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Fabrication of Pomegranate Peel Extract/Honey Nanofibers Loaded with Bee Venom as Effective Antibacterial Wound Dressings

A Thesis Submitted to

The Biotechnology Graduate program

In partial fulfillment of the requirements for

the degree of Master of Science

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June 2018

The American University in Cairo
School of Sciences and Engineering (SSE)
Fabrication of Pomegranate Peel Extract/Honey Nanofibers Loaded with Bee Venom as Effective Antibacterial Wound Dressings

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Has been approved by

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Dept. Chair/Director Date Dean Date
DEDEICATION

To the most important people in my life,

Dad, i love you. i wish i can always make you proud.

Mum, you are my true hero. None of this would have been possible without your endless help and support.

My Grandfather, thank you for your continuous care, compassion and love.

My Husband, thank you for your patience and for being there for me.

My Brothers, thank you for being supportive and caring throughout the way.

My sister, thank you for your comforting words and love in tough times.

My children, i extremely love you. You are my true blessing. Looking into your eyes makes everything look perfect.
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List of Abbreviations

3D: Three dimensional
BV: Bee venom
DMSO: Dimethylsulfoxide
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
MMP-9: Matrix metalloproteinase-9
MT: Masson Trichrome
MTT: (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide)
MW: Molecular weight
Oprf: Outer membrane protein F
PBS: Phosphate buffer solution
PCL: Poly(ε-caprolactone)
PGE2: Prostaglandin E2
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
TGF-p: Transforming growth factor-β
TGF-β1: Transforming growth factor β1
TNFα: Tumor necrosis factor-a
VAc: Vinyl acetate
VGEF: Vascular endothelial growth factor
Wt %: weight percent
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Abstract

In spite of the emerging advances in the field of wound dressings, there is still a deep need for the development of novel natural based dressings to offer a safe alternative for traditional wound dressings and most importantly fight against antibacterial resistance. The aim of this study is to develop novel honey based nanofibrous wound dressing for achieving both effective and safe treatment and investigate the dressings for antibacterial activity, cytotoxicity and in vivo wound healing effect in a rat wound model.

Natural products; Honey, Pomegranate peel extract (PPP), and bee venom (BV), known for their antibacterial and anti-inflammatory properties, were used in combination with Polyvinyl alcohol (PVA), to develop a novel natural-based nanofibrous wound dressing. Methanolic pomegranate peel extract was prepared and mixed with either Manuka honey (MH) or lyophilized multiflora honey powder (LH). By testing electrospun samples: (10% MH/1%PPP), (20% MH/2%PPP) and (25%/2.5%PPP) against S. aureus, it was shown that antibacterial activity increases with increasing the MH/PPP concentration. BV was added to the honey/PPP combination to prepare (25 % MH/2.5 % PPP/0.01 %BV) and (25 % LH/2.5 % PPP/0.01 %BV) nanofibers.

Scanning electron microscopy (SEM) showed that all samples had good morphology with no beads. Samples showed moderate swelling capacity in comparison to PVA while all samples showed better water loss capacity than PVA. Antibacterial tests showed significant antibacterial activity against both strains tested compared to both controls used (P< 0.0001). Against S. aureus, samples containing BV were slightly more effective than the sample without BV (P < 0.05). Against E. coli, sample (MH/PPP/BV) was slightly more effective than sample (LH/PPP/BV) (P < 0.05). In the cytotoxicity assay, all samples showed 100 - 120 % viability which indicated that the produced dressings have no significant cytotoxic effects. Results of the in vivo wound healing assay showed that treatment groups (25% MH/2.5%PPP), (25% MH/2.5%PPP/0.01 %BV), and (25% LH/2.5 %PPP/0.01 %BV) had a significantly decreased wound surface areas compared to both controls at days 3 and day 5 (P <0.0001). All treatment groups reached complete healing by day 10 compared to day 14 in the case of both controls. On the histological side, PVA control group showed poor healing compared
to all treatment groups. MH/PPP/BV sample micrographs showed excellent healing at day 10 resembling intact skin as shown by histological assessment. These results indicate that MH/PPP/BV combination can be considered as a promising formula to promote wound healing. However, further analysis is required to confirm the results and address the potential of the combination on more challenging wounds.
Chapter 1: Introduction and literature review

1.1. Introduction

Wounds are considered one of the critical public health problems in the world. In the United States alone, undertreated wounds and chronic wounds affected 6.5 million patients in 2009, with an annual cost in excess of 25 billion USD (Sen et al., 2009). In 2011, the global market of wound-care products produced 6,500 million USD with 7.5% annual growth rate. In 2023, the global advanced wound-care products market share is expected to reach nearly 16,300 million USD. Therefore, new technologies are essential to face this problem ((Sharma & Paul, 2015).

Nano material scaffolds are among the promising wound dressings to improve wound healing. These scaffolds have better properties compared to conventional dressings such as having high porosity, very small pore size and large surface area to volume ratio. These properties lead to higher exudate absorption, better wound permeation and prevention from further infection (Huang et al., 2003).

For decades, honey has been used as wound healing remedy in various forms, owing to its remarkable antimicrobial properties. Honey consists of water (20%), fructose (40%), glucose (30%), sucrose (5%), and other substances such as, minerals, vitamins, amino acids, and enzymes (Bagde et al., 2013). It was demonstrated from previous studies that honey has antibiotic, antimicrobial, and anti-inflammatory properties and inhibits a broad spectrum of bactericidal organisms (Chang et al., 2009, Pieper 2009). Thereby, it has the advantage of providing a moist environment that is suitable for the healing process without the fear of an occurring bacterial growth. Moreover, honey is acidic therefore it is able to provide fibroblasts with optimal environment for their activity.

The present study aims at testing multiple natural products loaded within honey/PVA nanofiber scaffold in order to improve wound healing activity in an animal model (Two types of honey were used MH and lyophilized multiflora honey powder).
1.2. Global impact

In 2011, the global market of wound-care products produced 6,500 million USD with 7.5% annual growth rate. This cost included nearly 230 million surgical procedures, chronic wounds (12–15 million leg ulcers, 9–11 million pressure sores) and an equal number of burn wounds yearly all over the world. Figure 1 shows the global prevalence of wounds according to wound type in 2011 and that predicted in 2025 (Sharma & Paul, 2015).

Despite the fact that surgical wounds showed the highest prevalence, it is expected that there will be a significant increase in number of chronic wounds whose wounds require advanced wound care products. The market share for advanced wound-care dressings is expected to increase in Germany, Japan, USA, France, UK, Italy, Brazil, Russia, Spain, China and India.

It is predicted that India will be the largest consumer for advanced wound care in the future. This might be attributed to the rise in lifestyle-related diseases such as diabetes as well as the raise in the economic status of the Indian population due to better health care policies and the fast-growing health insurance market. 3M, Smith & Nephew, Johnson & Johnson, Dr. Reddys and Beiserdorf are considered the main players in the field of wound-care dressings. In 2023, the global advanced wound-care products market share is expected to reach nearly 16,300 million USD. Another study, expected that the total global market share in 2014 that was worth 15,600 million USD will reach 18,300 million USD in 2019 (Sharma & Paul, 2015).

The predicted huge growth of the market stems from a raise in diabetes prevalence, presence of ageing populations, and obesity. These conditions lead to more chronic wounds and ulcers thereby causing considerable degree of possible mortality and morbidity, and also presenting a huge problem to the health care system as they are considered most costly wound types to treat.

1.3. Wounds and their classification

A wound can be defined as any cut or tear on the surface of the skin. Most commonly, wounds can be classified into acute wounds and chronic wounds, and into three types based on depth; first, superficial wounds that includes the epidermis layer, second, partial thickness wounds that include the epidermis along with the dermis layer and
third, full thickness wounds which include deeper layers being affected like the subcutaneous fat or even deeper layers (Figure 2).

Wounds can also be classified into wounds with tissue loss (e.g. burns, ulcer wounds, abrasions, pressure sores, iatrogenic wounds, trauma wounds) or without tissue loss like for example surgical wounds (Sharma & Paul, 2015).

Acute wounds normally heal on their own. For example, cuts, lacerations, penetrations, avulsion, bites and burns are considered different types of acute wounds. On the other side, chronic wounds do not heal in a timely and structured manner. Therefore, a continued management is needed in case of chronic wounds. Most common types of chronic wounds include; a) Diabetic ulcer. b) Pressure ulcers or bedsores that results from prolonged pressure to some body parts, and c) Arterial and venous ulcers resulting from avascular dysfunction mainly in lower limbs, leading to reduced blood flow and chronic inflammation (Sharma & Paul, 2015).

1.4. Wound healing process

The process of wound healing is a complex process starting with hemostasis, followed by inflammation, proliferation and finally, remodeling (Figure 3). Generally, healing time taken from the beginning to end is between two to three weeks unless other factors that may prolong healing time took place. In the inflammation step following a blood vessel injury, involves coagulation as well as an acute local inflammatory response where monocytes, neutrophils, lymphocytes and macrophages are recruited to the site of injury. In the proliferation step, all cytokines and factors that were previously released in the inflammatory step enhance the proliferation of progenitor cells. Fibroblasts, keratinocytes as well as endothelial cells are recruited in this step leading to migration and proliferation of cells (Sharma & Paul, 2015). In the remodeling stage, cells start to migrate in order to re-epithelialize the damaged tissue at the wound’s edges in a process known as epithelial mesenchymal transition. After this, a scar tissue is formed where the wounds starts to contract and myofibroblasts are formed as a result of fibroblasts differentiation. As a result of the occurring angiogenesis, newly formed blood vessels appear and nerves begin to sprout (Stein & Küchler, 2013, Sharma & Paul., 2015).
1.5. Modern wound healing products classification

Modern wound dressings were developed mainly to help in enhancing the healing process, rather than just creating a cover over the wound. These dressings are concerned with keeping wounds from dehydration as well as promoting healing. According to the nature of their action, modern wound healing products are classified into three main categories, passive, including traditional gauze or tulle wound dressings which represent the largest market segment, interactive and bioactive products. Among the disadvantages of these passive forms of dressings is that they can disrupt the wounds when removed as they tend to stick to the wounds. That’s why this type of wounds will only suit minor wounds. Although tulle which is made of petroleum jelly does not stick to the wound like gauze, it suits flat wounds with little exudate.

