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Fabrication of pomegranate/honey nanofibers for use as antibacterial wound dressings

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ABSTRACT

Honey, pomegranate peel extract and bee venom, were used in combination with polyvinyl alcohol to develop a novel nanofibrous wound dressing. Methanolic pomegranate peel extract was prepared and mixed with either Manuka honey or lyophilized multiflora honey powder together with bee venom. The formulas were tested for their antibacterial activity, cytotoxicity, and wound healing activity in an excisional wound rat model. Scanning electron microscopy showed that lyophilized honey fibers had smaller and more uniform diameter than Manuka honey fibers. Moderate swelling and higher weight loss capacities were detected when compared to polyvinyl alcohol mats. Antibacterial tests showed significant antibacterial activity against *S. aureus* and *E. coli* compared to negative controls ($P < 0.0001$). No cytotoxicity was observed. *In vivo* wound healing study showed that all treatment groups enhanced wound healing as shown by increased wound closure percentages compared to negative control groups at days 3,5 and 10 ($P < 0.0001$), and histological examination. In comparison to treatment groups, Medihoney® calcium alginate dressing significantly enhanced healing compared to negative controls at days 3 and 5. However, healing was delayed afterwards. These results indicate that Manuka honey/Pomegranate/Bee Venom nanofibers are promising for wound healing.

1. Introduction

Wounds are considered one of the critical public health problems in the world. In the United States, undertreated wounds and chronic wounds affected 6.5 million patients in 2009, with an annual cost in excess of 25 billion USD [1]. The global market of wound-care products is expected to reach nearly 16,300 million USD by 2023. Therefore, new technologies are essential to address this problem.

In the time of the serious increase of antibiotic resistance, natural products might become the last resort in order to deal with wounds.

Nanofibrous scaffolds are suggested to have advantageous properties over conventional dressings such as large surface area to volume ratio, high porosity and very small pore size. These properties led to higher exudate absorption compared to other polymer films, better wound permeation and prevention from further infection [2].

The use of honey in wound healing applications has been adopted owing to its remarkable antimicrobial properties. Honey consists of water (20 %), fructose (40 %), glucose (30 %), sucrose (5 %), and other substances (minerals, vitamins, amino acids, and enzymes) [3]. Honey was shown to have, antimicrobial, and anti-inflammatory properties. Moreover, honey is acidic therefore it is able to provide fibroblasts with optimal environment for their activity, making it difficult for bacterial survival [4,5]. Honey was shown to be superior to amniotic membrane dressing, and to silver sulfadiazine in treating partial thickness burns, and to ethoxy-diamino-acridine plus nitrofurazone dressing in pressure ulcer patients [6].

Poly vinyl alcohol (PVA) being a biocompatible, non-toxic, biodegradable polymer with good mechanical properties, has been proposed for various biomedical applications [7]. The combination of honey and PVA seems promising [8], in comparison to Aquacel Ag ® dressings,

Abbreviations: 3D, three dimensional; BV, bee venom; DMSO, dimethyl sulfoxide; *E. coli*, *Escherichia coli*; ECM, extracellular matrix; EGF, epidermal growth factor; GH, glutaraldehyde; H&E, hematoxylin and eosin; IL, interleukin; LH, lyophilized multiflora honey; MGO, methyl glyoxal; MH, Manuka honey; MT, Masson Trichrome; MTT, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide); MW, molecular weight; PBS, phosphate buffer solution; PCL, poly(ε-caprolactone); PPP, pomegranate peel powder extract; PVA, polyvinyl alcohol; PVAc, polyvinyl acetate; RPMI, Roswell Park Memorial Institute medium; *S. aureus*, *Staphylococcus aureus*; SEM, scanning electron microscope; VAc, vinyl acetate; Wt %, weight percent

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honey/PVA based scaffolds had similar wound closure rates while having better biocompatibility [9].

Pomegranate juice together with its peel contain remarkable amounts of polyphenolic compounds such as ellagic acid, ellagic tannins, flavanols, anthocyanins, catechin, procyanidins and gallic acid. Pomegranate peel constitutes 50 % of the pomegranate fruit weight. The peel was shown to have higher amounts of polyphenolic compounds than pomegranate juice and thereby possessing even stronger biological activity with promising wound healing potential [10]. Despite this, the peel is often discarded as waste [11]. Under European regulations, pomegranate peel waste should not be disposed neither on land nor in landfills because it holds a significant risk to watercourses. Therefore, pomegranate peel waste disposal and /or use is turning into a major industrial and scientific area [12]. PPP has shown promising results when used in wound healing as suggested by numerous studies [10,13]. Another interesting natural product that was included in our study is BV. The venom has been the focus of many research groups for years till date for its powerful therapeutic effect in various pathological conditions ranging from pain, rheumatic arthritis and skin diseases as well as tumors. Also, BV can be considered as a promising candidate for wound healing owing to its powerful antibacterial effect and potent anti-inflammatory properties [14,15].

In the present study, PPP and BV were loaded within honey/PVA nanofiber scaffolds in order to test their wound healing activity in an animal model of excisional wound. Two types of honey were used, Manuka honey (MH) and Lyophilized multiflora honey powder (LH).

