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Development of Honey/Chitosan Nanofibrous Scaffolds Loaded with Natural Materials and Bacteriophage: Evaluation of their Antimicrobial and Wound Healing Activities.

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To My Parents, Awad & Asmaa
To My Husband, Wael
To My Sons, Omar, Adam & Youssef
To my sister & brother, Basma & Ahmed
You Are my Heaven on Earth.
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Development of Honey/Chitosan Nanofibrous Scaffolds Loaded with Natural Materials and Bacteriophages: Evaluation of their Antimicrobial and Wound Healing Activities.

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Under the Supervision of Prof. Hassan M.E. Azzazy

Non-healing wounds represent a serious health care burden with major socioeconomic impacts. Bacterial infection of the wound site further complicates the healing process as it stimulates the immune system which in turn prolongs tissue inflammation thus further delaying the healing process. Moreover, wound associated bacterial contamination usually develops resistance to commonly used antibacterials leading to increased risk of systemic infections. Treatment of infected wounds is being achieved via different kinds of dressings in association with antibacterials, antiseptics and wound healing materials. Currently, however there has been a noticeable shift towards advanced antimicrobial wound care as a possible solution for the problem. Advanced antimicrobial wound care are dressings that can be loaded with either antibiotics or antiseptics and are able to reduce or eliminate the bacterial load at the wound site. However, one of the major challenges associated with such dressings is the continuous emergence of antibiotic resistant strains as well as the observed damage of healthy tissues in case of antiseptics. Moreover, it has been argued that the antimicrobial efficacy alone of an advanced dressing is insufficient and other properties that enhance the wound healing process are also required. To help provide a solution for this challenge, this study aims to investigate the development of a novel series of advanced antimicrobial wound dressings that are based on honey and chitosan and fabricated in the nanofibrous form. Honey and chitosan are well known for their wound healing and antibacterial properties. Moreover, developing the dressings in the nanofibrous structure allows enhancement of the wound healing process. Electrospinning technique was adopted to fabricate novel nanofibrous wound dressings based on high honey and chitosan concentrations (HPCS). Natural extracts namely: Cleome droserifolia (CE) and Allium sativum (AE) and apitherapeutics namely: bee venom (BV) and propolis (Pr) as well as bacteriophages (PS1) were loaded within the fabricated honey chitosan based nanofibrous dressings to enhance their antibacterial activity and extend it against resistant bacterial strains as well as increase their wound healing abilities. The fabricated series of nanofibrous dressings, HPCS, HPCS-AE, HPCS-CE, HPCS-AE/CE, HPCS-BV, HPCS-Pr and HPCS-BV/PS1 demonstrated enhanced wound healing abilities and variable antibacterial effects against the examined bacterial strains as compared to the commercial wound dressing Aquacel Ag. Most importantly, the developed series of nanofibrous dressings demonstrated enhanced biocompatibility as compared to the Aquacel Ag that demonstrated noticeable cytotoxicity. Thus, the developed series of nanofibrous wound dressings that are based on natural materials represent competitive candidates to be used as effective wound dressings.
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LIST OF ABBREVIATIONS

AE: Allium sativum aqueous extract  
BV: Bee venom  
CE: Cleome droserifolia aqueous extract  
CS: Chitosan  
ECM: Extracellular matrix  
FTIR: Fourier Transform Infra-Red Spectroscopy  
GA: Glutaraldehyde  
H: Honey  
P: PVA  
Pr: Propolis  
SEM: Scanning Electron Microscopy  
TEM: Transmission Electron Microscopy  
TGA: Thermogravimetric analysis  
XRD: X-Ray Diffraction
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1. BACKGROUND AND LITERATURE REVIEW

1.1 Wounds; Serious Health Care Burden

Non-healing wounds represent a significant health care burden with a major socioeconomic impact of over $25 billion as the cost of complicated and delayed wound healing (Sen et al., 2009). That is in addition to the mortality and morbidity risk posed to mankind due to chronic wound complications (Kirketerp-Møller et al., 2011). Chronic non-healing wounds are those that do not follow the normal physiological process in healing staying opened for several weeks reaching to months. The most commonly encountered chronic wounds include diabetic foot ulcers, arterial leg ulcers, pressure ulcers, venous leg ulcers and burns (Siddiqui & Bernstein, 2010). Pressure ulcers were found to affect nearly one-third of the population undergoing treatment in a critical care unit with an annual total treatment cost reaching to $11 billion in the United States. Moreover, $2.3–$3.6 billion are spent by the National Health Service (NHS) for the treatment of pressure ulcers and the cost per each case of pressure ulcer treatment ranges from $5,000 to $65,000 per case. Diabetic foot ulcers which are a leading cause of foot amputations, are considered among the most commonly encountered complications with diabetes affecting nearly 15% of the diabetic population. The prevalence of diabetes is expected to reach 333 million by 2025, from which 10% are expected to suffer from diabetic ulcers according to the International Diabetes Federation. Diabetic foot ulcers are considered one of the most serious chronic wounds leading to major socioeconomic implications as it takes about 12 to 14 weeks for a diabetic wound to heal. It was concluded from a recent study conducted in Sweden that the costs per case for the treatment of diabetic foot-ulcer associated complications range from $16,500 for patients suffering from severely impaired circulations to nearly $63,000 with patients undergoing amputation (Abrigo et al., 2014).

Healing of normal or acute wounds proceeds via a series of physiological phases that end up in functional and anatomic skin restoration (Figure 1). Normally, the wound healing process follows four distinct phases: hemostasis, inflammation, proliferation and tissue remodeling (Werner & Gorse, 2003). The hemostasis phase involves an immediate vascular and cellular response to the disruption of the skin surface. The arterial vessels constrict rapidly under the effect of various vasoactive mediators (norepinephrine, epinephrine, prostaglandins, serotonin and thromboxane)
which lead to hypoxia and acidosis. Moreover, the exposed sub endothelium activates the aggregation of the platelets leading to clot formation. Platelet aggregation results in their activation which enables them to release growth and chemotactic factors as proteases, platelet-derived growth factor (PDGF) and other vasoactive agents (eg, histamine, serotonin). Consequently, this mediates a reflex vasodilation and increase in the vascular permeability which facilitates the entrance of the inflammatory cells and the beginning of the inflammatory phase in the healing process (Hunt, 1988). The cellular components of the inflammatory phase include macrophages, lymphocytes and neutrophils. Neutrophils are the predominant cell type at the beginning of the inflammatory phase, and while it is not essential to the wound healing process, its importance originates in its ability to cleanse the wound site from bacteria and necrotic matter. Macrophages on the other hand, are essential components in the early phase of the process of wound healing as they phagocytose bacteria and debris and release elastases, collagenases and PDGF which stimulates proliferation and chemotaxis of fibroblasts and cells of the smooth muscle. Additionally, macrophages produce substances that attract endothelial cells and stimulate their proliferation at the wound site to mediate angiogenesis. T lymphocytes play an important role in production of antibodies as well as cellular immunity (Broughton et al., 2006).

The proliferation phase involves the formation of granulation tissue which includes fibroblasts, neovascular and inflammatory cells within a matrix of collagen, fibronectin, proteoglycans and glycosaminoglycans. Additionally, the proliferation phase involves epithelization, fibroplasia, angiogenesis and contraction. Epithelialization involves the formation of epithelium which acts as a seal between the environment and the wound. Moreover, the epidermal cells secrete collagenases which stimulate plasmin production that promotes the dissolution of the clot. Migrating epithelial cells promote the keratinocyte adhesion in order to guide such cells across the base of the wound. In the fibroplasia phase, fibroblast and mesenchymal cells differentiate, moreover fibroblasts produce collagen, fibronectin, elastin, proteases and glycosaminoglycans. Fibroblasts grow and proliferate with the reduction in the number of inflammatory cells. Angiogenesis which is promoted via the endothelial growth factor basic fibroblast growth factor, is important to sustain a blood supply to the formed tissue, which in turn lead to increased perfusion of various healing factors. As a final step in the proliferative phase the wound edge contract facilitating the closure of the skin defect at the wound site (Kirsner & Eaglstein, 1993).
Tissue remodeling represents the final phase of the wound healing process, during which collagen undergoes increased organization. This proceeds via gradual disappearance of fibronectin and replacement of hyaluronic acid and glycosaminoglycans with proteoglycans. Type I collagen replaces type III collagen and water resorption occurs from the scar. This allows collagen crosslinking and decreasing the thickness of the scar (Hunt, 1988).

Both acute and chronic wounds pass through the previously demonstrated phases, however chronic wounds exhibit delayed healing. In chronic wounds the normal series of physiological processes involved in healing is interrupted by the presence of persistent inflammatory stimuli. Such inflammatory stimuli result in increased production of metalloproteinases (MMPs) which lead to degradation of the extracellular matrix which in turn decreases the migration of cells and reduce the deposition of new connective tissue. Most importantly, such MMPs lead to the degradation of the growth factors, such growth factors that are essential and very important mediators for the normal series of mechanisms that are involved in all phases of the wound healing process. Thus, eventually leading to the disruption of the wound healing process (Bjarnsholt et al., 2008; Chen et al., 1999). This problem is overcomplicated by the increased level of contamination of chronic wounds by different kinds of bacteria. Such bacterial infection stimulates the immune system which in turn prolongs tissue inflammation thus further delaying the healing process. Moreover, chronic wound associated bacterial infection usually develops resistance to commonly used antibacterials showing great difficulty and resistance to be treated with common antibiotics and antibacterials, thus, leading to increased risk of systemic infections (James et al., 2008).

A bacterial wound infection demonstrates various clinical symptoms that include edema, pain, erythema and purulent exudates (Robson et al., 1990). The quantity of bacteria at the wound site has been correlated to infection, where it has been suggested that bacterial counts of more than $10^5$ CFU/g may be indicative of infection at the wound site. However, this varies according to the type of the organism, its virulence and pathogenicity, as well as the interaction of the organism with the surrounding microflora and the hosts’ immune response (Bowler, 2003). Generally, the wound microbiology could be described in different phases: contamination, colonization and infection which could spread and lead to invasive infection and septicemia (Figure 2). Contamination of the wound site means presence of bacteria that are not replicating, whereas, colonization is the presence of bacteria that are replicating at the wound site without associated tissue damage. An
infected wound is often associated with clinical signs of infection as well as tissue damage (Edwards & Harding, 2004). Therefore, wound infection results in delay in the process of wound healing.

Different kinds of bacteria are involved in the process of wound healing, and they may be present at the wound site either as single bacterial species or as a polymicrobial load of two or more bacterial species. It was observed that the most common bacterial isolates were Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Corynebacterium spp and Proteus mirabilis. Whereas, the most common species that are found in a polymicrobial infection were S. aureus, P. mirabilis, and P. aeruginosa with the most predominant associated combination between S. aureus/P. aeruginosa (Bessa et al., 2015).

It was reported that Staphylococcus aureus was the most common Gram positive bacteria (40-60% of the total load of bacteria) isolated from variable kinds of wounds (Bowler, 2003; Brook & Frazier, 1998; Gjødsbøl et al., 2006; Davies et al., 2004; Urbancic-Rovan et al., 2000; Korber et al., 2010). Whereas, P. aeruginosa was the most predominant Gram negative isolate (Gjødsbøl et al., 2006; Davies et al., 2004; Halbert, 1992; Madsen, 1996; Burmølle, 2010). It has been observed that both P. aeruginosa and S. aureus produce destructive virulence factors that were found responsible for infection prolongation and delay in the wound healing process (Bessa et al., 2015). The production of different virulence factors as clumping-factor A, coagulase, leuocidines and catalase by S. aureus has been linked to clinically significant wound infections (Dissemond, 2009). Similarly, P. aeruginosa elastase has been linked to its pathogenicity at the wound site (Schmidtchen, 2003). Additionally, it was observed that a relevant percentage of the isolated S. aureus (Bessa et al., 2015) were methicillin resistant S. aureus (MRAS), and that MRSA is becoming a more prevalent wound pathogen (Demling & Waterhouse, 2007) thus further complicating the problem of an infected wound. This is because a chronic wound infected with MRSA represents a source of a resistant nosocomial infection, additionally the presence of MRSA in a wound increases the risk further complication of the wound infection to septicemia (Demling & Waterhouse, 2007).

Thus, the success in managing bacteria in wounds is of utmost importance. Generally, treatment of chronic wound infections is achieved via antimicrobial therapeutics either systemic or topical (Lipsky & Hoey, 2009). It was observed that > 60% of chronic wound patients received antibiotic
treatments for a long periods of time (Howell-Jones et al., 2005; Tammelin et al., 1998). Although antibiotic therapy revolutionized wound care, the increased and continuous emergence of resistant bacterial strains together with the marked decrease in the discovery of new antibiotics has necessitated the need to discover alternative antimicrobials (Cooper, 2004). Thus, the use of topical antiseptic treatments has increased leading to reduction of antibiotic use in chronic wound treatments. The commonly used wound dressings in the treatment of chronic wound infections are traditional gauzes and bandages which are considered the most primitive and simple kinds of dressings that generally work only on achieving wound protection from trauma and bacterial contamination. Such traditional dressings are usually used in chronic wounds in association with other antibacterials, antiseptics and wound healing materials. Currently, chronic wound treatment is witnessing an increasing shift towards advanced wound dressings. Advanced wound dressings, are dressings that aid and accelerate the wound healing process via exudate management, the ability to control infections and providing essential growth factors that enhance the healing process (Frost & Sullivan, 2014).

Among the advanced wound dressings, antimicrobial wound dressings stand as an important sector that is most beneficial in the treatment of chronic wound infections (Abrigo et al., 2014). Such antimicrobial advanced wound dressings allow the sustained release of the loaded antimicrobials thus allowing the realization of their antibacterial activity while maintaining a healthy concentration to the healing tissues (Vowden et al., 2011).

The most commonly available antimicrobial wound dressings are dressings loaded with antiseptics such as, as silver and iodine. Silver-based dressings stand as one of the most common and effective antimicrobial dressings used. However, despite their enhanced broad spectrum of activity, development of resistance has unfortunately been reported together with some undesirable side effects of silver (Lansdown, 2002). Thus, research into development of effective antimicrobial wound dressings based on effective antimicrobials that are more biocompatible and able to be effective against resistant bacterial strains is of great necessity.

Among the advanced wound dressings nanofibrous advanced wound dressings stand as an important emerging sector (Zahedi et al., 2010). Nanofibrous based dressings allow pronounced wound healing ability as compared to other traditional dressings due to the high surface to volume ratio, morphological resemblance to the extracellular matrix (ECM) in the skin and thus promoting
cell adhesion, proliferation, and migration. Additionally, nanofibrous dressings exhibit haemostasis capability and increased ability to absorb exudates as well as enhanced cell respiration due to high porosity. Most importantly, nanofibrous based dressings are easily loaded with different materials as extracts, drugs, antiseptics or others thus allowing enhancement of their wound healing and antibacterial abilities (Zahedi et al., 2010; Zhang et al., 2005; Kanani & Bahrami, 2010).

1.2 Electrospun Nanofibers; Emerging Advanced Wound Dressings.

Nanofibers are a class of nanobased materials with different and broad applications in different fields including environmental engineering, optical electronics, nanocatalysis, defense and security as well as pharmaceutical and biomedical applications. Nanofibers demonstrate multiple advantages that allowed it to be of great interest in different applications. Nanofibers exhibit increased surface to volume ratio, increased porosity, in addition to the ease of fabrication of the nanofibers from multiple polymers either natural, synthetic or combination of both and the feasibility of loading the nanofibers with different materials alone or in combinations (Cui et al., 2006; Ramakrishna et al., 2006; Luu et al., 2003; Welle et al., 2007; Subbiah et al., 2005).

Electrospinning represents a facile, cost effective and versatile technique for fabrication of nanofibers compared to other nanofiber fabrication techniques (i.e. phase separation or self-assembly). In the electrospinning process, the polymeric solution is contained in a capillary, subsequently a droplet of the polymer solution forms at the capillary tip under a certain flow rate. A high voltage is then applied to the polymeric droplet and the droplet gets electrified leading to accumulation of charge at the droplet surface. At a certain applied voltage the electrostatic forces are able to overcome the polymeric solutions’ surface tension and the accumulated charge at the droplet surface causing the deformation of the formed droplet into a cone (Taylor cone), from which an ultrafine polymer jet is produced from the tip. The polymeric solutions’ charged jets travel towards the used collector and the solvent evaporates rapidly followed by the collection of ultra-fine dry fibers on the collector (Altstädt et al., 2008). The morphology of the collected fibers can be controlled via the adjustment of the different parameters of the electrospinning process (Teo & Ramakrishna., 2006). A detailed illustration of the electrospinning process as well as the different parameters affecting the fabricated nanofibers was provided via Bhardwaj and Kundu (Bhardwaj & Kundu., 2010). The basic setup for the electrospinner is shown in figure 3, whereas,
Sahay et al. and Migliaresi et al. provided a detailed review for more complex electrospinning set-ups (Sahay et al., 2011 & Migliaresi et al., 2012).

Currently, one of the main drivers in the wound dressing field is developing wound dressings in the nanofibrous form (Zhang et al., 2005). Nanofibrous wound dressings exhibit a number of intrinsic properties that make them of particular interest in wound healing applications. An ideal wound dressing should be capable of mimicking the natural extra-cellular matrix (ECM). The ECM is non-cellular constituent present in all tissues, during wound healing it acts as a scaffold that allow cell attachment, differentiation and proliferation (Martins et al., 2007). Nanofibers due to their nanometer size in addition to their random alignment within the nanofibrous mesh mimic the architecture of the ECM. Thus, wound dressings in the nanofibrous form provide the cells with an artificial ECM (Figure 4) promoting healing by encouraging tissue growth and leading to reduction in scar tissue formation as well as the required time for healing (Bhattarai et al., 2005). Moreover, the nano-pore sizes also assist in protecting injured tissues from bacteria and the high porosity and surface area enhance the absorption of fluids and the hemostasis. The nanofibrous mats exhibit high interconnected porosity (60-90%) (Kanani & Bahrami, 2010) thus, allowing high-gas permeation and cell respiration which prevent the dehydration of wounds (Zhang et al., 2005). The high surface area provided by the nanofibrous structure enhance the delivery of wound healing and antimicrobial agents, thus, eventually encourages wound healing (Diegelmann & Evans, 2004; Bhardwaj & Kundu, 2010).

Electrospun nanofibers for wound healing applications have been fabricated from synthetic and natural polymers as well as combinations of both of them (Zahedi et al., 2010). Synthetic polymers facilitate the process of electrospinning and enhance the mechanical properties of the developed nanofibrous mats, whereas, natural polymers increase the biocompatibility of the resulting nanofibrous mats and enhance the ability of the nanofibers to interact with the biomolecules that are involved in the wound healing process (Gunn & Zhang, 2010).

Initially, research involving development of nanofibrous wound dressings focused on optimizing the parameters of the process of electrospinning for fabrication of wound dressings with suitable mechanical, morphological and physico-chemical properties for providing dressings with suitable barrier properties that allow tissue protection and maintain the moisture level of the wound bed, such dressings that were referred to as passive dressings according to Abrigo et al. (Abrigo et al.,
Within this context, different types of nanofibers were fabricated from poly (urethane) (Khil et al., 2003), poly (vinyl alchol) (Phachamud & Phiriyawirut, 2011) and hyaluronic acid (Uppal et al., 2011), and both the morphological characterization and the invivo testing demonstrated their potential application as wound dressings. The hyaluronic acid nanofibers were proved to be more beneficial in treating full thickness wounds as compared to commercial wound dressings (Uppal et al., 2011).