Interactive products are made from foams and polymeric films. They are considered permeable to water vapor and oxygen while being impermeable to bacteria. Foams has better exudate absorption than films and amorphous gels, that’s why they can be used for moderate exudate absorption unlike films and gels which are suitable for low exudate wounds. A third type of products is represented by bioactive products, which delivers active components to the wound which contributes in the healing process. These components may include collagen, proteins, growth factors, alginates (Dhivya et al, 2015).

Other types of modern wound dressings may include tissue engineered skin substitutes which can be made up of cells, ECM or combination of both. They can be divided into cellular and acellular skin substitutes (Rubaiy et al., 2009). Medicated dressings generally are drugs-incorporated dressings. They help in the healing process either directly or indirectly by removing of the necrotic tissues. The dressings may contain antimicrobial agents that prevents infection or growth factors and enzymes which enhances regeneration. Composite dressing, can be called combination dressings that have multiple layers. Most composite dressings usually have three layers. An outer layer is designed to protect the wound against infection. A layer in the middle is usually composed of absorptive material which helps in autolytic debridement and in maintaining moisture environment while the third layer is supposed to be composed of non-adherent material so that it will not stick to the wound (Dhivya. et al, 2015).
1.6. Challenges in wound healing

Management of wounds with a delayed healing time i.e. chronic or non-healing wounds is considered as the main challenge to wound care practitioners and researchers. The main reasons to delayed healing include systemic diseases, arterial insufficiency and aging. The factors affecting the healing process can vary between local and systemic factors. Among the local factors is infection, ischemia, tissue maceration and presence of foreign bodies inside the wound area. Among the systemic factors, there is malnutrition, diabetes, age and renal diseases. Controlling the effects of these factors is important for normal healing process to take place.

In chronic wounds for example, there is a decreased level of growth factors such as Epidermal Growth Factor (EGF), platelet driven growth factor, basic fibroblast growth factor and transforming growth factor that are crucial for the healing process to work properly possibly due to degradation or becoming in active. In aging, fibroblasts have poor responsiveness to nutrients and growth factors which result in chronic wounds. Abnormal degradation of the extracellular matrix (ECM) can happen as result of over expression of the matrix metalloproteinase due to an increased proteinase activity (Clark et al., 2007, Sharma & Paul, 2015). Failure of chronic wounds to heal in comparison to the acute healing process is shown in figure 4, on the left of the figure, acute injury of a tissue activates blood clotting, aggregation of platelets and migration of macrophages and neutrophils to the location of the injury. First, a blood clot is formed of fibrin and fibronectin which acts as a scaffold for platelets and cell migration where growth factors are released. At day 3, the formed clot accumulates neutrophils which in turn help phagocytose micro-organisms. On the upper right of the figure, by day 5 after injury and under normal conditions, the wound continue the healing process with the help of the growing granulation tissue that is formed mainly of fibroblasts, more macrophages as well as neovasculature. The epidermis starts migrating towards newly forming tissue. On the lower right of the figure, when the wound fails to heal due to an underlying pathobiology or as a result of a microorganism invasion, the healing process is interrupted leading to a chronic wound or ulcer formation. Many factors hinder the acute healing process including venous insufficiency which in turn leads to transudation of fluids and fibrin cuffing of venules as a result to increased venous system hydrostatic pressure. Other factor might be diabetes mellitus where high
glucose levels which leads to dysfunction of ECM due to the occurring non-enzymatic glycation. Among the factors also there is arterial occlusion or increased external pressure that results in hypoxia of tissues and cell dysfunction. Bacteria might start to colonize the wound leading to the formation of biofilm which in turn worsen the condition by protecting the colonies of bacteria, becoming relatively resistant to phagocytic cells and antibiotics. Moreover, desperate phagocytes release proteases and toxic free oxygen radicals into the wound. This will worsen the condition even more as released agents start destroying the tissue cells, ECM, and growth factors in the wound. As a consequence of all these events, granulation tissue in growth and epidermal migration is absent in chronic wounds.

Although that there a huge advancement in the knowledge and the science of wound healing together with the technical innovation, involvement of pharmaceutical companies, an array of resources ranging from dressings, tissue replacement and cell based treatment options being available however, the biggest challenge is dealing with chronic wounds in terms of practical training with the condition. Also, Scarring prevention as well as achieving a cosmetically acceptable way of wound healing remains a challenge for wound care professionals. Over the past four decades, an extensive research has been done on wound healing and wound dressings. The main reasons for applying a dressing can be summarized in leading to rapid as well as a cosmetically accepted way of healing, remove odor, prevent infection or fight infection, decrease pain, contain exudates, cover a wound for cosmetically accepted appearance, causing minimal distress to the patients (Sharma & Paul, 2015).

1.7. Honey based dressings and Manuka honey

Having a history in being an ancient remedy for healing burns and wounds, honey is still believed to help heal wounds faster and fight against infections. It is suggested that ancient Egyptians used honey around 3000 BC. The “three healing gestures” papyrus mentioned honey and included it as an integral part within the ancient healing process. The first wound dressings are believed to be made from honey, vegetable fiber and animal fat. In modern history, recognition of honey as a topical antibacterial agent started in 1892.

Recently, there has been an interest in re-adopting the use of honey in wound healing especially with the emergence of the antibacterial resistance problem, in 2007, The US
Food and Drug Administration (FDA) approved the honey-based dressings MEDIHONEY® (Derma Sciences, Priceton, NJ), gel sheet where calcium alginate gelled with honey forming a rubbery layer similar hydrocolloids. The layer forms a softer gel when it absorbs the exudate and leaves a layer of honey in contact with the wound (Sharma & Paul, 2015). Other products like Algivon® dressings (Advancis Medical, Nottingham, UK) and Apinate TM dressings (Comvita® (Tepuke, New Zealand), honey- mechanically bonded calcium alginate fiber dressing), make a barrier protecting against pathogens. The outflow of exudates, induced by osmotic action, removes the wound bacteria, endotoxin debris and slough (Sharma & Paul, 2015).

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 (Carter et al., 2009). MH is typically dark in color and considered markedly viscous compared to other honey types. This property is due to the presence of a colloid or protein which is considered as main visually defining character.

Typically, honey contains many components that is responsible for its antimicrobial activity. As mentioned before in the introduction, honey has a high degree of osmolality owing to its high sugar content. In addition to this, the majority of honeys produce hydrogen peroxide (H$_2$O$_2$) upon dilution which leads to the production of reactive oxygen species. Despite, the potent activity displayed by H$_2$O$_2$, its activity can be inhibited by catalase enzyme which is a common component in the wound environment, where catalase is released from skin tissues, this may lead to a decrease in the antimicrobial activity. Other components can be described as immune modulatory molecules include: phenolic compounds, bee defensive 1 and flavonoid compounds, can also contribute to activity in some types of honeys (Alvarez et al., 2014). Role of honey and suggested mechanisms are summarized in table 1.

On the other side, MH unlike other honey types is able to retain its antimicrobial activity in the presence of catalase. MH has been famous with its non- peroxide antimicrobial effect owing to the presence of methylglyoxal (MGO) (Structure shown in figure 5) when research groups identified independently the presence of MGO in MH in 2008 (Adams et al., 2008, Mavricks et al., 2008). MGO is an organic compound that
can be called also 2-oxopropanal or pyruvaldehyde. MGO has the formula: \( \text{CH}_3\text{C(O)CHO} \). In the gaseous state, MGO has 2 carbonyl groups, an aldehyde and a ketone. However, in the presence of water, it is present as hydrates as well as oligomers. MGO is considered to be a pyruvic acid reduced derivative. MGO is formed as a side-product of several metabolic pathways in organisms. MGO is a component within MH as it was shown to have activity against both \( \text{E. coli} \) and \( \text{S. aureus} \) and has been shown to be responsible for most of the manuka honey’s antimicrobial activity (Mavric. et al., 2008, Israeliet al., 2014) and may help the formation of biofilms formed by \( \text{P. aeruginosa} \) (Israeliet al., 2014).

Interestingly, the mechanism by which MH exhibits its antibacterial activity has been investigated in \( \text{S. aureus} \) and \( \text{Pseudomonas aeruginosa} \) (see figure 6 and figure 7 respectively). It was shown that MH inhibits \( \text{S. aureus} \) by interfering with cell division, whereas it leads to lysis of \( \text{P. aeruginosa} \) cells due to the reduction of a key structural protein (Jenkins et al., 2015).

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 (Molan 2006, Tonks et al., 2007). MH was shown to help in stimulating toll-like receptor 4 on monocytes which in turn leads to the stimulation of the production of IL1b, IL6 and TNFα from monocytes which are important in tissue repair and regeneration (Tonks et al., 2001, 2003, 2007). Other effects that aid in the healing process were mentioned such as lowering the pH of wound area. It was reported that MH decreased pH of cutaneous wounds (Gethin et al., 2008). 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–haemoglobin 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 (Greener et al. 2003, Githen et al., 2008). Suggested mechanisms for honey as well as their clinical effects in healing wounds are represented in figure 8.

