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In vivo – Wound healing studies of *Leptospermum scoparium* honey loaded chitosan bioactive wound dressing

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ABSTRACT

The research aimed to develop a bioactive wound dressing using chitosan and *Leptospermum scoparium* honey, which is commonly known as Manuka Honey. The wound dressing was developed using solvent- casting technique with the honey as a drug. The wound healing ability of the developed dressing was evaluated against three different wound models namely excision, incision and burn wound model in terms of wound contraction percentage and histopathological examination. The results of the study revealed that the performance of the developed honey loaded chitosan dressing was comparable with commercial dressing. In the case of excision and incision wound models, complete healing was observed in on 18th day and in the case of burn wound model on 21 st day. The wound contraction percentage of CH dressed excision wound model is noted significantly better ($0.05 > p$; 94%) than the commercial dressing (90%). In incision wound model, the tensile strength of healing skin was noted significantly higher ($0.05 > p$) for CH dressed wounds than the commercial dressing. Similarly, for the burn wound also a significantly improved performance was noted it is noted ($0.05 > p$; 96%) for CH dressing than the commercial dressing (85%). The results of the study indicated that the CH dressing have higher potential for the wound healing application.

1. Introduction

Honey produced from a plant type called manuka (*Leptospermum scoparium*) is a monofloral honey that exhibits broad-spectrum antimicrobial activity against a diverse range of bacterial and yeast pathogens, and is equally effective against multi-drug resistant bacteria [1–3]. The antimicrobial activity of the honey influenced by various factors including its osmolarity, low pH, and the enzymatic production of hydrogen peroxide [4] Further, the researchers mentioned that the antibacterial ability also dependent on the processing and storage conditions, the physiology of the floral species, and bee-related factors such as age or colony health etc [5,6].

The hygroscopic nature of the honey is one of the main factors for the antimicrobial activity of the manuka honey. On the localized application, the honey absorbs the moistures from the skin and wound environment and dehydrates bacteria. As mentioned earlier, the hydrogen peroxide and gluconic acid is produced from the glucose oxidase enzyme when honey is in contact with wound surface. As the pH value of honey is low, the application of the honey on wound restricts the pH level of wound exudates and so reduces the possibilities of bacterial colonization and wound infection. This type of honey's also used in the wound malodor reduction as the malodor is the cause of critical colonization and partial tissue necrosis [7].

Few research works mentioned that the application of manuka honey reduces the quantity of exudates developed from the wound and so the oedema and pain in wounds. The high amount of sugar content of the honey maintains the wound surface moist and since the oedema is mobilized to surrounding tissues [8,9]. Anti-inflammatory effects of manuka honey were reported in clinical trials and animal models [10].

The wound healing mechanism of manuka honey is not yet completely explored. The literature records difference opinions from the researchers. Tonks et al and mentioned the healing mechanism of the honey is due to the Stimulation of the inflammatory response in leukocytes [8,9]. This inflammation triggers cellular events, which give rise to growth factors production, controls angiogenesis and proliferation of fibroblasts and epithelial cells. In 2007, same researcher reported that the 5.8-kDa component present in the honey is responsible for cytokine induction in human monocytes [11].

Abbreviations: PVA, poly vinyl alcohol; M, mole; S, seconds; cm, centimeter; °C, degree celsius; ml, milliliter; CHDressing/Dress, chitosan- manuka honey film dressing; HE, hematoxylin–eosin; MPa, Mega Pascal

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The higher level of pH value in the wound bed or wound exudates is the sole reason for the bacterial colonization and recalcitrant wound healing situations. This high pH level increases the protease activity slows down or prevents the healing by destroying growth factors, protein fibers and fibronectin in the wound matrix. Hence, fibroblasts activation and migration of epithelial cells are affected. Excessive inflammation causes the activity of protease. After application of honey, the lower pH, acidifies the wound bed and increases the amount of oxygen off-loaded from hemoglobin in the capillaries, and accelerates healing process [12]. Molan mentioned that the debriding action of honey removes slough and hence the source of bacteria. This prevents the stimulation of inflammatory response [13].

Chitosan, a natural biopolymer is extracted from the shells of crustaceans and it is termed as 2-amino-2-deoxy-b-D-glucopyranose with the molecular formula $(C_6H_{11}O_4N)_n$. The structure is similar to cellulose, in which the C-2 hydroxyl groups are replaced by acetamido residue. The chitosan's amino group with pKa value of ≈6.5 leads to a protonation in acidic to neutral solution. Hence, chitosan is water soluble and bioadhesive, thereby readily binds to the negatively charged surfaces like mucosal membranes. Thus, the transport of polar drugs across epithelial surfaces is enhanced, and chitosan is termed as biocompatible and biodegradable. Due to its low toxicity and good biocompatibility, chitosan is used in wide range of pharmaceutical applications [14]. Many researchers reported the wound healing efficiency of chitosan and experimentally proved. In comparison with Omiderm, chitosan-acetic acid and chitosan lactic acid films were used for punch biopsy wounds in rats. It was proved that a significance difference was observed in chitosan film treated wounds in terms of wound closure, scar formation and period of epithelialization [15].

In a study conducted with chitosan and gelatin composite film shown improved tensile strength, water absorption, wound contraction, visual healing and histopathological observation [16]. In another study reported the efficiency of chitosan gelatin film as wound dressing was investigated. With 1%, 2%, 3%, 4% of chitosan used for the study to develop chitosan films, mechanical properties, morphology, antibacterial, allergic or irritation were tested. The wound healing activities on mice mode reveled that chitosan films were very good in bacterial inhibitory efficacy, wound healing without any swelling or allergic reaction to the skin [17]. The chitosan film with minocycline hydrochloride was compared with commercial polyurethane film (Tegaderm) used as a backing in dressing. This was applied to burn wounds in the rat model and observed excellent effects [18]. In another study, chitosan-PVA-alginate film was prepared using casting/solvent evaporation technique. The three layer film exhibited good water vapor transmission rate, excellent light transmittance and fluid drainage ability. It showed effective antimicrobial activity against *S. aureus* and *E. coli* with great advantage of being used as a wound dressing material [19]. A novel wound dressing material was fabricated using chitosan and hyaluronic acid [20]. The composite films showed high transparency and desirable characteristics of wound dressings. In vivo animal studies revealed that chitosan/hyaluronic acid film accelerated wound healing against control dressing. Chitosan and alginates as polyelectrolyte complex membranes were prepared and recommended for highly exuding wounds with bacterial prevention capability [21].