More recent developments in nanofibrous wound dressing research involved development of nanofibrous dressings that are capable of accelerating the wound healing process and treating or preventing bacterial infection. Abrigo et al., referred to such dressings as interactive dressings that are able to combine the optimal physical and morphological requirements needed for wound healing and the value-added ability to limit the proliferation of bacteria and address the optimal environment for cell proliferation and migration (Abrigo et al., 2014). Such dressings were achieved via developing the nanofibrous wound dressings from a combination of polymers of synthetic origin and biopolymers that have antibacterial ability and enhanced affinity towards the wound environment. A wide variety of reports could be found in literature addressing the use of synthetic and natural combinations in the fabrication of nanofibrous wound dressings. Kim et al, developed nanofibrous meshes from a combination of polyurethane and gelatin that demonstrated their potential effectiveness as wound dressings (Kim et al., 2009). Similarly, nanofibrous meshes from keratin and poly (hydroxybutylate-co-hydroxyvalerate) have been fabricated and were proved to enhance the process of wound healing (Yuan et al., 2015). Cheng et al., developed nanofibrous mats from the combination of poly (ethylene oxide), type I collagen and chitosan. Such dressings demonstrated enhanced wound healing ability invivo compared to traditional wound dressings (Chen et al., 2008). Additionally, different reports demonstrated fabrication of nanofibrous meshes as potential wound dressings from combinations of synthetic polymers and natural materials as honey, natural extracts and essential oils (Jin et al., 2013). Further development within the nanofibrous wound dressing research involves loading of such interactive dressings with antimicrobials, drugs and wound healing agents to further enhance the wound healing and antibacterial properties of the developed wound dressings, such dressings that were referred to as advanced interactive wound dressings (Abrigo et al., 2014) were reported in a detailed review via Meinel et al., (Meinel et al., 2012). Despite that advanced interactive wound dressings are able to control bacterial infection at the wound bed, initial burst release of the loaded antibiotics and drugs
causes toxic effects to the healing tissue (Sill & von Recum, 2008; Agarwal et al., 2008). Thus, development of advanced interactive wound dressings that demonstrate no toxic effects to the healing tissues as well as enhanced antimicrobial and wound healing ability is of great demand.

### 1.3 Honey and Chitosan; Powerful Wound Dressing Materials

Honey and chitosan, two natural materials that possess a variety of favorable effects and exhibit particular importance in pharmaceutical and medical applications. Chitosan [(1–4)-linked 2-amino-2-deoxy-D-glucopyranose], is a natural polymer that is derived from chitin which is considered as one of the most abundant polysaccharides found in nature (Ohkawa et al., 2004; Prashanth & Tharanathan, 2007; Sun & Li, 2011). Chitosan is a biocompatible, biodegradable polymer with wound healing and antibacterial capabilities (Schiffman & Schauer, 2007), that’s in addition to its ability to promote tissue regeneration and help achieve hemostasis (Zhou et al., 2007). Such properties allowed chitosan to be the ideal polymer in different fields and industrial applications (Schiffman, & Schauer, 2007) as food (Tripathi et al., 2009), paper coatings (Vartiainen et al., 2004), textiles (Lim & Hudson, 2003), ophthalmology (Alonso & Sánchez, 2003), agriculture (Hanshou et al., 2000) as well as different biomedical applications (Jayakumar et al., 2010). Thus, numerous studies have electrospun chitosan into fibers with diameter of ∼100 nm (Zhou et al., 2007). However, the main drawback with such fibers was the inability to electrospin chitosan from its aqueous solution because of its high viscosity in addition to the strong hydrogen bonds forming in 3D networks and thus preventing the movement of the polymeric chains of chitosan under the influence of the electrical field (Homayoni et al., 2009; Zhou et al., 2007). Such a character that forced either the use of toxic solvents to allow spinning of chitosan in considerable high concentrations reaching to 7% (Geng et al., 2005) upon using (90%) concentrated acetic acid, or reaching 3% upon using trifluroacetic acid and dichloromethane (Ohkawa et al., 2004). Residues of such solvents are not favorable especially in applications where totally biocompatible material is required as drug delivery, wound dressings and tissue engineering. To decrease the toxicity of the solvents (Charernsriwilaiwat et al. 2010, Charernsriwilaiwat et al., 2011) prepared chitosan in aqueous salt as CS–hydroxybenzotriazole
PVA and CS–ethylenediaminetetraacetic acid /PVA, however the amount of incorporated chitosan did not exceed 1%.

Another approach for spinning chitosan was through co-spinning with other easily spun polymers in more biocompatible solvents. Among them, the composite Poly (vinyl alcohol)/chitosan has received great scientific interest either alone (Chuang et al., 1999; Paipitak et al., 2011; Zhang et al., 2007) or loaded/mixed with different materials to alter their characters to be suitable for different applications (Liao et al., 2011; Yan et al., 2012). Still such approach did not allow spinning of high chitosan concentrations.

Chitosan nanofibrous mats have demonstrated enhanced antibacterial and wound healing effects via different studies. Chen et al., demonstrated that electrospun chitosan, collagen type I and poly(ethylene oxide) nanofibrous mats exhibited enhanced wound healing activity as compared to traditional dressings (Chen et al., 2008). Spasova et al. utilized chitosan for coating poly(L-lactide) and composite poly L lactic acid/poly(ethylene glycol) nanofibrous mats which resulted in enhanced antibacterial activity against S.aureus and well observed hemostatic ability, thus demonstrating the developed nanofibrous mats as possible wound dressings (Spasova et al., 2008). Chitosan has also been electrospun with silk fibroin and presented a possible candidate for wound dressing due to the demonstrated antibacterial effects and the ability of the developed nanofibrous mat to enhance fibroblast proliferation (Cai et al., 2010). Chitosan is commonly chosen for fabrication of nanofibrous wound dressings because it accelerates the wound healing process via its activation to the polymorphonuclear cells, its hemostatic and antibacterial abilities, in addition to its ability to promote fibroblast proliferation (Li et al., 2012; Chen et al., 2008; Duan et al., 2006).

Honey is another natural material of striking medical importance that has always played an important role in traditional medicine and is now seriously witnessing a revival in modern care medicine.

Honey is a viscous hypersaturated sugar liquid mainly composed of fructose and glucose in addition to other compounds, especially phenolic compounds. Honey is a natural source of micro- and macro-nutrients with profound medicinal and nutritional properties (Khan et al., 2007). Honeys’ composition varies according to the floral source, season as well as other environmental factors. Honey exhibits anti-inflammatory, antioxidant and antimicrobial properties. Moreover, honey has strong wound healing activity (Molan, 2006) this is because of its debriding,
deodorizing, antioxidant, antimicrobial and anti-inflammatory properties (Vandamme et al., 2013). Additionally, honey exhibits acidic nature that provides the optimal environment for fibroblast cell proliferation (Bardy et al., 2008). Currently, the therapeutic protocols undertaken in wound care depend on the use of silver. Such therapeutic protocols are considered useful in limiting the bacterial infections, however, due to the presence of excessive concentrations of ionic silver at the wound site, undesirable side effects have been recorded. Thus, this has initiated a new approach in wound healing that depends on the use of natural antimicrobials in everyday clinical practice. Consequently, despite its traditional daily use, honey is now licensed as a medical device either combined with a sterile dressing or sterile tubes (Dai et al., 2014).

Unfortunately, electrospinning natural materials like honey is not possible as the process will result in either electrospraying at low concentrations or spinneret occlusion at high concentrations of the electrospun materials (Lin et al., 2013). Such natural materials are only electrospun into nanofibers when they are blended with other polymers.

In 2013, Maleki et al. were able to fabricate honey/polyvinyl alcohol nanofibers. Unfortunately, the maximum concentration that could be incorporated within the electrospun nanofibers was 2.25% honey of the total weight of the nanofibrous mat (Maleki et al., 2013). Recently, Wang and He, worked on fabrication of high honey concentration nanofibers, however, the maximum concentration of included honey was 9% with 10% polyvinyl alcohol of the total weight of the nanofibrous mat (Wang & He, 2013). This is because of the decreased viscosity of the honey/PVA solution where increasing the concentration of honey results in remarkable decrease in viscosity, making it quite difficult to electrospin because the degree of chain entanglements is not high enough to tolerate the cumbic stretching force that the charged jet is subjected to during electrospinning so results in bead formation and at higher honey concentrations inability for fiber formation (Maleki et al., 2013). Thus, there is a need to fabricate nanofibers composed primarily of high honey concentrations. Such concentrations will maximize the therapeutic and nutritional benefits of honey nanofibrous formulations in smaller dosage forms.
1.4 Poly (Vinyl Alcohol); Biocompatible Synthetic Polymer for Co-Electrospinning.

Poly (vinyl alcohol) (PVA) is an artificial polymer that has been widely used in commercial, food, medical and industrial sectors. PVA is made via the hydrolysis of polyvinyl acetate resulting in a biodegradable, biocompatible polymer (Gaaz et al., 2015). PVA is a semi crystalline, hydrophilic, nontoxic polymer that has acquired increased attention due to its enhanced thermal stability, good resistance to chemicals, cost effectiveness and biocompatibility. Additionally, PVA readily forms a three-dimensional networks in aqueous media due to its hydrophilic nature, thus increasing its swelling capabilities (Supaphol & Chuangchote, 2008).

PVA exhibits the extra advantage of excellent electrospinnability. Through the past few years, extensive literature have studied electrospinning PVA nanofibers and the different parameters that affect the electrospinning process (Zhang et al., 2005).

Due to its advantageous properties PVA has been extensively used in co-spinning natural polymers, synthetic polymers, natural materials and biomolecules. Recently, Li and Yang have fabricated wool keratin /PVA blend nanofibers, which exhibited good interactions and better mechanical properties (Li & Yang, 2014). Similarly, silk fibroin protein and curcumin were made into nanofibers via co-electrospinning with PVA (Lin et al., 2015). Chitosan is a polymer that exhibits difficulty in electrospinning, thus it was electrospun with PVA in different studies in different ratios that affected the properties of the fabricated nanofibrous mats. Alhosseni et al., electrospun (PVA)/chitosan nanofibrous mats that exhibit large pore sizes for its application in nervous tissue engineering and observed that the fabricated mats exhibited the most optimum properties required to meet the main requirements for nerve cell proliferation (Alhosseni et al., 2012).

1.5 Natural Materials for Enhancing the Wound Healing and Antibacterial Properties of the Nanofibrous Wound Dressings.

1.5.1 Natural Plant Extracts: Cleome Droserifolia and Allium Sativum.

Recent research has realized the extent of dependence of the developed world on medicinal plants, where quarter of the prescriptions dispensed in the USA usually contains one or more ingredients
derived from medicinal plants (Lewington, 1993). Plants exhibit a broad spectrum of activities making them useful in the treatment of different kinds of diseases. According to the WHO 80% of the health problems of worlds’ population could be treated by herbal medicinal drugs (Etkin 1981; WHO, 2003).

*Cleome droserifolia* (Forssk.)Del. is a plant belonging to the Cleomaceae family and is plant of striking medical importance and a long history of ethnomedecinal use (Rahman et al., 2004; Muhaidat et al., 2015). Different species of this family are of considerable interest for the human health and nutrition (Jeruto et al., 2008; Gupta & Rao, 2012). *Cleome L.* (Cleomaceae), is a genus of ca. 200 perennial and annual herbs (Simpson, 2010) among them nine species are found in Egypt (Ezzat & Motaal, 2012). *Cleome droserifolia* which is an aromatic herb having orbicular sticky leaves, is the most famous member of the *Cleome* genus in Egypt (Boulos, 1999). *Cleome droserifolia* is traditionally widely used in Egypt by the Bedouins in the treatment of diabetes. The antihyperglycemic effects of the plant has been proven through different studies (El-Khawaga et al., 2010; Abdel-Kawy et al., 2000). *Cleome droserifolia* is also traditionally used for treating rheumatism, scabies and inflammation (Hussein et al., 1994) that’s in addition to its proven antioxidant activity (El-Shenawy & Abdel-Nabi, 2004). The phytochemical studies revealed enrichment of *Cleome droserifolia* with different beneficial compounds including phenolics, flavonoids, terpenoids and alkaloids (Aboushoer et al., 2010; Abdel-Monem, 2012; Jane and Patil, 2012). *Cleome droserifolia* has also demonstrated cytotoxic effects against different cancer cell lines (Ezzat & Motaal, 2012). However, studies regarding Cleome biological activity as well as its phytochemistry are still far from being complete (Muhaidat et al., 2015).

*Cleome droserifolia* has recently been characterized for its antibacterial activity, Muhaidat et al, demonstrated the antibacterial activity of *Cleome droserifolia* oil against a wide range of bacteria (Muhaidat et al., 2015). The wound healing ability of *Cleome droserifolia* has been realized and utilized by the Bedouins who use a decoction of the herb and apply it to the wound site. However, no research studies have yet been conducted to evaluate the wound healing ability of *Cleome droserifolia*.

*Allium sativum* (Garlic) is another medicinal plant that has been extensively explored and linked historically via different societies to its ability to treat different health problems (Durairaj et al., 2009). *Allium sativum* has been used for different medicinal uses since about 5000 years ago, and
was included in Chinese medicine from nearly 3,000 years. Moreover, *Allium sativums*’ medicinal value was recognized by different civilizations as the Greeks, Egyptians, Babylonians and Romans. In 1858, *Allium sativums*’ antibacterial activity was observed by Pasteur, additionally it was utilized as an antiseptic during World War I and II to prevent gangrene (Singh et al., 2008; Londhe, 2014). Allium is a genus that comprises more than 500 species that belong to family liliaceae (Pazyar & Feily, 2011). *Allium sativm* mainly contains enzymes, sulfur-containing compounds, minerals and amino acids. Other constituents as oligosaccharides, selenium, arginine and flavonoids are also found in *Allium sativum* (Aviello et al., 2009). The sulfur compounds found in *Allium sativum* are responsible for a large number of its medicinal properties (Pazyar & Feily, 2011).

*Allium sativum* is currently well known for its use in different medicinal purposes and these include lowering of the blood pressure and cholesterol, prevention and treatment of cardiovascular diseases, antimicrobial activity as well as protective agent from cancer. (Cutler & Wilson, 2004; Tsao & Yin, 2001; Bakri & Douglas, 2005; Iwalokun et al., 2004). Such therapeutic effect of *Allium sativum* is mainly attributed to the water soluble and oil soluble organosulfur compounds.

*Allium sativums*’ antimicrobial activity has been addressed extensively in literature against wide range of bacteria (Benkeblia, 2004; Ankri & Mirelman, 1999; Cellini, 1996; Ekwenye & Elegalam, 2005). Such antibacterial activity is essentially important in wound healing due to the harmful effects of bacterial infection on the wound environment leading to delay in the process of wound healing.

Despite the huge literature on *Allium sativums*’ different medicinal effects, its role as a topical treatment in wound healing was just recently investigated. In 2006, Sidik et al, have examined the effect of aqueous extracts of Allium and honey on the wound healing rate in rats. Their results demonstrated the enhanced wound healing rates upon application of the Allium and honey combination as compared to the use of honey only, thus elucidating the effect of *Allium sativum* on enhancing the wound healing rate. According to our knowledge this was the first investigation on the effect of *Allium sativum* on the wound healing process (Sidik et al., 2006). The effect of the concentration of the *Allium sativum* aqueous extract on the wound healing process was investigated via examination of the wound closure rate and subsequent histological examination of the wounds. The results demonstrated that 10% extract allowed enhanced wound healing rate compared to 5%
extract and the positive control solcoseryl jelly, whereas, the histopathological examination revealed that the 10% extract and the solcoseryl jelly allowed enhanced tissue regeneration (Rokik et al., 2009). Aged *Allium sativum* extract was also investigated for the mechanism of its wound healing activity on skin wound on chicken and it was observed that *Allium sativum* increases the re-epithelialization process and allows for a profuse dose-dependent neovascularization (Ejaz et al., 2009). Thus, scientific evidence is introducing *Allium sativum* as a new frontier in wound healing agents which exhibits enhanced wound healing ability in addition to well observed antibacterial ability against different kinds of bacteria.

It was observed that none of *Cleome droserifolia* and *Allium sativum*, have been co-spun into nanofibers. And with the enhanced medicinal properties of both materials such an approach should be investigated in different medicinal applications.

### 1.5.2 Apitherapeutics: Bee venom and Propolis.

Apitherapy involves the use of different bee products in the treatment of different human diseases. Apitherapeutics include honey, royal jelly, pollen, propolis, beeswax and bee venom. The use of apitherapeutics in human treatment dates back to thousands of years ago, and it was mentioned in different religious texts as the bible and the Quran, as well as several historical medical documents as the papyrus of Ebers (1550 BC) (Gupta et al., 2014). Propolis and bee venom stand as two of the most important apitherapeutics that have been used in the treatment of different diseases in traditional medicine and their biomedical value is now documented via different research studies.

Bee venom is produced via the venom glands associating the sting apparatus of both the queens and the workers. Storing of the produced venom occurs in the reservoir of the bee venom and injection of the venom during the stinging process occurs through the sting apparatus (Schmidt & Buchmann., 1999). It was observed that approximately 0.15 – 0.30 mg of bee venom is injected via the bees’ stinger (Schumacher et al., 1989). Bee venom was recognized as being safe for human therapies, where the median lethal dose (LD50) is 2.8mg of venom per Kg of body weight for the adult human (Ali, 2012).

Bee venom contains several pharmacologically and biochemically active compounds, with some of them well studied and their mechanism of action elucidated (Bellik, 2015). Bee venom is mainly a mixture of enzymes, peptides and amines. It was observed that bee venom stimulated the immune
system (Ali, 2012; Ram et al., 2014). Melittin represents the major component of beevenom and was found to block neutrophil superoxide production (Somerfield et al., 1986) and suppress the inflammation via inhibition of the activity of the phospholipase enzyme (Saini et al., 1997). Such enzyme that is extensively released in severe inflammatory disorders and was found to cause degradation of tissues and organs (Mihelich & Schevitz, 1999). Bee venom is utilized in the treatment of different diseases as rheumatoid arthritis, lupus, sciatica, arthritis, multiple sclerosis, cancerous tumors, low back pain and others (Ali, 2012; Kwon et al., 2002; Kim et al., 2013). Additionally, bee venom exhibits antimicrobial, analgesic and antioxidant effects, thus suggesting that it could be a successful candidate for enhancement of the wound healing process (Khatun & Mukhopadhyay, 2013). Amin et al., have observed accelerated wound healing rates due to application of chitosan blend film containing bee venom on wounds which was related to the anti-inflammatory activity of bee venom (Amin et al., 2008). Similar results were observed via Han et al., who compared the wound healing process in full thickness wounds after application of bee venom cream and the commercial silver sulfadiazine. The results demonstrated that the bee venom cream exhibited wound healing rates similar to that of the commercial silver sulfadiazine, moreover, the bee venom cream demonstrated enhanced anti-inflammatory effect (Han et al 2012). The wound healing effects of bee venom was also extended to diabetic foot infections. A hydrogel based on poly(vinyl alcohol) and chitosan and loaded with bee venom was tested on diabetic rats and the results showed accelerated rate of wound healing as compared to the negative control and similar anti-inflammatory effect to diclofenac gel (Amin & Abdel-Raheem, 2014). Despite the favorable effects observed for bee venom on the wound healing process, developing combination nanofibrous wound dressings loaded with bee venom was not yet explored.

Propolis is the generic name of a combination of resinous materials collected by the honey bees from plants, exudates and buds, then mixed with the bee enzymes and bee wax. The term propolis was coined by Aristotle from the Greek words pro meaning before and polis meaning city, which refers to before the city or defender of the city (Abu-Seida, 2015). Propolis is normally used by the bees to protect the bee hive via coating the inner walls, which provides a shelter against rain and wind and against the entrance of different intruders as insects, lizards and snakes and also prevents the bacterial and fungal growth. The amount of propolis collected via a bees’s colony in one year ranges from 150 to 200 g of propolis (Martinotti & Ranzato, 2015). The major components of propolis include 50% resin, 30% bee wax, 10% aromatic and essential oils. 5%
pollen and 5% other substances (Burdock, 1998). With the realization of advanced identification techniques as high-performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectroscopy (GC-MS) numerous compounds have been characterized in propolis (Huang et al., 2014) including aromatic and aliphatic acids, esters, aldehydes, ketones, carbohydrates, vitamins and others. Among the different compounds found in propolis flavonoids stand as one of the most important compounds that possess increasing research interest (Marcucci, 1995). However, the composition of propolis varies according to the phyto-geographic character of the surroundings of the bee hive (Alves de Souza, 2013).

Propolis exhibits different biological activities with respect to the human body that allowed it to be useful in the treatment of different diseases. Propolis is considered non-toxic to humans with safe concentration of approximately 1.4 mg/kg day or 70 mg/day (Wagh, 2013) Propolis content of polyphenols allowed it to exhibit strong antioxidant activity (Gulcin, 2012; Olczyk et al., 2013; Castaldo, 2002) as well as anti-inflammatory activity in both acute and chronic inflammatory processes (Kuropatnicki et al., 2013; Sawicka et al., 2012). Propolis demonstrated strong ability to inhibit the development of different kinds of cancer (Xuan et al., 2014; Szliszka et al., 2011). Moreover, anti-hyperglycemic effect of propolis was observed in different studies (Matsui et al., 2004; Fuliang et al., 2005). The antibacterial, antiviral, antifungal effects of propolis have also been documented (Tosi et al., 1996; Grange & Davey, 1990; Marcucci et al., 2001). Propolis also exhibits antiseptic, astringent, spasmolytic, anesthetic, antiulcer, and immunomodulatory effects (Kuropatnicki et al., 2013).