In a way to rate MH, a grading system called UMF (Unique Manuka Factor) rating was started by the UMF association. The system is supposed to measure the non-hydrogen
peroxide antibacterial potency of MH. The higher the UMF number is, the higher the efficacy and anti-bacterial activity is. Another rating is (MGO) rating made by the Active Manuka Honey Association (AMHA), as MGO content increases as the potency and activity of MH increases. UMF rating typically ranges from 10 to 25 while MGO rating ranges from 100+ to 850+ (Atrott at al., 2009, Jenkins et al., 2015).

1.8. Electro-spun nanofibrous scaffolds for wound healing

Electro-spun nanofibrous scaffolds have many applications in the biomedical field especially in wound healing and tissue engineering. Main advantages of these special fibers include high surface area to volume ratio, high porosity. Other advantages related to the electro-spinning process include the wide range of electro-spinable polymers that can be used, versatility and flexibility of the electro-spinning process itself and the possibility of loading the fibers with bioactive agents, antibacterial agents or functional nanoparticles. All these advantages contribute to expanding the use of electro-spun nanofibers in wound healing application (Liu et al, 2017).

A desired feature in nanofibrous mat dressings that they can allows for providing a temporary structure for skin cells that resembles the native ECM functions, act as an adhesive layer for cells, guide the growing tissue and act as mechanical support. The three-dimensional (3D) electro-spun nanofibrous scaffolds have been developed to produce dressings that resembles the ECM nano sized structure. The highly porous nature of electro-spun matrices that resemble a natural ECM are important for the cells to restore their shape and restore their normal behavior (Puppi et al., 2011).

Electro-spinning, is a technology used for the formation for electrostatic fibers. The resulting fibers usually have diameters that range from several nanometers to several micrometers where high voltage electric fields are used in the process. A typical electro-spinner includes the following: high voltage power supply, spinneret system as well as a collecting screen as shown in figure 9. In the process, there are parameters that affect the transformation of polymer solutions into nanofibers. These parameters include: a) variables like flow rate, voltage used and distance from the needle tip to the collecting surface, b) solution properties such as concentration, viscosity, elasticity, conductivity and surface tension, (c) ambient parameters like humidity, solution temperature and air
velocity in the chamber of the electro-spinner (Huang, Z. M., Zhang, 2003, Vellayappan et al., 2016). All parameters are presented in figure 10.

There are many types of drug loading prior to the electro-spinning process (as shown in figure 10). The new electro-spinning techniques such as sequential electro-spinning and co-axial electro-spinning can contribute in the formation of drug delivery systems as they can produce prolonged release of various biomolecules or drugs. In addition, near field electro-spinning can produce aligned fibers which can be formed into 2 or 3-D scaffolds. These scaffolds are similar to the ECM, and are suggested to help scientists to make a tissue engineering breakthrough.

1.9 Poly vinyl alcohol, structure, synthesis and applications

Poly(vinyl alcohol) is among the famous hydrophilic synthetic polymers possessing good chemical as well as thermal stabilities. It has the chemical formula [CH$_2$CH(OH)]$_n$. The structure of PVA is considered a relatively simple structure (shown in Figure 11). The monomer (vinyl alcohol) doesn’t exist in a stable state where PVA is produced by polymerizing vinyl acetate (VAc) to polyvinyl acetate (PVAC) then this step is followed by hydrolysis to PVA. PVA is generally white in color and odorless. It requires heat (> 70°C) to obtain complete dissolution in water. The degree of hydrolysis affect chemical properties, crystallinity and water solubility of the polymer. Commercial grades of the polymer exist with high degrees of hydrolysis (nearly > 98.5%) (Hassan & Peppas, 2000).

Due to its non-toxicity, biocompatibility and good mechanical properties, PVA has been used in many biomedical applications, such as wound dressings, cardiovascular devices, drug delivery systems and artificial skin, and. Moreover, PVA is considered one of the famous electro-spinnable polymers that can be used to develop highly porous nanofibrous meshes. This made it easy to load the polymer solutions with a variety of other water soluble drugs or polymers taking in consideration adjusting the parameters that influences morphology of the formed fibers, such as polymer concentration, voltage, flow rate as well as the distance between the tip of the needle and the collecting surface (Puppi et al., 2011, Taepaiboon et al., 2006).
1.10. Pomegranate peel powder extract

Pomegranate (Punica granatum L.) is one of the fruits that gained researcher’s interest in recent years due to its enormous biological activities. Pomegranate fruit is rich in tannins and phenolics which were shown to be responsible for its antioxidant activity. Pomegranate juice together with its peel contain remarkable amounts of polyphenolic compounds such as ellagic acid, ellagic tannins, flavonols, anthocyanins, catechin, procyanidins and gallic acid. The fruit has been used in many pharmaceutical preparations like cosmetics, tinctures and other therapeutic formulas.

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. Despite this, the peel is often discarded as waste. That is why it gained concern in many studies being an abundant and cheap source for natural antioxidants. While there is an increased demand on processing of pomegranate. Larger amounts of wastes are produced. For each 1 ton of pomegranates being processed, 500-550 kg waste peels are produced as a major by-product (Qu et al., 2009, Tenkler et al., 2018). 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 is turning into a major problem mainly for pomegranate processing factories (Goula & Lazarides, 2015).

Several studies reported that pomegranate peel powder extract (PPP) had markedly higher antioxidant capacity than the pomegranate juice extract in scavenging against hydroxyl, peroxyl radicals and superoxide anion. In addition to its antioxidant activity, PPP was shown to have a range of other biological actions like anti-cancer activity, antimicrobial activity, anti-inflammatory and anti-diabetic activities. A diagram for the PPP extracted biomolecules and its suggested ethnopharmacological potential in many health conditions ranging from acting as a cardiovascular protective agent, anti-inflammatory and anti-allergic, anti-influenza as well as an antimalarial and effective wound healing agent is shown in figure 12 (Ismail et al., 2012).

In a way to investigate more into PPP wound healing mechanisms, Aslam et al., suggested that PPP activated the synthesis of type I procollagen and led to the inhibition
of the production of matrix metalloproteinase-1 (MMP-1; interstitial collagenase by dermal fibroblast. (Aslam et al., 2006).

1.11. Bee Venom

In the past, bee venom (BV) was not only considered as the main way by which bees defend themselves against predators but also it was popular for therapeutic value in many ancient civilizations (Babylon, Egypt, Greece and China) (Urtubey, N., 2005).

Apis mellifera honey BV grabbed attention for the first time in modern history in the late 19th century. In 1950, Aberman and Neuman suggested that the venom’s biological activity is attributed to the presence of protein and peptide content. In 1963, Benton et al. presented an ingenious way for the collection of venom in large quantities. Since then BV has been the focus of several scientists unravelling BV composition and the biological effects of its components (Banks & Shipolini, R. A. 1986).

The main activity of BV is attributed to melittin protein which constitutes from 40% to 50% of the dry weight of the venom. Melittin itself has been the focus of many studies owing to its potent antimicrobial as well as lytic properties (Banks & Shipolini, 1986). Mellitin was found to have cytotoxic activity towards cancer cells as well as being acting as a potent anti-inflammatory and anti-arthritic in very small amounts. However large doses were reported to be inflammatory and hemolytic. (Oršolić, 2013)

Many recent studies considered bee venom (BV) as a promising candidate for wound healing owing to its powerful antibacterial effect (Fennell, J. F. et al., 1967, Lubke et al., 1997, Isam et al., 2015, Lazarev, V., 2005) and potent anti-inflammatory properties (Kang et al., 2002, Lee et al., 2005, Lee et al., 2010). Another study also suggested that BV in concentration less than 100 μg/ml did not show cytotoxic effects. Bee Venom also, activates human epidermal keratinocyte migration and proliferation in vitro (Han et al., 2013).

1.12. Honey powder

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. In contrast to liquid honey which is viscous and difficult to disperse, honey powder was a good alternative. Tong et al., suggested that honey powder can be used to improve the dough quality and shelf life in bread making (Tong et al., 2010).
Among the methods to prepare honey powder is spray drying, vacuum drying and freeze drying/lyophilization. Extreme stickiness and high viscosity of liquid honey have been the main drive for the increasing demand for the production of dried honey powder by food manufacturers and consumers. However, getting honey into the free flowing powder form either by freeze or spray drying was reported to be a challenging process due to its stickiness and strong hygroscopic property (Adhikari et al., 2009, Kruszewski et al., 2014). Some papers reported that inclusion of high molecular weight carrier material like whey protein within honey helped to solve this problem (Shi et al., 2013, Samborska et al., 2015a,b) as whey protein can modify the surface properties of the atomised particles as well as the film-forming ability of the particles during spray drying (Islam et al., 2013, Wang et al., 2013).

Despite the challenge in achieving the powdered honey form without including additives, numerous companies sell pure honey powder. Also, although honey powder use is limited to food industry, its efficacy in other fields like wound healing, needs investigation. Taking in consideration ease of transportation and storage, being a concentrated form of honey and relative cheap price compared to other honey types, 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.
Chapter 2: Materials and Methods

2.1. Materials
Poly (vinyl alcohol) (PVA) with MW (molecular weight) ~ 125,0000 and commercial name “Mowiol 20-98” was purchased from Sigma Aldrich, Germany. Lyophilized multiflora honey was purchased from Xiaocaokeji, China. Manuka honey MGO 550+, was purchased from Manuka Health, New Zealand. Methanol (Ultra) gradient HPLC grade was purchased from J.T. Baker, Philipsburg, NJ, USA. Pomegranate peel powder was purchased from local condiments store. Glutaraldehyde (GH) solution 50% was purchased from Acros Organics, Belgium. RPMI media with L-glutamine, Alkaline Phosphate buffer (PBS) and trypsin were purchased from Lonza, Belgium. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma Aldrich, Germany. Difco Nutrient broth and Difco Nutrient agar were purchased from Thermo Fisher, Germany.

