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Research Article

ANALYSIS OF MANUKA (*LEPTOSPERMUM SCOPARIUM*) HONEY AND DEVELOPMENT OF CHITOSAN-MANUKA HONEY FILM FOR WOUND DRESSING APPLICATIONS

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ABSTRACT

This research focuses on the chromatographic identification of unique manuka compounds responsible for antimicrobial and wound healing properties. And also the Minimum Inhibitory Concentration (MIC) of manuka honey against *Staphylococcus aureus* (*S.aureus)* and *Escherichia Coli* (*E.coli*) was elucidated. The analyzed manuka honey sample with Unique Manuka Factor (UMF) of 18+ was selected for the formation of films for the wound contact layer with chitosan. MIC of manuka honey sample was derived using broth dilution method against the two wound pathogens *S.aureus* (gram positive) and *E.coli* (gram negative). Out of the ten concentrations (22.5%, 20%, 17.5%, 15%,12.5%, 10%, 8.5%, 7%, 5.5% and 4%) tested, the results depict that the MIC_{95} (atleast 95% inhibition) of manuka honey against gram positive *S.aureus* is 10%, and gram negative *E.Coli* is 12.5%. The antimicrobial and wound healing studies were also performed from the developed films. Chitosan-honey films exhibited higher bacterial inhibition against both *S.aureus* and *E.coli* compared to blank chitosan film. The inhibition zone of chitosan-honey films against *S.aureus* and *E.coli* was observed as 28mm and 24mm respectively. Chitosan film showed the same activity of 24 mm against *E.coli* and lower activity of 22 mm against *S.aureus*. Similarly, the *in vivo* wound healing studies revealed that the chitosan-honey film has 95% wound closure on day 18.

KEYWORDS: Antibacterial, Chitosan, Chromatography, Excision Wound healing, Film, Manuka honey, Minimum Inhibitory Concentration.

INTRODUCTION

Manuka honey (*Leptospermum scoparium)* has been reported to exhibit inhibition against 80 species of bacteria and well characterized for its broad spectrum of antibacterial property[1-4]. Manuka Honey exerts non-peroxide antibacterial activity, which emphasizes the reason for Unique Manuka Factor (UMF), which is a phenol equivalent factor, to *Leptospermum scoparium* honeys[5]. Numerous compounds were identified as a major contributor for the antimicrobial and wound healing potential of manuka honey[6-8]. Given the importance of manuka honey (*Leptospermum Scoparium*) as an antibacterial and wound healing agent, the compositional analysis of manuka honey was performed using GC-MS. The bioactivities of honey might be due to its phenolic and flavonoid compounds and also because of few unique compounds. Despite the advantages of chitosan and manuka honey in wound management, a combination of chitosan with manuka honey is not yet reported in this field. Honey in its nature becomes a running liquid, when it comes to contact with the skin[9]. Hence, with due advantage of holding honey on wound surface, the manuka honey is combined with chitosan along with glycerol and prepared as a film to be used for wound dressing applications.

MATERIALS AND METHODS

Materials Used

Medium molecular weight chitosan, with degree of acetylation greater than 75% was procured from Sigma Aldrich Chemicals Ltd. (Bangalore, India). All other chemicals were procured from HI-PURE Chem Industries, (Chennai, India). The deionized water was obtained from Kumaraguru College of Technology, Coimbatore, which was prepared by Electro-deionization (EDI) method. Manuka honey with UMF 18+ was obtained from MediHoney, Canada, Derma Sciences Inc. and the other reagents used were of analytical grade.

Microorganisms Used

The bacterial cultures used in this research work, gram positive *S.aureus* (MTCC 737) & gram negative *E.coli* (MTCC 1687) were obtained from MTCC, IMTech, Chandigarh.

Sterilization Procedure

The samples were uncontaminated under Ethylene Oxide (EO) sterilization procedure in an EO Gas Sterilizer Series 3 Anderson Sterilizers Inc. Samples were wrapped in the required size covers and placed inside a sterilization bag. In this system 100% Ethylene oxide gas sealed cartridge, an RH stabilizer chip (Humidichip) and an EO Gas Dosimeter (AN1087) were kept along with the prepared sample. Then the sterilization bag was

sealed, and the gas was released inside the sterilization bag by turning the knob in the cartridge. The samples were then sterilized for 16 hours at 50ºC in a standard operating mode.