Previous research workers evaluated the performance of chitosan-honey dressing against burn wound using *in-vitro* and *in-vivo* method and produced a positive result for 75% honey-chitosan wound dressing [22]. *In-vitro* antibacterial ability of the chitosan – honey wound dressing was successfully evaluated against the most common bacterial species like *Staphylococcus aureus and Escherichia coli* [23]. It is also reported that Honey/Chitosan nanofiber wound dressing also possessed significant amount of wound healing ability both *in-vitro* and *in-vivo* method [24]. Though, there are few studies represented the wound healing ability of the chitosan hydro gels, films sheets and honey individually, the combined effects were meagerly evaluated. Hence, in this study, the manuka (*Leptospermum scoparium*) honey loaded chitosan bio active wound dressing is prepared and evaluated for their wound healing ability using *In-vivo* wound model. The developed wound dressing was evaluated against the three different wound models namely excision, incision and burn wound model in terms of wound contraction and histopathological examination.

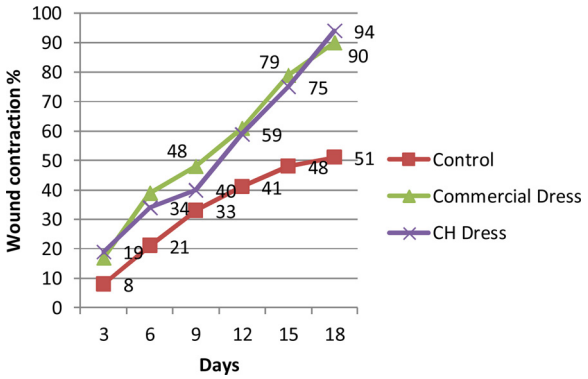

Fig. 1. Excision wound contraction % in rats of control, commercial and CH dress group.

2. Material and methods

2.1. Materials

Glycerol, sodium bicarbonate, sodium chloride and Lactic acid were purchased from HIPURE chem industries, (Chennai, India) and Manuka honey was obtained from MediHoney, Canada, Derma Sciences Inc. All the chemicals used were of analytical grade.

2.2. Methods

2.2.1. Bioactive wound dressing preparation

The films were prepared using casting technique, modification of the method described by Zhong and Xia (2008). Briefly, a chitosan aqueous solution was prepared in distilled water with 25% concentration, that contained lactic acid 1% (w/v) as solvent and stirred for 2 h. Then drop wise addition of 5 ml of 5% sodium bicarbonate was carried out. The solution was stirred using magnetic stirrer for 15 h after addition of glycerol (13% concentration) and honey (15% concentration) based on previous research results [25]. The resultant solution was filtered out and left to stand until the air bubbles get disappeared. Then required quantity of the solution was casted onto the petri dish. The petri dish was maintained in the oven at 40 °C for 24 h. The prepared films were rinsed with 500 ml of 1 M NaOH solution and then washed with distilled water. Then, these transparent, flexible films were stored at 25 ± 1 °C and relative humidity 60–65% in an airtight glass container until further use.

2.2.2. In vivo wound healing studies

Wound healing studies were carried out in Wister rats. The experimental models of excision, incision and burn wounds are discussed in this part.

i) Experimental Animals and Study Design

Thirty five female Wister rats weighing 200–250 grams were used for this study. 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 rats were divided into groups randomly and used for the study as given in the experimental protocol.

