>198.0</td>
<td>12.0</td>
<td>145.8</td>
<td>46.0</td>
<td>50.0</td>
<td>77.8</td>
</tr>
<tr>
<td>Sample CH</td>
<td>186.2</td>
<td>17.2</td>
<td>79.5</td>
<td>64.6</td>
<td>12.5</td>
<td>94.4</td>
</tr>
</tbody>
</table>

Wound healing acceleration of chitosan and chitosan-honey samples were compared with control and a standard commercially available cipladin ointment. The rate of wound contraction was assessed by determination of unclosed area as a function of time. On day 4, there was no significant difference between control and all the samples. Healing was rapid in CH sample on day 8 and this is due to the effect of honey in wound care. Wound healing occurs by granulation tissue filling in any cavity, and epithelial cells migrating across this from the surviving epithelium at the margin of the wound to create new skin cover. Manuka honey dressing, which plays a vital role in healing of wound at a faster rate creates moist healing environment, absence of microbes and supply of glycogen to the epithelial cells. The antioxidant phytochemical components play a vital role in reducing the inflammation. On day 12, the wound closure reached 94.4% in CH sample applied wounds. In contrast, there was still about 35% wound area unclosed in control wound and 15% in standard ointment applied wound.

**CONCLUSION**

In conclusion, the hydrogel sheet produced from chitosan-honey satisfies all the requirements of wound dressings like thickness, weight, folding endurance, degradation, water vapour transmission, water absorption, tensile strength and elongation. The dressing also has good amount of antibacterial activity against *S. aureus* and *E. coli*, which also arrests microbes transmitting from outside environment to wound bed. The dressing also promotes significant wound contraction, and accelerates wound closure and healing process. Thus, chitosan-honey hydrogel film may be a promising new dressing and further studies will be required to clarify its more clinical significance and this material can also be envisioned to different wound types.

**REFERENCES**


29. Van der Weyden E, The use of honey for the treatment of two patients with pressure