2. Materials and methods

2.1. Preparation of pomegranate peel extract

Preparation of the pomegranate peel powder (PPP), was performed according to Singh et al., 2002 [16]. Briefly, 25 g of the pomegranate powder were suspended in 100 ml Methanol (Ultra) gradient HPLC grade (J.T. Baker, Philipsburg, NJ, USA), and stirred at room temperature for 1 h. The extract was centrifuged at 4000 rpm for 3 min. The supernatant was diluted with distilled water (80:20) to change the freezing point from −97 ℃ to −20 ℃. The resulting solution was lyophilized using a freeze-drying machine (TOPT – 10 ℃ Freeze Dryer, China), for 48 h. The resulting powder was weighed and stored until used.

2.2. Preparation of polymer solutions

Solutions containing different ratios of Manuka honey MH MGO 550+, Manuka Health, New Zealand, Lyophilized multiflora honey LH powder Xiaocaokeji, China, PPP, Bee Venom BV, Insect Research Lab, Cairo, Egypt, and Poly vinyl alcohol PVA, molecular weight MW ~1,250,000 Mowiol 20–98, Sigma Aldrich, Germany) were prepared in the following concentrations: MH/PPP/PVA (10 %/1 %/12 %), (MH/PPP/PVA) (20 %/2 %/10.5 %), (MH/PPPPVA) (25 %/2.5 %/9.7 %), (MH/PPP/BV/PVA) (25 %/2.5 %/0.01 %/9.7 %), (LH/PPP/BV/PVA) (25 %/2.5 %/0.01 %/9.7 %). PVA was dissolved in deionized water by stirring at 90 ℃ for 3 h and then honey, PPP and BV were added.

2.3. Electro-spinning

Different voltages were applied to the polymer solutions (Gamma High Voltage Power Supply, USA) in order to determine the best voltage for each solution. Adjustment of the flow rate was carried out at 1 ml/min. The distance between the collecting plate and the nozzle of the electro-spinner (Sustaincubator, Cairo, Egypt) was adjusted at 12 cm throughout the spinning process. The concentration of the components of each formula was represented in wt %. After stirring, each prepared polymeric solution was taken into a 20 ml syringe that was attached to a needle. 16–22 KV voltage range was applied to the tip of the needle. The electro-spun fibers were collected on an aluminum foil sheet covering a non-moving collector. Electro-spinning parameters were adjusted for each solution, to obtain the optimum conditions.

2.4. Cross linking of fiber mats

Cross linking was achieved chemically using Glutaraldehyde (GH) 25 % (Acros Organics, Belgium). The fibers were placed in a desiccator after being saturated with GH vapors. Followed by heating under vacuum in a vacuum oven (Jeiotech, OV-11, South Korea) at 40 °C for 24 h.

2.5. Scanning electron microscopy

Nanofibers' morphology was observed using scanning electron microscope (SEM: FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany) at an accelerating voltage of 3 KV. For better quality of the resulting images, some of the scaffolds were sputter-coated with a gold layer before SEM examination (JEOL JFC-1600 Auto fine coater, Japan). All SEM images were then analyzed using image analysis software (Image J, National Institutes of Health, USA) where the average fiber diameter was determined. 50 random nanofibers from each image were used to determine the mean and the standard deviation of fibers diameters.

2.6. Swelling capacity

Swelling or water retention capacity of each fibrous sample was measured using a phosphate buffer saline (PBS) solution at (37 ˚C) for 30 min, 3 h and 7 days, where three replicas were used. The mats were weighed after wiping off excess PBS solution that adhered on the fibrous mats using a filter paper. Percentages of swelling of the nanofibrous samples were calculated using the following equation: swelling (Sw–I) /Sw × 100 where Sw is the weight of the swollen sample that was dried by the help of a filter paper and I is the initial mass of sample.

2.7. Weight loss capacity

Weight loss capacity of each sample was measured in PBS (Alkaline Phosphate buffer, Thermo Fisher, Germany) at the physiological temperature (37 ˚C) for 1 h, 7 days and 2 months using three replicas. After immersion, fibrous mats were left to dry on filter paper before weighing. The percentages of weight loss of the fibrous samples were calculated using the following equation: weight loss % = (I – Sd)/I × 100 where Sd is the dried mass of the sample after being suspended in PBS and dried at 40 ˚C. I is the initial mass of the sample.

2.8. In vitro antibacterial assessment

Viable cell count method [17] was used in order to evaluate the antibacterial activity of the electro-spun fibrous mats. The antibacterial activity was evaluated against *Staphylococcus aureus* and *Escherichia coli*. Each strain was added into 20 ml Difco nutrient broth (Thermo Fisher, Germany) that was adjusted to an optical density of 0.1 at wavelength of 625 nm. The following samples were tested: MH/PPP (25 %/2.5 %), MH/PPP/BV (25 %/2.5 %/0.01 %) and LH/PPP/BV (25 %/2.5 %/0.01 %) against both *S. aureus* and *E. coli,* where 0.01 g of each fibrous sample was added to each of bacterial organisms' test tubes having 1 ml from the nutrient media and bacterial strain mixture. All the fibrous scaffolds were UV sterilized for 1 h on each side before the test. *S. aureus* and *E. coli* tubes containing the samples together with a negative control tube were incubated at room temperature with shaking at 150 rpm. Samples were serially diluted in the nutrient broth medium, and 25 µl from selected dilutions were spread evenly on nutrient agar (Thermo Fisher, Germany) plates which were then incubated at 37 C for 24 h. Surviving colonies were counted and compared to the number of

the control tube colonies. The experiment was done in triplicates. The degree of growth inhibition for each sample was calculated according to the equation: Degree of Growth Inhibition (%) = C – S/C × 100 where C is the number of colonies from the negative control (bacteria in the tube without the fibrous sample) while S is the number of colonies of the bacteria in the tube with the samples.