Gas Chromatography-Mass Spectrometry (GC – MS) Analysis

Honey compound analysis was carried out in Thermo GC-Trace Ultra Ver.: 5.0 and Thermo MS DSQ II, with ZB 5- MS Capillary Standard Non-Polar Column. Helium was used as a carrier gas with the flow of 1.0ml/min. Temperature at interface and injector port was 250 ºC and the source was maintained at temperature of 200 ºC. The initial oven temperature was set as 70ºC and raised to 260ºC at 6ºC per minute rate. Injection volume was 1 Micro liter, and the ionization energy was 70 eV. The spectrum was matched with available libraries and the compounds with highest spectral fit were considered. NIST mass spectral library was used to confirm the identities, their name, molecular weight, and structure.

Minimum Inhibitory Concentration (MIC) Test

Broth dilution method was used to determine the MIC of Manuka honey. The experiment was carried out in 48- well micro titer sterile plates. Using cation-adjusted Mueller Hinton II broth (CAHMB), 25% stock solutions of honey were prepared. The solution is further diluted and obtained concentrations of 22.5%, 20%, 17.5%, 15%, 12.5%, 10%, 8.5%, 7%, 5.5% and 4%. Bacterial colonies were isolated from an overnight culture on blood agar. Turbidity of 0.5McFarland (approximately $1x10^8$ CFU/ml) was achieved by inoculating the bacterial colonies into peptone water. The final concentration of the inoculums was reached to $5x10^5$ CFU/ml by further dilution of bacterial suspension. Test well was made to the volume of 1 ml (0.5ml bacterial inoculums and 0.5 ml diluted honey) and incubated for 18hours at 35ºC. Three test wells were maintained for each assay; without honey and inoculums (only broth); without honey (broth and inoculums) and without inoculums (broth and honey).

For each bacterium, the test wells were observed for lack of turbidity against the negative control by visual inspection and optical density method. Thus, the minimum concentration that prevents 95% growth of each bacterium (MIC_{95}) was noted down. After visual reading, spectrophotometer was used to measure the optical density (OD) at 620nm, and the growth inhibition was determined using the formula given in Equation (1).

$$Inhibition\ \% = \frac{\left[1-(Test\ well\ OD - negative\ control\ well\ OD)\right]}{(Viability\ control\ well\ OD - Broth\ only\ well\ OD)} \times 100 \quad (1)$$

Experiment was done in triplicate to find out the average zone of inhibition[10].

***In vitro* Antibacterial Activity Assessment**

Antibacterial activity of the samples was assessed by agar diffusion qualitative method as per SN 195 920 standard. The test organisms used were *S. aureus* and *E. coli*.

Ethical Committee Clearance for *in vivo* Wound Healing Studies

All the *in vivo* wound healing studies were performed in compliance with animal ethical committee. The proposal was approved by Institutional Animal Ethics Committee, PSG Institute of Medical Sciences & Research (232/2014/IAEC), Coimbatore, India.

Experimental Animals and Study Design

Eight female Wister rats weighing 200-250 grams were taken for the excision wound experimental study and divided into four groups of two animals each (n=2). The rats were divided into groups randomly and used for the study as given in the experimental protocol. Throughout the study period, the rats were housed in individual cages and were provided with a source of water and a standard commercial rat diet. The degree of wound healing was studied by tracing the raw wound area subsequently. The size reduction and percentage of wound closure was recorded.

Anesthesia and Surgical Protocol - Skin Preparation

The dorsum of the rats was shaved and cleaned with povidine iodine and 70% alcohol. Then the rats were anesthetized with anesthetic ether and a sterile towel was used to isolate the operation site.

Excision Wound Model

Anaesthetized animals were placed in the operation table in its natural position. A square wound of about 1.0cmx 1.0cm x 0.2cm (depth) was made on depilated ethanol-sterilized dorsal thoracic region of rat[11]. Samples were topically applied as per the protocol till the epithelialization was complete.