2.2. Experimental Procedures

2.2.1. Extraction of the pomegranate peel powder
Extraction of the pomegranate peel powder was done according to a previously published protocol (Singh & Jayaprakasha, 2002). The first step was suspending 25 g of the pomegranate powder in 100 ml methanol. Stirring was done at room temperature for 1 h. The extract was then centrifuged at 4000 rpm for 3 min. The supernatant was then diluted with distilled water to ratio (80:20) to decrease the freezing point from -97°C to -20°C. The resulting solution was lyophilized using a freeze drying machine (TOPT – 10 C Freeze Dryer, China). The process was achieved after keeping the freeze drying machine operating for 48 h. The resulting powder was weighed and kept in an eppendorf tubes to be used later on.

2.2.2. Preparation of polymer solutions
Solutions containing different ratios of MH, LH, PPP, BV and PVA were prepared in the following concentrations: (10% MH/ 1% PPP/ 12% PVA), (20% MH/ 2% PPP/ 10.5% PVA), (25% MH/2.5% PPP, 9.7% PVA), (30%/MH/3%PPP/9.7%PVA), (25% MH/ 2.5% PPP/ 0.01% BV, 9.7% PVA), (25% LH/ 2.5% PPP/ 0.01%/ BV, 9.7% PVA). Honey concentrations used were chosen according to previous data (Sarhan & Azzazy...
2016). As for BV, it was shown that in concentration less than 100 μg/ml did not show cytotoxic effect while activated human epidermal keratinocyte migration and proliferation in vitro (Han et al., 2013). According to the later suggestion, BV concentration was fixed at 0.01 % in all samples. In our study, PPP concentration was used up to 2.5%. The extract was not loaded before into nanofibers. However, it has been shown that 2.5% PPP gel decreased wound healing time in animal model (Chidambara Murthy et al., 2004). PVA was dissolved in deionized water by stirring at 90°C for 3 h and then honey and PPP were added where stirring was maintained at room temperature for 1 h. After adding BV, the polymer jars were covered with aluminum foil at room temperature until electro-spinning.

2.2.3. 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 (Sustaimcubator, 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 that was covering a non-moving collector. Electro-spinning parameters were adjusted for each solution, depending on trial and error, to obtain the optimum conditions (Table 3). This is displayed in the fibers’ morphology as examined by the SEM. All the electro-spinning processes were performed at ~30% humidity and ~30°C and. After collection, the fibers were kept in a dry and cool place to be ready for further steps.

2.2.4. Cross linking of fiber mats
This step was achieved chemically using GH 25% solution. The fibers were placed in a desiccator. The desiccator was further closed after being saturated with GH vapors. Fibers’ exposure to the vapors was done at different time intervals (6, 12 and 24 hr). After this step, heating was done under vacuum in a vacuum oven (Jeiotech, OV-11, South Korea) at 45°C for 24 h.
2.3. Characterization of the prepared electro-spun fibrous meshes

2.3.1. 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 samples were sputter-coated with a gold layer before SEM examination (JEOL JFC-1600 Auto fine coater, Japan). Some samples were examined using (SEM Model Quanta 250 FEG (Field Emission Gun), FEI Company, Netherlands). 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 for each image were used to determine the mean and the standard deviation of fibers diameters.

2.3.2. Swelling and water retention 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 thee replicas were used. The mats were weighed after wiping off excess PBS solution that adhered on the fibrous mat using a filter paper. Percentages of swelling of the nanofibrous samples were calculated using the following equation:

\[ \text{Degree of swelling (\% of water uptake)} = \frac{(\text{Sw} - I)}{\text{Sw}} \times 100 \]

Where \( \text{Sw} \) 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.

2.3.3. Water loss capacity
Water loss capacity of each sample was measured in a phosphate buffer solution 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 water loss of the fibrous samples were calculated using the following equation:

\[ \text{Degree of water loss (\% of water loss)} = \frac{(I - Sd)/I)}{100} \]
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.

2.4. *In vitro* antibacterial assessment

Viable cell count method was used in order to evaluate the antibacterial activity of the electro-spun fibrous mats. The antibacterial activity was evaluated against both bacterial strains, *Staphylococcus aureus* and *Escherichia coli*. Each strain was added into 20 ml Difco nutrient broth that was adjusted to an optical density of 0.1 at wavelength of 625 nm. The following samples were tested against *S. aureus*: (10% MH/1% PPP), (20% MH/2% PPP), (25% MH/2.5% PPP). Based on the results of this experiment, the following samples: (25% MH/2.5% PPP), (25% MH/2.5% PPP/0.01% BV) and (25% LH/2.5% PPP/0.01%, BV) were tested 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 1ml from the nutrient media and bacterial strain mixture. All the fibrous scaffolds were UV sterilized for 1 h on each side before the test. The *S. aureus* and *E. coli* tubes containing the nanofibrous mats and a control tube were incubated at room temperature with shaking at 150 rpm. The samples from the bacterial broth as well as the control were serially diluted in the nutrient broth medium. After this step, 25 µL from some dilutions were spread evenly on nutrient agar plates which were then incubated at room temperature for one day. Surviving colonies were counted and compared to the control tube colonies numbers. The experiment was done in triplicate.

2.5. Cytotoxicity assay

L929 mouse fibroblast cells (American Type Culture Collection (ATCC) were used in this study to evaluate the toxicity of MH/PPP/PVA, MH/PPP/BV/PVA, LH/PPP/BV/PVA nanofibers. The cross-linked and non-cross-linked nanofibrous scaffolds were tested. Crosslinking was done through exposing the fibers to GH vapor for 24 h, then heating was done under vacuum at 40°C. The scaffolds were cut into 1 mg sheets and sterilized by exposing each side of the fibers to UV light for 1 h. The extracts were prepared through soaking the scaffolds in culture media containing RPMI with L-glutamine and 5 % antibiotic (pen-strept) for one day at room temperature. The fibers’ extracts were prepared at a concentration of 1mg/ml. Cytotoxicity was
investigated through adding 1 ml of the scaffolds extracts to the cells cultured in a 24-well plate. The cytotoxicity was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. L929 fibroblast cells 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% CO2 for 2 days at room temperature before treatment with the fibers’ extracts. Then, the extract for each fibrous sample was added to the cell monolayer inside each well. This step was followed by 1 day incubation of the cells in CO2 incubator at room temperature and 5% CO2. Triplicate wells were chosen for each sample. The assay depends on reducing the yellow tetrazolium salt to the purple crystals of formazan as a result of dehydrogenase enzymes that are released by the mitochondria of the live and active cells (Vatankhah et al, 2014). The number of viable cells present in each well is proportional to the amount of purple formazan crystals in this well. After 24 h of incubation, the medium in each well was aspirated. Then, 1 ml of MTT solution (5 mg/ml in PBS) was added to each well. The plate was incubated at 37 °C for 3 hours, the medium was then aspirated where 1 ml DMSO/well were added as a subsequent step. DMSO was supposed to dissolve the formazan crystals. 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:

\[
\text{Survival rate} \% = \frac{\text{Ab sample} - \text{Ab blank}}{\text{Ab control} - \text{Ab blank}} \times 100
\]

Where Ab sample is the sample absorbance, Ab blank is the absorbance of blank, Ab control is the absorbance of the control.

2.6. In vivo wound healing assay

Adult female Sprague-Dawley rats (Rattus norvegicus albinus) weighing ≈150-200 gm were used and divided into 6 groups. Each group having 4 -7 rats. All groups are represented in table 4All 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.
Rats were anesthetized with the help of intraperitoneal injection of Ketamine/Xylazine (ketamine 40-100 mg/kg IP, xylazine 5-13 mg/kg), while the dorsal hairs of the rats were shaved. To create a wound on each animal, a scissors was used to create an excisional wound on the dorsal side of each rat’s skin. One circular wound with a surface area of ~ 45 mm$^2$ was created on the back surface of each rat. Four groups of the rats received treatment dressings while two groups received control dressings (No treatment) and (PVA). Photographs were taken for the wounds at different time intervals (0, 3, 5, 10 and 14). The wound areas were then 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.

For histological analysis, wounded tissues were Hematoxylin and Eosin (H&E) as well as Masson Trichrome (MT) stained. Their histopathology was observed at days 5 and 10. Tissue samples were evaluated for enhanced healing for all treatment groups and compared to the PVA control group. In addition, a scoring system was developed for the groups where the least score was considered the best in terms of enhanced healing process.

