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Fabrication and characterization of antibacterial herbal drug-loaded polylactic acid/cellulose acetate composite nanofibers for wound dressing applications

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Chemistry

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List of Abbreviation

PLA: Poly (Lactic-acid)
CA: Cellulose acetate
TQ: Thymoquinone
DCM: Dichloromethane
DMF: Dimethyformamide
SDS: Sodium dodecyl sulfate
E-spun: Electrospun
NFs: Nanofibers
PBS: Phosphate buffer solution
EDTA: Ethylenediamintetraacetic acid
WVP: Water vapor permeability
SEM: Scanning electron microscope
ECM: Extracellular matrix
MW: Molecular weight
DMEM: Dulbecco's Modified Eagle's medium
MTT: (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide)
MH: Muller-Hinton nutrient agar
DMSO: Dimethylsulfoxide
TCPs: Tissue culture polystyrene plates

SFM: Serum-free-medium

UV-Vis: Ultra violet-visible spectroscopy

FT-IR: Fourier transform-Infra red spectroscopy

W%: weight percent

W/V: weight per volume ratio

V/V: volume per volume ratio

3-D: 3-dimensional

* S. aureues: Staphylococcus aureues

* E. coli: Escherichia coli

MDF: Mouse dermal fibroblast
Abstract

Polylactic acid (PLA) is a versatile biopolymer that is widely used as a biomaterial. However, one of the major issues which limit its further application in tissue engineering purposes is its hydrophobic nature and poor cellular interaction. Modification of PLA properties can be achieved by polymer blending techniques. Polymer blending is a simple yet attractive method to combine and optimize polymeric physical properties of interest. In this study, an antibacterial electrospun nanofibrous scaffolds, with diameters around 400–1000 nm, were prepared by physical blending PLA with a hydrophylic biopolymer, cellulose acetate (CA). In this stage, PLA was used as the main polymer, blended with CA, at two main ratios (9:1 and 7:3 w/w), to achieve desirable properties such as better hydrophilicity, excellent cell attachment and proliferation. For preventing common clinical infections, an antimicrobial agent, Thymoquinone, TQ was incorporated into the electrospun fibers. TQ is the active ingredient of Nigella sativa and it is well known for its antibacterial properties. The potentiality of the prepared scaffolds, regarding being used as an interactive wound dressing, has been investigated including, swelling behavior, WVP and porosity. The release profile of TQ from the prepared scaffolds was also examined at the physiological pH (7.4) and temperature (37 °C). The antimicrobial efficiency of the prepared scaffolds against gram negative and gram positive bacteria were determined by the agar diffusion assay. The interaction between fibroblasts and the TQ-loaded PLA: CA scaffolds such as viability, proliferation, and attachment were characterized. TQ-loaded PLA: CA scaffolds showed burst TQ release after 24 h, compared with medicated PLA scaffolds, followed by a sustained release rate for 9 successive days. The results also indicated that medicated PLA: CA nanocomposite scaffolds showed a significant antibacterial activity against both gram positive and gram negative bacteria. Furthermore, the prepared scaffolds enhanced cell viability, attachment and proliferation, as compared to medicated PLA nanofibers. The presence of CA in the nanofiberous scaffolds improved its hydrophilicity, bioactivity, and water uptake capacity. Furthermore, it created a moist environment for the wound, which can accelerate wound recovery. A preliminary in vivo study performed on normal full
thickness mice skin wound models demonstrated that TQ-loaded PLA: CA (7:3) scaffolds significantly accelerated the wound healing process by promoting angiogenesis, increasing re-epithelialization and controlling granulation tissue formation. Our results suggest that TQ-loaded PLA: CA nanocomposite mat could be an ideal biomaterial for wound dressing applications.
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Chapter 1
Introduction
Introduction

1.1 Chronic Wounds: clinical complications
Skin, the largest organ of the body, consists of different types of tissues including: connective, nervous, muscular, and epidermal. Such composition is responsible for the multifunctional properties of the skin. Skin not only responsible for sensation or physical protection of the body, but it can also perform number of biochemical, metabolic and immune functions. When our skin barrier is disrupted through chronic wounds, a series of complex physiochemical process takes place in an attempt to repair the damage. Such processes can be classified into inflammatory reactions (immune response to prevent infection) and proliferative reactions (regeneration of tissues) [1].

Chronic wounds are defined as the wounds that do not show a 20–40% reduction in area after two to four weeks of treatment. Such delay is chronic wounds fail to heal through the natural physiological processes described before. This is attributed to the fact that all chronic wounds are characterized by one or more persistent inflammatory stimuli, including; repeated trauma, or ischemia. Such inflammatory stimuli are continues and never overcome, thus impair the physiological progression toward healing. The most common chronic wounds are diabetic foot ulcers, pressure ulcers, venous leg ulcers, and arterial leg ulcers. In most patients, the origins of delayed healing comes from a metabolic dysfunction such as obesity, diabetes, malnutrition or, immunological defects due to genetic defects or cancer [1, 2].