- Anesthesia and Surgical Protocol - Skin Preparation

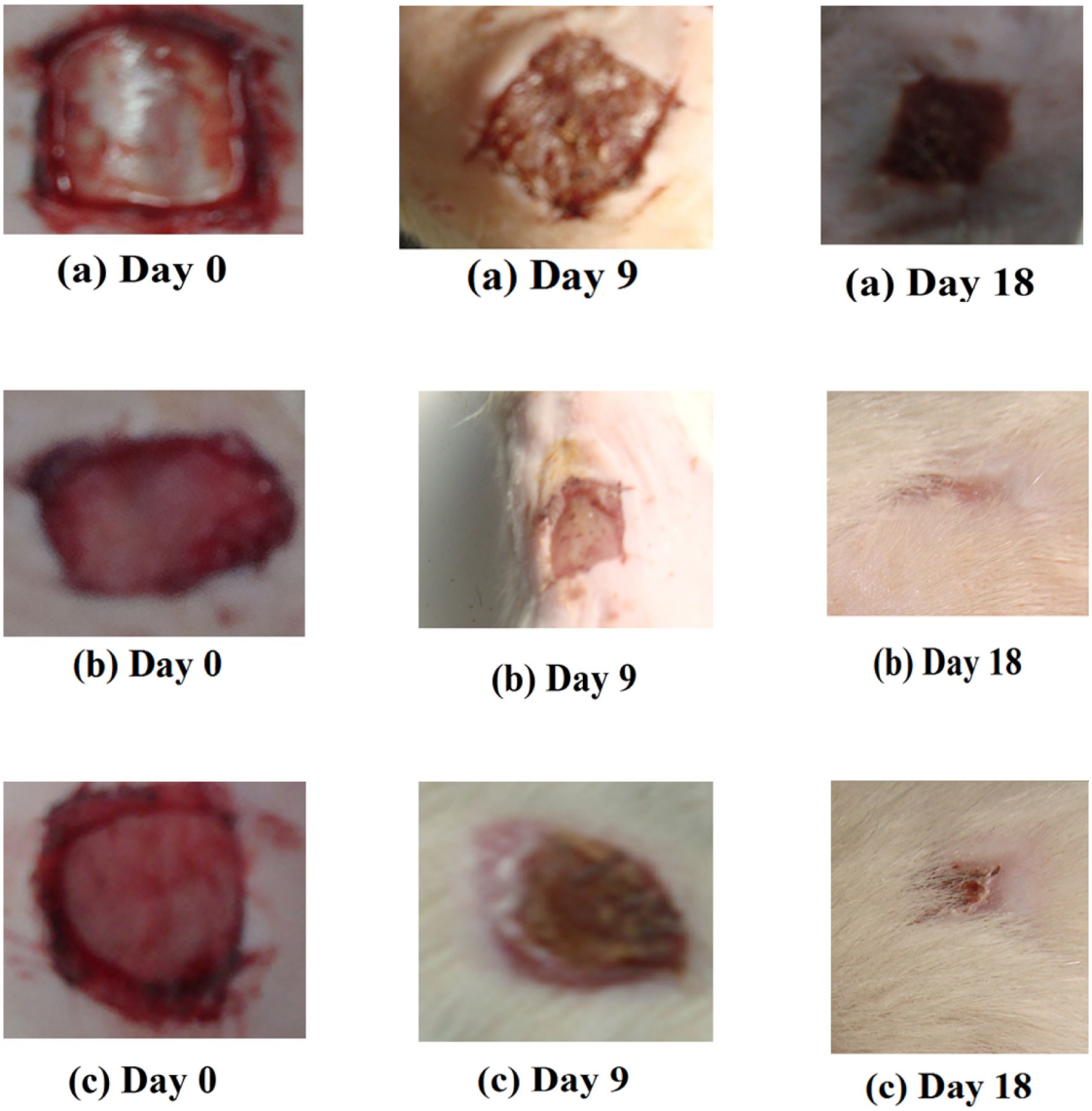

Fig. 2. The photographs showing the typical changes of excision wound contraction of (a) control group (b) commercial dressing (c) CH dress on day 0, 9 and 18.

Table 1
Statistical analysis results of Excision wound contraction between the wound dressing.

ANOVA for Excision wound contraction %						
Source of Variation	SS	df	MS	F	P-value	F crit
Between dressings	980.9333	2	490.4667	21.27838	0.000627	4.45897
Between Days	4920.4	4	1230.1	53.36659	0.000008	3.837853
Error	184.4	8	23.05			
Total	6085.733	14				

For all three types of wound models, 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

Excision wound was created as per Morton and Malone [26]. Anaesthetized animals were placed in the operation table in its natural position. A square wound of about 1.0 cm × 1.0 cm × 0.2 cm (depth) was made on depilated ethanol-sterilized dorsal thoracic region of rats. Samples were topically applied as per the protocol till the epithelialization was complete. The degree of wound healing was studied by tracing the raw wound area subsequently. The size reduction and percentage of wound closure was recorded.

- Incision Wound Model

A longitudinal incision of 2 cm length and 2 mm depth was made through the skin using a surgical knife. No systemic antimicrobials were used throughout the experimental study. Ligature was not used for suturing. The parted skin was brought together and stitched with black silk suture at 0.5 cm interval by a curved needle. Both the edges of the threads were tightened for

good closure of the wound. The wounds were then dressed as per the experimental protocol by considering the wounding day as Day 0. The skin breaking strength was measured with a tensiometer on day 10 [27].

- Burn Wound Model

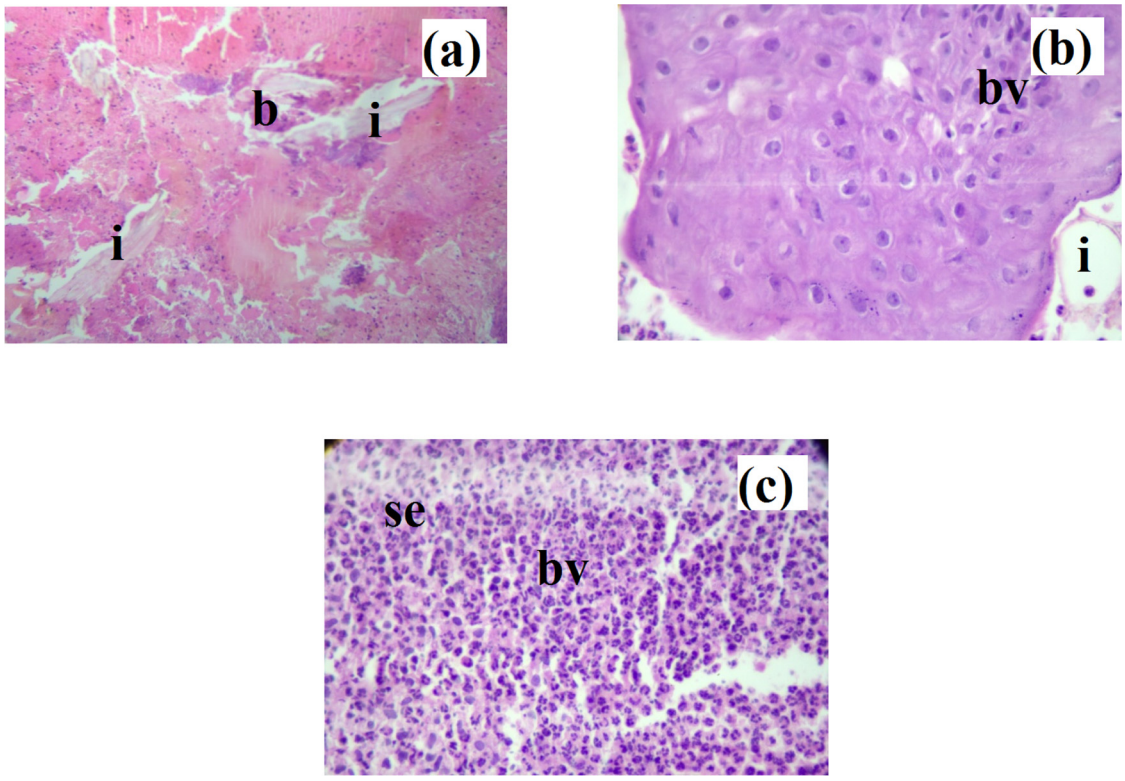

Fig. 3. Photomicrographs of excision wound tissues (a) control (b) commercial (c) CH dress; i-intermediary spaces, b-blood clots, bv-blood vessels, se-squamous epithelium.