**2.7. Statistical analysis**

GraphPad PRISM software was used for determining significance using anova for multiple comparisons among samples/groups and t-test for determining significance between each two samples/groups. Moreover, all graphs were done by the help of the software and error bars were added to the bar charts after determining mean and standard deviation for each sample. The analysis was done on the antibacterial assessment results as well as the *in vivo* wound healing assay results.
Chapter 3: Results

3.1. Morphological characterization

As shown from the SEM images that under the optimized spinning conditions, the fibers had random orientation, smooth surface, looked round shaped. No beads were observed in the fibers. LH sample showed smaller and more uniform diameter than those samples containing MH under the same spinning conditions. Range of fibers’ diameter and mean of diameter size is represented in table 4. Figure 13 shows the morphology of the samples: (10% MH/ 1% PPP/ 12% PVA), (25% MH/2.5% PPP/10.5% PVA), (25% MH/2.5% PPP/ 9.7% PVA), (25% MH/ 2.5% PPP/ 0.01% BV, 9.7% PVA) and (25% LH/ 2.5% PPP/ 0.01%/BV/ 9.7% PVA) and PVA (10%) together with the histogram graphs showing a relationship between the frequency and fibers’ diameter in nm. Figure 14 and 15 shows samples (25% MH/2.5% PPP/ 9.7% PVA) and (25% MH/ 2.5% PPP/ 0.01% BV, 9.7% PVA) respectively after cross linking by exposure to GH vapour for 24 h followed by drying the samples under vacuum for another 24 h. The images show the success of crosslinking. Figure 16 shows samples (10% MH/ 1% PPP), (20% MH/ 2% PPP/) and (25% MH/2.5% PPP) as viewed under SEM.

3.2. Swelling and water retention capacity

The percentages of water uptake capacity of the following samples: (25% MH/2.5% PPP/9.7% PVA), (25% MH/ 2.5% PPP/0.01% BV/9.7% PVA), (25% LH/ 2.5% PPP/0.01%, BV/9.7% PVA) and PVA (10%) are given in Table 5. The three samples show moderate capability for water uptake in comparison to PVA scaffolds which had more swelling capacity (see figure 17).

3.3. Water loss capacity

The percentages of water loss of the following samples (25% MH/2.5% PPP/ 9.7% PVA), (25% MH/ 2.5% PPP/0.01% BV/9.7% PVA), (25% LH/ 2.5% PPP/0.01%, BV/9.7% PVA) are given in Table 3. It was shown from the results, an increase in water loss capacity in honey incorporated scaffolds in comparison with PVA that showed less water loss capacity (see figure 18)
3.4. *In vitro* antibacterial assessment

In the starting experiment against *S. aureus*, it was shown that the number of colonies decreased with increasing the MH/PPP concentration (see figure 19). Based on this result, the sample with the highest concentration (25% MH/2.5% PPP) was selected for further experiments.

In the following experiment, the bacterial colonies were counted in triplicates for each of the samples: (25% MH/2.5% PPP), (25% MH/2.5% PPP/0.01% BV), (25% LH/2.5% PPP/0.01%) against *S. aureus* where the average was determined. All samples used in the experiments significantly decreased the number of colonies against *S. aureus* in comparison to PVA and the control tested (P<0.0001). Figure 20 a) shows a graph showing a relationship between the numbers of *S. aureus* colonies vs. samples tested while figure 20 b) shows the agar plates after 24 h incubation for each sample including the control. Samples containing BV were slightly more effective than the sample without BV (P = 0.0013) while there was no significance observed between both samples containing BV (MH/PPP/BV), (LH/PPP/BV).

When testing the same samples, the average number of *E. coli* colonies was also evaluated in triplicates and a relationship between the average number of *E. coli* colonies and different samples was plotted (see figure 21 a). It was clear from the results that the three samples tested showed significant decrease in the number of colonies compared to both controls used in the experiments (P<0.0001). Figure 21 b) shows the agar plates after 24 h incubation for each sample and the control. There was no significance between samples (MH/PPP) and (MH/PPP/BV) (P=0.561). However, sample (MH/PPP/BV) was slightly more effective than sample (LH/PPP/BV) (P=0.0382).

3.5. Cytotoxicity assay:

Figure 22 shows the cytotoxicities of the following samples: MH/PPP (uncross linked), MH/PPP (cross-linked), MH/PPP/BV (uncross linked), MH/PPP/BV (cross-linked), LH/PPP/BV (uncross linked), LH/PPP/BV (cross-linked) scaffolds as assessed by the MTT test.

In the cytotoxicity experiment, the extraction medium of the previously prepared fibers for each sample was used to evaluate their cytotoxicities against L929 fibroblast cells. All samples showed percent viability ranging from 120 % to 104 %.
3.6. *In vivo* wound healing assay

The average wound surface area for each group was measured and represented in bar charts at three time points (day 3, day 5 and day 10) in figure 23. It was shown from the results that MH 25%/PPP 2.5%, MH 25%/PPP 2.5%/BV 0.01% and LH 25%/PPP 2.5%/BV 0.01% samples significantly decreased wound surface area compared to both controls tested at day 3, day 5 and day 10 (P<0.0001). All treatment groups (MH 10%/PPP 1%, MH 25%/PPP 2.5%, MH 25%/PPP 2.5%/BV, LH 25%/PPP 2.5%/BV) achieved complete healing by day 10 compared to day 14 in case of both controls tested. The appearance of each wound was examined at different time points starting with day 0, passing by days 3, 5, 7, 10 and ending with day 14 of treatment. Figure 24 shows a macroscopic observation for each wound at day 0, day 5 and day 10 for the six groups tested (group 1, 2, 3, 4, 5, 6). Two control groups were used in the study PVA (group 5) and No treatment group (group 6). A representative figure shows a delayed healing in the two control groups compared to the treatment groups (see figure 24).

Histopathological assessment using H&E stain showed that treatment samples had better healing than the PVA control group. MH/PPP/BV micrograph showed great resemblance to normal skin at day 10 (see figure 25). MT micrographs revealed that at day 5 less dense collagen fibers were seen in all groups while denser collagen deposition of the treated samples was observed at day 10 when compared to the PVA control group at the same time point (see figure 26). A histological scoring system for the stained samples is presented in table 8. The scores are represented as follows: − = absent, + = scanty, ++ = moderate, +++ = profound. The scoring of the histologic data showed that sample 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 the same time points while MH/PPP (25%/2.5%) and sample MH/PPP (10%/1%) the same scores at both day 5 and day 10. All nanofiber dressings provided decrease in the inflammatory phase, allowed for earlier granulation tissue formation and earlier epithelialization.
Chapter 4: Discussion

Wounds problem together with their complications put a huge economic burden on health care systems especially in developing countries. The current study is an attempt to develop an effective nanofibrous wound dressings which meets the demands. Many natural components were the focus of our project. Honey, pomegranate peel extract, and bee venom are the three natural products that are known for their antibacterial and anti-inflammatory properties and have been used throughout history in wound healing.

Among the interesting fruit extracts being known for its superior antioxidant and antibacterial effect, is pomegranate peel extract (PPP). The fruit peel is usually being discarded as waste in spite of possessing higher amounts of polyphenolic compounds than the fruit’s juice and thereby having better biological activity. In the current study, multiple formulas were prepared using PPP owing to its anti-inflammatory, antioxidant, and antibacterial characteristics that was loaded in honey/PVA nanofiber scaffold in different concentrations in order to provide an added wound healing activity to the PVA/honey formula.

The methanolic extract of PPP was prepared and used in our study. According to Chidambara Murthy et al., methanol extracted the highest amount of antioxidant polyphenolic compounds from pomegranate peel in comparison with ethanol and water as solvents (Chidambara Murthy et al., 2002).

In wound healing, 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 (Chidambara et al., 2004). 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 (Hayouni et al, 2011).

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. Moreover, PPP gel-treated diabetic rats showed promoted the EGF, VGEF and TGF-β1. It also led to a raise in contents of hydroxyproline and promoted NO production (Yan et al., 2013).

Later on, MH together with pomegranate peel extract (PPP) were used to prepare four formulas having an increasing (MH/PPP) concentration. The formulas were electrospun and characterized using SEM. The samples were (10% MH/ 1% PPP/12% PVA), (20% MH/ 2% PPP/10.5 %PVA), (25% / 2.5%PPP/9.7%PVA) (30%MH/3%PPP/9.7% PVA). SEM analysis showed that fibers displayed a good morphology while the fibers’ diameter increased with increasing the MH/PPP concentration. Observing an increase in fibers diameter as MH concentration increases is matching previous studies (Minden-Birkenmaier et al, 2015, Yang et al., 2017).

After examining fibers’ morphology of samples (10% MH/ 1% PPP/12% PVA), (20% MH/ 2% PPP/10.5 %PVA), (25%MH/ 2.5%PPP/9.7% PVA), they were tested against *S. aureus*. The results that the antibacterial activity of the MH/PPP combination increases as the concentration increases. That is why sample (25%MH/ 2.5% PPP/9.7% PVA) was selected for further tests.

Concerning honey, there were numerous studies that compared conventional drugs used in wound healing and honey. In these studies honey showed successful results. For example, in comparison to silver sulfadiazine, the first drug of choice in treatment of burns, honey lead to shorter healing process in partial thickness burns. Also, honey had faster healing in comparison with ethoxy- diamino-acridine plus nitrofurazone dressing in pressure ulcer patients (Günes & Eser, 2007). In comparison to amniotic membrane
dressing, honey impregnated gauze showed faster healing in patients with partial thickness burns (9.4 days vs. 17 days) (Subrahmanyam, 1994).

Manuka honey (MH), was used due to its superior activity over other honey types and popularity in literature in healing wounds owing to the non-peroxide antimicrobial activity (Carter et al., 2016) and being a main component in several FDA approved dressings. In 2017, Yang et al., developed MH/Silk Fibroin (SF) fibrous antimicrobial wound dressing. The study showed that antibacterial effect of the dressings against, *E. coli, S. aureus, P. aeruginosa* and *MRSA* increased with increasing the MH concentration (from 10 wt % to 70 wt %) without affecting the excellent biocompatible properties of SF and finally enhanced wound healing in animal models while having similar results to Aquacel Ag dressings (Yang et al, 2017). Although having very high percent of MH (70%) incorporated within the meshes, MH used in this study was of low grade MH with UMF 5.