Chronic wounds are highly susceptible to the risk of bacterial infection. This is because the longer the wound remains opened and unhealed, the more likely it will be colonized by microorganisms coming from different sources (external environment, surrounding skin, and endogenous sources). Chronic wound infections not only increase patient discomfort or cause anxiety, but it can also lead to high risk of systemic illness. This is attributed to the fact that when the pathogens enter chronic wounds, they develop infection by the formation of a biofilm. Within the biofilm, bacteria is isolated form the immune system and can develop high resistance against antibacterial agents. Thus, it can result into
systemic infection which can further complicate illness. For that reason, chronic wound infections can be considered as the main causes of mortality and morbidity in developing countries [2-4].

1.2 Nanofiberous membranes as wound dressing material.

Ideal wound dressing for complicated chronic wounds must not only act as a physical barrier against mechanical trauma, it shall also accelerate the healing process, preventing bacterial infections and allowing skin to restore its structure (Figure 1.1).

![Figure 1.1: Requirements for an effective wound dressing material [5].](image)

Conventional wound dressings that are commercially available including, natural or synthetic bandages, cotton wool, lint, and gauze, do not meet the properties required for rapid healing of chronic wounds. They act as a cover to the wound and do not have any special properties, such as gas permeability, water uptake (limited swelling capacity) or bacterial infiltration. When it comes to chronic wounds, such poor performance can result in delaying the healing process, or even in more complications, such as causing ischemia/necrosis [3, 5].
A multifunctional device that is able to treat different types of chronic wounds by minimizing the risk of infection and wound recurrence is not currently available to patients. Research in this field currently focuses on developing dressings that can combine two essential properties: First, is to control the physiological parameter on which the healing process is based. This includes the gas and moisture permeability and exudates adsorbing capacity. Second, is to incorporate an antibacterial agent with a controlled release manner. A wound dressing that is capable of delivering all these requirements would both stimulate the healing process while preventing infections. This class of wound dressing materials can be classified as the bioactive dressings. Bioactive dressings include those incorporating drug delivery systems skin substitutes, and growth factors. Consequently, they can play an active role in the healing process by activating or driving cellular responses. Bioactive dressings represent an essential step forward toward the development of effective systems capable of delivery an accelerated healing for chronic wounds while eliminating their clinical complications [3, 6-8]. However, research is still very intensive in this regard.

1.2.1 Nanofibers Properties
Polymeric Nanofibrous meshes refers to the structures made of ultra-fine polymeric fibers. The diameters of such fibers ranges from several micrometers down to few nanometers. Such very small diameter results in a relatively high surface area to volume ratio (approximately 100m²/g for a fiber with a diameter 100 nm) [9]. The increase in surface area per unit ratio can greatly alter the physio-chemical and morphological properties of the obtained nanofiber mats including:

*High porosity and small pore size:* The way nanofibers are synthesized allow self-assembled nanofibers to align themselves into a characteristic 3D pattern, such as a honey comb meshes. Because of the small fiber diameter and high surface area to volume ratio, tiny pores in the range of 0.5 nm are formed within the nanofiber mesh, resulting in highly porous 3D structure (Figure 1.2) [9][10].
Figure 1.2: SEM micrographs showed highly porous structure with interconnected pores and bead-free PCL NFs [9].

Owing to their several intrinsic properties, including high surface area to volume ratio and high porosity, research drivers in the field of wound care development are now focusing on the manufacture of wound dressings in the form of nanofibrous membranes. Such unique properties allow them to satisfy the essential requirements for an ideal wound dressing material [9].

1- Accelerating wound healing: for the wound dressing material to accelerate wound healing, it needs to support cellular attachment, migration and proliferation to encourage tissue regeneration. Nanofibrous structure has the ability to support cellular growth. This is attributed to their unique structure that mimic the structure of the extracellular matrix (ECM). ECM is the non-cellular component present within all tissues. It is characterized by being a 3-D structure with high porosity, and sufficient mechanical properties. Thus, it acts as a natural scaffolds that physically support cells and provide them with the conditions required for attachment, proliferation, migration and differentiation. Nanofibrous membranes can offer an ideal alternative for the native ECM that has been destroyed by injury. Owing to their 3-D structure nano-size and high porosity, it can imitates the architecture of the native ECM. Furthermore, it can act as a synthetic scaffolds that
can further reproduce the structure of the natural injured ECM. Another advantage is the ability to electrospun natural polymers, such as protein and polysaccharides. ECM in its composition, includes interconnected structures of proteins (keratin, collagen and elastin), polysaccharides (glycoaminoglycans). Thus, the ability to incorporate natural components within the fibrous mesh can further improve biocompatibility and cellular response [3, 5, 7, 11].

2- **Gas and water vapor permeability**: An essential parameter in the healing process is the permeability of the wound dressing to air. High permeability to air result in better healing performance, since it allow for cellular respiration. The water vapor permeability is also essential, because moisture can also accelerate the healing process. Nanofibrous structure shows high porosity (60-90%). Thus it can allow for cellular respiration and high gas and moisture permeation [3, 11, 12, 13].