Table 2
Tensile strength value of experimental group on rat model.

Experimental Groups	Tensile Strength (MPa)	
	4th day	18th day
Control	0.79	1.31
Commercial	1.21	2.02
CH Dress	1.67	2.27

Table 3
Statistical analysis results of Tensile strength value of Incision wound between the wound dressing.

ANOVA for Tensile strength value of Incision wound						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Wound dressing	0.8611	2	0.43055	38.38484	0.02539	19
Between Days	0.620817	1	0.620817	55.3477	0.017592	18.51282
Error	0.022433	2	0.011217			
Total	1.50435	5				

Partial thickness burn wounds were created in the dorsum region of the rats with the help of a piece of hot metal, which is placed perpendicularly on the rats for 30 s. The size of the wound was maintained as approximately 2 cm diameter.

- Treatments

As per the experimental protocol, the rats were treated with proper medication and change of dressing. During the study period, the rats were maintained in individual cages and fed with a regular diet and water. To avoid the loss of dressings during the movement of the rats, perforated gauze cloths were used to hold the dressings in place.

2.3. Evaluation of wounds

Each rat was observed daily, and the condition of each wound was examined on specific days with proper intervals. Wounds were traced with the help of a transparency sheet, and photographs were taken.

2.3.1. Assessment of degree of healing

Wound contraction was measured by degree of wound healing % by measuring the wound size using trace paper as given in Equation.

$$\%\ \text{of wound contraction} = \frac{(\textit{Wound area day } 0 \ - \ \textit{wound area day } (n))}{\textit{Wound area day } 0} \times 100$$

Epithelialization point was taken when the eschar fell off from the wound surface without any residual raw wound.

2.3.2. Histopathological examination

Specimen skin of 0.5 cm × 0.5 cm was taken for the histopathology test from the wound surface during the completion of healing process. The tissues were preserved in 10% phosphate-buffered formalin. The sections were then stained with HE reagent and examined under light microscope.

2.3.3. Statistical analysis

The Statistical difference between the control, commercial and developed wound dressing was analysed using ANOVA in Microsoft Excel platform.

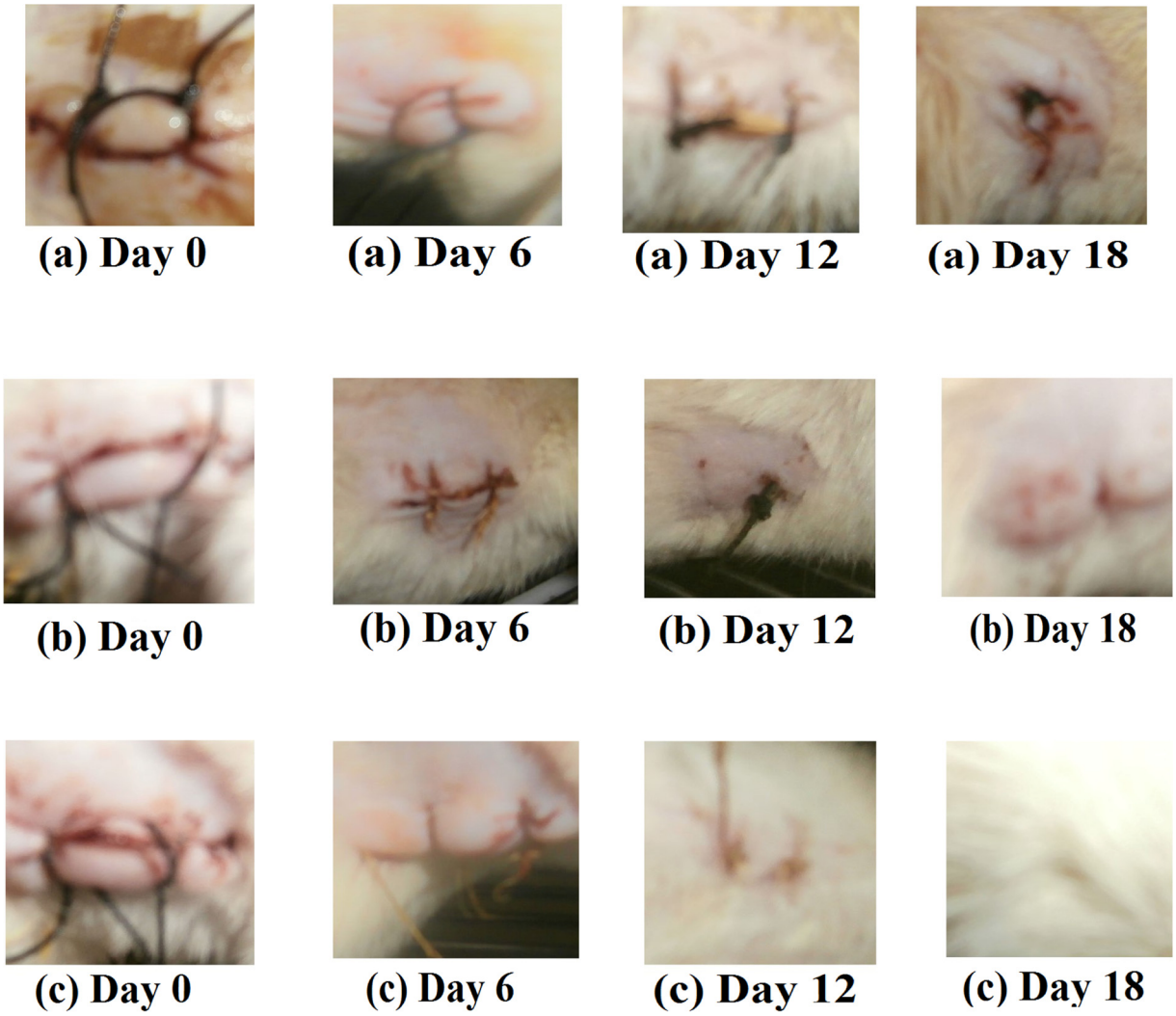

Fig. 4. The photographs showing the typical changes of incision wound contraction of (a) control group (b) commercial dressing (c) CH dress on Day 0, 6, 12 and 18.