2.9. Cytotoxicity assay

The cytotoxicity of each of the fibrous meshes: MH/PPP/PVA, MH/PPP/BV/PVA, LH/PPP/BV/PVA was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Thiazolyl blue tetrazolium bromide (MTT), Sigma Aldrich, Germany). The cross-linked meshes were sterilized by exposing each side of the fibers to UV light for 1 h. The fibers were soaked in culture media containing RPMI (RPMI media with L-glutamine, Lonza, Belgium) with L-glutamine and 5 % antibiotic (pen-strept) for 24 h at room temperature. The final concentrations were prepared as 1 mg/ml. L929 mouse fibroblast cells (ATCC) were seeded in a 24 well plate (TCPS; Costar®, Corning, NY, USA) at a density of 10,000 cells/well and then were incubated in a humidified incubator with 5 % CO_2 for 48 h at 27 C before treatment with the fibers' extracts. Cytotoxicity was tested by adding 1 ml of the scaffolds conditioned media to the cultured cells in triplicates. After 24 h of incubation at 37 ° C, the medium in each well was aspirated, followed by adding 1 ml of MTT solution (5 mg/ml in PBS) to each well. The plate was incubated again at 37° C for 3 h, the medium was then removed and 1 ml Dimethyl-sulfoxide (DMSO, Lonza, Belgium) was added to each well. The optical absorbance was measured at 595 nm using a plate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). Cells not treated with the extracts were used as the negative control. Cell viability (%) was calculated based on the following equation: Survival % = Ab sample–Ab blank/ Ab control – Ab blank × 100 where Ab sample is the sample absorbance, Ab blank is the absorbance of blank, Ab control is the absorbance of the control.

2.10. In vivo wound healing assay

Adult female Sprague-Dawley rats (*Rattus norvegicus albinus*) weighing ≈150 − 200 g, were randomized into 5 groups. All animal procedures and care were performed in compliance with National Research Council's Guide for the Care Use of Laboratory Animals, and with the national institute of health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1985). Rats were anesthetized using Ketamine/Xylazine intraperitoneal (ketamine 40 − 100 mg/kg IP, xylazine 5 − 13 mg/kg). The hair over the dorsal area was removed, skin disinfected, and an excision with a surface area of ~ 45 mm [2] was created using surgical scissors. Three groups of the rats received treatment dressings (MH/PPP, MH/PPP/BV, LH/PPP/BV) while three control groups received either PVA, No treatment or Medihoney® calcium alginate pad (Derma Sciences) with 95 % active MH honey and 5 % calcium alginate fibers. MH is produced by (*Apis mellifera*) honey bees foraging on the tea or Manuka tree (*Leptospermum scoparium*) which grows throughout New Zealand and southeastern Australia [18].Wounds were examined and photographed at different time intervals (0, 3, 5 and 10 days). The wound areas were measured with the help of Image J software (Image J, National Institutes of Health, USA) where the average surface area for each wound was determined. Wound closure was measured as a percentage based on the equation: (Wi-Wn)/Wi x 100, where Wi is the wound surface area at day 0 and Wn is the wound surface area at day of choice (day 3 or day 5 or day 10).

For histological analysis, skin samples were taken at days 5 and 10 and fixed in formalin, followed by paraffin embedding and Hematoxylin and Eosin (H&E) and Masson Trichrome (MT) staining. Tissue samples were evaluated for enhanced healing. In addition, a scoring system was developed for the groups where the least score was considered the best in terms of enhanced healing process.

2.11. Statistical analysis

Data points at 14 days were considered end points for the current experiment. Data sets were assessed for normality and data points outside 95 % confidence intervals were considered as outliers and excluded from analysis.

Statistical analyses were carried out using GraphPad PRISM ® software. All data were expressed as mean ± standard error of mean. The independent variables of individual comparisons were illustrated by using Least Significant Difference post-hoc test of one-way ANOVA to compare the differences of mean values between different groups. P values that were less than 0.05 are considered statistically significant.

3. Results

3.1. Morphological characterization

Scanning electron microscopy (SEM) showed that the fibers had random orientation, smooth surface and were round shaped. No beads were observed. LH incorporation led to smaller and more uniform diameter than MH scaffolds, under the same spinning conditions (Fig. 1).

3.2. Swelling and weight loss capacity

The three scaffolds, MH/PPP/PVA (25 %/2.5 %/10.5 %), MH/PPP/BV/PVA (25 %/2.5 %/0.01 %/9.7 %), and LH/PPP/BV/PVA (25 %/2.5 %/0.01 %/9.7 %) showed moderate capability for water uptake in comparison to PVA (10 %) scaffolds which had more swelling capacity. Honey incorporated scaffolds had higher weight loss capacity in comparison with PVA that showed less weight loss capacity as shown in Fig. 2.