3- **Absorbing excess wound exudates**: Moisture is an essential factor in wound healing. However, the presence of high amount of fluids in wound beds can further accelerate bacterial colonization. An ideal wound dressing material must provide sufficient amount of moisture to avoid wound dehydration while being able to swell and absorb excess secretions in wound bed. The high surface area and interconnected pores in the nanofiberous mesh structure allow them to absorb fluids [3, 4, 11].

4- **Obstructing or preventing bacterial invasion**: beside their high porosity, the size and dimensions of the pores is also an advantage for nanofibrous meshes. Nanofibrous structure have pores within the nanometers. The average diameter of spherical bacteria is 0.5-2.0 µm. For rod-shaped or filamentous bacteria, length is 1-10 µm and diameter is 0.25-1.0 µm. On the other hand, the approximate pore size diameter of nanofibrous mesh is 0.5 nm. Such minute size can prevent microbial infiltration from the external environment. Also, the ability to introduce bioactive agents during the fabrication process can further allow for incorporating antibacterial drugs within the mesh structure [3][11].
1.2.2 Nanofibers fabrication: Electrospinning technology
Various techniques are available for the fabrication of nanofibrous meshes, however, electrospinning is the most common technique used and this is attributed to number of advantages outlined in this sections.

Compared with other fabrication techniques, including phase separation and self-assembly, electrospinning provides a simple and cost-effective way to produce nanofibrous meshes with fiber diameter in the nanometer range. The main advantage for the electospinning technique beside its feasibility is that a wide range of polymers can be used. There are many natural polymers that were used fabricated into nanofibers in the sub-micron range using the electrospinning technique. This natural polymers includes proteins, such as collagen and gelatin, and polysaccharides, such as cellulose and its derivative cellulose acetate. This is considered an advantage when it comes to biomedical applications because, for biological purposes, the materials used need to be biocompatible. The two main classes of polymers that are used for biomedical applications are either natural polymers or biocompatible synthetic polymers, such as poly (β carpolactone) and poly (lactic acid). Another advantage for electrospinning is that it allows the combination of different synthetic and natural polymers to fabricate nanofiberous membranes of tailored properties. The possibility of large-scale production combined with simplicity and versatility makes the electrospinning technique very attractive for a wide range of applications [3, 9, 14, 15, 16, 17]

A typical electrospinning setup (Figure 1.3) consists of a high voltage power supply (up to 30 KV), a pump, to adjust the flow rate of the polymer solution, a syringe connected to a capillary needle through which the polymer solution pass, and a collector. The needle tip is connected to one electrode of the power supply, while the collector is connected to the opposite electrode. The basic principle for the electrospinning technique depends on applying a high voltage to a polymer droplet hanged at the needle tip. Such voltage will electrify the polymer molecules within the droplets with the same charge. Consequently, electrostatic repulsion will take place which will result in the deformation of the polymer...
droplet, forming a cone (taylor cone). Finally, polymeric jet ejects from the deformed one and moves toward the oppositely charged collector. During its journey, the solvent evaporates and the polymer jets finally deposit in the form of ultra-fine fibers mesh on the surface of collector. The basic configuration shown in figure is used for the fabrication of non-woven meshes composed of randomly aligned fibers [3, 14, 16, 18].
Figure 1.3: Schematic representation of electrospinning basic set up. a) Image of Taylor cone forming at the spinneret during the electrospinning process. b) Image of polymeric filament forming from Taylor cone and moving toward the collector [3].
1.3 Components of an effective wound dressing nanofibers

A wide range of polymers can be used to obtain ultrafine nanofibers in the submicron range. In the last decade, more than 200 polymers have been electrospun and characterized successfully to be introduced for different applications. When it comes to wound dressing applications, biopolymers become of huge interest. Also, an effective nanofiberous scaffolds, targeting wound care purposes, shell also include an active ingredient that can further enhance the healing process. Such active ingredient could be an antibacterial agent, or a growth factor [3, 19].

1.3.1 Biopolymers

Biopolymers are class of polymers that are produced by living organisms. Thus, they are considered as polymeric biomolecules. Biopolymers are widely used in number of biomedical applications, owing to their high cyto-compatibility. Biopolymers can be subdivided into two main categories: First, is the biodegradable polymers, derived from renewable and non-renewable resources. Second, is the materials that are non-biodegradable but derived from renewable resources. Biodegradable biopolymers are defined as those that undergo microbial induced chain scission leading to their mineralization. Biodegradable Biopolymers can be further sub-divided into two main groups. One group contains the biodegradable petroleum-based polyesters such as Poly-caprolactone (PCL). The other group contains polymers from common natural and renewable resources, like the thermoplastic starch, cellulose and cellulose derivatives, or poly-lactic-acid (PLA) [20, 21, 22].
1.3.1.1 Poly-lactic acid: Structure, synthesis and applications

**Structure.** Polylactic acid or polylactide (PLA) is the polymer of lactic acid. It represents a linear thermoplastic aliphatic polyester that is derived from natural and renewable resources, such as corn starch, tapioca roots, and sugarcane. It exists in two optical forms (Figure 1.4), L (+) Polymer and D (-) polymer. Both isomers are partially crystalline, while the racemic poly (D, L-lactic acid) is amorphous [23,24].