Table 4
Wound contraction % of burn wounds.

Experimental Groups	Wound Contraction %		
	Day 11	Day 18	Day 21
Control	39	43	58
Commercial Dressing	62	79	85
CH Dress	74	87	96

Table 5
Statistical analysis results of wound contraction % of Incision wound between the wound dressing.

ANOVA for burn wound model contraction %						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Dressings	2449.556	2	1224.778	105.4833	0.000346	6.944272
Between days	683.5556	2	341.7778	29.43541	0.004048	6.944272
Error	46.44444	4	11.61111			
Total	3179.556	8				

3. Results and discussions

3.1. In vivo wound healing studies

The developed CH dressing's was evaluated for their wound healing activity with commercial dressings in excision, incision and burn wound models. The percentage of wound contraction was assessed by measuring the unclosed area at different point of time.

3.2. Excision wound model

Nine female Wister rats were taken for the excision wound experimental study and divided into three groups of three animals each (n = 3). Group I served as untreated control sample; Group II animals were dressed with selected commercial dressing; Group III was treated with CH dressing. After wound creation, the animals were dressed daily and observed once in 3 days and till 18th day. Healing was visible in both commercial dressing and CH dress treated wound groups. The inflammation caused wound aggravation in control group and hence, the healing was delayed. Hence, there was a significant difference in wound size reduction in control and dressing applied groups. Fig. 1 shows the wound contraction % of three groups of animals on day 0, 3, 6, 9, 12, 15 and 18. The photographs of progress of wound contraction and closure were taken on day 0, 9 and 18 and given in the Fig. 2. The results were evaluated using statistical tool and it is found that, the

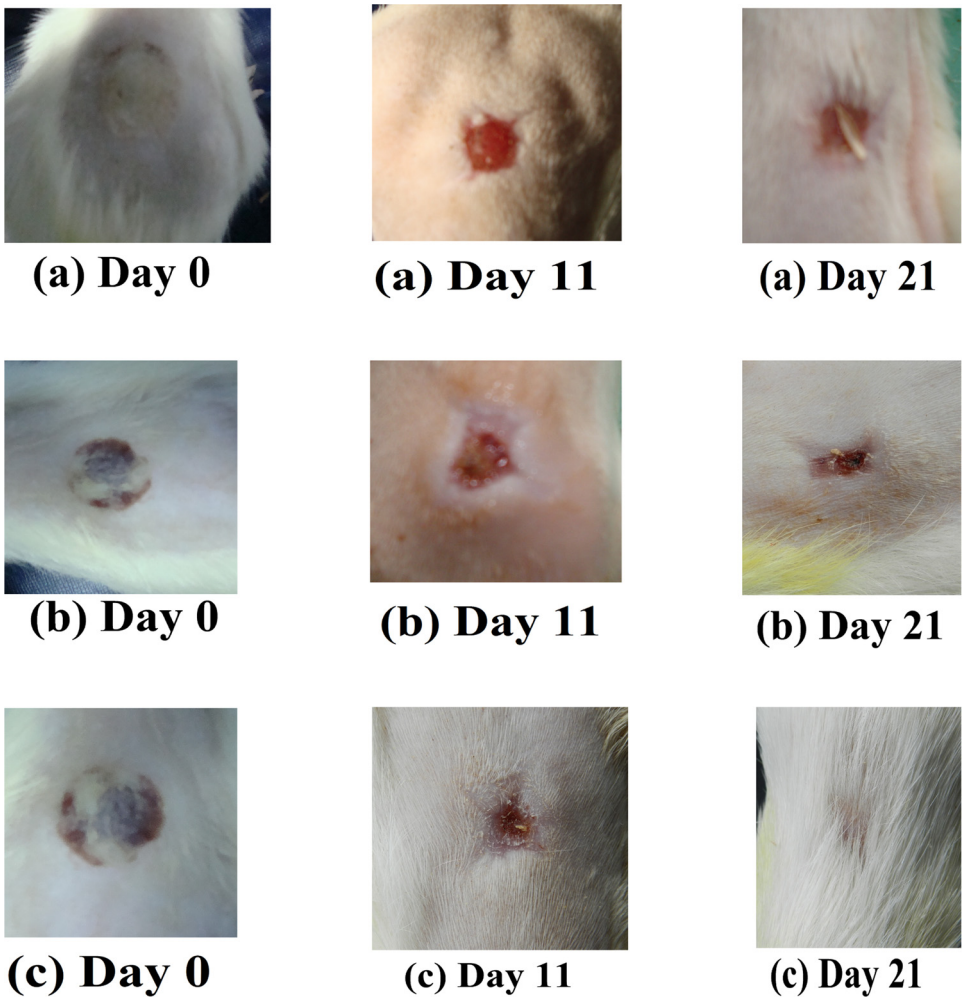

Fig. 5. The photographs showing the typical changes of burn wound contraction of (a) control group (b) commercial dressing (c) CH dress on day 0, 11 and 21.

excision wound closing percentage was significantly different ($0.05 > p$) among the control, commercial and developed dressing as provided in Table 1. The contraction percentage of the CH dressing were significantly higher contraction % at 18^{th} day (94%) than the control (51%) and commercial (90%) groups.

The wound exudates were absorbed by both commercial and CH dressing uniformly. Both the dressings were removed from the surface easily without damaging the newly generated tissues. The healing rate in the negative control group rats was very slow due to inflammation. On day 18, the degree of wound healing reached only 51%. In contrast, epithilialization was observed on day 15 in both commercial and CH dress treated wound. On day 18, commercial dressing applied wound showed 90% wound contraction, whereas chitosan-honey dressed wound showed 94% wound contraction. CH dress treated wounds exhibited higher degree of healing.