3.3. In vitro antibacterial assessment

Scaffolds used in the experiment, MH/PPP (25 %/2.5 %), MH/PPP/BV (25 %/2.5 %/0.01 %) and LH/PPP/BV (25 %/2.5 %/0.01 %) significantly inhibited bacterial growth for both *S. aureus* and *E. coli* in comparison to PVA and the negative control tested ($P < 0.0001$) as shown in Fig. 3 and in Supplementary data Fig. 1s. Both scaffolds containing BV were more effective against *S. aureus*, than those without BV ($P < 0.05$). MH/PPP/BV was more effective against *E. coli,* than (LH/PPP/BV) ($P < 0.05$).

3.4. Cytotoxicity assay

Using MTT assay, (Fig. 4), all mats showed percent viability ~ 100 %, when tested on L929 fibroblast cells.

3.5. In vivo wound healing assay

All three treatment scaffolds, MH/PPP (25 %/2.5 %), MH/PPP/BV (25 %/2.5 %/0.01 %) and LH/PPP/BV (25 %/2.5 %/0.01 %) significantly increased wound closure percentage compared to both PVA treated group and no treatment group at days 3, 5 and 10 ($P < 0.0001$) as shown in Fig. 5. At day 10, all treatment groups almost achieved complete healing, compared to PVA group and no treatment groups which showed delayed healing and complete wound closure was seen at days 13 and 14 respectively. Medihoney ® group significantly increased wound closure percentages at days 3 and 5 compared to PVA and no treatment groups but displayed slower healing at day 10. Complete healing was achieved at day 13.

Histopathological assessment using H&E staining (Supplementary data, Fig. 3s) showed that, PVA control group displayed severe

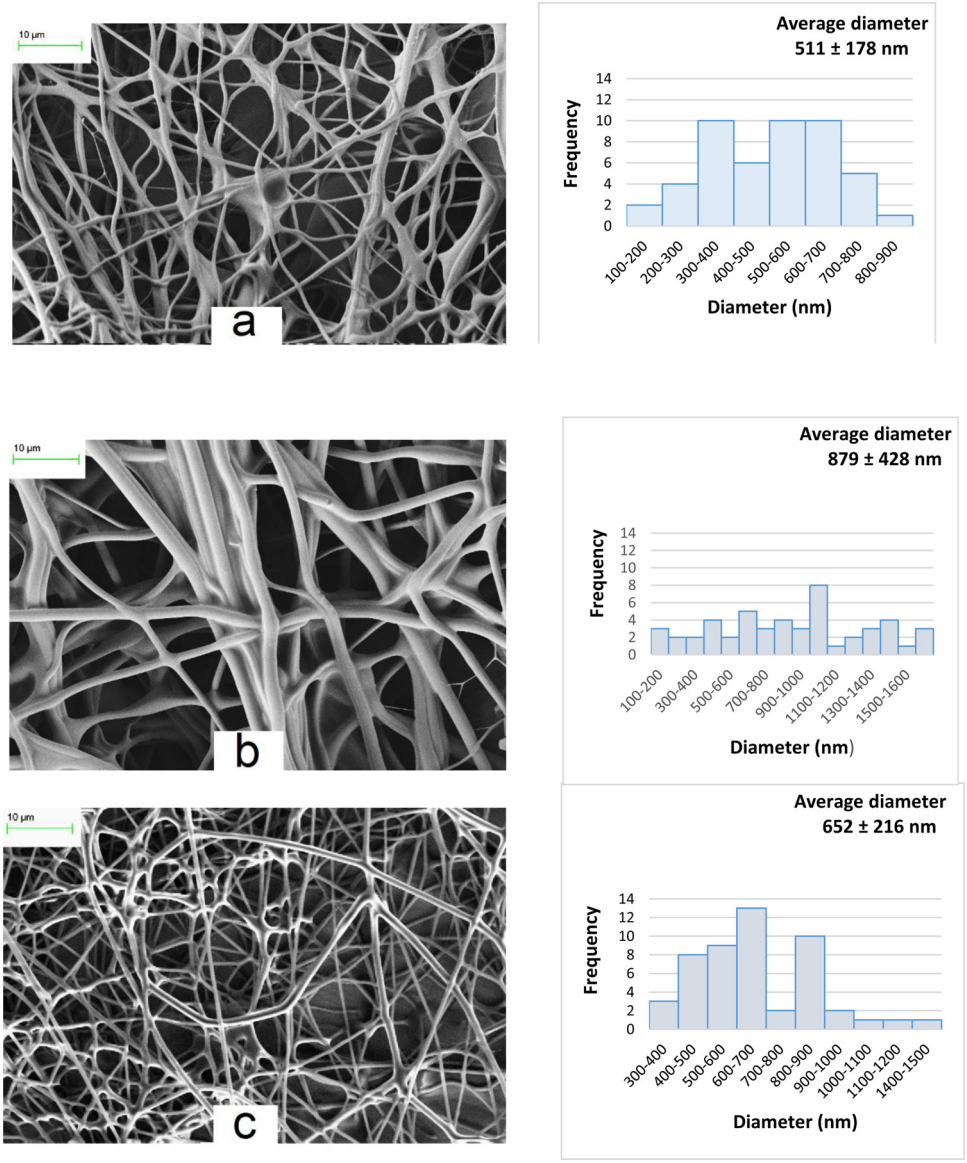


Fig. 1. Scanning electron photomicrographs showing, representative images of; (a) MH/PPP (10 %/1 %), (b) MH/PPP/BV (25 %/2.5 %/0.01 %), (c) LH/PPP/BV (25 %/2.5 %/0.01 %). LH incorporated fibrous scaffolds showed smaller and more uniform diameter than MH incorporated scaffolds. Histogram for the diameters of fibers (n ≈ 50) in each mat is shown.

epidermal necrosis, and ulceration with inflammatory cell infiltration, by day 5. Delayed healing with ulcer formation and necrosis remained see by day 10. The Medihoney ® group showed incomplete re-epithelialization with ulcer and scab formation and severe inflammatory infiltrations at day 5. This group also showed highly cellular granulation tissue with mild congestion of subcutaneous blood vessels. By day 10, Medihoney ® treated wounds showed incomplete healing with ulcer formation, along with necrosis and inflammation.