Table 1: Zone of inhibition of the samples against *S.aureus* and *E.coli*

Film Samples	Zone of Inhibition(mm)	
	S.aureus	*E.coli*
Chitosan	22	24
Chitosan-honey	28	24

Table 2: Percentage wound contraction of excision wound rat models

Sample	Wound contraction %		
	Day 6	**Day 12**	**Day 18**
Control	18.7	51	64.0
Std. Ointment	25.8	68.8	85.5
Sample C	22.0	56.0	77.8
Sample CH	27.2	74.6	95.0

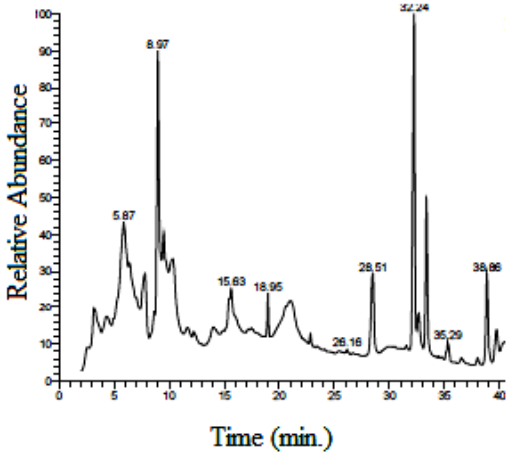

Figure 1: Chromatogram of manuka (*Leptospermum scoparium*) honey

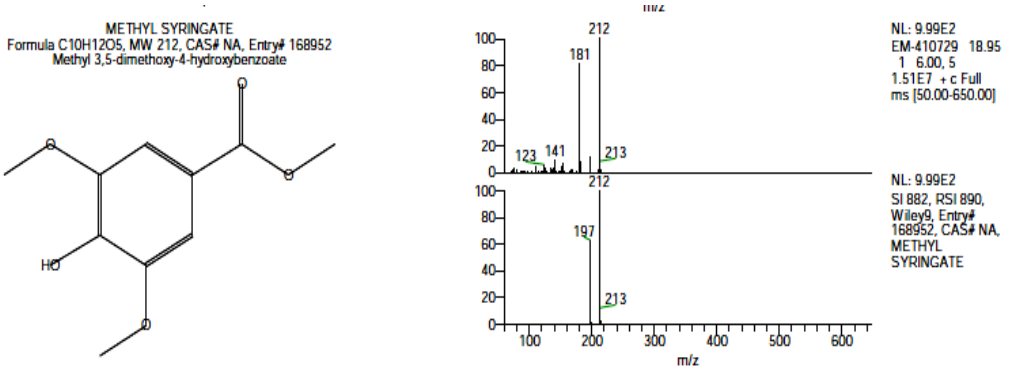

Figure 2: Molecular structure and fragmentation portion of Methyl Syringate

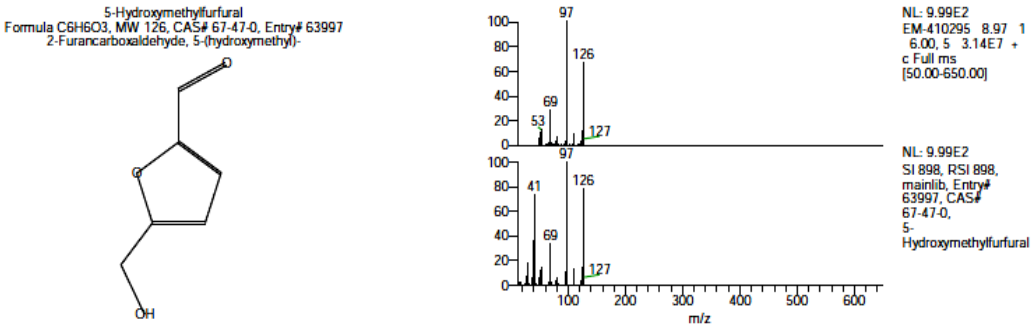

Figure 3: Molecular structure and fragmentation portion of 5- Hydorxymethylfurfural