Wound healing activity of manuka honey could be explained through its higher antibacterial efficacy and wound healing property. Antibacterial effect of honey is attributed to its osmotic effect due to high sugar content, non-peroxide factors and phenolic content. It also stimulates the mutagenesis of human-T and B-lymphocytes, thereby exhibits immune-stimulant activity [28]. In case of occlusive dressings like hydrogel, films and hydrocolloids, they keep the wound bed moist. Thus the wound fluid is always in contact with the wound. It is experimented that certain cytokines (growth factors) present in the wound exudates modulate connective tissue formation, thus epidermal migration [29,30]. Also peptides such as interleukin-1, epidermal growth factor, transforming growth factor-beta and platelet-derived growth factor are expected to be at the wound site. Acute wounds occluded with commercial have the chemical and immunoglobulin fluid composition, which is same as that of serum. Hence, better healing is claimed in case of commercial dressings [31].

3.2.1. *Histopathology studies of excision wound tissues*

Photomicrograph of control sample was shown in Fig. 3. a. The sections exhibited hemorrhage and necrosis. It also showed blood clot with areas of inflammatory exudates. The fragments of squamous epithelium with granulation tissue composed of neutrophilic abscess. This was attributed to incomplete re-epithelialization. Fig. 3.b exhibited the histological observations of commercial dressing treated wound tissues, which showed high power of squamous epithelium. The sections were also noted for fragments of squamous epithelium with granulation tissue composed of neutrophils and monomuclear cells with plenty of proliferating blood vessels. Foci showed collection of epitheloid cells

The CH dress treated sample showed fragments of stratified squamous epithelium with granulation tissue, which is a new connective tissue with tiny blood vessels that forms on the surface of wound during healing process with foci of calcification and brown structures. Complete epithelial regeneration was observed from tissues (Fig. 3.c). Histopathological studies confirms the better healing of CH dress treated excision wound compared to control and commercial dressing treated wounds

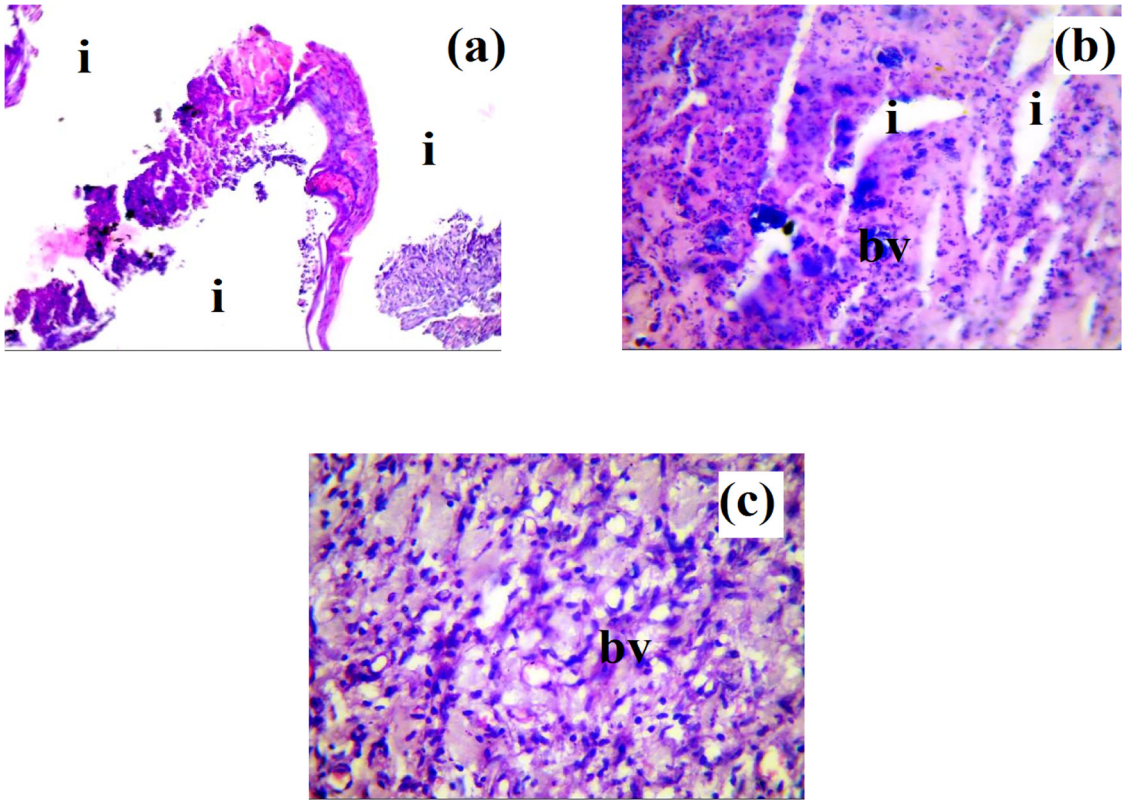

Fig. 6. Photomicrographs of burn wound tissues (a) control (b) commercial (c) CH dress i-intermediary spaces, bv- tiny blood vessels.

3.3. Wound healing activity of incision wound model

Nine female Wister rats were taken for the incision wound experimental study and used as mentioned in the previous section. After incision wound creation and suturing, the animals were dressed daily and observed once in 4 days and till 18th day. Wound tissues were collected on day 4 and day 18, to analyze the tensile strength. Table 2 shows the breaking strength values of incision wound rat model. For all the three dressing treated wounds, tensile strength increased significantly from day 4 to day 18. On day 18, the commercial and CH dress sample shows higher breaking strength of 2.02 MPa and 2.27 MPa respectively. This may be due to the formation of epithelial cells in the wound sites of commercial and CH dress treated wounds. The data were analysed using ANOVA and the results were presented in Table 3. From the results it can be noted that the tensile strength of the CH dressed Incision wound model is significantly higher ($0.05 > p$) than the commercial dressed wound and control wound.