**Synthesis.** PLA is synthesized by a two-step process consisting of fermentation, for production of lactic acid from the plant base, and chemical process for polymerization. Generally, there are two methods for PLA polymerization process (Figure 1.5).

![Chemical structure of poly-lactic acid (PLA) and its constituent monomers][23]

*Figure 1.4: Chemical structure of poly-lactic acid (PLA) and its constituent monomers [23].*
- **Direct condensation polymerization**: This reaction refers to the direct condensation of lactic acid and it can be considered the least expensive route of synthesis. Low molecular weight (MW) PLAs (< 3 000 g/mol) can be produced easily by direct condensation. However, one of the main drawbacks of this process is that it cannot be used for the synthesis of high molecular weight PLA. This is because the reaction taking place is an equilibrium reaction with water produced as by-product. Thus, it is essential to remove water continuously to drive the reaction forward. In this regard, it is very difficult to obtain a solvent-free high molecular weight polymer by this route. In order to overcome such limitation, chain coupling agents are used, which further adds cost and complexity to the process.

- **Polymerization through lactide formation**: Lactide is a cyclic dimer that is produced by the dehydration of lactic acid. First, lactic acid is pre-polymerized to obtain low molecular weight (MW) PLA as an intermediate. Second, the intermediate PLA is catalytically depolymerized through an intramolecular trans-esterification reaction to produce the cyclic lactide. Finally, cyclic lactide undergoes ring-opening polymerization to form the high (MW) PLA [20, 23, 24].
Properties and Applications. PLA is a hydrophobic polymer with limited solubility in water and common alcohols, such as ethanol. The methyl group in the lactic acid is responsible for such hydrophobicity. It is also presents a steric hindrance that is responsible for the high solubility of PLA in organic solvents. Although, low molecular weight PLAs are partially hydrophilic and contain oligomers with some aqueous solubility. The presence of ester linkages in the polymer backbone (Figure 1.4.) allows hydrolytic degradation of the polymer in an aqueous environment. The degradation rate is dependent on several parameters such as crystallinity (crystalline PLA degrades slower), molecular weight (MW) (low MW PLAs degrade faster), environment (pH, ionic strength, temperature) and particle morphology (porous structures degrade faster) [24, 25, 26].
In 2010, PLA was the classified as the second most important bio-plastic in the world regarding its consumption volume. Owing to their desirable properties, Polylactic acid (PLA) has been considered as a good alternative to petroleum-based plastic in food and medical application. Such properties includes biodegradability (PLA is 100% biodegradable), renewability (PLA can be recycled over 7-10 times), biocompatibility, and low toxicity to humans. The degradation products, resulting from PLA aqueous hydrolysis, are biocompatible and metabolizable; they are removed from the body by the citric acid cycle. Of the two PLA enantiomers, the polymeric L-lactic acid is commonly used because it can be degraded into the endogenous lactic acid produced by the body. Also PLA possesses enhanced physical properties such as transparency, good thermal, mechanical properties, and good processing properties [23, 24, 25].

With their excellent biocompatibility, polylactic acid (PLA), as well as its copolymers with glycolic acids (PLGA), are becoming the most commonly used synthetic biodegradable polymers in the medical field. PLA was approved by the US Food and Drug Administration in the 1970s and since has been widely employed in sutures, clips, plates and screws, in drug delivery devices, and in food packaging applications [25].

1.3.1.2 Cellulose acetate: structure, synthesis and applications

**Structure.** Cellulose acetate (CA) is ester form of cellulose. Cellulose is a natural polymer, consisting of anhydroglucose as repeating units, and has three hydroxy groups per unit (Figure 1.6). Cellulose acetate can be easily obtained from natural resources and recycled in environment by biodegradation. CA with different properties are obtained depending on the esterification degree (degree of substitution). The degree of substitution is also expressed as acetyl value (combined acetic acid (%)) [27, 28].
**Synthesis.** Cellulose acetate is produced through two-step reactions involving: i) the esterification of acetic acid with cellulose and ii) the partial hydrolysis of the resultant ester groups (Figure 1.7). The synthesis process starts by the activation of raw cellulose by a pre-treatment step. Usually wood pulp or cotton linters are used as the cellulose raw material. In the subsequent step, acetylation reaction takes place by adding acetic anhydride or acetic acid to the pretreated cellulose. Sulfuric acid is used as a catalyst to carry out the esterification reaction. In the acetylation step, cellulose triacetate is generated, in which almost all the hydroxy groups have been esterified. The final step, known as the ripening step, water is added to the prepared cellulose triacetate. This results in the partial hydrolyzes of the ester groups to result in cellulose acetate with a desired acetyl value [29, 30].