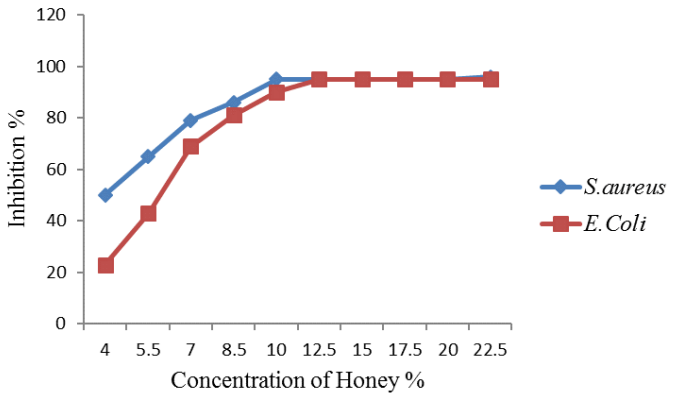

Figure 4: Bacterial Inhibition % at different honey concentrations

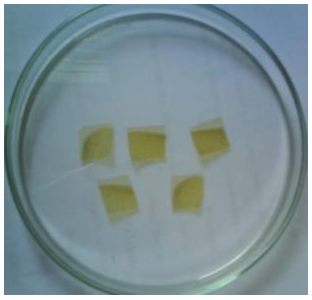

Figure 5: Photographic image of chitosan-honey films

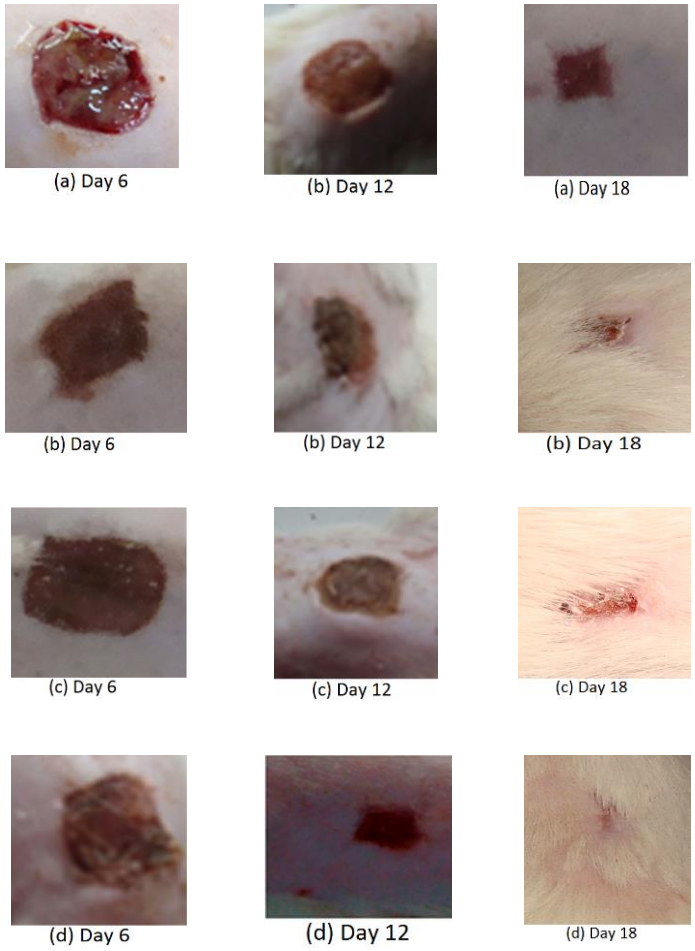

Figure 6: Visual appearance of the excision wound during the animal study (a) control (b) standard ointment (c) chitosan film (d) chitosan-honey film

RESULTS AND DISCUSSIONS

Component Analysis of honey by Gas Chromatography-Mass Spectrometry (GC-MS)

Manuka honey sample with UMF 18+ was taken for this study. It was reported that manuka honey with UMF 10+ is used for wound care. Honey sample stored in dark glass container at 5°C to prevent photo degradation was used for the experimental work. Methanol extract of honey was injected into gas chromatography, and the result was analyzed using mass spectrometry to trace the compounds.

Identification of Compounds

Mass spectrum of GC-MS was interpreted using the database of NIST4 library. The component spectrum was compared to the known components pre-stored in the inbuilt library.