Propolis has also demonstrated strong wound healing abilities where it allows a favorable environment for re-epithelization (Olczyk et al., 2014) it enhances skin cell proliferation (Sehn et al., 2009) and it was recently observed to be able to quench free radicals, thus allowing its safe application in burn treatment (Olczyk et al., 2013). Propolis was also observed to speed up the repair of burned tissue via stimulating the remodeling of the wound bed matrix, which could be linked to propolis flavonoid content which reduces lipid peroxidation and prevents cell necrosis (Olczyk et al., 2014). Additionally, invitro immunomodulatory and immunostimulatory effects on macrophages has been observed (Toreti et al., 2013). Another important property of propolis that enhances the wound healing process is its antibacterial activity. Different studies revealed the strong antibacterial activity of propolis, especially against Gram positive bacterial strains (Grange
& Davey, 1990) Such antibacterial activities could be due to the synergistic activities of the different compounds found in propolis the most important of which is propolis flavonoid content (Abu-Seida, 2015). Thus, propolis role in enhancing the wound healing process could be attributed to its anti-inflammatory, antimicrobial and immunomodulatory properties (Castaldo., 2002). McLennan et al, demonstrated that a single topical application of propolis increases the wound healing rate of a full thickness cutaneous wound in a diabetic rodent model (McLennan et al., 2008). Abu-Seida et al, observed enhanced healing of full thickness skin wound in dogs after application of propolis (Abu-Seida, 2015).

Recently, propolis was electrospun into polymeric nanofibers via electrospinning polyurethane and propolis solution, and the results demonstrated that the inclusion of propolis within the nanofibrous mats allowed enhanced cell compatibility and increased hydrophilicity and antibacterial activities (Kim et al., 2014). Similarly, Sutjarittangtham et al fabricated nanofibers of polycaprolactone and ethanolic extracts of propolis via electrospinning (Sutjarittangtham et al., 2012). Propolis was also electrospun with polyactic acid forming nanofibers that exhibited bactericidal activity (Sutjarittangtham et al., 2014). Recently, Adomavičiūtė et al., combined propolis and silver nanoparticles in poly (vinyl pyrrolidone) nanofibers and demonstrated the enhanced antibacterial activities of the developed nanofibers against a wide range of bacteria (Adomavičiūtė et al., 2016). This illustrates that propolis has been electrospun into nanofibers with different polymers, however none of the developed nanofibers were tested for their wound healing activities despite the previously reported enhanced effect of propolis on wound healing.

1.5.3 Bacteriophages; Viruses Killing Bacteria

Bacteriophages (phages), (Figure 5) are viruses that infect and rapidly destroy bacteria (Brüssow & Hendrix, 2002). Phages are considered as the most abundant microorganisms with an average number of $10^{30}$-$10^{32}$ particles of bacteriophages. Humans are continuously exposed to phages via unprocessed food and water. Additionally, phages are found in the saliva, dental plaque and the intestinal tract. One milliliter of unpolluted water contains about $2 \times 10^8$ bacteriophage particles (Endersen et al., 2014).

Felix d’Herelle introduced the term bacteriophage in 1917 and was the first to examine phage therapy. d’Herelle used phages against infections of livestock and even examined phages’
antibacterial therapeutic efficiency on himself. It was then noticed that Twort described the same phenomenon in 1915 and also Hankin in 1896 against *Vibrio cholerae* in the Ganges River (Twort, 1915). In the 1920s, d’Hérelle utilized bacteriophages to fight different bacterial infections introducing new discipline that was defined as “phage therapy”.

Phage therapy was utilized in different countries as the major antibacterial. Additionally, different pharmaceutical companies including E.R. Squibb & Sons (Princeton, NJ, USA), Swan-Myers/Abbott laboratories, and Eli Lilly (Indianapolis, IN, USA) produced different bacteriophage commercial preparations (Monk et al., 2010). However, due to the poor understanding of phage properties in addition to the limited knowledge about different human diseases, variable outcomes were associated with phage therapy and thus their benefit was questioned via many specialists. It was in the 1940s when the utilization of bacteriophage ceased especially in Western countries with the introduction of the miracle antibiotics (Monk et al., 2010). However, with the alarming and continuous rise in bacterial resistance during the last two decades, together with the decrease in the introduction of new antibiotics, interest in phage therapy has been revived. An interest that has resulted in a number of companies in different countries for phage based products in food, diagnostics, agriculture, and therapeutics.

Bacteriophages were proved efficient antibacterials through numerous studies and against different kinds of bacteria, they were also proven to be more efficient than potent antibiotics in the market as vancomycin, linzolid, ampicillins and trimethoprim (Smith & Huggins, 1982; Smith & Huggins, 1983; Smith et al., 1987; Chibani-Chennoufi et al., 2004; Soothill, 1992). Additionally, the studies revealed that phages therapeutic efficiency is extended also against resistant bacterial strains as vancomycin-resistant *Enterococcus faecium* strain (Biswas et al., 2002) and methicillin- resistant *S. aureus* (MRSA) (Matsuzaki et al., 2005).

Bacteriophages were proved effective in enhancing the wound healing process via their ability to treat persistent bacterial infections in chronic wounds. Mendes et al., observed that the application of a topical bacteriophage cocktail to a wound in a diabetic animal model resulted in noticeable decrease in bacterial count and subsequent enhancement in the wound healing process (Mendes et al., 2013). Similar results were observed upon application of bacteriophages for the treatment of pseudomonas infections in burn wounds (Soothill, 1994; Ahmad, 2002). Bacteriophages were also efficient in eradication of bacterial biofilms in wound (Seth et al., 2013), this is considered very
important as biofilms represent communities of surface associated bacteria that are enveloped in a hydrated extracellular matrix. Thus, they are considered as a source of persistent infection to the wound site particularly because of their resistance to conventional antibiotics.

Due to the increased role of bacteriophages in human life there is a need for better storage techniques that will enable more stable and effective formulations for bacteriophage storage and transportation. Currently, freeze drying represents the most effective method for long term storage of bacteriophages (Miyamoto-Shinohara et al., 2000). However, freeze drying was found to be expensive and time consuming (Dai et al., 2014). Electrospinning of bio-composite nanofibers was utilized as a novel technique for storing bacteriophages (Korehei & Kadla, 2013; Salalha et al., 2006; Lee & Belcher, 2004). However, none of the electrospun bio-composite nanofibers loaded with bacteriophage was further tested in different biomedical applications.

Despite the promising research results of bacteriophages and despite the approval of the FDA for the utilization of phage in food preservation, different challenges are still facing the regulatory approval of bacteriophage based therapeutics. Among these challenges bacteriophages narrow host range stands as an important challenge that calls for innovative solutions. Each phage strain infects only one bacterial type (Sulakvelidze, 2011). In most situations cocktails of bacteriophages are utilized to broaden the host range and enhance the therapeutic efficiency. Nevertheless, approval of phage cocktails via the regulatory bodies is problematic, where they are more likely to approve a single phage strain thus other methods have also to be developed.

Thus, there is a need to develop broad spectrum bacteriophage formulations that are not based on phage cocktails. Within this context developing a broad spectrum bacteriophage wound dressing will satisfy an urgent need of developing bacteriophage preparations that could achieve approval of different regulatory bodies.
2. AIM & OBJECTIVES

The aim of this study is to develop biocompatible nanofibrous antimicrobial wound dressing based on natural materials and to load the developed nanofibrous wound dressing with different natural materials (natural extracts, apitherapeutics and bacteriophages) for the aim of achieving enhanced biocompatibility and wound healing activity as well as enhanced broad spectrum antimicrobial activity as compared to existing antimicrobial wound dressings. The objectives of the project are as follows:

1- Electrospinning of uniform nanofibers based on high concentrations of honey and chitosan via biocompatible solvents. Evaluation of the effect of changing the honey concentration on the properties of the developed nanofibers.

2- Fabrication of electrospun honey/chitosan based nanofibrous mats loaded with natural extracts to enhance the wound healing and antibacterial properties of the developed nanofibers.

3- Fabrication of electrospun honey/chitosan based nanofibrous mats loaded with apitherapeutics to enhance the wound healing and antibacterial properties of the developed nanofibers.

4- According to the results of objectives 2 and 3, one of the fabricated nanofibrous dressings will be further loaded with bacteriophages to achieve enhanced, broad spectrum antibacterial activity. At the same time, this will allow developing a broad spectrum bacteriophage formulation as a solution to one of the challenges of phage based therapeutics.
3. MATERIALS AND METHODS

3.1 Materials

Chitosan (Mw, 240 kDa and DDA of 84%, Chitoclear, cg110, TM 3728) was purchased from Primex, Siglufjordur, Iceland. Fresh bulbs of Allium sativum (AE) were purchased from a local vendor. Cleome droserifolia (CE) were collected from the mountains of Sinai, Egypt. Poly (vinyl alcohol) (85,000 Da Mwt), ethanol (absolute, ≥99.8%), gluteraldehyde (25% in H₂O) and Polyethylene glycol (6000 Da Mwt) were purchased from Sigma Aldrich (St. Louis, USA). Acetic acid (glacial, 99-100% purity) was supplied from Merck (Wadeville, South Africa). Muller Hinton broth, Nutrient broth, Nutrient agar, Luria-Bertani broth and agar-agar, were supplied from Oxoid (Basingstocke, UK). Aquacel ® Ag (ConvaTec Inc) was purchased from local pharmacy in Egypt. Fetal bovine serum (FBS), thiazolyl blue tetrazoliumbromide–MTT (M2128-1G), Phosphate buffered saline (PBS), Dulbecco’s modified eagle medium (DMEM) and triton X were supplied from Sigma Aldrich (St. Louis, USA). Honey (clover) (H) [viscosity: 15,300 mPas, total soluble solid content: 81%], Bee venom (BV) [amino acid content (histidine; 12.6%, alanine; 7.9%, cysteine; 6.44%, glutamic; 3.81%, tyrosine; 3.28%, valine; 3.02%, leucine; 2.87%, and methionine; 2.69%) protein and peptide content (Milittin; 51.6%, phospholipase A2; 15.4%, mastocyte degranulating peptide; 3.5%, apamin; 3.3%, minimine; 3.2%, hyaluronidase; 2.5%, adolopin; 1.6% and unkowns; 18.9)(Ahmed, 2006)] and propolis (Pr) [Total flavonone and di hydroxyl flavonal; 1.330 ± 0.140, total flavones and flavonals; 3.200 ± 0.162, total phenolic content 30.847 ± 0.064, Phenolic content (mg/100g) (phenol; 37.57, parahydroxy benzoic acid 9.18, p.coumaric acid; 1.25, chrys; 67.03, galangin; 70.13, daidzin; 42.97, acacetin; 48.32) total insoluble content (41.03 ± 0.16) and volatile substances content (4.10 ± 0.10) (Aly, 2012)] were provided from the faculty of Agriculture, Cairo University (Egypt).

3.2 Methods

3.2.1 Preparation of Aqueous Extracts of Allium sativum (AE), Cleome droserifolia (CE), and propolis (Pr).

Bulbs of fresh AE were extracted according to the method of Al-Astal (Al-Astal, 2003). Fresh bulbs of AE (10 g) were peeled, washed with distilled water several times, then the AE was homogenized aseptically using a sterile mortar and a pestle. Subsequently, a Whatman No. 1 paper
was used to filter the homogenized mixture. The obtained filtrate was then directly used in the preparation of the electrospinning solutions. On the other hand, dried leaves of CE were extracted according to established protocols (Ezzat & Motaal, 2012). The air dried aerial parts of CE were powdered and extracted via boiling in distilled water for two minutes and then allowed to stand for ten minutes before filtration. Whatman No. 1 paper was used to filter the boiled mixture. Subsequently, a rotary evaporator was used to remove the water and the remaining extract was dried in a vacuum oven at 40°C (Jeiotech, OV-11, South Korea) until a dry powder of CE extract was obtained. The powder was weighed and stored until further use.

Aqueous extracts of propolis were prepared via covering propolis (500 g) with 1 liter of 20% aqueous ethanol solution as the solvent and placed in an amber glass container. The mixture was allowed to stand at room temperature for two weeks with periodic agitation. Subsequently, the mixture was filtered via Whatman No. 1 filter paper. Dry propolis powder was achieved via incubating the filtrate at 70 °C. The dry powder achieved represents the water soluble propolis utilized in this study (Sosnowski, 1983).

3.2.2 Preparation of the electrospinning solutions

Different solutions were prepared with the aim of incorporation of the highest honey and chitosan concentrations using biocompatible solvents. Different weight ratios of PCS and HP as well as HPCS were prepared as follows: PCS (7%:1.5%, 7%:2.5% and 7%:3.5%); HP (20%:10% and 30%:10%), and HPCS (30%:7%:1.5%, 30%:7%:3.5%, 30%:5%:5.5%, 30%:5%:4.5%, 20%:7%:3.5%, and 40%:7%:3.5%). Solutions were prepared in 1% acetic acid. The HPCS solutions incorporating high honey and chitosan concentrations exhibited very high viscosity at the time of preparation thus they were aged at room temperature for different time intervals. Subsequently, HPCS solutions with different honey concentrations were prepared for studying the effect of changing the honey concentration on the prepared nanofibers. The solutions were prepared using the following weight% ratios; (10:7:3.5), (20:7:3.5), and (30:7:3.5) of honey, poly(vinyl alcohol), and chitosan, respectively dissolved in 1% acetic acid. Then, the as-prepared solutions were allowed to age at room temperature.

For enhancing the antibacterial and wound healing activities of the fabricated nanofibers, natural extracts, namely *Allium sativum* (AE) and *Cleome droserifolia* (CE) and apitherapeutics, namely bee venom and propolis were loaded within HPCS nanofibers.
Blend solutions of honey/poly (vinyl alcohol)/ chitosan/bee venom (HPCS-BV) and honey/poly (vinyl alcohol)/ chitosan/ aqueous propolis extract (HPCS-Pr) were prepared in the following concentrations weight % ratios (30:7:3.5:0.01) and (30:7:3.5:10) respectively. The HPCS-BV and HPCS-Pr blend solutions were prepared in 1% acetic acid. Solutions were allowed to age at room temperature.

Various blend solutions of honey/poly (vinyl alcohol)/ chitosan/ *Allium sativum* extract (HPCS-AE), honey/poly (vinyl alcohol)/ chitosan/ *Cleome droserifolia* extract (HPCS-CE), and honey/poly (vinyl alcohol)/ chitosan/*Allium sativum* extract/*Cleome droserifolia* extract (HPCS-AE/CE) were prepared. In the preparation of the blend solution of (HPCS-AE), AE was used as 50% of the solvent to which honey (30 w/v), chitosan (3.5 w/v) and poly (vinyl alcohol) (7 w/v) were dissolved. Both the blend solutions of HPCS and HPCS-AE were prepared in 1% of aqueous acetic acid. Both solutions were allowed to age at room temperature for 1 week. *Cleome droserifolia* dry powder extract (CE) (10 w/v) was added to both the as-prepared HPCS and HPCS-AE blend solutions before electrospinning and stirred for 1h to form the blend solutions of HPCS-CE (30:7:3.5:10 w%) and HPCS-AE/CE(30:7:3.5:10 w%) in 50% AE as the solvent, respectively. During preparation of all blend solutions, poly(vinyl alcohol) was dissolved separately in half the volume of the solvent at 100°C with stirring followed by addition of the remaining volume of the solvent with the other constituents to the cooled solutions to avoid any degradation of the active constituents due to exposure to elevated temperatures.

### 3.2.3 Viscosity measurements.

The viscosity of the poly(vinyl alcohol) (7%), PCS (7%:3.5%), HP (30%:7%), and HPCS (30%:7%:3.5% and 10%:7%:3.5%) samples were determined. The aqueous AE extract replaced 50% of the solvent of the HPCS blend solution, thus, its effect on the viscosity of the blend solution had to be examined. The viscosity of the HPCS-AE (30%:7%:3.5%:50%) blend solution was determined using a viscometer (Myr; VR-3000, Viscotech Hispania, Tarragona, Spain). The solutions were aged for a week and the viscosity was determined at different time intervals (0, 24, 48 h and 1 week). The average value of three measurements was reported as mean ± SD.
3.2.4 Electrospinning of the as-prepared solutions

The as-prepared solutions were loaded in a 5ml plastic syringe attached to a stainless steel needle (22 gauge) as the nozzle for electrospinning (E-spin, NanoTech, Kalyan-pur, India). Two electrospiners were utilized (NANON-O1A, MECC, Japan & E-spin, NanoTech, Kalyanpur, India). The nozzle was connected at a high electric potential and the distance between the nozzle and the collector, the flow rate and the voltage that allowed the most uniform nanofiber deposition were selected. A ground collector wrapped with aluminum foil and cotton gauze were used for collection of the samples.

3.2.5 Cross-linking of fiber mats

Physical and chemical methods were used to crosslink the nanofibrous mats of HPCS. Glutaraldehyde (GA) was used for chemical crosslinking. The fiber mats were placed in a closed desiccator that was saturated with GA vapors (40 ml). Exposure of the nanofibrous mats to the GA vapors was done for different time intervals (30, 60, 120 and 180 min as well as 48 h and 72 h). Subsequently, enhancement of the crosslinking reaction and removal of unreacted (GA) were done via heating the nanofibrous mats in an oven under vacuum at 40°C for 24 h. Physical crosslinking was performed by freezing/thawing and heating techniques. Freezing and thawing was performed via freezing the fiber mats for 15 min in liquid nitrogen followed by thawing at room temperature for 15 min for three successive cycles. Heating was carried out under vacuum in an oven (Jeiotech, OV-11, South Korea) at both 110°C, 100°C for 15 min and 80°C for 25 min as well as at 70°C for 24 h.

3.2.6 Characterization and measurements

The morphologies of the electrospun nanofibers were observed using scanning electron microscopy (FESEM, Leo Supra 55, Zeiss Inc., Oberkochen, Germany) and transmission electron microscopy (Jeol, Musashino, Akishima, Tokyo, Japan). Image-J software was used for measurement of the diameters of the collected nanofibers. From three different images 100 fibers were measured for each of the developed nanofibrous mats. Subsequently, the average diameter and diameter distribution were determined. Fourier transform infrared spectroscopy (FTIR) was performed for the raw poly(vinyl alcohol) and chitosan and the HPCS nanofibrous mats (30%:7%:3.5%) using FTIR (Thermoscientific, Nicolet 380, USA). The transmission mode with KBr pellets was used for bulk chitosan and poly(vinyl alcohol) as well as and HPCS nanofibrous
mats. The X-ray diffraction patterns of the HPCS nanofibers (30%:7%:3.5%) with increasing honey concentrations (10%H, 20%H & 30% H) were obtained using an XRD diffractometer (Bruker 4040, Karlsruhe, Germany) with a wavelength, λ=0.154 nm at 40 kV, 150 mA, and at a scan speed of 4° per minute in the 2θ range of 5°–80°. Moreover, thermogravimetric analysis of the nanofibers was performed with a TGA analyzer (TGA Q50, TA Instruments). Samples were heated in a platinum pan under nitrogen atmosphere (60ml/min) up to 700 ºC, at a heating rate of 10ºC/min. The stability of the nanofibrous structure was evaluated via SEM examination of the morphology of the crosslinked and non-crosslinked samples after storage for 1 year on shelf. Crosslinking of the stored samples was performed via exposure of the nanofibers to GA vapours for 1h and 3h followed by heating at 40ºC under vacuum.

3.2.7 Evaluation of swelling and weight loss capabilities of the HPCS nanofibers
The swelling and weight loss abilities of the developed HPCS nanofibrous mats with increasing honey concentrations; 10:7:3.5, 20:7:3.5, and 30:7:3.5 (W%) as well as the HPCS nanofibers loaded with the natural extracts and apitherapeutics were evaluated. The mats were placed in phosphate buffered saline, PBS of a pH 7.4 at 37°C. The following relationships were used for determination of the swelling ability of the nanofibrous mats at 1, 4 and 24 h, and their weight loss at 24 h:

\[
\text{Degree of swelling} \, \% = \left[ M - M_i / M_i \right] \times 100 \quad (1)
\]

\[
\text{Weight loss} \, \% = \left[ M_i - M_d / M_i \right] \times 100 \quad (2)
\]

Where M is the weight of the swollen nanofibrous mats after plotting their surface with filter paper, \( M_d \) is the weight of the dried nanofibrous mats after being removed from the phosphate buffer saline. The swollen nanofibrous mats were dried in an oven at 40ºC until constant weight was achieved. \( M_i \) is the initial dry weight of the electrospun nanofibrous mats.