Another study also compared Aquacel Ag dressings to honey/chitosan nanofibers scaffolds had similar wound closure rates while having better biocompatibility (Sarhan and Azzazy, 2016). In 2015, another study investigated the potential of MH/ poly(e-caprolactone (PCL) scaffolds in terms of *in vitro* wound healing effects. The study demonstrated that MH incorporation (10 1, 5, 10, and 20% v/v) within the scaffold promoted cell infiltration into it and increased fibroblast proliferation when in direct contact with the scaffold dressing. Disc diffusion test demonstrated significant antibacterial effect against *E. coli* (Minden-Birkenmaier et al, 2015)

We also tested a second type of honey, lyophilized multiflora honey powder (LH). Using lyophilized honey was interesting in terms of being a concentrated form of honey that its use is capturing food industry interest to increase quality of some products. Many companies sell the pure form of LH where the price is considered to be cheap in comparison to other more expensive honey types like MH. Although it was not reported that honey powder was used in field other than food industry. Its interesting features in terms of ease of storing and transportation, relative cheap price and being more concentrated captured our attention to include in our study. We wanted to formulate both MH and LH and observe the differences in the results.
Another interesting component that captured attention to include in our study was Bee Venom (BV). The venom has been the focus of many research groups for years for its powerful therapeutic effect in various pathological conditions ranging from pain, rheumatic arthritis and skin diseases as well as tumors. In spite of offering a great potential in dealing with many incurable conditions, the venom is considered as cytotoxic to eukaryotic cells. That’s why adopting new attempts to move around these limitations is important in order to offer effective solutions for treating many challenging disorders all over the world. For example, nanotechnology provides promising techniques aiming at providing solutions by which cytotoxic effects of BV to eukaryotic cells can be controlled (Oršolić et al, 2012).

BV was added at the least concentration of (0.01 %) was selected for fear of possible cytotoxicity. According to one study, BV in concentration >100 μg/ml did not show cytotoxic effects. Moreover, it promoted human epidermal keratinocyte migration and proliferation (Han et al., 2013).

In a subsequent characterization step, fibrous samples were further tested for swelling as well as water loss capacity. All samples showed moderate swelling capacity when compared to PVA which had higher swelling capability while honey containing scaffolds showed better water loss capacity than PVA. The reason to the observed moderate swelling might be due to the fact that honey has a high solubility in water. At first, Despite honey is supposed to promote water uptake (MohdZohdi et al., 2011), its high solubility in water causes an increase in fibers’ degradation rate. Similar to this a study done by Wang et al., 2012 were given the same results in honey incorporated gelatine/chitosan hydrogel (Wang, Zhu, Xue, & Wu, 2012). Also, Wessam Sarhan and Hassan Azzazy obtained similar results when investigating swelling capacity as well as water loss capacity of Honey/Chitosan/ PVA nanofibrous scaffolds. Where honey inclusion within the scaffold increased the water loss capacity of the PVA scaffolds while showing moderate water uptake capacity in the swelling test in comparison to honey free PVA scaffolds (Sarhan and Azzazy, 2015)

In the current study we report that honey based nanofibers showed strong antibacterial activity against both gram positive and gram negative organisms tested. Having strong
activity of honey based dressings against *S. aureus* is consistent with a previous study that demonstrated that honey/chitosan nanofibers showed pronounced antibacterial activity against *S. aureus*. However, honey/chitosan nanofibers had mild antibacterial activity against *E. coli* although honey used was at higher concentration (40%) than our current study (Sarhan and Azzazy, 2015). An explanation to this may be attributed to the possible synergistic effect between honey and PPP or may be due to MH incorporation. It was reported that MH has a strong antibacterial activity towards *E. coli* (Jenkins et al., 2015). This is consistent with our study’s results where MH incorporated samples were slightly more effective than LH incorporated samples. However, since a good antibacterial effect was also observed in LH samples, that’s why we suggest a possible synergistic effect between honey and PPP that led to a strong antibacterial effect against *E. coli*.

Also it was observed that despite the significant antibacterial activity in comparison to both controls tested. Samples containing BV showed a slight increase of activity against *S. aureus* but had no added effect against *E. coli*. This can be explained from the previously reported from data supporting the increased activity of BV against gram positive bacteria than gram negative bacteria (Fennell et al., 1967).

In crosslinking using GH, 12, 24 h exposures were favored over 6 h exposure. The reason to this is attributed to the observed easy dissolution of the fibers in PBS when exposed to GH vapors for 6 h. While 12-24 h exposure assured the desired crosslinking result as fibers retained their structure and did not dissolve instantly when submerged in PBS.

The three samples were further evaluated for cytotoxicity using MTT assay. Our results show that, all samples had 100-120 % cell viability which indicated that the produced fibrous dressings have no significant cytotoxicity towards skin cells.

Based on the cytotoxicity tests, the following samples were chosen for further testing on animals: (10% MH/1% PPP), (25% MH/2.5%PPP), (25% MH/2.5%PPP/0.01%BV) (25% LH/2.5%PPP/0.01%BV) (no treatment) and (PVA).
In the wound healing assay that was done using excision wound models. Treatment groups ((25% MH/2.5%PPP), (25% MH/2.5%PPP/0.01%BV) (25% LH/2.5 %PPP/0.01% BV)) led to a significant decrease in wound surface areas when compared to both controls used at day 3 and day 5. However, all treatment groups had wound surface area < 2mm and thereby reached complete healing by day 10 compared to day 14 in case of both controls.

On the histological side, all treatment groups showed better healing when compared to the 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.

Taking together our results and previous data, based on the antibacterial test and the clinical assessment of the in vivo wound healing assay, we suggest that the use of lyophilized honey can also be adopted for the perpetration of future dressings. LH incorporated nanofibers were smaller than those having MH and had more uniform diameter. Taking in consideration the difference in price, LH can be considered as a much cheaper alternative for MH. We also suggest that MH/PPP/BV combination can be considered as an effective combination to be used for wound healing. BV at 0.01% led to a slight increase in activity against S. aureus and in combination with MH, it displayed better healing pattern histologically (MH/PPP/BV). We suggest that more experiments should be done in order to confirm the enhanced healing effect of MH/PPP/BV combination histologically. Also in further work, BV can be added at higher concentrations, with caution and samples should be monitored for possible cytotoxicity. Despite both types of honey MH and LH showed similar results in terms of decreasing the wound surface area; however a slight increase in activity was observed against E. coli in the MH incorporated sample (MH/PPP/BV) than the sample containing LH (LH/PPP/BV) in the antibacterial assessment. Moreover, based on the histology results, MH/PPP/BV showed slightly better healing pattern than LH/PPP/BV. However, further studies are necessary to investigate this effect.
Future directions

Further analysis is needed to investigate the potential of honey based nanofibrous dressings on more challenging wound types like chronic and diabetic wounds taking in consideration comparing MH to other alternative honey types. Further studies are also needed for addressing the potential of using LH powder in preparing electro-spun nanofiber dressings and wound healing. Natural products used in this study were promising. Interestingly, varying the concentration of each component might be having a direct impact on both fibers’ morphology as well as, dressings’ effectiveness and toxicity. For example, BV concentration can be increased while assessing possible cytotoxicity. Also, increasing PPP concentration while decreasing honey concentration might display fibers with different morphology and possible increased or decreased antibacterial activity. In addition, each component can be loaded alone in increasing concentrations within each nanofibrous dressing to have better understanding of each component potential in terms of accelerating wound healing and fighting infections. Taking in consideration histology results, MH/PPP/BV sample showed the best healing pattern. However, further histological analysis should be done to confirm this effect.
Table 1. Role of honey in wound healing and suggested mechanisms (Oryan et al., 2016)

<table>
<thead>
<tr>
<th>Role of honey</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-microbial</strong></td>
<td>H2O2 in all honey types and MGO in case of MH were shown to responsible for the antimicrobial activity</td>
</tr>
<tr>
<td></td>
<td>High osmolality</td>
</tr>
<tr>
<td></td>
<td>High osmotic pressure of honey withdraws wound exudate into the surface</td>
</tr>
<tr>
<td></td>
<td>Nitric oxide (NO) production</td>
</tr>
<tr>
<td></td>
<td>Presence of NO in honey was suggested to aid in antimicrobial, antifungal and antiviral effects of honey</td>
</tr>
<tr>
<td></td>
<td>Increase lymphocytic activity and phagocytosis</td>
</tr>
<tr>
<td></td>
<td>Honey was shown to promote T- lymphocytes and B -lymphocytes generation as well as activate neutrophils’ phagocytosis <em>in vitro</em>. In addition, it was shown that honey also stimulates monocytes to produce cytokines, interleukin-6 (IL6), interleukin-1 (IL1Type equation here.1) and tumor necrosis factor-a (TNFα) which thereby stimulates the immune response to infection.</td>
</tr>
<tr>
<td></td>
<td>Antibody production</td>
</tr>
<tr>
<td></td>
<td>Honey was shown to increase antibodies production</td>
</tr>
<tr>
<td></td>
<td>Prostaglandins</td>
</tr>
<tr>
<td></td>
<td>Honey reduces prostaglandin concentrations and its inhibitory effect was found to increase with time</td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td>Honey was found to decrease the damage caused by reactive oxygen species during the inflammatory phase of wound healing thus reducing further tissue necrosis and scarring. Moreover, it was shown that honey reduces inflammation via inhibiting the NF-kB pathway.</td>
</tr>
<tr>
<td><strong>Wound debridement</strong></td>
<td>Applying honey dressing over a wound allows for creating a moist environment allowing rapid debridement where high osmotic pressure of honey and stimulation of proteases by H2O2 may be among the main factors contributing to this effect.</td>
</tr>
<tr>
<td><strong>Boosting the immune system</strong></td>
<td>Proteins and peptides within honey can help in boosting the immune system like the 55 kDa glycoprotein called major royal jelly protein 1 and another protein called type II arabinogalactan. They were suggested to promote the release of TNFα from monocytes. In addition, NO present in honey enables it to enhance the immune system. Another factor that contributes to this effect is that honey causes a reduction in prostaglandins’ concentration which in turn promotes antibody production. Moreover, prebiotic oligosaccharides in honey help to enhance the immune responses. Recently, a study by Tonks et al. suggested that a identified a 5.8 kDa protein within MH honey and reported that it is responsible for stimulating the immune system.</td>
</tr>
</tbody>
</table>
Table 2. Manuka honey antibacterial strength and their corresponding values using MGO and UMF ratings. (Atrott et al., 2009)