Chromatogram Analysis

The chromatogram of manuka honey exhibits a wide range of eluted portions, which depicts that honey has more diverse composition. The eluted compounds were classified as phenolic derivatives and flavonoids. Figure 1 shows the chromatography of manuka honey, which was analyzed for 40mins. Notable peaks in various places indicate the specificity of compounds that are available in manuka honey.

Methyl syringate is described as the effective index of antibacterial activity in manuka honey. The fragmentation portion of methyl syringate is given in Figure 2. The retention time of methyl syringate is 18.95 minutes, and peak area is 1.82%.

Figure 3 shows the fragmentation portion of 5-Hydroxymethylfurfural (HMF). HMF peak is eluted at the retention time of 8.97 minutes, and the area is 13.93%. HMF is an organic compound derived from dehydration of sugars. 5-hydroxymethylfurfural (HMF) is responsible for UMF activity in manuka honey.

The principal compound responsible for antimicrobial activity in manuka honey is methylglyoxal (MGO), which is also termed as manuka enriched component, which arises spontaneously from dihydroxyacetone, and is available in the nectar of the manuka flowers. This increases with controlled heating or aging. There exists a perfect linear correlation between MGO and antibacterial properties of manuka honey and hence it is labeled as unique bioactivity factor. Measurement of MGO is difficult because it gets converted into quinoxaline before it is measured. MGO compound could not be directly interpreted in our chromatography elucidation but by obtaining the elution of N-(pentafluoropropionyl)-N-(3,3-diphenylpropyl)-à-methyl at retention time of 32.24 minutes and area of 19.76 %. 3, 4, 5, trimethoxybenzoic acid- esterified maltose molecule, which has antibacterial activity against the gram positive *S.aureus*, was also identified. In the given chromatogram, a very broad class of methoxylated or hydroxylated benzoic acid derivatives attached to maltose or glucose is represented.

Manuka honeys have a significant level of phenolic compounds and methoxylated derivatives. Phenolic compounds are responsible for bioactivity, and they could be a part of free radical scavenging. Also, immune response can be modulated by phenolic compounds, which promotes wound healing, which can subsequently affect their metabolism. 1,2-Unsaturated pyrrolizidine alkaloids contents are of great interest in the honeys intended for the wound care applications. Methyl-4-O-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-3,5-dimethoxybenzoate (MGGD) is a new compound, found in manuka honey, is responsible for myeloperoxidase (MPO) inhibition activity, which is a glucoside of methyl syringate.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration indicates the lowest concentration of the honey sample that resists the test bacteria. In contrast with Minimum Bacteriocidal Concentration (MBC), MIC reflects the bacteriostatic activity. There are wide variations in the published results of MIC value of manuka honey against *S.aureus* and *E.coli*. Hence, this study aims at spotting the MIC value of manuka honey used in this study. As per previous literatures, MIC of Manuka honey was in the range of 4% to 25% for different bacteria. Hence, the concentrations tested were in the range of 4 to 22.5%.

MIC values were assessed both by visual inspection and using a spectrophotometer. In visual method, manuka honey exhibits lower MIC against *S.aureus* than *E.coli.* MIC, when tested against *S.aureus*, shows 12.5%, and *E.coli* shows 17.5%. This indicates the better activity of manuka honey against *S.aureus* in lower concentrations. Figure 4 shows the spectrometric inhibition % of both gram positive and gram negative bacteria in a wide range of honey%. Spectrophotometric readings exhibited the MIC_{95} (at least 95% inhibition), against *S.aureus* is 10% and *E.coli* is 12.5%.

Chitosan-Honey Film Preparation for Wound Contact Layer

Films were prepared by casting technique with and without honey. Chitosan honey films were prepared using chitosan (2%), honey (13%) and glycerol (15%) and prepared film is shown in Figure 5.

***In vitro* Antibacterial Studies**

Chitosan honey films exhibited higher bacterial inhibition against both *S.aureus* and *E.coli* compared to blank chitosan film (Table 1), which can be explained by the inherent antibacterial property of chitosan and honey. The inhibition zone of chitosan-honey films against *S.aureus* and *E.coli* was observed as 28 mm and 24 mm respectively. Chitosan film showed the same activity of 24 mm against *E.coli* and lower activity of 22 mm against *S.aureus*.