3.2.8 Evaluation of the Antibacterial activity
The viable cell count technique was used for the evaluation of the antibacterial properties of the developed nanofibrous mats. The collected nanofibrous mats were sterilized via exposure to the UV for 20 minutes except for the bacteriophage loaded nanofibers that were aseptically collected and stored. Both of the developed nanofibrous mats and the Aquacel Ag (0.05 gm) were added to 3ml sterile Muller Hinton broth. Subsequently an overnight bacterial suspension (30 ul) from each
of the tested bacteria that was adjusted to 0.5 McFarland standard (1x 10^8 c.f.u./ml) was added to them. The tubes and a negative control were incubated at 37°C with agitation at 100 rpm for 24 h. Following the 2h incubation 10 ul from each treated bacterial suspension as well as the positive and negative controls were subjected to serial dilution. From every dilution 50 ul were added to nutrient agar plates and incubated for 24 h at 37°C. The surviving colonies on the nutrient agar plates were then recorded in plates that allowed counting from 10 to 150 CFU. The experiment was repeated three times and the mean value of CFU was determined (Gallant-Behm et al., 2005). Aquacel Ag (ConvaTec Inc) was evaluated for its antibacterial activity and utilized as the positive control.

The antibacterial activity of the developed HPCS nanofibers with different honey concentrations were evaluated against *Escherichia coli, Staphylococcus aureus* at two different bacterial counts (1x 10^8 cfu/ml & 1x 10^7 cfu/ml). Upon loading the HPCS nanofibers with natural extracts, apitherapeutics and bacteriophages, the antibacterial activity was evaluated against *Escherichia coli, Staphylococcus aureus*, and resistant bacterial strains: Methicillin resistant *Staphylococcus aureus* (MRSA) and Multi drug resistant (MDR) *Pseudomonas aeruginosa* at the bacterial count of (1x 10^8 cfu/ml).

### 3.2.9 In vivo wound healing studies

The wound healing abilities of the developed nanofibrous mats were evaluated invivo on male mice weighing 25g. All animals were anaesthetized with a mixture of ketamin HCl (50mg/kg) and xylene HCl (20mg/kg) and their backs shaved followed by creating a 9 mm wound on the back of every mice using a biopsy puncher. The tested nanofibrous mats were UV sterilized for 20 min before placing them on the wound site except for the bacteriophage loaded nanofibers that were aseptically collected. Aquacel Ag (ConvaTec Inc) was utilized as a positive control, whereas untreated wounds covered with a cotton gauze were utilized as a negative control. The change in the wound size was evaluated at 3, 5, 7, 10 and 12 days. The wound area (%) that remained exposed represented the ability of wound healing for each of the examined samples. Three mice were evaluated for each of sample and the controls and the mean value for three measurements was recorded.

\[
\text{Wound area (\%)} = \left[ \frac{W (3, 5, 7, 10, 12)}{W (0)} \right] \times 100
\]

*Where W (0) and W (3, 5, 7, 10, 12) represents the exposed wound areas of the wounds on days 0 and 3, 5, 7, 10 and 12 respectively.*
3.2. 10 Histological examination and the scoring system used for the histologic outcomes

The wound site and the surrounding skin and muscle were cut and fixed with buffered formalin (10%) then the samples were put in paraffin followed by sectioning. Five mice were treated with each sample and the positive control as well as five mice untreated. Tissue samples were taken from each mice at each time interval. The collected samples were subsequently stained via Hematoxylin and Eosin (H&E) staining and Masson’s Trichrome staining (MT). Samples collected on days 3, 5, 7, 10 and 12 were subjected to H&E staining whereas the MT staining was performed for samples collected on day 10. The stained tissue sections were evaluated according to the following histological outcomes: necrosis, hemorrhage, granulation tissue, amount of inflammatory infiltrates, epithelization and thickness of the epidermis and collagen deposition. A histologic scoring system was utilized to evaluate every parameter and a 0-3 score was assigned for every sample. Necrosis, inflammatory infiltrates, hemorrhage, epithelization, epidermis thickness and collagen deposition were graded as 0 (none), 1 (scant), 2 (moderate) and 3 (abundant). Inflammation severity was scored as follows: 0 – (no inflammatory cells) no inflammation; 1 – (scant inflammatory cells) 2 – (moderate inflammatory cells) 3 – (abundant inflammatory cells). The maturation of the granulation tissue was graded as: 0 (immature), 1 (mild maturation), 2 (matured), 3 (fully matured with collagen deposition). Collagen distribution (based on the distribution of the collagen fibers in the microscopic fields) was graded as: 0 (no collagen distributed), 1 (non-uniform distribution), 2 (mild uniformity in distribution), 3 (uniform distribution) (Xie et al., 2013)

3.2.11 Cell viability assay

The developed nanofibrous mats as well as the commercial dressing Aquacel Ag as a positive control were evaluated for their cytotoxicity. The nanofibrous samples were sterilized for 30 min using the UV and then washed and soaked in PBS solution. The extract solutions of the tested samples were then filtered via sterile disposable filters (0.20 mm, Merck, Darmstadt, Germany). DMEM media was then used to make several dilutions of the extract (0, 25, 50 and 100%). Human fibroblast cells (HFD4, ATCC; crl-2522) (1x10^4 cells per well) were incubated for 24 h in a 96 well plate. The different dilutions of the extract solutions were added to the plate followed by incubation at 37°C in a CO₂ incubator. After 24h, the human fibroblast cells were incubated with
the extract solution for 48 h. Subsequently, 20 μl of the MTT [MTT: 3-(4, 5-dimethylthiazol-2-
yl)-2, 5-diphenyltetrazolium bromide)] solution was added to every well and incubated for 4h. To
evaluate the viability of the cells, the formazan crystals formed were dissolved in 200 μl DMSO
and the optical density was recorded (595 nm). The results of the examined samples and the
positive control were compared to those of an untreated control (Son et al., 2016).

3.2.12 Cell proliferation.
The effect of the developed nanofibrous mats as well as Aquacel Ag (positive control) on cell
proliferation ability was evaluated. The human fibroblast cells (HFD4, ATCC; crl-2522)
(1x10^4 cells/well) were seeded on the examined samples followed by incubation for 1 and 3 days.
At every time point the examined samples were taken from the original plate to another 24
culture plate containing 1 ml fresh media and 100 μl MTT solution per well and then incubated
for 4h. The formed dark blue formazan crystals were dissolved in DMSO and the optical density
was measured at wave length 595 nm (Son et al., 2016).

3.2.13 Bacteriophage isolation, purification and characterization
According to the previous antibacterial evaluation one of the examined bacteria was selected and
used for enriching and isolating a virulent bacteriophage. Briefly, sewage samples were collected
from different Egyptian hospitals and used for bacteriophage isolation. Samples (5 gm) were
suspended in Luria-Bertani broth (30 ml) and 30 ul from an overnight culture of the selected
bacteria was added and incubated with the mixture for 6h at 35°C with constant shaking in order
to enrich the bacteria specific bacteriophage. Subsequently, choloroform drops were added to the
mixture that was allowed to stand for 15 min and then filtered via Whatman No.1 filter paper to
remove any solid particles. Bacterial cells and debris were removed via centrifugation of the filtrate
for 5 min at 11,000 g. For amplification of the isolated bacteriophage, polyethylene glycol 6000
(PEG 6000) (10%) and sodium chloride (1M) were put on the supernatant followed by incubation
of the solution overnight at 4 °C and then centrifugation at 11,000 g for 20 min. The pellet was
dissolved in PBS (1ml) and then filtered via 0.22 μm filter for removal of the residual bacterial
cells. Phage plaque assay was performed via mixing the amplified bacteriophage solution with
exponential growth culture of the selected bacteria and allowing them to stand for 15 mins and
then plating them with semi-solid agar medium (0.6%) followed by incubation at 35 °C for 4h.
From the resulting plates a single plaque was selected and used for purification and amplification.
(Stenholm et al., 2008; Carey-Smith et al., 2006). This resulted in the collection of a concentrated phage stock solution ($10^9$-$10^{10}$ PFU mL $^{-1}$)

For characterization of the isolated bacteriophage, an aliquot of the amplified phage suspension was put on a copper EM grid (400 mesh size) having a nitrocellulose surface backed with carbon. After 10 s of incubation, the copper grid was blotted using filter paper and 2% uranyl acetate and lead-citrate were used for staining. Subsequently, the copper grid was blotted again and then dried in air. Samples were observed via a Plus Transmission Electron Microscope (Jeol, Musashino, Akishima, Tokyo, Japan).

3. 2.14 Electrospinning and antibacterial evaluation of the bacteriophage loaded nanofibers

According to the antibacterial results of the HPCS nanofibers loaded with natural extracts and apitherapeutics, one of the nanofiber solutions is selected for subsequent loading with the isolated bacteriophage. The bacteriophage stock solution (1ml) was added to the selected nanofiber solution (9 ml) and agitated at 70 rpm for 1h. Subsequently, the mixture was subjected to electrospinning. The applied voltage, flow rate as well as the distance between the needle and the collector were selected based on the values that allowed the most uniform nanofiber deposition. The collected nanofibres were then subjected to antibacterial evaluation together with the nanofibers without the bacteriophage and Aquacel Ag as a positive control. The viable cell count technique was utilized to evaluate the antibacterial activity. The same steps followed in the previous antibacterial evaluation were undertaken.

2.2.15 Statistical Analysis

The results of the quantitative data are presented as mean ± standard deviation (SD). For in vitro experiments, average values were reported from three independently prepared samples. Results were evaluated statistically using students t-test was and a p-value of less than 0.05 was considered significant.
4. RESULTS & DISCUSSION

4.1 Fabrication of Uniform Electrospun Nanofibers Based on High Concentrations of Honey and Chitosan (Sarhan & Azazzy, 2015a).

4.1.1 Preparation of the electrospinning solutions

Solutions of poly(vinyl alcohol)/chitosan (PCS), honey/poly(vinyl alcohol) (HP) and honey/poly(vinyl alcohol)/chitosan (HPCS) were prepared and tested for viscosity at different time intervals as shown in table 1. At zero time, the (HP; 30%:7%) exhibited very low viscosity (175 mPas) and the (PCS; 7%:3.5%) exhibited extremely high viscosity (85,440 mPas) making both solutions impossible to spin. Whereas, the combination of (HPCS; 30%:7%:3.5%) exhibited at day zero 34,000 mPas. However, such viscosity value was still above the optimum viscosity needed for electrospinning. Consequently, the HPCS solutions were allowed to age for one week at room temperature.

Interestingly, it was observed that the viscosity of the HPCS solutions decreased noticeably upon aging. This was unlike the PCS and the HP solutions that demonstrated increased viscosities after aging (Table 1). The decrease in viscosity with time that was observed with the HPCS solutions could be attributed to enzymatic degradation of chitosan by the enzymes that are found in honey. Honey contains small amounts of enzymes, including enzymes that are able to transform polysaccharides into smaller products as amylase. Such enzymes most likely degrade chitosan into its oligosaccharides (Xie et al., 2011). Additionally, hydrogen peroxide, which is a major component of honey, may have contributed to the enzymatic degradation of the chitosan backbone (Brudzynski, 2006). Interestingly, it was observed that increasing the honey concentration within the HPCS mixtures allowed further reduction in the viscosity of the solutions as demonstrated in table 1.

4.1.2 Electrospinning and characterization of the morphology and functional groups of the developed nanofibers.
Different concentrations of the PCS, HP and HPCS were electrospun (E-spin, NanoTech, Kalyanpur, India). For the PCS combinations, the highest concentration of chitosan that could be electrospun with polyvinyl alcohol using 1% acetic acid, was 1.5% at 20 kV, 10ul/min as the flow rate and 15 cm distance between the collector and the needle. On the other hand, for the combinations of HP, the highest honey concentration that could be electrospun with polyvinyl alcohol was 20% honey (Figure 6a) at 22 kV, 10ul/min as the flow rate and 15 cm distance between the collector and the needle. However, clusters were observed within the electrospun nanofibers, which are most probably honey clusters that could not be included within the HP nanofibers. Remarkably, upon addition of 3.5% chitosan to the same combination of HP, uniform nanofibers were achieved (Figure 6b). This is attributed to the favorable effect of chitosan on solution viscosity, allowing it to reach to the optimum degree required for chain entanglements needed to form uniform nanofibers. However, upon increasing the concentration of honey to 30% within the HP combination, the clusters of honey increased extensively even after changing the parameters of electrospinning (Figure 6c). This indicates that the poly(vinyl alcohol) polymer is incapable of incorporating higher honey concentrations even at increased concentrations of poly(vinyl alcohol), where the reduction in viscosity imparted by honey on the HP combinations could not be overcome by increasing the poly(vinyl alcohol) concentration. On the other hand, increasing the concentration of chitosan within the PCS combinations resulted in extremely viscous solution that was impossible to electrospin (Table 1). Interestingly, aging the combination of HPCS (30%:7%:3.5%) for more than 2 days allowed it to reach to the optimum viscosity required for easy electrospinning and collection of uniform nanofibers at 24 kV, 10ul/min as the flow rate and 15 cm distance between the collector and the needle (Figure 6d). The combination of HPCS allowed for the first time the fabrication of biocompatible nanofibers containing high honey and chitosan concentrations using biocompatible solvents.

Realizing the synergistic effect of the combination of chitosan and honey on the HPCS solutions’ viscosity, attempts were made to increase incorporated honey and chitosan concentrations. Electrospinning 35% and 40% honey within the PCS (3.5%: 7%) combination was successful (Figures 7a and b). Moreover, electrospinning 4.5% and 5.5% chitosan within the HP combinations containing 30% honey was achieved (Figures 7c and d). However, the concentration of incorporated P was decreased to 5% because of the increased viscosity of the solution due to the increased concentration of incorporated chitosan.
In previous attempts to fabricate nanofibers with high concentration of honey, the maximum concentration of honey that was electrospun with poly(vinyl alcohol) was 9% (Wang & He, 2013). This is due to the remarkable decrease in the solution viscosity upon increasing the honey concentration, thus making it impossible to electropsin. This is the first report to fabricate nanofibers with concentrations of honey reaching to 40% of the weight of the nanofibrous mats. Furthermore, the favorable effect of honey on the chitosan solution viscosity upon aging allowed for incorporating higher chitosan concentrations reaching to 5.5% for the first time via biocompatible solvents.

Despite the success achieved in electrospinning HPCS nanofibers containing 35% and 40% honey, the electrospinning rate of such nanofibers was very slow leading to an increase in the collection time of the nanofibrous mats, especially with the single needle prototype electrospinner utilized in this study. Thus, the HPCS nanofibers with 30% honey were selected for completing the study due to the feasibility of their collection with the current electrospinner. However, with the advancement witnessed with multi-needle, needleless and large scale electrospinners, collection of nanofibers with 40% and more honey should be feasible. On the other hand, HPCS nanofibers with 3.5% chitosan were selected for completing the study, because of the longtime of aging required for the 5.5% chitosan solution to allow it to reach to the viscosity optimum for electrospinning, in addition to the slow electrospinning rate.

The FTIR spectra of the powders of CS and P as well as the HPCS nanofibers were analyzed. Chitosan showed characteristic bands at 3429 cm$^{-1}$ and 1655 cm$^{-1}$ corresponding to both the OH and the amide O C NH$_2$ groups. The CH$_3$ and CH$_3$O groups showed bands between 1000 and 2000 cm$^{-1}$ (Paipitak et al., 2011). Poly(vinyl alcohol) exhibited bands at 3429 cm$^{-1}$, 2923 cm$^{-1}$, and 1444 cm$^{-1}$ corresponding to the characteristic bands of OH, CH$_2$, and CH OH groups (Yan et al., 2012).

The previous characteristic bands observed for both P and CS were preserved in the resulting HPCS nanofibers. However, it was realized that the absorption peak at 3429 cm$^{-1}$ and 1655 cm$^{-1}$ corresponding to both the OH and amide O C NH$_2$ groups shifted to a lower wave number in the hybrid HPCS nanofibers. The characteristic peak observed in the HPCS nanofibers at 1058 cm$^{-1}$
could be attributed to the COC symmetric stretching and COH bending vibrations of the proteins found in honey. Whereas, the amide band of the protein found in honey could be realized at 1641 cm$^{-1}$ (Philip, 2009). Moreover, the peaks observed between 900 cm$^{-1}$ and 750 cm$^{-1}$ were attributed to the anomeric region, which is characteristic of the saccharide configuration of honey (Jaganathan & Mandal, 2009; Philip, 2010).

4.1.3 Crosslinking and characterization of the morphology of the nanofibers before and after cross-linking treatment.

It was observed that the HPCS nanofibrous mats lost their nanofibrous structure upon being in contact with aqueous media. Thus, different crosslinking techniques were undertaken, in order to achieve sufficient crosslinking degree without jeopardizing the nanofibers’ biocompatibility. During crosslinking care was taken not to expose the nanofibers to temperatures exceeding 110° C to avoid the reduction in the quality of honey with increase in the hydroxymethylfurfural content upon exposure to elevated temperatures (Tosi et al., 2004). It was reported that exposure of honey to 40° C for up to 96h does not affect any of the biomolecules found in honey (Molan, 1992).

The fabricated nanofibers were chemically crosslinked via exposure to the GA vapors for different time intervals followed by heating at 40° C for 24 h under vacuum to enhance the crosslinking efficiency and remove any unreacted residues of the GA. Figure 9 illustrates the images of the nanofibers that were chemically cross-linked after being immersed in PBS for 15 min. It was observed that the nanofibers subjected for three days to GA vapors demonstrated superior crosslinking (Figure 9a) where their original shapes were maintained with no swelling observed. Whereas, the nanofibers exposed for two days to GA vapors demonstrated similar results, however slight swelling was noticed (Figure 9b). Interestingly, it was realized that the nanofibrous structure could still be maintained with some swelling observed after exposure to GA vapors to three hours (Figure 9c). Meanwhile, upon reduction of the exposure time to GA vapors for 1 h (Figure 9d) lower crosslinking efficiency could be realized (Figure 9d), where partial degradation of the surface layers of the nanofibers began together with noticeable swelling of the nanofibers. It is worth mentioning, that the crosslinking efficiency noticeably decreased upon reducing the exposure time to the GA vapors for thirty minutes, where the nanofibrous morphology of the surface layers was nearly lost, probably because of the increased percentage of degraded fibers.
The different physical crosslinking procedures undertaken were heating at different temperatures for different periods of time and different cycles of freezing and thawing in liquid nitrogen for different time intervals. It was observed that crosslinked nanofibers were achieved upon heating the nanofibrous mats at 110°C for 15 min (Figure 9e), with noticeable swelling realized. Meanwhile, heating for 24 h at 70°C demonstrated partial degradation of the swollen nanofibers (Figure 9f). Heating causes induction of the crystallization of the polymers forming the nanofibers (Kang et al., 2010). On the other hand, freezing and thawing did not allow maintaining of the nanofibrous structure. It is worth noting that the physical crosslinking techniques employed made the nanofibrous mats brittle and easily liable to cracking. Moreover, heating resulted in a color change from white to light brown. The same effect was realized upon storage of the nanofibrous mats for several months. Such change in color may be attributed to possible interactions between the amino groups in chitosan and the GA aldehyde group.

4.1.4 Evaluation of the stability of the nanofibrous structure of the crosslinked and noncrosslinked nanofibers.

The developed HPCS (30%:7%:3.5%) were evaluated for the stability of their nanofibrous morphology before and after crosslinking. Samples were crosslinked via exposure to GA vapors for 1h and 3h followed by heating at 40°C for 24h. The crosslinked and non-crosslinked samples were placed in petri dishes closed with parafilm and stored on shelf at room temperature for 1 year and then the stability of the nanofibrous structure was examined via SEM. As seen in figure 10, the nanofibrous structure could still be realized in both the crosslinked and non-crosslinked samples after 1 year, however, the crosslinked samples allowed better preservation of the nanofibrous structure. In the non-crosslinked samples the outer layer of the nanofibrous mat seem to have degraded and the nanofibrous structure of the underlying layers could be observed beneath the upper degraded layer (Figure 10a & 10b). In the crosslinked samples, degradation of the outermost layer was not observed, however, significant swelling of the nanofibers could be observed. The degradation of the outermost layer in the non-crosslinked sample and the swelling observed in the crosslinked sample could be attributed to the adsorption of moisture from the surrounding environment.
It is of note that the increase in the crosslinking time from 1h to 3h exposure to GA vapours did not result in a noticeable change in the morphology of the stored nanofibers.

4.2 Evaluation of the effect of changing the honey concentration on the properties of the developed HPCS nanofibers (Sarhan et al., 2016a).