The Manuka Honey/Pomegranate treated group showed incomplete epidermal re-epithelialization at day 5 and enhanced healing with complete epithelial bridging across wound gap by day 10. Animals treated with bee venom incorporated Manuka Honey/Pomegranate showed better healing with complete epithelial bridging across wound gap by day 5, and complete epithelial bridging, with minimal inflammatory infiltration and activated hair follicles by day 10. Lyophilized honey/pomegranate/bee venom group showed ulcer formation and necrotic tissue at day 5, and enhanced healing and complete epithelial bridging by day 10.

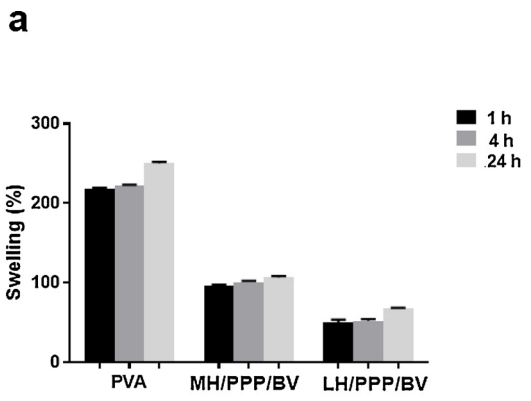
Overall, treatment groups showed variable rate of healing compared to PVA controls. The best was MH/PPP/BV treated group, in which the skin showed close resemblance to normal skin at day 10.

Mason trichrome (MT) staining (Supplementary data, Fig. 4s) revealed that at day 5 lower dense collagen fiber deposition in all groups compared to normal skin. Dense collagen deposition was observed at day 10 in all treatment groups compared to the PVA control group, as well as Medihoney® group at the same time point.

A detailed histological scoring system for the samples is presented in supplementary data table 1. The scoring of the histologic data (Fig. 6) showed that MH/PPP/BV group had the best score in terms of most enhanced healing at days 5 and 10, followed by LH/PPP/BV and MH/PPP at the same time points. All treatment fibers provided a decrease in the inflammatory phase, and allowed for earlier granulation tissue formation and earlier epithelialization.

4. Discussion

Wound healing problem represent an increasing economic burden on health care systems especially in developing countries. The current



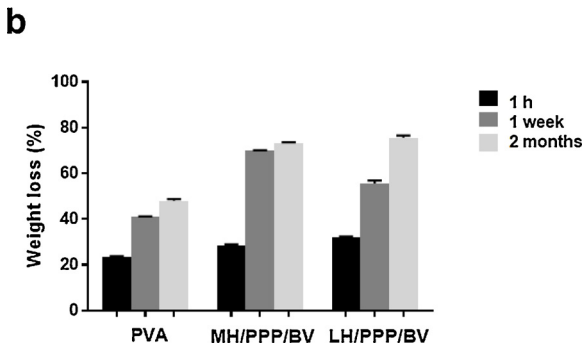


Fig. 2. Swelling behavior (a) and weight loss behavior (b) of the electrospun scaffolds at different time points. Honey incorporated scaffolds showed moderate water uptake in comparison to PVA scaffolds which had more swelling capacity. Honey incorporated scaffolds had higher weight loss capacity in comparison to PVA, data shown as mean ± SD (n = 4).

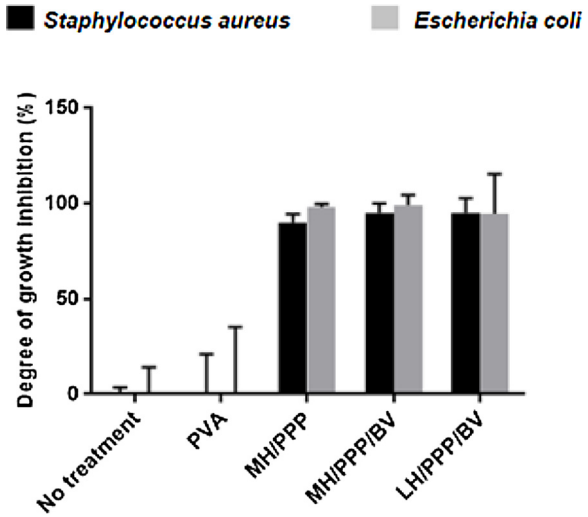


Fig. 3. Antibacterial activity against *S. aureus* and *E. coli.* a) Degree of growth inhibition of different fibrous scaffolds against *S. aureus* and *E. coli*. Data shown as mean and standard deviation (n = 3). All mats significantly inhibited (90%–98 % growth inhibition) bacterial growth compared to PVA and the negative control ($P < 0.0001$). BV incorporated scaffolds were more effective against *S. aureus* than those without BV ($P < 0.05$). MH/PPP/BV was more effective against *E. coli,* than (LH/PPP/BV) ($P < 0.05$). Data shown as Mean ± SD (n = 5).