Fig. 4 shows the photographs of incision wounds observed on various days. The images show that the wound healing rate of control group was lower compared to commercial and CH dress treated groups. There existed a notable difference in healing of commercial and CH dress treated wounds. On day 18, CH dress wound healed at a faster rate with almost maximum closure of wounds than commercial dressing group, where 20% of area of wound still remains unclosed. This could be attributed to the effect of chitosan and manuka honey in healing of incision wounds due to their accelerated wound healing properties

3.4. Wound healing activity of burn wound model

Nine female Wister rats were taken for the burn wound study and divided into three groups of three animals each ($n = 3$). Group I served as untreated control sample; Group II animals were dressed with selected commercial dressing; Group III was treated with CH dressing. After burn wound creation, the animals were dressed daily and observed till 21st day. Wound contraction % of burn wounds are shown in Table 4. It is observed that the CH Dress treated burns healed at a highest rate. On day 6, there was a notable difference between wound contraction % of commercial dressing and CH dress treated wounds. The healing in control groups was very slow. On day 11, wound contraction of CH dress increased to 74%, 12% higher than commercial and 35% higher than control group. On day 18, the wound contraction % of CH dress and commercial dressing was 87% and 79% respectively. On the day 21, the wound contraction % was noted as 85% and 96% in commercial dressing treated and CH dress treated wounds respectively. The statistical results were in support with the above findings that the CH dressed wounds have significantly ($0.05 > p$) better contraction that the commercial and control wound as presented in Table 5.

Photographic images captured on day 0, 11 and 21 during the healing of burn wound are shown in Fig. 5. On day 21, almost complete healing was observed in CH dress treated wounds with intact epidermis. This confirmed that chitosan honey films greatly accelerated the wound healing percentage. Comparatively, the healing rate was lower in case of commercial dressing treated wounds and very slow healing was observed in control groups.

The treatment of burns involves two major considerations, prevention of infection and promotion of epithelial cells proliferation. Hence, an ideal wound dressing should have a balance between cytotoxicity, moist environment and antibacterial efficacy to enhance epithelialization. According to these desirable properties, chitosan-honey films are expected to have better wound healing effect. Chitosan possesses favorable properties for dermal regeneration. It is also responsible for stimulating type IV collagen synthesis, giant cell migration, cytokine production and fibroblast activation [32]. Due to higher hydrophilicity, chitosan and honey possesses production of extracellular matrices. Fibroblast proliferation and synthesis of hyaluronic acid are attributed to the depolymerisation of chitosan into N-acetyl-B-D-glucosamine [33]. The acceleration of wound healing by honey in hydrogel matrix was well explained by Norimah [34]. Honey contributes in rapid

debridement of wound, rapid clearance of infections, minimization of scarring, tissue granulation, stimulation of angiogenesis and epidermal growth [35].

3.4.1. Histopathology studies of burn wound

Histological features of burn wound control group are illustrated in Fig. 6a. Histopathological analysis of the tissue sections taken from the burn wounds after 20 days showed an incomplete arrangement of flattened epithelial cells covered with neutrophilic abscess and necrotic debris. The wound surface was covered with exudates. Presence of large intermediary spaces confirms there is no healing in the burn wounds after 20 days. The wound treated with commercial dressings exhibited the neutrophilic infiltration and the accumulation of proteinaceous fluidy material in the dermis region and the thin blood vessels which confirms the wound healing. Eventhough the healing was observed, presence of the foci of necrotic debris and intermediary spaces in meagre amount confirms the incomplete healing of wounds treated with commercial dressing. (Fig. 6b).

The CH dress treated wound showed the dense proliferation of blood vessels and regenerated epithelium. Formation of dense fibrocollagenous tissue and the deposition of refractive proteinaceous substances confirms the healing was better compared to the untreated wounds (control) and the wounds treated with commercial dressings (Fig. 6c).

4. Conclusions

In this research, *Leptospermum scoparium* Honey loaded Chitosan Bioactive wound dressing was developed and its wound healing ability was evaluated by *in -vivo* wound healing studies. The wound healing efficacy was analysed using wound contraction percentage and histopathology results. The results of the analysis revealed that the developed bioactive dressing having a better wound healing efficacy in all three types of wound models statistically ($0.05 > p$). In excision and incision wound models, complete healing was observed in on 18th day and in burn wound model on 21st day. In both excision and incision wound models, the results of commercial and CH dressing was comparable. In the case of burn wound model, CH dressing exhibited a better wound healing results than commercial dress statistically ($0.05 > p$). Thus it can be concluded that the prepared CH dressing can be used as a potential wound dressing material for excision, incision and burn wounds with light to moderately exudation and it is a potential dressing for burn wounds, due to the effect of manuka honey.

Ethical statement

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.

Financial disclosure

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Conflict of interest

None.