Novel honey/chitosan/ poly(vinyl alcohol) (HPCS) electrospun nanofibers based on high concentrations of honey reaching to 40% were successfully fabricated. Thus, honey is considered the major component of the developed HPCS nanofibers. Consequently, the effect of changing the honey concentration on the properties of the developed nanofibers was studied. Increasing concentrations of honey (10%, 20% & 30%) were included within the PCS (7%:3.5%) nanofibers and examined for the effect of changing the honey concentrations on the morphology, crystallinity, thermal behavior, swelling, degradation and antibacterial abilities of the developed HPCS nanofibers.

4.2.1 Effect of changing the honey concentration on the morphology of the electrospun HPCS nanofibers.

As apparent from Figure 11, it was noted that increasing the honey concentration led to increasing the diameter of the nanofibers. For instance, the HPCS nanofibers with 10% honey exhibited a mean fibre diameter of 284 ± 97 nm (Figures 11a & 11b) which increased to 371 ± 110 nm, and 464 ± 185 nm upon increasing the honey concentration to 20% (Figures 11c & 11d), and 30% (Figures 11e & 11f), respectively.

The increase in the fiber diameter is a direct consequence for increasing the amount of honey loaded within the nanofibers as can be observed from Figure 12a & 12b, where it is apparent that honey is embedded within the chitosan/poly(vinyl alcohol) nanofibers. It was also observed that the amount of honey loaded within the nanofibers influences the fiber diameter distribution. As seen in Figure 11d, addition of 20% honey to the chitosan/poly(vinyl alcohol) nanofibers allowed for the most focused fiber diameter distribution, as most of the nanofibers exhibited diameters between 300 nm and 450 nm (Figure 11d). Whereas, the addition of 10% and 30% honey to the
chitosan/polyvinyl alcohol nanofibers resulted in broad distribution of the diameters of the fibers (Figures 11b & 11f).

4.2.2 Evaluation of the effect of changing the honey concentration on the crystallization of the HPCS nanofibers

The XRD diffraction patterns of pure poly(vinyl alcohol) and chitosan have been previously reported (Nakane et al., 1999; Samuels, 1981). Moreover, the XRD patterns of polyvinyl alcohol/chitosan (PCS) nanofibers and films were reported by Jia et al., who observed that nanofibers of the PCS exhibited deteriorated crystalline structure compared to the films (Jia et al., 2007).

Figure 13 illustrates the XRD diffraction patterns of the prepared HPCS nanofibers with increasing honey concentrations. The HPCS nanofibers exhibited an amorphous microstructure with a single broad peak around $2\theta = 20^\circ$. Such XRD patterns are in coherence with those observed for the previously prepared poly(vinyl alcohol)/chitosan nanofibers (Jia et al., 2007). Thus the addition of honey did not affect the diffraction model of the poly(vinyl alcohol)/chitosan nanofibers and consequently the increase in the honey concentration within the HPCS nanofibers had no effect on their diffraction pattern. The deterioration of the crystalline structure of the electrospun nanofibers was previously reported (Deitzel et al., 2001; Zong et al., 2002). Such deterioration could be attributed to the fast deposition and drying of the elongated electrospun nanofibers thus hindering the crystallization (Jia et al., 2007).

4.2.3 Evaluation of the effect of changing the honey concentration on the thermal stability of the HPCS nanofibers

Thermogravimetric analysis (TGA) analysis of the HPCS nanofibers with increasing honey concentrations (10%, 20% and 30%) was performed. As observed in figure 14, the examined samples demonstrated similar thermal degradation process that takes place in several steps. The first step of weight loss is attributed to moisture elimination which resulted in loss of less than 10% of the weight of the examined nanofibers below 120 °C.

It is of note that at 120 °C the HPCS nanofibers having 10% honey exhibited the highest weight loss of ~8% whereas the weight loss decreased by increasing the amount of honey within the HPCS
nanofibers to ~6% and ~3% with the 20% and 30% honey, respectively. This indicates that the HPCS nanofibers with higher honey concentrations exhibited higher initial moisture content, which is result of the hygroscopic nature of honey. The second and major weight loss of approximately 50% of the weight occurred after 120 °C till 400°C and is mainly attributed to the thermal decomposition of the polymer structure as well as degradation of the honey components followed by carbonization of the honey content (Figure 14). In the final step of the thermal decomposition at temperatures above 500°C, the polymer backbone has been ruptured in addition to the oxidation of the organic matter found in honey. Similar observations have been previously reported (Chauhan et al., 2014; Felsner et al., 2004). The thermogravimetric analysis clearly demonstrates that the fabricated HPCS nanofibers with different honey concentrations exhibit good thermal stability below 120°C.

4.2.4 Evaluation of the effect of changing the honey concentration on the swelling and weight loss abilities of the nanofibers.

Honey and chitosan nanofibrous mats represent top candidates for wound dressing applications and determining their swelling capabilities would allow prediction of their exudate management ability (Li et al., 2013). The effect of changing the honey concentration was studied at two mild crosslinking degrees. These include exposing the nanofibers to GA vapours for 1h and 3h with subsequent heating at 40°C to enhance the crosslinking and remove any residual GA.

As observed in figure 15, increasing the honey concentration within the nanofibers decreased its swelling ability at both the tested crosslinking degrees (Figures 15a & 15b). It could be observed from the figures that the HPCS nanofibers with 10% honey and 1h of crosslinking with the GA vapours exhibited superior swelling capabilities reaching to 520% at 1h and 300% after 24 h (Figure 15a).

On the other hand, the effect of the crosslinking time on the swelling capabilities of the HPCS nanofibers varied according to their incorporated honey concentration. For the HPCS nanofibers with 10% honey, increasing the crosslinking time from 1h to 3h decreased their swelling capabilities noticeably from 520% to 273%. Whereas, HPCS nanofibers with 20% honey exhibited increased swelling ability with the increase in the crosslinking time from 1h to 3h. Noticeably, the HPCS nanofibers with 30% honey demonstrated the lowest swelling ability at both crosslinking times. Although honey is known for its high water uptake ability (MohdZohdi et al., 2011), it also
has high water solubility. Such high water solubility results in increasing the degradation rates of the nanofibers and consequently losing their compact porous structure that can hold in water (Wang et al., 2012). Thus, this eventually results in massive decrease in swelling ability. This was observed by the very low swelling abilities of the HPCS nanofibers with 30% honey. Interestingly, the increase in the crosslinking efficiency by increasing the exposure time to the GA vapours allows the nanofibers to maintain a more compact nanofibrous structure (Li et al., 2013; Kim et al., 1992) thus, the percent of released and solubilized honey decreases. This allows the honey to be maintained within the nanofibers for longer periods of time, and thus its water uptake capabilities could be realised.

On the other hand, the increase in the crosslinking degree decreases the swelling ability as it hinders the intermolecular motion and chain disentanglements within the nanofibrous scaffold. These two opposite effects on the swelling abilities of the nanofibrous scaffolds could be observed in the results presented in figures 15a & 15b.

In the HPCS nanofibers with 10% honey, the amount of honey within the nanofibers is small thus the effect of the swelling hindering due to crosslinking was more pronounced than the water uptake ability of the maintained honey. Whereas, when the concentration of honey increased to 20%, the water uptake ability of the maintained honey in this case exceeded the hindering effect of crosslinking on swelling which allowed the HPCS nanofibers with 20% honey to exhibit a noticeable increase in swelling ability even at 24 h by increasing the crosslinking time to 3h. The HPCS nanofibers with 30% honey however showed slight decrease in the swelling ability with increasing the crosslinking time after 24h. This is because at such high concentration of honey such crosslinking treatments could not overcome the increased solubility of the HPCS nanofibers with 30% honey which affects the compact structure of the nanofibrous scaffold. Such results were confirmed with the weight loss results of the HPCS nanofibers with increasing honey concentrations (Figure 15c), where the increase in the honey concentration within the nanofibers resulted in increased weight loss at both the tested crosslinking degrees.

These results reveal the importance of optimization of the crosslinking degree as well as the honey concentrations within the developed HPCS nanofibers to adjust the water uptake ability as well as the weight loss according to the desired application.
4.2.5 Effect of increasing the honey concentration on the antibacterial activity of the developed HPCS nanofibers

It is the aim of the current study to develop nanofibers that could be utilized as effective antimicrobial wound dressings, thus, the antibacterial activity of the fabricated HPCS nanofibers was screened against Gram positive; *S. aureus* and Gram negative; *E. coli* as they are two of the most common pathogens found in infected wounds (Bessa et al., 2015). Moreover, two different bacterial counts were utilized to study the efficacy of the developed nanofibrous mats in inhibition of high bacterial loads.

Both honey and chitosan exhibit antibacterial activity. Honey exerts its antibacterial activity via its acidity, high sugar content as well as its ability for hydrogen peroxide production (Vandamme et al., 2013). Whereas, the polycationic nature of chitosan allows it to interact with the negatively charged membranes of bacteria leading to loss in the permeability of the membrane with subsequent cell leakage and death (Muzzarelli et al., 1990).

The effect of changing the honey concentration within the HPCS nanofibers on the antibacterial activity of the developed nanofibers was investigated as shown in figure 16. The increase in the honey concentration within the HPCS nanofibers enhanced their antibacterial activities against both *S. aureus* and *E. coli* at $1 \times 10^7$ CFU/ml (Figures 16a & 16b). However, upon increasing the bacterial count to $1 \times 10^8$ CFU/ml the increase in the honey concentration resulted in an increase in the antibacterial activity against *S. aureus*, whereas nearly no antibacterial effect was realized against *E. coli*. These results are in agreement with No, et al (2002) who demonstrated the weak antibacterial activity exhibited by chitosan against Gram negative bacteria (No et al., 2002). The nanofibrous structure allowed enhancement in the antibacterial activity of the components included within the nanofibers. The examined samples (0.05 g) contain chitosan less than 10 ppm and ~ 0.0875% honey and demonstrated pronounced antibacterial effect against *S. aureus* compared to no antibacterial effect at the same concentrations for both chitosan and honey alone (Goy et al., 2009; Islam et al., 2011I; Liu et al., 2006; Mandal & Mandal, 2011). Such results could be due to the massive increase in the surface to volume ratio of the nanofibers.

Thus, due to the enhanced antibacterial activity of the HPCS nanofibers loaded with 30% honey they will be selected to be further loaded with other antimicrobials. Despite the increased swelling
ability of the 10% honey HPCS nanofibers, the main aim of the present study is to develop an efficient antimicrobial wound dressing. Consequently, the HPCS formula that was selected to be further loaded with natural materials and optimized as a nanofibrous antimicrobial wound dressing is 7% PVA, 3.5% Chitosan & 30% Honey.
4.3 Evaluation of HPCS Nanofibers Loaded with Natural Extracts as Antimicrobial Wound Dressings (Sarhan et al., 2016b).

To enhance the antibacterial activity of the HPCS (30%:7%:3.5%) nanofibers, they were loaded with two natural extracts that have demonstrated antibacterial and potential wound healing capability through previous literature. Thus, the HPCS (30%:7%:3.5%) nanofibers were loaded with aqueous extracts of *Allium sativum* (AE) and *Cleome drosirifolia* (CE) and the resulting nanofibrous mats were examined for their swelling, weight loss, antibacterial, cytotoxicity, wound healing abilities as well as their abilities to enhance cell proliferation.

4.3.1 Fabrication of the electrospun HPCS, HPCS-AE, HPCS-CE, and HPCS-AE/CE nanofibers.

As previously demonstrated, electrospinning HPCS nanofibers containing high concentrations of H and CS was only possible by aging the solution of PCS with 30% H for a week. *Allium sativum* aqueous extract was included in the HPCS combination via substituting 50% of the solvent in which the HPCS were prepared resulting in the formation of HPCS-AE solution that was subsequently electrospun into HPCS-AE nanofibers. Upon preparation of the HPCS-AE it was observed that the solution exhibited high viscosity that was inappropriate for electrospinning, thus the solution was aged for a week at room temperature while observing the viscosity of the solution at different time intervals.

It was observed that the substitution of 50% of the solvent of the HPCS with aqueous extract of AE resulted in a massive reduction in its viscosity as compared to the HPCS solution which was utilized as the control (table 2). The observed reduction in viscosity could be due to degradation of the CS polysaccharide into its lower molecular weight oligomers due to the addition of the AE aqueous extracts.

As demonstrated in table 2, the reduction in viscosity imparted by the addition of the aqueous AE extract was very sharp and was observed from the first two hours (1410 mPas) and reached maximum decrease in viscosity after 48 h reaching to 420 mPas as compared to 3660 mPas in the case of the HPCS solution without the aqueous AE extract. This indicates the vital role played via
the AE aqueous extract in the CS degradation with the subsequent reduction in the solution viscosity.

Before electrospinning and after both the HPCS and HPCS-AE solutions have reached to the optimum viscosity required for electrospinning, the dry powder of CE (10%) was added as stirred for 1h. It should be noted that addition of the CE extract to both the HPCS and HPCS-AE solutions was not possible before aging because of the increased viscosities of the solutions that were difficult to be stirred. It was realized that the addition of the CE dry powder extract did not affect the required viscosity for electrospinning even after aging.

The as-prepared solutions of HPCS and HPCS-AE were electrospun (NANON-O1A, MECC, Japan) and collected as nanofibrous mats for further examinations. It was observed that the addition of AE aqueous extracts to the HPCS solution has facilitated the electrospinning process, which was attributed to the reduction in viscosity imparted by the AE on the solution. However, due to the massive decrease in viscosity undesirable dripping has occurred. The parameters used during the electrospinning of the HPCS-AE nanofibers and that allowed for a continuous and steady jet were a voltage of 27 kV, a flow rate of 0.5 ml/h, and 13 cm as the distance between the needle and the collector. Collection of the nanofibrous mats continued for 4.5h and their surface morphology was observed using SEM (Figure 17). Both the HPCS and HPCS-AE demonstrated a compact, uniform, smooth, and bead-free morphology. Additionally, it was realized that the HPCS-AE exhibited the least nanofiber diameter and the most focused diameter distribution among the tested nanofibrous mats (145 ± 58nm).

Upon the addition of the dry powder of CE to both the HPCS-AE and the HPCS solutions, the process of electrospinning became more difficult until optimizing the parameters of electrospinning to be 28kv, 0.7 ml/h flow rate and 14 cm as the distance between the needle and the collector. The nanofibers were collected for 3.5 hours, however it was still critical to achieve a uniform nanofiber deposition. This may be due to the sticky nature of Cleome droserifolia (CE) as it exhibits glandular sticky leaves (Płachno et al., 2009).

Figure 17 demonstrates a bimodal diameter distribution of both the HPCS-AE/CE and HPCS-CE nanofibers, because of the addition of high CE concentration in addition to its sticky nature. Moreover, noticeable branching was observed with a noticeable formation of clusters at the
branching points within the collected nanofibers (Xu et al., 2011). This branching could be attributed to spinning highly concentrated solution (Reneker & Yarin., 2005) using a high voltage combined with the sticky nature of electrospun solution. Electrospinning high concentrated solutions results in a jet with large diameter, which could result in the formation of branches (Yarin et al., 2005). This in turn leads to large inter-fiber spaces, which proofed to be more beneficial in cell related applications such as tissue engineering and wound healing (Gu et al., 2013; Shokrgozar et al., 2011) taking into consideration the density of the electrospun nanofibrous mat.

**4.3.2 Evaluation of the swelling and weight loss abilities of the fabricated nanofibers.**

The fabricated nanofibers of HPCS-CE, HPCS-AE and HPCS-AE/CE were crosslinked and examined for their swelling capabilities after immersion in PBS (pH 7.4) for 1, 4, and 24 h. Crosslinking of the fabricated electrospun nanofibers was done by exposing the nanofibers to GA vapors for 1 hour and 3 hours with subsequent heating at 40°C in order to remove any residues of GA and enhance the crosslinking of the nanofibers.

As observed in figure 18, the HPCS-CE and the HPCS-AE at one hour crosslinking, demonstrated similar swelling capabilities with a slight decrease in the swelling (%) of both the HPCS-CE and HPCS-AE as compared to the previously examined HPCS (30%H) (Figure 15), especially after 24 hours of immersion in the PBS (Figure 18a). At three hours crosslinking noticeable decrease in the swelling ability of the HPCS-CE was observed. On the other hand, the low swelling (%) of the HPCS-AE/CE nanofibrous mats was observed at one and three hours crosslinking times, demonstrating values of less than 15% swelling as compared to ~90% swelling in the case of the HPCS-AE and previously examined HPCS (30%H) (Figure 15) after immersion in PBS buffer for 24 h (Figure 18b). Such results illustrate that the swelling abilities of both the HPCS and the HPCS-AE nanofibers greatly decreased upon addition of the CE. This could be attributed to the CE sticky nature which hinders the chain disentanglements and intermolecular motion of the fabricated nanofibers and thus hinders their swelling abilities (Plachno et al., 2009).

Increasing the time of crosslinking permits maintaining a more compact nanofibrous structure that allows the water uptake ability of the porous structure of the nanofibers to be realized. At the same time, the extent of crosslinking should not be increased to the point that hinders the chain entanglements and intermolecular motion within the nanofibers (Li et al., 2013; Kim et al., 1992).
Upon increasing the time of crosslinking to 3 h, the swelling ability of both the HPCS-AE and HPCS-AE/CE increased, whereas, nearly no effect was realized on the HPCS-CE nanofibers, which demonstrated similar swelling (%) at both the crosslinking times (1 h and 3 h) examined. Such results illustrate that the increase in the time of crosslinking affected only the nanofibrous mats containing the aqueous AE. This could be due to the fact that the AE containing nanofibers demonstrated increased weight loss as compared to the HPCS-CE nanofibers (Figure 18c). Thus, upon crosslinking the AE containing nanofibers a more compact structure could be maintained for longer periods of time resulting in the enhancement observed in their swelling capabilities after three hours crosslinking.

According to the swelling results of the examined nanofibers (Figure 15 & 18), it is expected that the HPCS-AE/CE nanofibers would demonstrate nearly no capability for management of exudates. Whereas, the HPCS-CE, HPCS (30%H) and HPCS-AE nanofibrous wound dressings would demonstrate moderate capability for exudate management. The HPCS, HPCS-AE and HPCS-CE samples demonstrated moderate swelling capabilities as compared to nanofibers previously electrospun lacking honey (Jannesari et al., 2011). This could be due to honeys’ high water solubility which results in increasing the weight loss of the electrospun nanofibers. Moreover, despite that chitosan enhances the water uptake capability of the electrospun nanofibers, increasing the chitosan concentration results in an opposite effect. This was previously illustrated by Son et al., who observed that in nanofibrous mats of chitosan/poly(vinyl alcohol) having low chitosan concentration, polymeric hydrogels are easily formed via the hydrophilic poly(vinyl alcohol) in solutions thus leading to enhanced swelling. However, upon increasing the concentration of chitosan, the intermolecular forces between the amine groups and the side chains of chitosan increase and decrease the swelling capability (Son et al., 2009).

### 4.3.3 Evaluation of the antibacterial abilities of the fabricated nanofibers.

Research into development of effective antimicrobial wound dressings represents an increasing trend within the wound dressing market. This is because of the major complications associated with infected wounds that are resistant to current treatment protocols. Chronic non-healing wounds are usually treated with antimicrobial therapeutics either systemic or topical (Lipsky & Hoey., 2009). It was observed that > 60% of these patients received antibiotic treatments for a prolonged period of time (Howell-Jones et al., 2005; Tammelin et al., 1998). With the alarming rise in
antibiotic resistance alternative antibacterials are of great necessity. Silver-based dressings represent one of the most common alternative antibacterials now effectively used in wound treatment. Unfortunately, resistance against silver as well as undesirable side effects have been reported (Lansdown, 2002).

The developed HPCS nanofibrous mats exhibited mild antibacterial activity against *S. aureus* and weak antibacterial activity against *E. coli*. To enhance the antibacterial activity of the developed HPCS nanofibers, CE, AE and their combination were loaded within the HPCS nanofibers and examined for their antibacterial abilities against *S. aureus*, *E. coli* and two resistant strains; MDR *P. aeruginosa* and MRSA. The selected bacterial strains for the study are considered among the most common bacterial pathogens encountered at the wound site (Bessa et al., 2015). Aquacel Ag was examined for its antibacterial effect and compared to the antibacterial activities of the fabricated nanofibrous wound dressings. Recently, it was reported that the Aquacel Ag demonstrated the strongest antibacterial activity among other silver-based wound dressings in the market (Yunoki et al., 2015).