study is an attempt to develop an effective and safe nanofibrous wound dressings which meets the demands and fights against bacterial infections. Pomegranate is an interesting fruit known for its superior

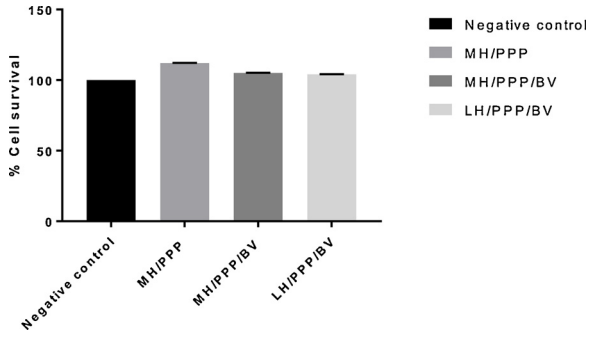


Fig. 4. Cytotoxicity of scaffolds. Crosslinked scaffolds; MH/PPP, MH/PPP/BV, LH/ PPP/BV, showed no effect on cell viability in comparison to the negative control used. Data shown as mean ± SD (n = 4 in triplicates).

antioxidant and antibacterial effects. The fruit's peel is usually discarded as waste. In the present study, methanol was used for the extraction of pomegranate peel powder. According to Chidambara et al. [16], methanol extracted the highest amount of antioxidant poly phenolic compounds from pomegranate peel in comparison to ethyl acetate and water [16].

Pomegranate (PPP) was investigated in enhancing wound healing in several research projects. One study demonstrated that the methanolic extract of the fruit's peel contained a high content of phenolic compounds as well as other constituents. The study further aimed at preparing a 10 % (wt/wt) gel and investigated its efficacy on Wistar rats' excision wounds. The results showed that rats that received 5.0 % gel treatment showed complete healing after 10 days compared to 12 days in those rats treated with 2.5 % and 16–18 days in the rats that received a blank gel [19]. In 2011, Hayouni et al., investigated the efficacy 5 % (w/w) of the methanolic extract of a pomegranate peel based-ointment on guinea pigs. The results demonstrated that the PPP ointment significantly promoted wound contraction as well as the period of epithelialization as assessed by the biochemical, mechanical and histopathological characteristics [20]. Another study evaluated the activity of PPP gel in cutaneous wounds in diabetic rat models. The results showed that the gel significantly shortened healing time as shown by histological examination as its use promoted in collagen regeneration, fibroblast infiltration, vascularization, and epithelialization [21]. However, PPP was not loaded before into nanofibers. In our study, 2.5 % concentration was used based on previous data suggesting that standardized PPP (5 and 2.5 %) increased incision wounds' tensile strength in a rat model and had superior results over ellagic acid (pomegranate peel extract main anti-oxidant component) in increasing the synthesis of collagen and inhibition of the infiltration of neutrophils [13]

In the present work, we report that honey-based nanofibers showed strong antibacterial activity against both gram positive and gram-negative organisms tested. Previous studies using higher concentration (40 %) of honey, demonstrated that honey/chitosan nanofibers showed pronounced antibacterial activity against *S. aureus*, and moderate activity against *E. coli* [8]. The use of MH can possibly explain the differences with the current study as a strong antibacterial effect of MH against *E. coli* was demonstrated previously [22]. In our hands, MH mats were more effective than those with LH against *E. coli*. However, since a good antibacterial effect was also observed in LH mats, we suggest a possible synergistic effect between honey and PPP that led to a strong antibacterial effect against *E. coli*. Previously, the antibacterial effect of PPP has been demonstrated against wide range of bacteria including *S. aureus* and *E. coli* [23].

Unlike other honey types, MH is able to retain its antimicrobial activity in the presence of catalase enzyme which is commonly present in the wound tissue. MH has been famous with its non-peroxide antimicrobial effect owing to the presence of methylglyoxal (MGO) which