<table>
<thead>
<tr>
<th>Strength</th>
<th>MGO</th>
<th>UMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>120+</td>
<td>10-12</td>
</tr>
<tr>
<td>Medium</td>
<td>220+</td>
<td>13-5</td>
</tr>
<tr>
<td>High</td>
<td>400+</td>
<td>18-20</td>
</tr>
<tr>
<td>Ultra - high</td>
<td>900+</td>
<td>32-35</td>
</tr>
</tbody>
</table>
Table 3. Electro-spinning parameters that were used for fabricating the nanofibrous samples

<table>
<thead>
<tr>
<th>Concentration (wt %)</th>
<th>Voltage (KV)</th>
<th>Flow rate (ml/h)</th>
<th>Nozzle (mm)</th>
<th>Distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% MH/1% PPP</td>
<td>16</td>
<td>1</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>20% MH/2% PPP, 12% PVA</td>
<td>22</td>
<td>1</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>25% MH/2.5% PPP/10.5% PVA</td>
<td>22</td>
<td>1</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>25% MH/2.5% PPP/0.01% BV/9.7% PVA</td>
<td>22</td>
<td>1</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>25% LH/2.5% PPP/0.01%/PVA 9.7%</td>
<td>22</td>
<td>1</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>PVA 10%</td>
<td>16</td>
<td>1</td>
<td>25</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 4. All groups of rats used in the *in vivo* wound healing assay

The table shows four treatment groups and two control groups (PVA and No treatment), the number of rats used per group and the corresponding samples used for each group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (5 rats)</td>
<td>MH/PPP (25/2.5) wt%</td>
</tr>
<tr>
<td>Group 2 (5 rats)</td>
<td>MH/PPP (10/1) wt%</td>
</tr>
<tr>
<td>Group 3 (7 rats)</td>
<td>MH/PPP/BV (25/2.5/0.01) wt%</td>
</tr>
<tr>
<td>Group 4 (6 rats)</td>
<td>LH/PPP/BV (25/2.5/0.01) wt%</td>
</tr>
<tr>
<td>Group 5 (7 rats)</td>
<td>PVA (10) wt%</td>
</tr>
<tr>
<td>Group 6 (4 rats)</td>
<td>No treatment</td>
</tr>
</tbody>
</table>
Table 5. Range of diameter size in nm as well as mean diameter size for the nanofibrous samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Range of diameter size (nm)</th>
<th>Mean diameter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% MH/1% PPP</td>
<td>230 - 880</td>
<td>511 ± 178</td>
</tr>
<tr>
<td>25% MH/2.5% PPP</td>
<td>315 - 1678</td>
<td>728.032 ± 320</td>
</tr>
<tr>
<td>25% MH/2.5% PPP/0.01% BV</td>
<td>322 - 1848</td>
<td>742.1755 ± 380</td>
</tr>
<tr>
<td>25% LH/2.5% PPP/0.01%/BV</td>
<td>371-1030</td>
<td>652 ± 216</td>
</tr>
<tr>
<td>PVA</td>
<td>212 - 638</td>
<td>412 ±12</td>
</tr>
</tbody>
</table>
Table 6. Swelling capacity of selected nanofibrous samples

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>25% MH/2.5% PPP/ 0.01BV</th>
<th>25% LH/ 2.5% PPP/ 0.01%, BV</th>
<th>PVA 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96 %</td>
<td>50 %</td>
<td>218 %</td>
</tr>
<tr>
<td>4</td>
<td>101 %</td>
<td>52 %</td>
<td>222 %</td>
</tr>
<tr>
<td>24</td>
<td>106.6 %</td>
<td>68 %</td>
<td>250 %</td>
</tr>
</tbody>
</table>
Table 7. Water loss capacity of selected nanofibrous samples

<table>
<thead>
<tr>
<th>Time</th>
<th>25% MH/ 2.5% PPP/ 0.01% BV</th>
<th>25% LH/ 2.5% PPP/ 0.01%, BV</th>
<th>PVA 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>28.5 %</td>
<td>32%</td>
<td>15.5 %</td>
</tr>
<tr>
<td>7 days</td>
<td>66%</td>
<td>55.5%</td>
<td>41%</td>
</tr>
<tr>
<td>2 months</td>
<td>73%</td>
<td>75.5 %</td>
<td>48%</td>
</tr>
</tbody>
</table>
Table 8. Histological scoring system for H&E and MT stained wounded tissues