The antibacterial effect of *Allium sativum* has been attributed to its content of thiosulfinates including diallyl sulphide, allyl methyl sulphide, and diallyl disulphide, where they disrupt cell components and block the pathways of various bacterial enzymes (Elsom, 2000; Chen et al., 1999). Whereas Cleome’s antibacterial activity has been attributed to its content of various terpenes including the β-eudesmol, sesquiterpenes carotol, and δ-cadinene (Muhaidat et al., 2015).

Figure 19 represents the antibacterial activities of the developed HPCS, HPCS-CE, HPCS-AE and HPCS-AE/CE nanofibrous wound dressings in comparison with the commercial Aquacel Ag wound dressing. It was observed that complete inhibition of *S. aureus* was achieved via both the HPCS-AE and HPCS-AE/CE nanofibrous mats compared to noticeable reduction in bacterial count with the Aquacel Ag wound dressing (Figure 19a). Such effect is mainly attributed to the inclusion of the AE aqueous extracts within the HPCS nanofibrous mats. Additionally, it was realized that among the fabricated nanofibrous mats, only the HPCS-AE/CE demonstrated mild antibacterial activity against MRSA, thus illustrating that the combined effects of both the CE and AE was needed to achieve antibacterial effect against MRSA (Figure 19b). It is of note that the achieved antibacterial effect against MRSA was not significant as compared to the negative control (p < 0.05) and less than that observed with the Aquacel Ag. On the other hand, no antibacterial
activity was demonstrated against both the *E. coli* and the *MDR P. aeruginosa*, whereas, the Aquacel Ag exhibited bactericidal activity against *E. coli* and enhanced bacterial inhibition against MDR *P. aeruginosa* (Figure 19 c & 19 d).

The antibacterial activities of the chitosan, honey, *Cleome droseifolia* and *Allium sativum* were reported against both Gram negative and Gram positive bacteria (Muhaidat et al., 2015; Muzzarelli et al., 1990; Gaherwal et al., 2004). Additionally, increased antibacterial effect against *S. aureus* compared to *E. coli* was observed for chitosan (No et al., 2002) and *Cleome droseifolia* oil (Muhaidat et al., 2015) as well as the honey and *Allium sativum* mixture (Andualem, 2013). This agrees with the results reported here regarding the enhanced antibacterial activity against the examined *S.aureus* and MRSA strains.

**4.3.4 Evaluation of the wound healing abilities of the fabricated nanofibers.**

The fabricated nanofibrous wound dressings and the commercial dressing Aquacel Ag were applied on a 9 mm excisional wound on the dorsal back of mice. For determination of the change in wound size over time, photographs of the wound area were taken on days 3, 5, 7, 10 and 12 (Figure 20). The wound healing ability of the examined nanofibrous wound dressings was determined via measurement of the percentage of the wound size remaining exposed at each time point as compared to the wound size on day 0 (Figure 20).

Honey and chitosan have well proven ability to enhance the wound healing process (Mandel & Mandel, 2011; Dai et al., 2011; Seckam & Cooper, 2013). Chitosan has been observed to beneficially influence every stage in the wound healing process (Dai et al., 2011). Chitosan indirectly enhances cell proliferation (Azuma et al., 2015) and stimulates migration of both the polymorphonuclear cells (PMN) and mononuclear cells (MN). Both of which showed the ability to degrade chitosan into its low molecular weight oligomers that exhibit profound capability to promote cell migration (Minami et al., 1997). Moreover, chitosan was observed for its stimulatory effects on macrophage nitric oxide production (Peluso et al., 1994). Honeys’ wound healing ability has been historically recognized since ancient times. Such wound healing ability is related to honeys’ ability to provide a moist wound healing environment, fast autolytic debridement and pros- as well as anti-inflammatory effects, in addition to honeys’ antibacterial and antioxidant activities (Tonks et al., 2003, Majtan et al., 2006, Molan & Rhodes., 2015). *Allium sativum* have been recently studied for its effect on enhancing the wound healing process where it was reported that
it increases the re-epithelialization and neovascularization (Sidik et al., 2006). On the other hand, *Cleome droserrifolia*, has not yet been evaluated for its effect on the wound healing process, however, Cleomes’ antioxidant activity have been well observed (El-Khawaga et al., 2010; El-Shenawy & Abdel-Nabi, 2004) and antioxidants have been known for their ability to enhance the wound healing process via prevention of the overexposure of the wound site to oxidative stress which leads to delay in the wound healing process (Fitzmaurice et al., 2011).

As demonstrated in figure 20, the wound size decreased noticeably on day 3 as observed with the HPCS, HPCS-AE and HPCS-AE/CE nanofibrous dressings as compared to the wounds of the negative control. Additionally, all the examined wound dressings as well as the Aquacel Ag exhibited significant decrease in the wound size as well as the Aquacel Ag as compared to the negative control on days 5 and 7. At the same time there was nearly no reduction in the wound size of the negative control at day 5 (Figure 20).

It was observed that the rate of wound closure enhanced greatly with the HPCS nanofibrous mats and upon addition of aqueous extracts of AE in the HPCS-AE nanofibrous mats, the rate of wound closure increased. On the other hand, the rate of wound closure decreased upon addition of the dry extract of CE to the HPCS-Ce nanofibrous mats. Whereas, the inclusion of the combination of both extracts within the nanofibrous mats of HPCS-AE/CE resulted in similar rates of wound closure to the HPCS nanofibrous dressings (Figure 20). Interestingly, it was observed that the HPCS-AE demonstrated enhanced wound closure rate as compared to the Aquacel Ag, whereas, both the HPCS and the HPCS-AE/CE demonstrated similar rates of wound closure to the commercial Aquacel Ag wound dressing.

The histopathology of the wound tissue was subsequently examined to observe the effect of the fabricated nanofibrous dressings on the different stages involved in the wound healing process. The wound tissues were H&E stained and their histopathology studied and scored at days 3, 5, 7, 10 and 12. Moreover, the collagen deposition in the wounded tissues was examined and scored at day 10 after staining the wound tissue with the MT stain (Figures 21 & 22, and Table 3). Necrotic tissue is usually accumulated in chronic wounds. Necrotic tissue is defined as dead tissue which most frequently results from inadequate blood supply. As observed in figure 21 and table 3, the application of all of the developed nanofibrous wound dressings as well as the positive control Aquacel Ag to the wound site reduced the necrosis as compared to the negative control, with the
most enhanced reduction in necrosis observed with the HPCS-CE since day 5. Additionally, the
number of inflammatory cells reduced as compared to the negative control upon application of the
developed nanofibrous wound dressings, where they completely disappeared at day 10 with the
HPCS-AE/CE nanofibrous dressing. This indicates that the fabricated nanofibrous wound
dressings prevented the prolongation of the inflammatory phase, which could be attributed to the
anti-inflammatory effects of the materials of the wound dressings as honey and Allium sativum.
Moreover, it was realized that the number of macrophage cells was greater than the number of
neutrophils in the treated wounds.

Early epithelization was observed in all the treated wounds as compared to the negative control
with the AquacelAg, HPCS-AE/CE and HPCS-AE nanofibrous wound dressings demonstrating
the earliest epithelization as well as formation of thicker epidermis (Figure 21 and Table 3).
Additionally, both the HPCS-AE/CE and the Aquacel Ag allowed for earlier formation of
granulation tissue. Moreover, the examined nanofibrous wound dressings as well as the Aquacel
Ag allowed mature formation of granulation tissue together with dense collagen deposition (Figure
21 and Table 3). This was confirmed by the MT staining of the wound tissues at day 10 which
showed that the regenerated collagen in the treated wounds was denser as compared to the negative
control, and that both the nanofibrous mats of HPCS and the Aquacel Ag demonstrated the most
dense deposition of collagen. It was also observed that the HPCS-AE, HPCS-AE/CE, HPCS, and
the Aquacel Ag exhibited the most uniform distribution of collagen (Figure 22 and Table 3).
Overall, the scoring of the data of the histologic examination revealed that among the examined
nanofibrous wound dressings, the HPCS-AE/CE exhibited the most enhanced effect on the wound
healing process followed by the HPCS nanofibrous wound dressing having scores very similar to
the Aquacel Ag. Both nanofibrous dressings allowed decrease in the inflammatory phase, and
earlier formation of granulation tissue as well as earlier epithelization and deposition of thicker
epidermis. Additionally, both nanofibrous wound dressing’s induced uniform and dense deposition
of collagen.
4.3.5 Evaluation of the fibroblast cytotoxicity of the fabricated nanofibers and their effect on fibroblast cell proliferation.

The fabricated HPCS, HPCS-CE, HPCS-AE and HPCS-AE/CE nanofibrous mats as well as the commercial wound dressing Aquacel Ag were tested for their cytotoxicity using the MTT assay. Additionally, the effect of the fabricated nanofibrous dressings and the commercial wound dressing Aquacel Ag on fibroblast cell proliferation was evaluated via the MTT assay. Oxidoreductase cellular enzymes reflect the number of viable cells via reduction of the soluble tetrazolium dye (MTT; 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to the insoluble purple formazan salt. The developed nanofibers were extracted and diluted to yield different extract concentrations; 100%, 75%, 50% and 25%, that were tested for their cytotoxicity. The fibroblast cells were cultured in the different dilutions of the extract and the cytotoxicity was determined via estimation of the viable cells after 48h (Figure 23a).

It was realized that both the HPCS-CE and the HPCS nanofibrous mats demonstrated the highest fibroblast cell viability of 90% and 87%, respectively in the 100% extract solution. Whereas, significant reduction in cell viability of 68% \( (p < 0.05) \) was observed with the HPCS-AE that increased to 75% upon adding CE to the HPCS-AE/CE nanofibers in the 100% extract solution (Figure 23a). On the other hand, it was observed that the commercial dressing Aquacel Ag at all tested dilutions exhibited increased cytotoxicity to the cultured fibroblasts \( (p < 0.05) \) and showed viable fibroblast cell counts of approximately 9% similar to the results observed with the cytotoxic control (Figure 23a).

Figure 23b demonstrates fibroblast cell proliferation results at 1 and 3 days as determined via the MTT assay. The OD values of the HPCS-AE/CE, HPCS-CE and HPCS nanofiber mats increased with the increase in culture time. Both the HPCS and the HPCS-CE nanofibrous mats exhibited the most significant enhancement \( (p < 0.05) \) in proliferation after 3 days of incubation (Figure 23b). Whereas the HPCS-AE nanofibrous mats exhibited nearly the same OD values at 1 & 3 days. On the other hand, the Aquacel Ag exhibited significant cytotoxic effect on the proliferation of the fibroblast cells as observed from the low OD values (Figure 23b). Such results confirm the previously reported cytotoxicity for the Aquacel Ag wound dressing in the previous evaluation of the cytotoxicity (Figure 23a). The observed cytotoxicity of the commercial wound dressing Aquacel Ag was previously reported in different studies (Yunoki et al., 2015; Burd et al., 2007).
Generally, the HPCS-CE and HPCS nanofibrous dressings exhibited the highest levels of cell proliferation and viability within the examined nanofibrous dressings. The addition of CE dry extract to the nanofibers of the HPCS-AE has increased their proliferation and cell viability results. Interestingly, all the fabricated nanofibrous mats exhibited major increase in cell proliferation and viability as compared to the Aquacel Ag commercial wound dressing (Figures 23a and 23b).
4.4 Evaluation of HPCS Nanofibers Loaded with Apitherapeutics and Bacteriophages as Antimicrobial Wound Dressings.

Apitherapeutics were loaded within the HPCS (30%:7%:3.5%) nanofibers as an alternative approach to enhance the antibacterial abilities of the developed nanofibers. Two apitherapeutics, namely: bee venom and propolis were loaded within the HPCS (30%:7%:3.5%) nanofibers and tested for their antibacterial abilities. Additionally, the nanofibrous mat that demonstrated the most enhanced antibacterial activity among the developed HPCS nanofibers loaded with natural extracts and apitherapeutics was selected and further loaded with bacteriophage. The bacteriophage was isolated against a bacteria resistant to the selected nanofibrous mat and electrospun with the selected combination of the nanofibrous mat, thus extending the spectrum of antibacterial activity of the selected nanofibrous mat. The developed apitherapeutic and bacteriophage loaded nanofibrous mats were examined for their swelling, weight loss, antibacterial, cytotoxicity, wound healing abilities as well as their abilities to enhance cell proliferation.

4.4.1 Fabrication of the electrospun HPCS-Pr and HPCS-BV nanofibers.

Recently, propolis (Pr) has been co-spun into polymeric nanofibers and examined for its effect on the mechanical and antibacterial properties of the electrospun nanofibers. Ethanolic and aqueous propolis extracts have been loaded within the nanofibers in different concentrations ranging from 2 to 10% (Sutjarittangtham et al., 2012; Sutjarittangtham et al., 2014), however it was observed that at concentrations above 8% of ethanolic or aqueous propolis extract the solution could not be electrospun (Sutjarittangtham et al., 2014). Thus, in the current study the ability to fabricate uniform nanofibers loaded with 10% propolis was examined within the HPCS nanofibers.

Bee venom on the other hand has not yet been formulated in the nanofibrous form, however its diverse biomedical properties have been documented via different studies (Ali, 2012; Kwon et al., 2002; Kim et al., 2013). It was observed that bee venoms’ antibacterial property was achieved via concentrations ranging from 12.5 to 25 ug/ml for Gram-positive bacteria and 1 to 10 mg/ml for Gram-negative bacteria (Lowenstein et al., 1997). Thus, bee venom was loaded within the HPCS nanofibers in the concentration of 1 mg/ml to be able to target both Gram positive and Gram negative bacteria.
The aqueous extracts of Pr and the dry powder of BV were added to the prepared solutions of HPCS prior to electrospinning. The electrospun mats were characterized via SEM (Figure 24) then subjected to analysis of the diameter of the nanofibers via image J. The HPCS solutions were previously spun into uniform bead free nanofibers of 464 ± 185 nm in diameter. As observed in figure 24, electrospinning of 10% Pr within the HPCS solution allowed for the collection of dense nanofibrous mats. However, due to the high concentration of Pr included within the nanofibers, noticeable branching was observed together with cluster formation within the nanofibers. Additionally, an increase in the diameter of the nanofibers over that of the previously spun HPCS was observed, where the average nanofiber diameter reached to 737 ± 260 nm with broad diameter distribution.

On the other hand, the inclusion of the BV within the HPCS nanofibers allowed for uniform deposition of the nanofibers with average diameter of 459 ± 140 nm and a focused diameter distribution (Figure 24). The inclusion of BV did not result in variation in the morphology of the nanofibers over the previously collected HPCS nanofibers. This could be attributed to the minute concentration of the included BV.

The parameters that were utilized in electrospinning both the HPCS-BV and HPCS-Pr solutions were a high electric potential of 27 kV (E-spin, NanoTech, Kalyan-pur, India) and a constant flow rate of 0.5 ml/h was maintained, whereas, the distance between the nozzle and the collector was maintained at 15 cm.

4.4.2 Evaluation of the swelling and weight loss abilities of the fabricated nanofibers.

The developed nanofibers of HPCS-Pr and HPCS-BV were examined for their swelling abilities after immersion in PBS (pH 7.4) for 1, 4, and 24 h. Crosslinking was performed by exposing the nanofibers to GA vapors for 1 & 3 hours with subsequent heating at 40°C.

As observed in figure 25, the HPCS-BV demonstrated enhanced swelling values as compared to the HPCS-Pr nanofibers at 1, 4 and 24h. The HPCS-BV exhibited swelling values similar to those previously reported for the HPCS nanofibers. This is because of the small concentration of BV.
added to the HPCS nanofibers, thus, such low concentration did not result in variation of the swelling properties of the HPCS nanofibers.

The HPCS-Pr nanofibers exhibited very small swelling values, especially at 4h and 24h. This may be attributed to the high concentration of the included propolis (10%), which decreased the available void left for water uptake and swelling. Moreover, the observed increase in weight loss (Figure 25c) of the HPCS-Pr nanofibers is another reason for the decreased swelling values recorded for the HPCS-Pr due to the loss of the compact structure that allows the water uptake (Figure 25).

The increase in the crosslinking time resulted in slight effect on the swelling abilities of the HPCS-BV nanofibers, where a slight decrease in the swelling ability was observed at 24h. This could be attributed to the effect of crosslinking on hindering of the chain entanglements and intermolecular motion within the nanofibers (Kim et al., 1992). The effect of crosslinking was more pronounced on the HPCS-Pr nanofibers, where the swelling abilities of the HPCS-Pr nanofibers decreased noticeably. On the other hand, weight loss was slightly decreased upon increasing the crosslinking time to 3h.

4.4.3 Evaluation of the antibacterial abilities of the fabricated nanofibers.

Propolis and bee venom are two natural apitherapeutics that have demonstrated effective antibacterial activity against different kinds of bacteria (Hegazi et al., 2015; Popova et al., 2005; Kujumgiev et al., 1999). Bee venom’s antibacterial activity is related to a number of peptides like melittin, adolapin, apamin and mast cell degranulating peptides as well as biologically active amines and non-peptide components (Kwon et al., 2002; Fennel., 1968). Whereas propolis antibacterial activity is mainly attributed to its content of flavonoids and cinnamic acid (Sharaf et al., 2013; Popova et al., 2005). It was observed that propolis prevents cell wall division, and causes disorganization of the cytoplasm, cell wall and the cytoplasmic membrane leading to inhibition of protein synthesis and bacteriolysis (Lotfy, 2006).

In this research propolis and bee venom have been loaded within the previously spun HPCS nanofibers and examined for their antibacterial activities against S. aureus, E. coli and two resistant strains; MDR P. aeruginosa and MRSA. The commercial wound dressing Aquacel Ag was used as a positive control, whereas untreated bacterial broth was utilized as a negative control.
As observed in figure 26, the inclusion of bee venom within the HPCS nanofibers in the HPCS-BV nanofibers allowed for noticeable enhancement in the antibacterial activity even against resistant bacterial strains. The HPCS-BV nanofibers allowed for complete bacterial inhibition of \textit{E.coli} similar to the effect of the commercial Aquacel Ag. Such results agree with the previously reported enhanced antibacterial activity of bee venom against \textit{E.coli} (Hegazi et al., 2014). Additionally the HPCS-BV nanofibers exhibited enhanced antibacterial activity over the commercial Aquacel Ag against the tested Gram positive strains where it demonstrated ~ 6 log and 5 log reduction in the bacterial count of \textit{S.aureus} and MRSA respectively as compared to ~ 4 log and 2 log reduction in case of the Aquacel Ag. However, the HPCS-BV nanofibers exhibited no antibacterial activity against \textit{P. aeruginosa}, whereas, the Aquacel Ag demonstrated enhanced antibacterial activity against it (Figure 26).

The HPCS-Pr nanofibers on the other hand exhibited weaker antibacterial activity than the HPCS-BV nanofibers. As compared to the Aquacel Ag, the HPCS-Pr demonstrated enhanced antibacterial activity against both \textit{S.aureus} and MRSA, whereas against \textit{E.coli} and MDR \textit{P. aeruginosa} it exhibited nearly no antibacterial activity compared to enhanced antibacterial activity with the Aquacel Ag (Figure 26).

According to the antibacterial results of the HPCS nanofibers loaded with the natural extracts (Figure 19) and apitherapeutics (Figure 26), it was observed that the HPCS-BV nanofibers exhibited the most enhanced antibacterial activity among the developed nanofibers. The HPCS-BV nanofibers demonstrated enhanced antibacterial activity against \textit{E.coli}, \textit{S.aureus}, and MRSA, stronger than that observed with the commercial wound dressing Aquacel Ag. However, it exhibited nearly no antibacterial activity against \textit{P. aeruginosa}, unlike the Aquacel Ag that demonstrated enhanced antibacterial activity against it. \textit{P. aeruginosa} is considered the most commonly encountered Gram negative bacteria in wounds (Gjødsbøl et al., 2006; Burmølle, 2010). Thus, HPCS-BV was selected to be further loaded with a bacteriophage against \textit{P. aeruginosa} in order to develop a nanofibrous wound dressing with broad spectrum antibacterial activity against the most common bacterial pathogens encountered at the wound site.
4.4.4 Isolation, electrospinning and antibacterial evaluation of the bacteriophage loaded nanofibers

The alarming rise in bacterial resistance has revived the interest in bacteriophage therapy. Bacteriophages are viruses that specifically infect and rapidly destroy bacteria. Although bacteriophages are now witnessing increased research and are applied in food preservation, integration of phage therapy in human therapeutics is still facing many challenges among them is their narrow host range (Sarhan & Azazzy., 2015b). Utilization of bacteriophage in wound care has been examined in a number of studies (Rhoads et al., 2009; Seth et al., 2013), however, its integration into nanofibrous wound dressing with broad spectrum antibacterial activity has not yet been realized.