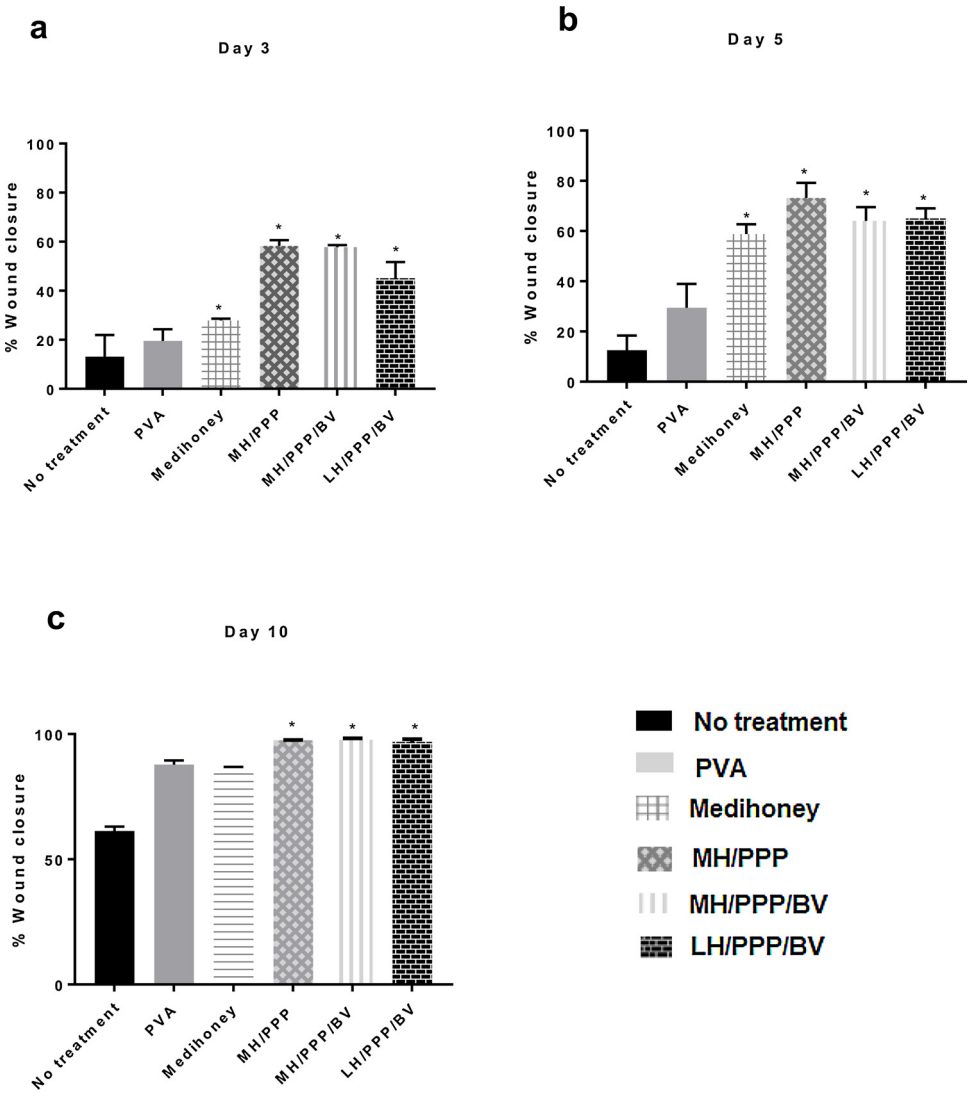


Fig. 5. Bar chart showing wound closure in different groups. All treatment groups reached almost 98 % reduction in wound size by day 10 (c) compared to PVA and no treatment controls. Data shown as mean and standard deviations (n = 4–6). Results were significant in all three treatment groups when compared to PVA and No treatment groups (* $P < 0.0001$). In Medihoney® treated group, wound closure was significantly better than controls at days 3 (a) and 5 (b). However, healing was delayed afterwards.

was discovered in 2008 [24,25]. MGO is responsible for most of the manuka honey's antimicrobial activity [26]. In addition to its non-peroxide antimicrobial activity, it was suggested that MH has a role in modulating the initial inflammatory response by promoting the production of cytokines that regulate the production and the angiogenesis of fibroblasts [27,28]. MH was shown to help in stimulating toll-like receptor 4 on monocytes which in turn leads to the stimulation of the production of interleukin 1 beta, interleukin 6 and tumor necrosis factor alpha from monocytes which are important in tissue repair and regeneration [28]. Other effects that aid in the healing process were

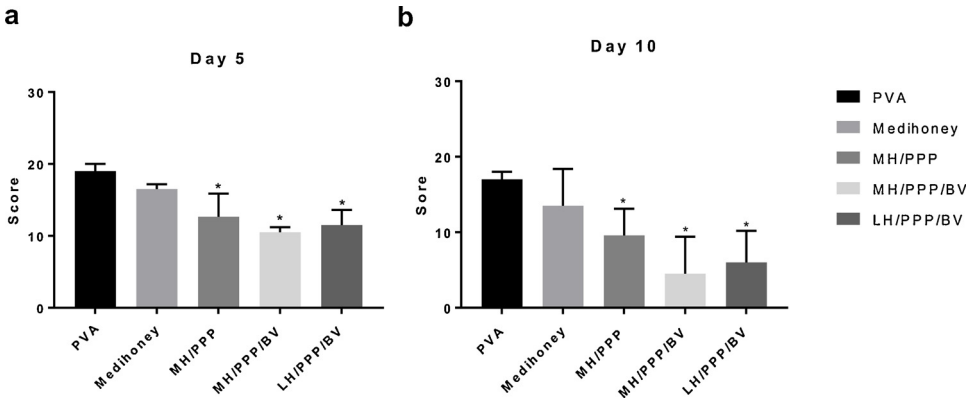


Fig. 6. Histological scoring system for H&E stained wounded tissues for day 5 (a) and day 10 (b). MH/PPP/BV had the best score in terms of most enhanced healing effect at day 5 and 10 followed by LH/PPP/BV at day 5 and day 10. The three treatment groups had better scores when compared to PVA and Medihoney groups (* $P < 0.05$). There was no significance between PVA and Medihoney groups at both time points tested.

mentioned such as lowering the pH of wound area. It was reported that MH decreased pH of cutaneous wounds [29]. Raising pH of the wound towards acidic was suggested to have many effects starting from including a shift to the right to what is called the oxygen–hemoglobin dissociation curve, leading to an increase in oxygen release, decreased toxicity of end products of bacteria such as ammonia, decreased protease activity, activated destruction of abnormal collagen, activated angiogenesis, enhanced fibroblast and macrophage activity as well as controlled the enzyme activity [29].