From the literature, it is understood that chitosan has intrinsic bacteriostatic property which hinders the growth of bacteria, through different mechanisms. Blank chitosan films exhibited higher inhibition zone against *E.coli* than *S.aureus*. This may be attributed to the significantly higher hydrophilicity of gram negative bacteria than gram positive bacteria, which makes it more sensitive to chitosan thereby causing more morphological changes. But, in contradiction to the above statement, the bacterial inhibition of chitosan honey films is higher in all the cases of *S.aureus* than *E.coli*.. Hence, it is clear that in chitosan-honey films, both chitosan and honey contributes to antibacterial property and the addition of honey increases the zone of inhibition. This also highlights the compatibility of chitosan-honey films. Since manuka honey is regarded as non-peroxide honey, it is proved that many factors are responsible for its antibacterial activity. Presence of methyl syringate, methyl glyoxal, high sugar content and acidic pH contributes the higher level of antibacterial activity in manuka honey. Amino groups in the chitosan forms ammonium salts in the acidic media attributed to higher antibacterial activity of chitosan-honey films.

***In vivo* Wound healing studies**

Group I served as untreated control sample; Group II as positive control treated with standard cipladin ointment; Group III animals were dressed with chitosan film; Group IV was treated with chitosan-honey film. After wound creation, the animals were dressed daily and observed once in 3 days and till 18^{th} day. The percentage of wound contraction in treated and control groups were analyzed in the excision wound sites, and the results are shown in Table 2 and Figure 6.

Wound healing acceleration of chitosan and chitosan-honey samples were compared with control and a standard commercially available cipladin ointment. There was no infection found in any of the test samples throughout the study. The rate of wound contraction was assessed by measuring the unclosed area during 6^{th}, 12^{th} and 18^{th} day. Healing was rapid in CH sample on day 12 and could be able to observe the epithelialization process obviously. During this process, filling of granulation tissue in any cavity, and migration of epithelial cells occurred and on day 18, the wound was covered with a horny layer with almost 95% wound contraction.

Manuka honey dressing, which plays a vital role in healing of the wound at a faster rate, is attributed to moist healing environment, absence of microbes and supply of glycogen to the epithelial cells. On day 18, the wound closure reached 95% in CH sample applied wounds with cell proliferation and organization of collagen. In contrast, there was still about 36% wound area unclosed in control wound and 15% in the standard ointment applied wound. On day 18, the % wound closure reached 78% in case of chitosan film treated wound. The

decrease in healing time of chitosan-honey film could be explained by the wound healing activity of chitosan and honey in the film form. By suppressing the production and propagation of inflammatory cells, stimulating the proinflammatory cytokines and by stimulating the proliferation of fibroblasts and epithelial cells, manuka honey reduces the healing time.

Manuka honey exhibits reduced inflammation attributed to the antioxidant phytochemical components and the high amount of phenolic compounds are responsible for its antioxidant activity. The low pH (3.5-4.5) of manuka honey is assigned to the inhibition of microbial growth on the wound surface, and reduction in protease activity. Fibroblast activity and oxygenation were also increased with increase in cellular activity, cell proliferation and migration. The acidic environment of chitosan honey films leads to the stimulation of growth factors, such as TGF-β, fibroblastic activity and hence stimulated wound healing.

CONCLUSION

GCMS tests of manuka honey sample reveals that significant level of bioactive compounds like methyl syringate, methylglyoxal and 5-Hydroxymethylfurfural (HMF), which are responsible for the antibacterial activity of honey are present in the sample. The presence of more bioactive compounds leads to the application of manuka honey in wound care medical applications. MIC results depict that the MIC_{95} (at least 95% inhibition) of manuka honey against gram positive *S.aureus* is 10%, and gram negative *E.Coli* is 12.5%. The dressing also has a good amount of antibacterial activity against *S.aureus* and *E.coli*, which also arrests microbes transmitting from the outside environment to the wound bed. The results of *in vivo* excision wound healing studies confirmed that on day 18, the wound closure reached 95% in chitosan-honey sample applied wounds with cell proliferation and organization of collagen. In contrast, there was still about 36% wound area unclosed in control wound and 15% in the standard ointment applied wound. This indicates that chitosan- manuka honey film can be effectively used as wound contact layer for wound dressings.

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