**Properties and applications.** CA is highly hydrophilic, with good liquid transport and water absorption abilities. It can be easily fabricated into films, membranes and fibers. CA has been widely used in membranes for separation and medical application because of its advantageous properties, such as good biocompatibility, biodegradability, regenerative properties, high affinity with other substances, high modulus, and adequate flexural and tensile strength [28, 29].

![Figure 1.6: Chemical structure of cellulose acetate [27].](image-url)
1.4. Natural extracts in nanofibrous wound dressings

As it was mentioned previously, active ingredients are widely used with in the nanofiberous membranes to prepare an interactive scaffolds for wound care. A main class of active ingredients is the natural products, especially plant and herbal extracts. By understanding the pharmacology of many ancient medicinal herbs increases day by day, the importance of alternative medicine also increase. Alternative medicine has gained more attention in modern science. Research has proved that herbal medicine can exert their medicinal effect on the human body through mechanisms identical to those already understood for chemical compounds in conventional drugs. In other words, herbal medicines do not differ greatly from conventional drugs regarding how they work. Thus, herbal medicines can be as
effective as conventional medicines. Various naturally occurring compounds are now used effectively as therapeutics agents such as the antimicrobial drug penicillin and the widely used chemotherapeutic agent, paclitaxel [31, 32, 33]

Thymoquinone (TQ), a phytochemical found in the volatile oil of *Nigella sativa* seeds, has shown potential as a novel therapeutic agent. The seeds have been used as a folk remedy for various ailments for thousands of years. The presence of rich biological active compounds in *Nigella sativa* volatile oil has highlighted its traditional medicinal use. Many active principles have been isolated from *Nigella sativa* seed, however, the most important ingredient, responsible for its medicinal properties, is the Thymoquinone (TQ). TQ (2-isopropyl-5-methyl-1, 4-benzoquinone) (Figure 1.8), shows a wide range of biological effects including: antibacterial, anti-cancer, anti-diabetic, and anti-inflammatory effects. TQ has low solubility in aqueous medium, which further limit its bioactivity. A lot of studies has been done on its medicinal properties [34, 35].

![Chemical structure of Thymoquinone](image)

*Figure 1.8: chemical structure of Thymoquinone, active principle of Nigella Sativa [34]*.
Chapter 2
Literature review
2 Literature review

2.1 Functional nanofibrous scaffolds for wound dressing applications

The initial phase of research for the fabrication of nanofibers-based wound dressing materials mainly focused on optimizing the different parameters of the electrospinning process in order to achieve the desired morphological, physico-chemical, and mechanical properties. The following phase of research aims at creating more advanced nanofibrous devices that is able to encourage the healing process and prevent infection. In this regard, wound dressing based on nanofibrous meshes can be classified into three main categories: passive; interactive and advanced. This classification reflects the evolution of nanofibrous wound dressings in terms of the selected materials, technique of fabrication, and functionalization of the fibers [3].

2.1.1 Passive Electrospun Meshes

Passive electrospun meshes are defined as the dressing material that can provide the minimal morphological and physical properties required for the healing process. This includes small diameter (within the nano-range), adequate membrane proposity, and gas and moisture permeability. Such system can maintain suitable levels of moisture in the wound bed and protect tissues from mechanical trauma. Passive electrospun meshes are fabricated from both natural and synthetic polymers [3]. Khil et al. successfully fabricated electrospun poly (urethane) membranes [36]. The morphological characterization and in vivo experiments showed that the matrix could be employed as wound dressings. Also, Phachamud and Phiriyawirut suggested the potential use of poly (vinyl alcohol) (PVA) fibers as wound dressing because of their swelling behavior and uniform fiber diameter [3]. Uppal et al. optimized the electrospinning conditions for the fabrication of hyaluronic acid (HA) nanofibrous [37]. In vivo studies indicated that the e-spun membranes offered the best treatment of full-thickness wounds when compared with four types of commercial
dressings (adhesive bandage, a sterilized HA film, gauze with Vaseline and an antibiotic dressing).

### 2.1.2 Interactive Electrospun Meshes

Interactive electrospun meshes combine the morphological and physical properties required for wound healing with the added value of being able to induce optimal cell responses and limit bacterial infiltration in the wound bed. The main strategy utilized to achieve such purpose is the combination of synthetic polymers and biopolymers. Synthetic polymers ensure easy processability and good mechanical properties for the mesh, while natural polymers improve the biocompatibility of the mesh so that it can actively interact with biomolecules involved in the healing process. Such combination targets the production of a multicomponent system more closely mimic the ECM (extracellular matrix). The main strategy used for combining natural and synthetic polymers within the same electrospun mesh is blending both natural and synthetic polymers in a single solution to be electropsun together, forming “poly-blended” nanofibers [3, 5].