Honey was used in concentrations up to 25 %, taking in consideration previous data suggesting the most effective and spinnable honey concentrations [8]. Concentrations higher than (MH/PPP/PVA) (25 %/2.5 %/9.7 %) were difficult to spin. SEM analysis showed that the fibers displayed a good morphology while fiber diameter increased with increasing the MH/PPP concentration, which is consistent with previous studies [30,31]. Yang et al. [30], developed MH/Silk Fibroin (SF) antimicrobial wound dressing, which showed antibacterial activity against, *E. coli, S. aureus, P. aeruginosa* and *MRSA,* and enhanced wound healing in animal models, although it was similar to Aquacel Ag ® dressings. Although a very high concentration of MH (70 %) was incorporated within the meshes, MH used was of a low unique manuka factor (UMF 5), unlike MH used in the present study (MGO 550+ equivalent to ~ UMF 20). Another study using MH/poly ε-caprolactone (PCL) scaffolds demonstrated that MH incorporation (1, 5, 10, and 20 % v/v) within the scaffolds promoted cell infiltration and fibroblast proliferation, as well as a significant antibacterial effect against *E. coli* [30].

Scaffolds containing BV showed a slight increase of antibacterial activity against *S. aureus* but had no added effect against *E. coli*. This is in line with previously reported data supporting the increased activity of BV against gram positive bacteria than gram negative bacteria [32].

Subsequent characterization steps showed that all honey containing scaffolds showed moderate swelling capacity when compared to PVA, possibly due to the fact that honey has a high solubility in water [33], which causes an increase in fibers' degradation rate. These finding are in line with, Wang et al. [34] who demonstrated a decrease in swelling behavior in honey incorporated gelatine/chitosan hydrogel. Others [8,35] also showed similar results when investigating swelling, and weight loss capacity of Honey/Chitosan/ PVA nanofibrous scaffolds.

Crosslinking using GH, for 12 and 24 h exposures were favored over 6 h exposure, as these showed easy dissolution of the fibers in PBS, while 12 − 24 h exposure achieved the desired crosslinking result as fibers retained their structure and did not dissolve instantly when submerged in PBS.

Cytotoxicity results show that, all scaffolds had ~ 100 % cell viability which indicated that the produced fibrous dressings have no significant cytotoxicity against skin cells. The results were consistent with previous data of honey incorporated nanofibrous dressings [9]. BV was added at the least concentration of (0.01 %) to avoid possible cytotoxicity. According to Han et al. [36], BV in concentration > 100 μg/ml did not show cytotoxic effects. Moreover, it promoted human epidermal keratinocyte migration and proliferation [36]. Another study claimed that pomegranate peel extract had potential toxicity and should be used with caution [37]. However, in the current study, the concentration used (2.5 %) did not show any cytotoxicity after *in vitro* testing on L929 cells.

Based on the cytotoxicity tests, the following scaffolds were chosen for further testing *in vivo*: (MH/PPP) (25 %/2.5 %), (MH/PPP/BV) (25 %/2.5 %/0.01 %), (LH/PPP/BV) (25 %/2.5 %/0.01 %), (no treatment) and (PVA). Treatment groups showed significant decrease in the percentage of wound closure, compared to both PVA and negative control at days 3 and 5. By day 10, all treatment groups had wound surface area < 2 mm and thereby reached complete healing compared to day 14 in case of the no treatment control. In comparison to treatment groups, Medihoney® calcium alginate pad accelerated wound healing in the initial part of healing at days 3 and 5. However, wound closure rates decreased during the second week of treatment and wounds did not heal completely at day 10. This could be attributed to the complete dissolution of the dressing matrix together with the loaded ingredients within few days as soon as the dressing was introduced to the aqueous media of the wound thereby leading to quicker release and not offering complete coverage during the second phase of treatment. In contrast to this, most probably due to cross linking, all nanofibrous scaffolds retained their structure, did not instantly dissolve within the wound's aqueous media, offered slower release of the loaded treatment and provided complete coverage for the wounds even after the last day of healing. Histologically, all treatment groups showed better healing than PVA control group, which displayed poor healing at the two time points tested (day 5 and day 10). MH/PPP/BV sample showed excellent healing at day 10 where the epidermis showed mature collagen deposition resembling normal intact skin. Medihoney® calcium alginate pad is an MH incorporated calcium alginate dressing (95 % active MH and 5 % calcium alginate fibers) and one of the alginate dressings known for being highly absorbent for wound exudate [38].

Honey powder is a concentrated form of honey lacking the 20 % moisture content. This type of honey captured attention for its use in food industry improve the dough quality and shelf life in bread making [39]. Taking in consideration ease of transportation and storage, being a concentrated form of honey and relative cheap price compared to other honey types, we suggest that the potential of this form of honey should be addressed through including it in more studies to observe its use in fields other than food industry.

In conclusion, Lyophilized honey can be adopted for the perpetration of future wound dressings. Lyophilized honey incorporated nanofibers were smaller and had uniform diameter than those with MH, and lyophilized honey is a much cheaper alternative for MH.

Bee Venom (BV) at 0.01 % showed an increased anti-bacterial activity against *S. aureus* and in combination with MH, animals displayed better healing pattern histologically.

Declaration of Competing Interest

Dr. Azzazy is a cofounder of a startup company which develops nanofibrous wound dressings. He is also a co-inventor of a patent on nanofibrous wound dressings. Other authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.wndm.2020.100181.

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