The phage plaque assay was utilized for the isolation of a bacteriophage against the MDR *P. aeruginosa* from the different sewage samples collected and then subsequently purified and amplified into a *P. aeruginosa* phage (PS1) suspension. The PS1 phage was then subjected to morphological characterization via TEM. The results of the TEM imaging revealed that the PS1 phage exhibits an icosahedral head of 71 nm in diameter and a contractile tail of 110–115 nm in length (Figure 27a). Thus, the bacterial virus was classified as a representative of the Myoviridae family (Soothill, 1992; Ackermann et al., 1994).

The PS1 phage stock solution (10⁹-10¹⁰ PFU mL⁻¹) was loaded within the HPCS-BV solution and subjected to electrospinning at 27 kV, 0.5 ml/h as flow rate, whereas the distance between the needle and the collector was maintained at 13 cm. The fibers collected were characterized via SEM which revealed a dense and uniform bead free deposition of the HPCS-BV/PS1 nanofibers (Figure 27b). The analysis of the fiber diameter distribution illustrated that the diameter distribution of the collected nanofibers did not show a noticeable difference from that of the HPCS-BV nanofibers (Figure 26) with an average diameter of 498 ±145 nm (Figure 27c) and a focused fiber diameter distribution.

The HPCS-BV/PS1 nanofibers were examined for their antibacterial activity against MDR *P. aeruginosa*. Bacteriophages are characterized by their instant antibacterial activity, where lysogenic phages are adsorbed on the surface of the bacterial cell followed by injection of the phages’ genetic material into the bacterial cell cytoplasm. The host cell machineries are then utilized for making new phages and then the host cell is killed at the end of the growth cycle.
To observe the antibacterial activity of the phage loaded nanofibers, the examined nanofibers as well as the controls were tested for the antibacterial activity after 24h. The results revealed strong antibacterial activity against MDR *P. aeruginosa* for the HPCS-BV/PS1 nanofibers which exceeded that of the Aquacel Ag (Figure 27d). Complete bacterial inhibition of the MDR *P. aeruginosa* was achieved via the HPCS-BV/PS1 nanofibers whereas the Aquacel Ag still showed week bacterial growth (Figure 27d).

Loading of the HPCS-BV nanofibers with the PS1 bacteriophage allowed for extension of the antibacterial activity of HPCS-BV against MDR *P. aeruginosa*. At the same time, the developed HPCS-BV/PS1 represents a broad spectrum bacteriophage formulation, where the combination of the natural materials of the nanofiber and the bacteriophage allowed the nanofibrous formulation to exhibit broad spectrum antibacterial activity. The observed results (figures 26 & 27d) illustrate that the HPCS-BV/PS1 nanofibers exhibit enhanced antibacterial activity over the commercial Aquacel Ag even against resistant bacterial strains. Aquacel Ag was recently reported to exhibit the strongest antibacterial activity among other silver based wound dressings in the market (Yunoki et al., 2015). Thus, this indicates the enhanced efficacy of the developed HPCS-BV/PS1 as an antibacterial formulation.

### 4.4.5 Evaluation of the wound healing abilities of the fabricated nanofibers.

The developed nanofibrous mats were evaluated for their effect in enhancing the wound healing process. Propolis has well proven ability to enhance the wound healing process (Mandel & Mandel, 2011; Seckam & Cooper, 2013; Dai et al., 2011; McLennan et al., 2008), whereas the wound healing ability of bee venom is recently recognized (Amin & Abdel-Raheem, 2014). Propolis, an important component of the bee hive was observed to exhibit wound healing ability due to its antimicrobial, antioxidant, immunomodulatory, anti-inflammatory and analgesic effects (Sforcin, 2007; Cardoso et al., 2010; Ramos et al., 2012). It was demonstrated that both caffeic and phenethyl ester present in propolis exhibit immunosuppressive activities on T-cells which play a significant role in several inflammatory diseases (Lotfy, 2006). Bee venom exhibits anti-inflammatory and antimicrobial activities that were proven beneficial in enhancing the wound healing process (Hider, 1988; Seo et al., 2008). It was recently observed that bee venom limits the prolongation of inflammation via regulating the levels of inflammatory cytokines (Kwon et al., 2002; Abu-Seida, 2015).
A 9 mm excisional wound was performed on the dorsal back of mice upon which the developed nanofibrous mats were applied. The wound region was photographed on days 3, 5, 7, 10 and 12 to illustrate the variation in the wound over time (Figure 28). Additionally, to determine the ability of the nanofibrous mats to enhance the wound closure rate, the wound size remaining exposed (%) was determined by comparing the wound size at each time point with the wound size at day 0 (Figure 28). Aquacel Ag was utilized as the positive control in the wound healing study of the developed nanofibrous mats and the subsequent histopathological examination.

As observed in figure 28, the HPCS-Pr, HPCS-BV and HPCS-BV/PS1 nanofibers as well as the Aquacel Ag exhibited enhanced wound closure rate as compared to the negative control covered with a cotton gauze.

Interestingly, it was observed that the HPCS-Pr demonstrated enhanced wound closure rate as compared to the commercial Aquacel Ag wound dressing. The enhanced effect of HPCS-Pr on wound healing was realized from day 3, with significant reduction in the wound size as compared to both the negative control and the Aquacel Ag. The HPCS-BV nanofibers, on the other hand exhibited similar wound closure rate to the positive control Aquacel Ag, whereas upon addition of PS1 to the HPCS-BV nanofibers a slight enhancement in the wound closure rate was observed (Figure 28). This could be attributed to the change in the weight loss rate of the HPCS-BV/PS1 nanofibers as compared to the HPCS-BV nanofibers because of the dilution of the HPCS-BV polymeric solution with 10% of the phage stock solution while electrospinning the HPCS-BV/PS1 nanofibers. This allows for the presence of increased concentration of the materials of the nanofibers at the wound site which leads to enhancement of the wound healing process due to the beneficial effects of the materials of the nanofibers on the wound healing process.

The reduction in the healing time could be attributed to the presence of the apitherapeutics, honey, propolis and bee venom as well as chitosan in the developed nanofibers. This is due to the anti-inflammatory effect of these materials that prevent prolongation of the inflammatory response that delays the wound healing process (Alvarez-Suarez et al., 2014). Moreover, the antibacterial properties of the utilized materials; honey, bee venom, propolis and chitosan prevent the presence of persistent inflammatory stimuli due to the presence of bacteria at the wound site and thus prevent the prolongation of the inflammatory phase (Bjarnsholt et al., 2008).
It is worth mentioning that the fabricated nanofibrous wound dressings easily attached to the wound site, eliminating the need for biological adhesives. This could be attributed to the hydrophilic nature of the CS, P and H in addition to the increased water solubility of the high honey concentration included within the nanofibers. Thus, the fabricated nanofibrous wound dressings allow to keep the wound desirably hydrated.

Samples of the wound tissue were H&E stained and their histopathology evaluated and scored at days 3, 5, 7, 10 and 12 days. Moreover, samples at day 10 were MT stained and examined for collagen deposition (Figures 29 & 30 and Table 4).

It was observed from the histological data (Figure 29 and Table 4) that the HPCS-Pr allowed for the most enhanced reduction in necrosis since day 5. At the same time, all the examined nanofibrous dressing and the Aquacel Ag decreased the necrosis as compared to the negative control. Such effect could be attributed to the anti-inflammatory effect of honey, chitosan, bee venom, and propolis which reduces the damage caused by the free radicals resulting from inflammation thus preventing further necrosis (Alvarez-Suarez et al., 2014).

Additionally, the application of the developed nanofibrous dressings to the wound site prevented prolongation of the inflammatory phase, especially with the HPCS-Pr and the HPCS-BV/PS1 nanofibrous wound dressings, where the inflammatory cells were last observed at day 7, whereas in the case of the negative control they persisted till day 12. Consequently, this allowed for early epithelization as well as formation of thick epidermis in the wounds treated with the nanofibrous dressings as well as the Aquacel Ag (Figure 29 and Table 4). Such results agree with previously reported results for bee venom, honey and propolis regarding their anti-inflammatory effect and thus their ability to decrease inflammation (Kwon et al., 2002; Molan, 2006; Han et al., 2012; Castaldo, 2002; Peng et al., 2008).

Earlier formation of granulation tissue was observed in the wounds treated with the Aquacel Ag, HPCS-Pr, and HPCS-BV/PS1, thus, indicating accelerated wound healing rate. Dense collagen deposition was observed with all the tested nanofibrous dressings and was confirmed via staining of the wounds with MT stain at day 10 and comparing them to the commercial Aquacel Ag due it its documented effect on enhancing the wound healing process (Barnea et al., 2010) (Figure 30 and Table 4).

As seen in figure 30 all the examined nanofibrous dressings allowed for dense collagen deposition as well as uniform collagen distribution similar to that observed with the Aquacel Ag. Previously,
it was reported that the presence of large amounts of collagen is correlated to adequate wound healing (Drucker et al., 1998).

Overall, it was observed that the developed nanofibrous dressings demonstrated enhanced wound healing rates as compared to the Aquacel Ag. In Fact, the HPCS-Pr demonstrated enhanced effect on wound healing more than that observed via the Aquacel Ag according to the histopathological examination and the scoring of the histologic data (Table 4), whereas, the HPCS-BV and HPCS-BV/PS1 nanofibrous dressings demonstrated similar results to the Aquacel Ag. Such results are of significant importance due to the current focus on honey and natural products with antimicrobial activity to be used as advanced antimicrobial wound care products in clinical practice (Alvarez-Suarez et al., 2014; Frost & Sullivan, 2014). Especially, that the current therapeutic protocols rely on silver, and despite their efficacy as antimicrobial wound dressings the undesirable side effects associated with silver are generating increasing concern (Demling & DeSanti, 2001; Alvarez-Suarez et al., 2014).

4.4.6 Evaluation of the fibroblast cytotoxicity of the fabricated nanofibers and their effect on fibroblast cell proliferation.

The developed HPCS-Pr, HPCS-BV and HPCS-BV/PS1 nanofibers were evaluated for their cytotoxicity on human dermal fibroblasts as well as for their effect on the cell proliferation via the MTT assay. Additionally, the effect of the commercial wound dressing Aquacel Ag on cell cytotoxicity and proliferation was evaluated and used as a positive control in both tests.

The developed nanofibers were extracted and diluted to yield different extract concentrations; 100%, 75%, 50% and 25%), that were tested for their cytotoxicity. The fibroblast cells were cultured in the different dilutions of the extract and the cytotoxicity was determined via estimation of the no of viable cells after 48h (Figure 31a).

As observed in figure 31a, the HPCS-BV nanofibers exhibited the highest cell viability even at 100% extract concentration thus indicating that loading of HPCS nanofibers with BV did not affect the biocompatibility of the nanofibers. Whereas, the HPCS-BV/PS1 nanofibers demonstrated a minor decrease in the viability of the fibroblast cells compared to the HPCS-BV nanofibers. This may be attributed to the increase in weight loss of the HPCS-BV/PS1 compared to the HPCS/BV nanofibers due to the loading of the nanofibers with 10% phage stock solution, where the loaded
solution affected the degradation rate of the polymeric based nanofibers, resulting in an increase in weight loss of the nanofibers. Thus, the amount of the released components including BV from the HPCS-BV/PS1 nanofibers increased which resulted in a minor change in cell viability. However, despite the reduction in cell viability due to the HPCS-BV/PS1 nanofibers it still exhibited significant \( (p < 0.05) \) enhancement in cell viability compared to the commercial Aquacel Ag dressing that demonstrated noticeable cytotoxic effects.

On the other hand, the HPCS-Pr nanofibers demonstrated a noticeable reduction in cell viability in the 100% and 75% extract solutions (Figure 31a). This may be due to the increased concentration of propolis loaded within the nanofibers. Propolis has been proven to exert cytotoxic effects on different tumor cell lines, however, it also demonstrated some cytotoxicity for non-tumor cell lines (da Silva et al., 2013; Calhelha et al., 2014). Moreover, Kim et al studied the cytotoxic effects of polyurethane nanofibers loaded with 5, 10 and 30% of propolis solution and demonstrated that the 5% propolis solution loaded nanofibers allowed for the most enhanced cell proliferation even after 7 days, whereas increasing the concentration of the loaded propolis solution to 30% resulted in decreased proliferation rates, taking into consideration that loading with 10% propolis solution extract contains less amount of propolis than loading with the same percent of dry powder propolis extract as was performed in the current study (Kim et al., 2014). Despite the reduction in cell viability observed with the HPCS-Pr nanofibers it still demonstrated significant \( (p < 0.05) \) enhancement in cell viability compared to the Aquacel Ag commercial dressing (Figure 31a).

The effect of the developed nanofibers as well as the Aquacel Ag on fibroblast cell proliferation was studied at 1 and 3 days. Figure 26b demonstrates the proliferation results as determined via the MTT assay. It was observed that HPCS-Pr demonstrated the lowest OD values among the developed nanofibers, whereas the HPCS-BV nanofibers exhibited the highest OD values followed by the HPCS-BV/PS1 nanofibers at 24h. However, none of the developed nanofibers allowed an enhancement in the proliferation of the fibroblast cells after 3 days of incubation. This may be related to the increased concentration of the released BV and Pr in the medium following prolonged incubation, especially that recent evaluation of the HPCS nanofibers on the fibroblast cell proliferation revealed significant enhancement in proliferation at 3 days of incubation. Compared to the commercial Aquacel Ag dressing, both the HPCS-BV and HPCS-BV/PS1 demonstrated significant increase in cell viability at both 24 and 72h as observed via the OD
values. The observed cytotoxicity of the Aquacel Ag was previously reported in different studies (Yunoki et al., 2015; Burd et al., 2007).

It is of note that the swelling capabilities of the nanofibrous mats and their capabilities to enhance cell proliferation and viability could be increased by increasing the pore diameter. This could be achieved by changing of the parameters utilized during the electrospinning process (Kazemi Pilehrood et al., 2014) or by inclusion of some treatments as carbon nanotubes before electrospinning (Shokrgozar et al., 2011). Additionally, the nanofibrous mats density must be taken into consideration as an increase in the density will result in reduction in the breathability of the fabricated nanofibrous mats and thus, lead to restriction in nutrient and metabolic waste transportation and reduction in cell viability as well as decrease in the swelling capability of the fabricated nanofibrous mats. Within this context it was observed that ultrasonication of the fabricated nanofibrous mats could help overcome such limitation (Lee et al., 2011). Moreover, collection of nanofibrous mats of low density on a substrate could be another approach to be undertaken to overcome such limitation. Thus, future work on the fabricated nanofibrous mats will consider optimization of the breathability of the nanofibrous mats by different approaches with subsequent testing of the effect of each approach on the cell proliferation and viability as well as the swelling capability of the fabricated nanofibers.
Non-healing wounds represent a pressing health care problem with major socioeconomic impacts. The success in managing bacteria in wounds is of utmost importance, this is because bacterial infection stimulates the immune system which in turn prolongs tissue inflammation thus further delaying the healing process. Moreover, wound associated bacterial infection usually develops resistance to commonly used antibacterials, thus leading to increased risk of systemic infections. Antimicrobial advanced wound dressings stand as an important sector in the treatment of wound infections. Silver-based dressings stand as one of the most common and effective antimicrobial dressings used. However, despite their enhanced broad spectrum antibacterial activity, development of resistance has been reported together with some undesirable side effects of silver. Thus, through the current research different approaches have been undertaken to develop a series of effective antimicrobial wound dressings based on effective antimicrobials that are more biocompatible and able to achieve enhanced antibacterial and wound healing activity.

The first objective in this study was to develop nanofibrous wound dressing based on high honey concentration. Different concentrations of honey (H) and chitosan (CS) were electrospun with poly(vinyl alcohol) (P) resulting in HPCS nanofibers having H concentrations up to 40% and CS concentration up to 5.5%. The combination of H and CS had a synergistic effect on the solution viscosity causing it to reach to the viscosity optimum for electrospinning. Such effect allowed for the first time for the development of nanofibers comprising 40% honey of their actual weight as compared to only 9% in previous attempts without the use of toxic solvents. Chemical and physical crosslinking of the fabricated HPCS nanofibers allowed different degrees of crosslinking, thus extending their areas of application.

Subsequently, different honey concentrations (10%, 20% and 30%) were electrospun within the chitosan (3.5%) /poly (vinyl alcohol) (7%) nanofibers to study the effect of changing the honey concentration on the different properties of the electrospun nanofibers. It was observed that increasing the honey concentration resulted in an increase in the fibre diameter from 284 ± 97 nm with 10% honey to 464 ± 185 nm with 30% honey. The swelling of the nanofibers was greatly influenced by the concentration of incorporated honey and the degree of crosslinking. Highest swelling extent was observed with HPCS nanofibers having 10% honey, and the least swelling...
was noted in the HPCS nanofibers having 30% honey. The crystallization and thermal stability of the nanofibers on the other hand were not affected by changing the honey concentration within the developed HPCS nanofibers. The antibacterial activities of the HPCS nanofibers with different honey concentrations was evaluated against *S. aureus* and *E. coli* at two different bacterial counts. It was observed that increasing the honey concentration within the HPCS nanofibers enhanced their antibacterial activity against both *S. aureus* and *E. coli* at $7 \times 10^7$ CFU/ml. Whereas, at $7 \times 10^8$ CFU/ml nearly no antibacterial effect was realized against *E. coli* at all honey concentrations included within the HPCS nanofibers. Due to the enhanced antibacterial activity of the HPCS nanofibers loaded with 30% honey they were selected to be further loaded with other antimicrobials.

The second objective was to load the developed HPCS nanofibers with natural extracts to enhance their antibacterial and wound healing abilities. *Allium sativum* (AE) and *Cleome droserifolia* (CE) were loaded within the selected HPCS nanofibers. Allium sativum aqueous extract substituted 50% of the solvent of the HPCS in the HPCS-AE nanofibers and 10% of dried aqueous extract of *Cleome droserifolia* were loaded within the HPCS in the HPCS-CE nanofibers whereas, the HPCS-AE/CE were loaded with both the AE and CE extracts. The HPCS, HPCS-CE, HPCS-AE and HPCS-AE/CE nanofibrous mats were characterized and examined for their weight loss, swelling, cytotoxicity and wound healing capabilities. Moreover, the antibacterial activities of the developed nanofibers were evaluated against *S. aureus, E. coli*, MRSA and MDR *P. aeruginosa*. The antibacterial, wound healing abilities and cytotoxicity results were compared to those of the commercial wound dressing Aquacel Ag. It was observed that substitution of 50% of the solvent with AE resulted in massive reduction in the HPCS solution viscosity. The HPCS-AE/CE nanofibrous mats demonstrated the lowest swelling capabilities and the highest weight loss among the fabricated nanofibers at two tested crosslinking degrees (1h and 3h exposure to GA vapours followed by heating at 40 °C for 24h) showing values of less than 90% weight loss and 15% swelling as compared to 60-70% weight loss and ~ 90% swelling in the case of HPCS and HPCS-AE after immersion in PBS buffer for 24h. The antibacterial evaluation demonstrated that the fabricated nanofibers exhibited no antibacterial activities against *E. coli* and MDR *P. aeruginosa*. However, complete bacterial inhibition of *S. aureus* better than that produced with the commercial dressing Aquacel Ag was achieved with both the HPCS-AE and the HPCS-AE/CE nanofibrous dressings. Moreover, the bacterial count of MRSA decreased by 1.5 log with the HPCS-AE/CE as
compared to 3.5 log decrease in bacterial count with the Aquacel Ag. On evaluation of the wound healing capabilities of the fabricated nanofibrous dressings as compared to the Aquacel Ag, it was observed that the HPCS-AE/CE and the HPCS exhibited similar wound closure rates whereas the HPCS-AE allowed enhancement in the wound closure over that exhibited via the Aquacel Ag. The scoring of the histopathological data showed that both the HPCS and the HPCS-AE/CE nanofibrous wound dressings demonstrated the most enhanced effects on the different stages in the wound healing process with scores very close to the Aquacel Ag. Most importantly, it was observed that the HPCS-CE, HPCS-AE/CE and HPCS exhibited the highest levels of proliferation and cell viability as compared to the commercial Aquacel Ag that demonstrated noticeable cytotoxicity.