A broad variety of reports based on the combination of natural and synthetic polymers is available in literature. Yuan et al. fabricated wound dressing meshes by electrospinning a blend of modified keratin and poly (hydroxybutylate-co-hydroxyvalerate) (PHBV) [38]. Keratin is a fibrous proteins that performs an essential structural role in skin and hair. Wound healing and histological tests revealed that the composite membranes accelerated wound recovery. Kim et al. fabricated electrospun meshes by blending polyurethane (PU) and gelatin and showed their potential application in wound healing [39]. Gelatin is a natural polymer derived from collagen. It is widely used for biomedical purposes since it is biodegradable, non-toxic, and easily available at low cost. Another composite nanofibrous meshes, consisting of type I collagen, chitosan, and poly (ethylene oxide) (PEO), has been fabricated by Cheng et al [40]. The resulted membrane showed better wound healing rates than conventional dressing when used in rat models. Chitosan is a polysaccharide that can function as a proliferation promoter, antibacterial agent, and wound healing accelerator.
Owing to their well-known repetition as an effective wound healing agents, a lot of focus is now given to alternative natural compounds, including essential oils and honey. Generally, natural substances cannot be electrospun into fibers because of their week mechanical and structural stability upon hydration. In this regard, natural compounds are blended with synthetic polymers in order to be electrospinned. PVA/PAA/PEG/PVP nanofibers with HPMC and aloe Vera were fabricated by electrospinning method. HPMC was added to the hybrid polymer solution as the water retention agent and aloe vera were also added to the system to promote the wound healing. The fabricated nanofibers mats were homogenous and linear with nanofiber diameters ranged between 80-480 nm [41]. Also, honey is a very attractive natural material for wound healing purposes due to its anti-inflammatory and antimicrobial properties. Sarhan, et al. were able to fabricate nanofibers with high honey concentrations (40%) and high chitosan concentrations (5.5%) of the total weight of the fibers using biocompatible solvents (1% acetic acid) [42]. The fabricated nanofibers showed pronounced antibacterial activity against Staphylococcus aureus but weak antibacterial activity against Escherichia coli. Also, the nanofibers revealed no cytotoxicity effects on cultured fibroblasts. Other natural products have been successfully electrospinned with natural and synthetic polymers. The data is summarized in Table 2.1.
Table 2.1: Some naturally derived substances used in interactive electrospun wound dressing

<table>
<thead>
<tr>
<th>Alternative - naturally occurring materials</th>
<th>Properties</th>
<th>Scaffold material and solvent</th>
<th>Electrospinning parameters</th>
<th>Fiber Characterization</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emu-oil</td>
<td>Facilitates wound healing and alleviates pain; analgesic, and anti-inflammatory properties</td>
<td>PU</td>
<td>Voltage=16 KV</td>
<td>Average diameters=400–600 nm</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DMF/THF</td>
<td>Flow rate = NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soya protein</td>
<td>Promotes tissue regrowth, by integrating into blood clots and stimulating collagen deposition</td>
<td>PEO</td>
<td>Voltage=12 kV;</td>
<td>Average diameters=200–1000 nm</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HFP</td>
<td>Flow rate= 0.8 ml/h</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Distance=15 cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silk-fibroin</td>
<td>Exhibits good oxygen and vapor permeability and minimal inflammatory reaction</td>
<td>PEO</td>
<td>Voltage=10–11 KV;</td>
<td>Fibers with flattened, ribbon-like morphology</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixed with EGF</td>
<td>Flow rate= 1.2 ml/h</td>
<td>Fibroblasts adhesion and proliferation within the fibers is shown</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Distance= 15–21 cm</td>
<td>Average fiber diameter=1000 nm</td>
<td></td>
</tr>
</tbody>
</table>
2.1.3 Advanced Interactive Electrospun Meshes

Advanced interactive electrospun meshes are capable of delivering antibacterial agents to prevent and treat infection [3]. Number of researches are now focusing on preparing drug-loaded nanofibrous meshes with controlled release property.