References

[1] B.G. Visavadia, J. Honeysett, M.H. Danford, Manuka honey dressing: an effective treatment for chronic wound infections, Br. J. Oral Maxillofacial Surg. 46 (2008) 55–56.
[2] S. Blair, N. Cokcetin, E. Harry, D. Carter, The unusual antibacterial activity of medical-grade *Leptospermum* honey: antibacterial spectrum, resistance and transcriptome analysis, Eur. J. Clin. Microbiol. Infect. Dis. 28 (2009) 1199–1208.
[3] R. Cooper, L. Jenkins, A. Henriques, R. Duggan, N. Burton, Absence of bacterial resistance to medical-grade manuka honey, Eur. J. Clin. Microbiol. Infect. Dis. 29 (10) (2010) 1237–1241.
[4] P. Molan, The antibacterial activity of honey. The nature of the antibacterial activity, Bee World 73 (1) (1992) 5–28.
[5] N.N. Cokcetin, M. Pappalardo, L.T. Campbell, P. Brooks, D.A. Carter, S.E. Blair, E.J. Harry, The antibacterial activity of australian *Leptospermum* honey correlates with methylglyoxal levels, PLoS One 11 (12) (2016) e0167780, , https://doi.org/10.1371/journal.pone.0167780.
[6] A. Janmaat, M. Winston, R. Ydenberg, Condition-dependent response to changes in pollen stores by honey bee (*Apis mellifera*) colonies with different parasitic loads, Behav. Ecol. Sociobiol. 47 (3) (2000) 171–179.
[7] J. Chambers, Topical manuka honey for MRSA-contaminated skin ulcers, J. Palliat. Med. 20 (2006) 557–563.
[8] A. Tonks, R.A. Cooper, A.J. Price, P.C. Molan, K.P. Jones, Stimulation of TNF-alpha release in monocytes by honey, Cytokine 14 (4) (2001) 240–242.
[9] A.J. Tonks, R.A. Cooper, K.P. Jones, S. Blair, J. Parton, A. Tonks, Honey stimulates inflammatory cytokine production from monocytes, Cytokine 21 (2003) 242–247.
[10] S. Gupta, H. Singh, A. Varshney, P. Prakash, Therapeutic efficacy of honey in infected wounds in buffaloes, Indian J. Anim. Sci. 62 (1992) 521–523.
[11] A.J. Tonks, E. Dudley, N.G. Porter, J. Parton, J. Brazier, E.L. Smith, A 5.8-kDa component of manuka honey stimulates immune cells via TLR4, J. Leukoc. Biol. 82 (2007) 1147–1155.
[12] R.A. Cooper, P.C. Molan, L. Krishnamoorthy, K.G. Harding, Manuka honey used to heal a recalcitrant surgical wound, Eur. J. Clin. Microbiol. Infect. Dis. 20 (10) (2001) 758–759.
[13] P.C. Molan, Re-introducing honey in the management of wounds and ulcers - theory and practice, Ostomy Wound Manag. 48 (2002) 28–40.
[14] H. Nagahama, T. Kashiki, N. New, R. Jayakumar, T. Furuike, H. Tamura, Preparation of biodegradable chitin/gelatin membranes with GlcNAc for tissue engineering applications, Carbohydr. Polym. 73 (2008) 456–463.
[15] A.T. Khan, K.K. Peh, A preliminary investigation of chitosan film as dressing for punch biopsy wounds in rats, J. Pharma. Sci. 6 (2003) 20–26.
[16] T.V.L. Hima Bindu, M. Vidyavathi, K. Kavitha, T.P. Sastry, R.V. Suresh Kumar, Preparation and evaluation of chitosan-gelatin composite films for wound healing activity, Trends Biomater. Artif. Organs 24 (3) (2010) 123–130.
[17] T.S. Moe, T.A. Khaing, T.Z. Han, H.M. Mon, Effects of chitosan films on wound healing and evaluation of their properties, The 3rd GMSARN International Conference (2008).
[18] S. Aoyagi, H. Onishi, Y. Machida, Novel chitosan wound dressing loaded with minocycline for the treatment of severe burn wounds, Int. J. Pharm. 330 (2007) 138–145.
[19] H.N. Pei, X.G. Chen, Y. Li, H.Y. Zhou, Characterization and ornidazole release in vitro of a novel composite film prepared with chitosan/poly(vinyl alcohol)/alginate, J. Biomed. Mater. Res. 85A (2008) 566–572.
[20] H. Xu, L. Ma, H. Shi, C. Gao, C. Han, Chitosan-hyaluronic acid hybrid film as a novel wound dressing: in vitro and in vivo studies, Polym. Adv. Technol. 18 (2007) 869–875.
[21] A.P. Rodrigues, E.M.S. Sanchez, A.C. daCosta, A.M. Moraes, The influence of preparation conditions on the characteristics of chitosan-alginate dressings for skin lesions, J. Appl. Polym. Sci. 109 (2008) 2703–2710.
[22] Reham F. El-Kased, Reham I. Amer, Dalia Attia, M.M. Elmazar, Honey-based hydrogel: in vitro and comparative in vivo evaluation for burn wound healing, Sci. Rep. 7 (2017) 9692.
[23] L. Sasikala, Bharathidurai, R. Rathinamoorthy, Manuka honey loaded chitosan hydrogel films for wound dressing applications, Int. J. Pharmtech. Res. 5 (4) (2013) 1774–1785.
[24] W.A. Sarhan, H.M. Azzazy, I.M. El-Sherbiny, Honey/Chitosan nanofiber wound dressing enriched with Allium sativum and Cleome droserifolia: enhanced antimicrobial and wound healing activity, ACS Appl. Mater. Interfaces 8 (10) (2016) 6379–6390.
[25] L. Sasikala, R. Rathinamoorthy, Bhaarathi Dhurai, Optimization of process conditions for chitosan-manuka honey film as wound contact layer for wound dressings, Wound Med. 23 (2018) 11–21.
[26] J.J. Morton, M.H. Malone, Evaluation of vulnery activity by an open wound procedure in rats, Arch. Int. Pharmacodyn. Ther. 196 (1972) 117–126.
[27] M. Singh, R. Govindarajan, V. Nath, Antimicrobial, wound healing and antioxidant activity of Plagiochasma appendiculatum Lehm. Et Lind, J. Ethnopharmacol. 107 (1) (2006) 67–72.
[28] Khaled E. El-Nagar, Nashwa M. Nagy, Mohamed A. Elgamal, Medical dressing treated with Honey/Chitosan microencapsules, Int. J. Chem. 4 (2) (2012) 79–89.
[29] I.A. Mckay, I.M. Leigh, Epidermal cytokines and their roles in cutaneous wound healing, Br. J. Dermatol. Syph. 124 (1991) 513–518.
[30] D.J. Whitby, The biology of wound healing, Surgery 1 (1995) 25–26.
[31] I.A. Buchan, J.K. Andrews, S.M. Lang, Laboratory investigation of the composition and properties of pigskin wound exudate under Op-Site, Burns 8 (1981) 39–46.
[32] R.A.A. Muzzarelli, Chitin and chitosans for the repair of wounded skin, nerve, cartilage and bone, Carbohyd. Polym. 76 (2009) 167–182.
[33] R. Jayakumar, M. Prabaharan, P.T. Sudheesh Kumar, S.V. Nair, T. Furuike, H. Tamura, Mr.Anthony Laskovski (Ed.), Novel Chitin and Chitosan Materials in Wound Dressing, Biomedical Engineering', Trends in Materials Science, InTech., 2011ISBN: 978-953-307-513-6.
[34] Norimah Yusofa, A.H. Ainul Hafizaa, Rozaini M. Zohdia, Md Zuki A. Bakarb, Development of honey hydrogel dressing for enhanced wound healing, Radiat. Phys. Chem. 76 (2007) 1767–1770.
[35] Claudia Basualdo, Veronica Sgroy, S. Finola Monica, Juan M. Marioli, Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds, Vet. Microbiol. 124 (2007) 375–381.