The third objective achieved in this study was to load the fabricated HPCS nanofibers with apitherapeutics as another approach to develop effective biocompatible antimicrobial wound dressings. The HPCS nanofibers were loaded with apitherapeutics; bee venom (0.01%) in the HPCS-BV nanofibers and propolis (10%) in the HPCS-Pr nanofibers. The developed nanofibers were characterized and examined for their swelling and weigh loss abilities as well as their antibacterial activities against *S. aureus*, *E. coli*, MRSA and MDR *P. aeruginosa*. It was observed that the diameter of the HPCS-Pr nanofibers was 737 ± 260 nm, whereas, the HPCS-BV exhibited nanofiber diameter of 459 ± 140 nm. Moreover, the lowest swelling values were observed with the HPCS-Pr nanofibers, showing values of 20% and 29% as compared to 76% and 90% with the HPCS-BV nanofibers at 1 and 3 h of crosslinking, respectively. Whereas, the lowest weight loss values 65-55% were exhibited by the HPCS-BV nanofibers after 24h in PBS at the two tested crosslinking degrees (1h and 3h exposure to GA vapours followed by heating at 40 °C for 24h). The results of the antibacterial study demonstrated strong antibacterial activity of the HPCS-Pr nanofibers against the tested Gram positive strains *S. aureus* and MRSA as compared to the commercial Aquacel Ag wound dressing. However, the HPCS-BV demonstrated enhanced antimicrobial activity over the HPCS-Pr nanofibers where it exhibited enhanced antibacterial activity against *S. aureus*, MRSA and *E. coli* more than that observed with the Aquacel Ag. Nevertheless, no antibacterial activity was achieved against MDR *P. aeruginosa* whereas the Aquacel Ag exhibited strong antibacterial activity against it. Thus, the HPCS-BV was selected to be further loaded with a bacteriophage against *P. aeruginosa* and achieve the forth objective of the current study. The bacteriophage PS1 was isolated against the examined MDR *P. aeruginosa* and
loaded within the HPCS-BV nanofibers extending the spectrum of antibacterial activity of the HPCS-BV/PS1 to include *P. aeruginosa* causing nearly complete inhibition of it. The developed HPCS-Pr, HPCS-BV and HPCS-PV/PS1 were further tested for their cytotoxicity and wound healing abilities. The wound healing study results demonstrated that the HPCS-Pr exhibited wound closure rates and histopathological scores better than those demonstrated with the Aquacel Ag, whereas the HPCS-BV and HPCS-BV/PS1 exhibited similar wound healing results to the Aquacel Ag. Most importantly, it was observed that the developed HPCS-BV and HPCS-BV/PS1 nanofibers demonstrated enhanced biocompatibility as compared to the Aquacel Ag that exhibited strong cytotoxicity. Whereas, the HPCS-Pr demonstrated some cytotoxicity at 100% and 75% extract solutions however, they were significantly lower than those observed with the Aquacel Ag.

Through the current study, a series of nanofibrous wound dressings based on natural materials were fabricated. The fabricated nanofibrous dressings, HPCS, HPCS-AE, HPCS-CE, HPCS-AE/CE, HPCS-BV, HPCS-Pr and HPCS-BV/PS1 demonstrated enhanced wound healing abilities and variable antibacterial effects against the examined bacterial strains as compared to the commercial Aquacel Ag. Most importantly the Aquacel Ag was proved to exhibit noticeable cytotoxicity on fibroblasts, whereas the fabricated nanofibrous dressings demonstrated enhanced biocompatibility, with the HPCS demonstrating the most enhanced cell viability and proliferation results. The HPCS nanofibrous dressing comprising 10% H demonstrated the highest swelling capability and thus the highest ability to absorb exudates. Among the developed nanofibrous dressings the HPCS-Pr demonstrated the most enhanced effect on wound healing, more pronounced than Aquacel Ag. Whereas, the HPCS-BV/PS1 demonstrated the most enhanced antibacterial activities exceeding the commercial Aquacel Ag and at the same time demonstrating similar wound healing effects and enhanced biocompatibility. Overall, the fabricated series of nanofibrous dressings exhibited antibacterial and wound healing abilities as well as enhanced biocompatibility, thus they represent competitive candidates to be used as effective wound dressings.
6. RECOMMENDATIONS AND FUTURE DIRECTIONS

Recommendations for future work on the developed series of honey based nanofibrous dressings include examination of the mechanical properties of the developed nanofibrous dressing and optimization of the mechanical properties of the developed nanofibrous dressings to be suitable for wound healing applications via the use of different fillers or different levels of crosslinking as well as utilization of polymers with enhanced mechanical properties. Furthermore, evaluation and optimization of the breathability of the developed series of honey based dressings will be undertaken. Additionally, the effect of loading the nanofibrous dressings with different natural materials as well as growth factors on the wound healing and antibacterial abilities of the developed nanofibrous dressings will be further explored. That’s in addition to evaluating the effect of co-spinning different kinds of polymers with honey and evaluating the effect of such combinations on the swelling, degradation, and mechanical properties of the developed nanofibers.

The possible effect of honey and *Allium sativum* aqueous extracts on the degradation of chitosan will be investigated via determination of the molecular weight of the chitosan after treatment with honey and *Allium sativum* aqueous extracts. Additionally, the kinetics of release of the natural components included within the nanofibers will be studied as well as the degradation rate of the developed nanofibrous mats. Moreover, more biocompatible methods of crosslinking will be investigated. Additionally, the wound healing abilities of the developed nanofibrous mats will be tested on chronic non-healing wounds as well as infected wounds.
## 7. TABLES

**Table 1.** Change in the viscosity (mPas) of the P, PCS and HPCS solutions upon aging (Sarhan & Azazzy, 2015).

<table>
<thead>
<tr>
<th>Sample</th>
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<th>24h</th>
<th>48h</th>
<th>168h</th>
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<td>404</td>
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**Table 2:** Change in the viscosity (mPas) of the HPCS and HPCS-AE solutions upon aging (Sarhan et al., 2016).

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Table 3. Histological scoring system for MT and H&E stained wound tissues for both the control and experimental groups (Sarhan et al., 2016).

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<th>Histopathological lesions</th>
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<th>Aquacel Ag</th>
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<th>HPCS</th>
<th>HPCS-AE/CE</th>
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Table 4. Histological scoring system for Masson’s trichome and H&E stained wound tissues for both the control and experimental groups.

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<th>HPCS-BV</th>
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<td>++++++ ++++ + -- --</td>
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8. FIGURES

**Fig 1**: Schematic illustration of the different phases involved in the wound healing process.

**Fig 2**: Schematic illustration of the wound bacterial microbiology.
Fig 3. Schematic presentation of the electrospinning process.

Fig 4. Schematic illustration of the resemblance of the nanofibrous structure to the extracellular matrix (ECM) of the skin.
Fig 5. Schematic illustration of bacteriophage.
Fig 6. SEM images of the electrospun nanofibrous mats with the highest honey concentration within the HP and the HPCS nanofibers: (a) HP (20%:10%), (b) HPCS (20%:7%:3.5%) (c) HP (30%:10%), (d) HPCS (30%:7%:3.5%) (Sarhan & Azazzy, 2015a).
Fig 7. SEM images of the electrospun nanofibers containing maximum concentration (%) of honey and chitosan within the HPCS nanofibers: (a) HPCS (35%:7%:3.5%), (b) HPCS (40%:7%:3.5%), (c) HPCS (30%:5%:4.5%), (d) HPCS (30%:5%:5.5%) (Sarhan & Azzazy., 2015a).
Fig 8. FTIR spectra for (a) chitosan (CS) (b) poly vinyl alcohol (P) and (C) HPCS nanofibers (30%:7%:3.5%).
Fig 9. SEM images of the chemically (a, b, c, d) and physically (e, f) cross-linked HPCS (30%:7%:3.5%) nanofibrous mats. Cross-linking was performed by exposure to GA vapors with subsequent heating under vacuum at 40°C for 24 h. Different mats were exposed to GA for different time intervals (a) 3 days, (b) 2 days, (c) 3 h, and (d) 1 h. Images (e) and (f) demonstrate the successful physical cross-linking attempts: (e) heating for 15 min at 110°C under vacuum and (f) heating for 24 h at 70°C under vacuum (Sarhan & Azzazy., 2015a)
Fig 10. SEM images of the crosslinked and non-crosslinked electrospun HPCS nanofibers (30%:7%:3.5%), after 1 year of storage on shelf. (a, b) non-crosslinked HPCS (c) crosslinked HPCS via 1hr exposure to GA (d) crosslinked HPCS via 3hr exposure to GA.
Fig 11. SEM images of the electrospun honey/polyvinyl alcohol/chitosan (HPCS) nanofibrous mats with increasing concentrations of honey (a, c, e) and their diameter distribution (b, d, f): (a, b) HPCS (10%:7%:3.5%), (c, d) HPCS (20%:7%:3.5%), and (e, f) HPCS (30%:7%:3.5%) (Sarhan et al., 2016a).
Fig 12. TEM (a) & SEM (b) images of honey/polyvinyl alcohol/chitosan (HPCS) nanofibers (30:7:3.5 w %) illustrating the inclusion of honey within the nanofibers (Sarhan et al., 2016a).

Fig 13. XRD diffraction patterns of the honey/polyvinyl alcohol/chitosan (HPCS) nanofibers with increasing honey concentrations. The weight blending ratios of the electrospun mats were 7% polyvinyl alcohol (P), 3.5% chitosan (CS), and increasing concentrations of honey (H): 10%, 20%, and 30% (Sarhan et al., 2016a).
Fig 14. TGA of the honey/polyvinyl alcohol/chitosan (HPCS) nanofibers with increasing honey concentrations. The weight blending ratios of the electrospun mats were 7% polyvinyl alcohol (P), 3.5% chitosan (CS), and increasing concentrations of honey (H): 10%, 20%, and 30% (Sarhan et al., 2016a)
Fig 15. Swelling % (a & b), and weight loss % (c) of the honey/polyvinyl alcohol/chitosan (HPCS) nanofibers mats with increasing honey concentrations. The weight blending ratios of the electrospun mats were 7% polyvinyl alcohol (P), 3.5% chitosan (CS), and increasing concentrations of honey (H) 10%, 20%, and 30%. The swelling abilities of the nanofibers (a) 1 h crosslinked (1 h CL) (b) 3 h crosslinked (3 h CL) were tested after immersion in PBS (pH 7.4) for 1, 4, and 24 h. The weight loss of the 1 h and 3 h crosslinked nanofibers (c) were tested after immersion in PBS (pH 7.4) for 24 h (Sarhan et al., 2016a).
Fig 16. The antibacterial activity of the electrospun honey/polyvinyl alcohol/chitosan (HPCS) nanofibrous mats against $1 \times 10^8$ CFU/ml and $1 \times 10^7$ CFU/ml of *E. coli* (a) and *S. aureus* (b) represented by reduction in the log (CFU) after 24 h. The weight blending ratios of the electrospun mats were 7% polyvinyl alcohol (P), 3.5% chitosan (CS), and increasing concentrations of honey (H); 10%, 20%, and 30% (n = 3, Student’s t-test, *p < 0.05 versus the negative control) (Sarhan et al., 2016a).
Fig 17. SEM images of the electrospun nanofibrous mats and their diameter distribution of HPCS, HPCS-AE, HPCS-CE, and HPCS-AE/CE (Sarhan et al., 2016b).
Fig 18. % Swelling (a & b) and % weight loss (c) of the HPCS-AE, HPCS-CE and HPCS-AE /CE. The swelling capabilities of the nanofibers (a) 1 h crosslinked (1h CL) (b) 3h crosslinked (3h CL) were examined after immersion in PBS (pH 7.4) for 1, 4, and 24 h. The weight loss of the 1h and 3h crosslinked nanofibers (c) were examined after immersion in PBS (pH 7.4) for 24h (Sarhan et al., 2016b).
Fig 19. The antibacterial activity of the electrospun mats of HPCS, HPCS-AE, HPCS-CE, (HPCS-AE /CE and Aquacel Ag wound dressing against S. aureus (a) MRSA (b) E. coli (c) and MDR P. aeruginosa (d) at 24 h on $7 \times 10^8$ CFU/ml bacteria. Aquacel Ag was utilized as the positive control and the negative control was kept untreated. Data represents mean ± SD (n = 3, Student’s t-test, *p < 0.05) (Sarhan et al., 2016b).
**Fig 20.** Photographic images of the extent of the wound closure (a) graphical demonstration of the changes in the size of wound (b) on days 3, 5, 7, 10 and 12 for the HPCS, HPCS-AE, HPCS-CE, HPCS-AE/CE nanofibrous mats and the Aquacel Ag wound dressing. The Aquacel Ag was utilized as the positive control and the negative control was kept untreated and covered with a cotton gauze (Sarhan et al., 2016b).
**Fig 21.** Histopathological evaluation of sections of the H&E stained wound tissue treated with the HPCS, HPCS-AE, HPCS-AE/CE, HPCS-CE nanofibrous mats and the Aquacel Ag wound dressing at days 3, 5, 7, 10 & 12 days (Original magnification 100). The Aquacel Ag was utilized as the positive control, and the negative control was untreated and covered with a cotton gauze.

**HPCS:** Micrographs of the central area of the wound treated with the HPCS nanofibrous dressing. Note the increased infiltration of the inflammatory cells at 3 & 5 days and the formation of matured granulation tissue with well oriented deposition of collagen as well as thick layer of epidermis at days 10 & 12 and the near disappearance of the inflammatory cells. **HPCS-AE:** Micrographs of the central area of the wound treated with the HPCS-AE nanofiber dressing. Note the massive inflammatory cell infiltration at days 3, 5 & 7 and the exudate observed at 5 & 7 days. **HPCS-AE/CE** and **HPCS-CE:** Similar to HPCS-AE but with additional characteristics specific to their compositions.
granulation tissue that is well vascularized and with good oriented collagen deposition at day 10 was observed. HPCS-CE: Micrographs of the central area of the wound treated with the HPCS-CE nanofiber dressing. Note the massive infiltration of inflammatory cell at 3, 5, & 7 days and the enhanced vascularization (note the newly formed blood capillaries) within the matured formed granulation tissue. Well oriented deposition of collagen was observed since day 10. HPCS-AE/CE: Micrographs of the central area of wounds treated with the HPCS-AE/CE nanofibrous dressing. Note the massive inflammatory cell infiltration at 3, 5 & 7 days and the well vascularized granulation tissue formed with well oriented deposition of collagen since day 10. Notice the deposition of thick layer of epidermis since day 10 and that the inflammatory cells is nearly diminished. Aquacel Ag: Micrographs of the central area of wounds treated with the positive control Aquacel Ag dressing. Note the massive inflammatory cell infiltration at days 3 & 5 and the formation of mature granulation tissue that is highly vascularized and note the deposition of collagen since day 7. Notice the thick epidermal layer deposition since day 10 and that the inflammatory cells are greatly diminished. -ve control: Micrographs of central wound area of untreated controls: Note the massive inflammatory cell infiltration and the observed hemorrhage at days 7 & 12 as well as the disorganized granulation tissue. Notice the epithelial layer absence at day 10 and the deposition of thin epidermal layer at day 12 (Sarhan et al., 2016b).
Fig 22. Histopathological evaluation of sections of the MT stained wound tissues treated with the HPCS, HPCS-AE, HPCS-AE/CE, HPCS-CE nanofibrous mats and the Aquacel Ag wound dressing at day 10 (Original magnification 100). The Aquacel Ag was utilized as the positive control and the negative control was kept untreated and covered with a cotton gauze. Notice deposition of dense collagen in the HPCS, HPCS-AE, HPCS-AE/CE, HPCS-CE nanofiber dressings and the Aquacel Ag as compared to the negative control (-ve control) (Sarhan et al., 2016b).
Fig 23. Fibroblast cell viability (a) and fibroblast cell proliferation (b) as determined via the MTT assay for the HPCS, HPCS-AE, HPCS-CE, HPCS-AE/CE and Aquacel Ag. Aquacel Ag was used as the positive control in the two assays. Data represents mean ± SD (n = 3, Student’s t-test, *p < 0.05 versus the HPCS mats, ***p< 0.05 versus culture times 24h) (Sarhan et al., 2016b).
Fig 24. SEM images of the HPCS-Pr and HPCS-BV nanofibers and their corresponding diameter distribution.
Fig 25. % Swelling (a & b) and % weight loss (c) of the HPCS-Pr and HPCS-BV nanofibrous mats. The swelling capabilities of the nanofibers (a) 1 h crosslinked (1h CL) (b) 3h crosslinked (3h CL) were examined after immersion in PBS (pH 7.4) for 1, 4, and 24 h. The weight loss of the 1h and 3h crosslinked nanofibers (c) were examined after immersion in PBS (pH 7.4) for 24h.
Fig 26. Illustration of the antibacterial activity of the HPCS-Pr, HPCS-BV nanofibrous mats and Aquacel Ag wound dressing. The antibacterial activity was tested against *E. coli* (a) *S. aureus* (b) MRSA (c) and MDR *P. aeruginosa* (d) at 24 h on $7 \times 10^8$ CFU/ml bacteria. Aquacel Ag was utilized as the positive control, whereas the negative control was kept untreated. Data represent mean ± SD (n = 3, Student’s t-test, *p < 0.05).
**Fig 27.** Illustration of the morphology of the isolated PS1 phage (a) and the electrospun HPCS-BV/PS1 nanofibers (b) and their diameter distribution (c). Illustration of the antibacterial activity of the HPCS-BV, HPCS-BV/PS1 nanofibers against MDR *P. aeruginosa* at 24 h on $7 \times 10^8$ CFU/ml bacteria (d). Aquacel Ag was utilized as the positive control, whereas the negative control was kept untreated. Data represent mean ± SD (n = 3, Student’s t-test, *p < 0.05).
Fig 28. Photographic images of the wound healing process (a) graphical illustration of the variation in the size of the wound (b) on days 3, 5, 7, 10 and 12 for the nanofibrous dressings HPCS-Pr, HPCS-BV, HPCS-BV/PS1 and the untreated negative control (-ve control) as well as the positive control treated with the commercial wound dressing Aquacel Ag.
Fig 29. Histopathological evaluation of sections of the H&E stained wound tissue treated with the HPCS-Pr, HPCS-BV, HPCS-BV/PS1 nanofibrous mats and the Aquacel Ag wound dressing at different time intervals (3, 5, 7, 10 & 12 days) (Original magnification 100). The Aquacel Ag was utilized as the positive control and the negative control was kept untreated and covered with a cotton gauze. HPCS-Pr: Micrographs of the central area of the wound treated with the HPCS-Pr nanofibrous dressing. Note the necrosis at day 3, and the massive infiltration of inflammatory cells at days 3, 5, & 7. Highly vascularized granulation tissue was noticed since day 5. Notice the epithelization and thick epidermal layer at days 10 & 12. HPCS-BV and HPCS-BV/PS1: Micrographs of the central area of the wound treated with the HPCS-BV and HPCS-BV/PS1 nanofiber dressing, respectively. Note the massive inflammatory cell infiltration at days 3, 5, & 7, necrosis was observed at days 3 and 5. Note the well vascularized granulation tissue since day 7. Notice the epithelization since day 10 and formation of thick epidermal layer at 10 & 12 days.
Aquacel Ag: Micrographs of the central area of wounds treated with the positive control Aquacel Ag dressing. Note the necrosis at days 3 & 5 and the massive inflammatory cell infiltration. Notice the highly vascularized well oriented granulation tissue since day 7 and the epithelization and formation of thick epidermis at days 10 & 12. –ve cont.: Micrographs of central wound area of untreated controls covered with a cotton gauze. Note the massive inflammatory cell infiltration and necrosis till day 7 and deposition of thin epidermal layer at day 12.

Fig 30. Histopathological evaluation of sections of the MT stained wounds treated with the HPCS-BV, HPCS-Pr, HPCS/BV/PS1 nanofibrous mats and Aquacel Ag wound dressing at day 10 (Original magnification 100). The Aquacel Ag was used as the positive control. Notice deposition of dense collagen in the wounds treated with the developed HPCS-Pr, HPCS-BV, HPCS-BV/PS1 nanofiber dressings similar to that observed with the positive control Aquacel Ag dressing. Note that the most uniform and dense collagen deposition was observed with the wound treated with the HPCS-Pr nanofibrous dressing.
Fig 31. Illustration of results of the MTT assay for determination of the fibroblast cell (ATCC; crl-2522) viability (a) and proliferation (b) for the HPCS-Pr, HPCS-BV, HPCS-BV/PS1 nanofibrous mats and the Aquacel Ag wound dressing. Aquacel Ag was used as the positive control in both assays. Data represent mean ± SD (n = 3, Student’s t-test, *p < 0.05 versus the Aquacel Ag dressings).
9. REFERENCES


10. APPENDIX