The scores are represented as follows: Necrosis: $- = (0)$ absent, $+ = (1)$ scanty, $++ = (2)$ moderate, $+++ (3) = profound$. Inflammatory cells $- = (0)$ absent, $+ = (1)$ scanty, $++ = (2)$ moderate, $+++ (3) = profound$. Hemorrhage: $- = (0)$ absent, $+ = (1)$ scanty, $++ = (2)$ moderate, $+++ (3) = profound$. Granulation tissue maturation: $- = (3)$ absent, $+ = (2)$ scanty, $++ = (1)$ moderate, $+++ (0) = profound$. Epithelialization: $- = (3)$ absent, $+ = (2)$ scanty, $++ = (1)$ moderate, $+++ (0) = profound$. Collagen fibers maturation: $- = (3)$ absent, $+ = (2)$ scanty, $++ = (1)$ moderate, $+++ (0) = profound$. Activated hair follicles: $- = (3)$ absent, $+ = (2)$ scanty, $++ = (1)$ moderate, $+++ (0) = profound$.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Necrosis</th>
<th>Inflammatory</th>
<th>Hemorrhage</th>
<th>Granulation</th>
<th>Epithelialization</th>
<th>Collagen fibers</th>
<th>Activated hair</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>PVA (10%) Day 5</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>PVA (10%) Day 10</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>MH/PPP (25%/2.5%) Day 5</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>MH/PPP (25%/2.5%) Day 10</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>MH/PPP (10%/1%) Day 5</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>MH/PPP (10%/1%) Day 10</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>MH/PPP/BV (25%/2.5%/0.01%) Day 5</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>MH/PPP/BV 25%/2.5%/0.01% Day 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>1</td>
</tr>
<tr>
<td>LH/PPP/BV 25%/2.5%/0.01% Day 5</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>LH/PPP/B (25%/2.5%/0.01%) Day 10</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 1. The global prevalence of wounds by type in 2011 and that expected for 2025. It is expected that there will be a significant increase in number of chronic wounds whose wounds requires an advanced wound care products (MedMarket diligence).
Figure 2. Stages of wound healing: (a) Inflammation step, followed by proliferation step and finally (c) remodeling. Inflammation step includes injury of a blood vessel followed by coagulation, and acute local inflammatory response with the formation of a clot that helps to protect against threatening infections. Proliferation step is characterized by cell migration, angiogenesis as well as granulation tissue formation (c) Tissue remodeling step involves the formation of a disorganized ECM as is shown in the figure in addition to a slightly elevated area with the lack of the normal skin appendages (Stein & Küchle, 2013).
**Figure 3. Tissue injury of acute wound vs. chronic wounds.** On the left, acute injury activates blood clotting, aggregation of platelets and migration of macrophages and neutrophils to the injury site and a blood clot is formed. On the upper right of the figure, by day 5 after injury and under normal conditions, the wound continue the normal healing process with the help of the growing granulation tissue. On the lower right of the figure, the wound fails to heal properly due to an underlying pathobiology or as a result of a microorganism invasion where the healing process is interrupted leading to a chronic wound or ulcer formation (Clark et al., 2007).
Figure 4. Methylglyoxal structure.
Figure 5. Suggested mechanism by which MH inhibits *S. aureus*. MH was found to influence cell division later stages where after speta formation is obtained, decreased production of murine hydrolase together with being sequestered into an inactive form lead to having the two daughter cells remaining attached as the septa are unable to become degraded. This eventually leads to cell death (Jenkins et al., 2015).
Figure 6. A suggested mechanism by which MH inhibits *P. aeruginosa*. MH initially causes cell envelope destabilization via the down-regulation of OprF which is a key protein involved in maintaining cell shape and cell wall stability. Loss of this structural protein leads to what is called, membrane blebbing which eventually leads to cell lysis (Jenkins et al., 2015).
Figure 7. Suggested modulatory effects of honey throughout the stages of wound healing. Clinical effects are on the left of the figure while their suggested mechanisms are on the right of the figure (Lee et al., 2011).
Figure 8. A diagram showing the process of polymeric nanofibers formation via electrospinning, different techniques for electro-spinning and different types of collectors. A typical electro-spinner include a high voltage power supply, spinneret system as well as a collecting screen. Based on the configuration of the syringe required for loading. There are different electro-spinning techniques ranging from co-electro-spinning and passing by side by side electro-spinning, coaxial spinning, multi-jet spinning, emulsion spinning and the technique of surface immobilization. Different collectors are displayed also which may range from fixed or static collector, rotating drum, grid and a rotating disc (Vellayappan et al., 2016).
**Figure 9. Parameters affecting the electrospinning process.** Parameters include parameters related to either the solution, process or surrounding conditions (Vellayappan et al., 2016).
Figure 10. PVA structure.
Figure 11. Potential of pomegranate peel extract (PPP) in treating various health conditions.
The figure shows graphical representation for the PPP extracted biomolecules and its suggested
entopharmacological potential as a cardiovascular protective agent, anti-inflammatory and anti-
allergic, anti-influenza as well as an antimalarial and effective wound healing agent. Moreover, PPP
was shown to have anti-cancer properties (Ismail, T. et al., 2012).
<table>
<thead>
<tr>
<th>Sample</th>
<th>SEM images</th>
<th>Histogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%MH/1.5 PPP</td>
<td>![SEM image]</td>
<td>![Histogram](Diameter nm)</td>
</tr>
<tr>
<td>25%MH/2.5 % PPP</td>
<td>![SEM image]</td>
<td>![Histogram](Diameter nm)</td>
</tr>
<tr>
<td>25%MH/2.5 % PPP/0.01% BV</td>
<td>![SEM image]</td>
<td>![Histogram](Diameter nm)</td>
</tr>
</tbody>
</table>
Figure 12. Representative SEM images of the corresponding fiber mats, including histogram for the diameters of fibers (n≈50).
Figure 13. Representative SEM images of the crosslinked (MH/PPP) sample. (a) Cross linked MH/PPP mat viewed by SEM: FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany) at magnification 1500 X. (b) Cross linked MH/PPP mat viewed by SEM Model Quanta 250 FEG (Field Emission Gun), FEI company, Netherlands) at magnification 1.500 X. (c) Cross linked MH/PPP mat viewed by SEM Model Quanta 250 FEG (Field Emission Gun), FEI company, Netherlands) at magnification of 3000 X.
Figure 14. Representative SEM image for MH/PPP/BV sample. The image reveals the success of the crosslinking process. The sample was viewed by SEM ((SEM: FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany)) SEM at magnification OF 1500 X.
Figure 15. Representative SEM images of the samples: a) (MH 10%/PPP 1%), b) (MH 20%/PPP 2%), and (MH 25%/PPP 2.5 %). Samples were viewed using SEM Model Quanta 250 FEG (Field Emission Gun), FEI Company, Netherlands.
Figure 16. A bar chart showing a comparison between the swelling behaviors of the electro-spun scaffolds. Honey incorporated samples showed moderate capability for water uptake in comparison to PVA scaffolds which had more swelling capacity.
Figure 17. A bar chart showing a comparison between the water loss behaviors of the electrospun samples. It was shown from the results, an increase in water loss capacity in honey incorporated scaffolds in comparison with PVA that showed less water loss capacity.
Figure 18. Antibacterial activity against *S. aureus* increases with increasing in the MH/PPP concentration. a) Bar chart showing the relationship between the numbers of *S. aureus* colonies detected vs. samples used in the experiment. It is shown from the chart that sample MH/PPP (25%/2.5%) had the greatest activity. b) Agar plates after the experiment. It is shown from the figure that as MH/PPP concentration increased, the number of bacterial colonies decreased compared to the control used in the experiment after plating the samples 6th dilutions. c) Agar plates after the experiment. It is shown from the figure that as MH/PPP concentration increased, the number of bacterial colonies decreased compared to the control used in the experiment (control and PVA) after plating the samples 7th dilutions.
Figure 19. Antibacterial activity against *S. aureus*. a) Bar chart showing the antibacterial activity against *S. aureus*. It is shown from the figure that the three scaffolds decreased number of bacterial colonies compared to both controls used in the experiment (control and PVA) (*P* < 0.0001). Samples containing BV were slightly more effective than the sample without BV (*P* < 0.05) b) Agar plates after the 24 h incubation. All honey based samples showed significant decrease in number of colonies in comparison to both controls tested.
Figure 20. Antibacterial activity against *E. coli*. a) Bar chart showing the antibacterial activity against *E. coli*. It is shown from the figure that the three scaffolds decreased the number of bacterial colonies compared to both controls used in the experiment (control and PVA) (P < 0.0001). Sample (MH/PPP/BV) was slightly more effective than sample (LH/PPP/BV) (P < 0.05). b) Agar plates after 24 h incubation. It is shown from the figure that the three scaffolds decreased the number of bacterial colonies compared to both controls used in the experiment (control and PVA).
Figure 21. **Cytotoxicity of electro-spun scaffolds.** Samples included the following samples MH/PPP (uncross-linked), MH/PPP (cross-linked), MH/PPP/BV (uncross-linked), MH/PPP/BV (cross-linked), LH/PPP/BV (uncross-linked), LH/PPP/BV (cross-linked) scaffold. All samples did not affect cell viability.
Figure 22. Representative graphs showing average wound surface area at day 3, 5 and 10. a) Bar chart showing average wound surface areas at day 3. It is shown from the figure that samples MH/PPP (25/2.5) wt %, MH/PPP/BV (25/2.5/0.01) wt%, LH/PPP/BV (25/2.5/0.01) wt%, significantly decreased wound surface area compared to both controls tested (P< 0.0001). b) Bar chart of the average wound surface areas at day 5. It is shown from the figure that samples MH/PPP (25/2.5) wt%, MH/PPP/BV (25/2.5/0.01) wt%, LH/PPP/BV (25/2.5/0.01) wt%, significantly decreased wound surface area compared to both controls tested (P<0.0001). c) Bar chart of the average wound surface areas at day 10. It is shown from the figure that all treatment groups (samples MH/PPP (25/2.5) wt%, MH/PPP(10/1) wt%, MH/PPP/BV (25/2.5/0.01) wt%, LH/PPP/BV (25/2.5/0.01) wt%) had a significantly decreased wound surface areas and almost achieved complete healing compared to both controls tested (P<0.0001).
Figure 23. Images showing healing progression. The images show healing progression from day 0 to day 10 for wounds treated with all treatment scaffolds vs. the 2 control scaffolds used in this study. It is clear from the pictures that healing was achieved by day 10 in all treatment groups unlike slower healing in PVA and no treatment groups.
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Figure 24. Representative histological assessment for all treatment groups vs. PVA control group at day 5 and day 10 as shown by H&E staining (magnification 100 x). a) A section in normal skin covered by an intact epidermis is shown. Normal histological structures of skin including intact cellular elements in epidermis (arrow), dermis (black star) and subcutaneous tissue are shown (red star). b) PVA control group at 5 days showed severe epidermal necrosis, ulceration with many necrotic tissue debris infiltrated with inflammatory cells (arrow) along with dermal layer severe inflammation. Cells infiltration with congested blood vessels and focal areas of hemorrhage were also observed. c) At 10 days the group
showed delayed healing with ulcer formation and necrotic tissue debris (arrow) and granulation tissue formation (star). d) MH/PPP (25%/2.5%) at day 5 demonstrated incomplete epidermal re-epithelialization (arrow) as well as ulcer formation. Diffuse dermal and subcutaneous infiltration of inflammatory cells were also observed. e) The same group at 10 days demonstrated enhanced healing with complete epithelial bridging across wound gap (arrow). A smaller area of highly cellular granulation tissue (star) was observed with moderate inflammatory cells infiltrations. f) MH/PPP (10%/1%) group showed ulcer formation and necrotic tissue debris (arrow) as well as granulation tissue formation in dermal layer at 5 days. At 10 days, the same group (g) demonstrated enhanced healing process with complete epithelial bridging across wound gap (arrow). A decreased area of highly cellular granulation tissue (star) was observed with moderate inflammatory cells infiltrations. Some hair follicles were also observed. MH/PPP/BV (25%/2.5%/0.01%) group showed good healing at day 5 with complete epithelial bridging across wound gap (arrow). However; focal hemorrhagic areas (star) at dermo-epidermal junction with extensive dermal inflammatory cells infiltration were observed. i) At 10 days the wound of this group revealed accelerated wound healing with complete epithelial bridging (arrow), minimal inflammatory cells were observed in dermal layer and more activated hair follicles growth (star). j) LH/PPP/BV (25%/2.5%/0.01%) group at 5 days showed ulcer formation and necrotic tissue debris (arrow) and granulation tissue formation (star). At 10 days (k) demonstrated enhanced healing, complete epithelial bridging (arrow). A small area of highly cellular granulation tissue (star) was observed with moderate inflammatory cells infiltrations along with some hair follicles growth.
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MH/PPP (25%/2.5 %)

MH/PPP / (10%/1 %)
Figure 25. Representative histological assessment for all treatment groups vs. PVA control group at day 5 and day 10 as shown by Masson Trichrome staining (magnification 100×). Abundant mature collagen fibers deposition in dermal layer can be observed in normal skin in (a). It is shown from the pictures that at day 5 less dense collagen fibers were seen in all groups as seen by grey shades instead of blue color while denser collagen deposition of the treated samples: e, g, I, k was observed at day 10 when compared to the control group (c) at the same time point.
References


American Type Culture Collection (ATCC): http://www.atcc.org


Lee, W. R., Kim, S. J., Park, J. H., Kim, K. H., Chang, Y. C., Park, Y. Y., ... & Park, K. K. (2010). Bee venom reduces atherosclerotic lesion formation via anti-


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