Poly (ε-caprolactone) (PCL) electrospun fibers containing the antibiotic, ampicillin sodium salt, were successfully twisted into nanofiber yarns. A significant reduction in the diameter of fibers and diversity was observed with the addition of ampicillin salt to the polymer solution. The authors attributed such effect to the increase in conductivity of the polymer solution associated with the addition of the antibiotic salt. Also the antibacterial results indicated that the released antibiotic maintained its antibacterial activity after fabrication against both gram-positive \textit{Staphylococcus aureus} and gram-negative \textit{Klebsiella pneumonia}. Therefore the as-spun yarns were antimicrobial active [46]. In another study done by Qia at al. antibiotic drug tetracycline hydrochloride (TCH)-loaded halloysite nanotubes was incorporated in PLGA matrix to (TCH/HNTs/PLGA) prepare composite nanofibers [47]. The results showed that the incorporation of TCH-loaded HNTs within the PLGA nanofibers is able to improve the tensile strength and maintain the three-dimensional structure of the nanofibrous mats. Also, the cytotoxicity and cell attachment results demonstrated that the prepared composite is biocompatible with mouse fibroblast cells cultured onto the fibrous scaffolds. More importantly, the TCH/HNTs/PLGA composite nanofibers were able to release the antibacterial drug TCH in a sustained manner for 42 days and display antimicrobial activity against \textit{S.aureus}. The potential use of another class of antibiotic, ciprofloxacin, was also explored by Unnithan et al [48]. The authors were able to prepare antibacterial nanofibers membranes via electrospinning of solution containing dextron, polysaccharide molecule, polyurethane (PU) and ciprofloxacin HCl (CipHCl) drug. The obtained nanofibers mats had good morphology. The in vitro viability and SEM morphology observation indicated that the cells interacted favorably with the scaffolds especially the drug-containing one. Moreover, the results of the inhibition zone tests showed good bactericidal activity against both of Gram-positive and Gram-negative bacteria.
Another class of antibacterial agents that have been introduced in the advanced interactive electrospun meshes is the silver. Silver, either in its nanoparticle form (NPs) or as silver nitrate (AgNO$_3$), has great bactericidal effect against wide range of bacterial. Duan et al. produced silver NPs-loaded antimicrobial nanofibers, intended as wound dressing, by electrospinning of a PCL solution with small amounts of silver-loaded zirconium phosphate nanoparticles (nano AgZr) [49]. The results of the antimicrobial tests showed that these fibers have maintained the strong killing abilities of Ag existed in the nano AgZr against the tested bacteria strains (Gram-positive) Staphylococcus aureus (ATCC 6538) and Gram-negative E. coli (ATCC). The cell proliferation results indicated that the human dermal fibroblast (HDF) cells were able to attach and proliferate as continuous layers on the nano AgZr-containing nanofibers and maintained the healthy morphology of HDF. Another technique for the preparation of silver NPs-loaded nanofibrous membrane was also investigated by Chen et al [50]. In this study, silver NPs-laoded nanofibers were prepared by electrospinning polyurethane (PU) solution containing silver ion, followed by UV treatment. The UV radiation reduce silver ion in the PU/AgNO$_3$ nanofibers to silver nanoparticles. SEM, TEM and XRD analysis proved the presence of Ag NPs impeded within the nanofibers structure. For promoting wound healing, collagen was grafted to fiber surface by low temperature and oxygen plasma treatment. This can improve surface hydrophilicity of the membrane and facilitate covalent binding of collagen molecules to its surface. Results showed that inhibition of bacterial growth improved to approximately 100%. This was also associated with the increase of membrane water absorption ability, which further facilitates its use as function wound dressing. Animal studies showed better healing performance for the prepared composite membrane in comparison to gauze and commercial collagen sponge.

2.1.3.1 Plant extract and phytochemicals in advanced electrospun meshes
Many medicinal plants have a long history of curative properties in wound healing. The capability of utilizing electrospinning technique to combine the advantages using these plant extracts in the form of nanofibrous mats and their potential application as skin graft substitutes has been investigated by number of researches. Four different plant extracts was
incorporated in PCL matrix and evaluated for their potential use in skin tissue engineering [51]. The plant species investigated in this study was *Indigofera aspalathoides*, *Azadirachta indica*, *Memecylon edule* (ME) and *Myristica andamanica*. The ability of human dermal fibroblasts (HDF) to proliferate on the electrospun nanofibrous scaffolds was evaluated via cell proliferation assay. The interaction of HDF with the electrospun scaffold was studied by F-actin and collagen staining studies. The results indicated that PCL/Me scaffolds possess the least cytotoxicity with HDF proliferation capacity 31% higher than the proliferation on PCL nanofibers after 9 days of cell culture. This percentage was the highest among all the other extract loaded-nanofibrous scaffolds. Therefore, the authors performed the epidermal differentiation of adipose derived stem cells on PCL/ME scaffolds and obtained early and intermediate stages of epidermal differentiation.

In another study, the feasibility to utilize PCL, as a drug-delivery carrier for curcumin were investigated [52]. Curcumin is a phenolic phytochemical that is extracted from the roots of *Curcuma longa* L and is responsible for its medicinal properties. The high-specific surface area and high porosity can enhance the functional properties of Cur. Bead-free nanofibers were obtained by direct electrospinning of PCL/curcumin solution (7% curcumin based on the weight of polymer). The nanofibrous scaffolds showed a sustained release of curcumin for 72 h. The release profile also indicated that the drug can be deliver with a dose much lower than the reported cytotoxic concentration while remaining bioactive. The cell viability assays also indicated more than 70% viability for the Human foreskin fibroblast cells (HFF-1) on curcumin-loaded nanofibres. The curcumin-loaded nanofibres were able to reduce the inflammatory induction. This was proved by the low levels of interleukin-6 released from mouse monocyte–macrophages seeded onto the scaffolds after being stimulated by *E. coli* derived lipopolysaccharide. The in vivo wound healing capability of the curcumin loaded PCL nanofibres was demonstrated by an increased rate of wound closure in a streptozotocin-induced diabetic mice model. These results demonstrate that the curcumin-loaded PCL nanofiber matrix is bioactive and has potential as a wound dressing with anti-oxidant and anti-inflammatory properties.
Crude bark extract of *Tecomella undulate*, a medicinal plant widely known for its traditional wound healing ability, were incorporated within PCL/PVP nanofibrous matrix [53]. Morphological characterization of the nanofibers by SEM showed that the incorporation of the herbal extract in the polymer media did not alter their morphology or size since both drug-loaded and drug-free nanofibers appeared to be smooth with the same diameter range. The composite scaffolds showed high loading efficiency for the herbal extract. Also, the zone inhibition activity against standard strains of *Pseudomonas aeruginosa* MTCC 2297, *Staphylococcus aureus* ATCC933, *Escherichia coli* (IP-406006) showed that the prepared scaffolds were able to inhibit the growth of the all the tested bacterial strains. On the other hand, incorporating aqueous extract (5% Wt) of *Grewia mollis Juss* within (PU) nanofibers affected both the morphology and size of PU nanofibers [54]. FE-SEM-EDX and FT-IR results confirmed both well orientation of nanofibers and good dispersion of aqueous extract within the fibers. The antimicrobial effects of aqueous extract loaded fiber mats was studied against *Escherichia coli* ATCC 52922 (Gram negative) and *Staphylococcus aureus* ATCC 29231 (Gram positive) in liquid medium. The scaffolds were able to inhibit the growth of the bacterial strains.

The capability of using PLA and CA as passive, interactive or advanced interactive electrospun meshes have been investigated through number of studies that will be discussed in the following sections.

### 2.3 Cellulose acetate application in wound dressing

The major component of fibrous extracellular matrix of dermis is composed of a complex combination of proteins and polysaccharides. Electrospuning of cellulose acetate, as a polysaccharide derivate, is considered an added value for tissue engineering purposes. Kontogiannopoulou et al. investigated the probability of incorporating Alkannin and shikonin (A/S), naturally occurring hydroxynaphthoquinones in plants, within cellulose acetate electrospun fibers to be used for wound healing applications. Both drugs were effectively loaded into the nanofibrous mesh [55]. The incorporation of drugs did not
considerably affect fibers morphology and both drug free and drug loaded nanofibers were smooth with diameter varied from 315 to 670 nm. High drug entrapment efficiencies (ranged from 74% to 95%) and appropriate release profiles were achieved, that render these fibers as potential topical/transdermal wound healing dressings.

Poly-blended NFs depending on cellulose acetate were also prepared and investigated for their potential use as topical/transdermal patches or as wound dressing [56]. In this study, a series of nanofibrous membranes were prepared from cellulose acetate (CA) and polyester urethane (PEU) blend-electrospinning. To prevent common clinical infections, an antimicrobial agent, polyhexamethylene biguanide (PHMB) was incorporated into the electrospun fibers. The presence of CA in the nanofibers membrane improved its hydrophilicity and permeability to air and moisture. CA-containing fibers showed better swelling behavior vapor permeability. In conclusion, inclusion of CA within the matrix not only increased the liquid uptake but also created a moist environment for the wound, which can further accelerate wound healing. The same conclusion, regarding improved hydrophilicity by incorporating CA, was also achieved by Unnithana, et al [57]. In this study, the author prepared an antibacterial CA-based nanofibrous scaffolds by electrospinning of solution of PU blended with CA and zein. PU was used as the foundation polymer while CA and zein was added to the matric to achieve desirable properties such as better hydrophilicity, excellent cell attachment, proliferation and blood clotting ability. An antibiotic, streptomycin sulfate, was incorporated into the electrospun fibers to prevent clinical infections. PU–CA–zein-drug composite nanoscaffold showed enhanced blood clotting ability in comparison with pristine PU nanofibers. The presence of CA and zein in the nanofiber membrane improved its hydrophilicity, bioactivity and created a moist environment for the wound. Also, the cell proliferation assessment revealed better cell growth and cell attachment on drug-loaded PU–CA and PU–CA–zein composite nanofibrous scaffolds than on PU nanofibers.
Vatankhah et al proved that combining both CA, as a polysaccharide derivative, and gelatin, as a protein, is an effective simulator of the structure and composition of native extracellular matrix (ECM) [58]. The authors successfully electrospun cellulose acetate/gelatin mats at different ratios and evaluated their performance as a scaffold for either skin tissue engineering or as a wound dressing. The results showed that the cell proliferation and adhesion capability is highly dependent on the composition of cellulose acetate and gelatin in the scaffolds. High proliferation of human dermal fibroblasts was obtained on electrospun cellulose acetate/gelatin 25:75. This confirmed the capability of such composite nanofibers as a tissue-engineered scaffold that can be used for full-thickness wounds. On the other hand, electrospun cellulose acetate/gelatin 75:25 can be a potential low-adherent wound dressing, which can be easily removed. Thus, it can be applied for superficial wounds.

2.4 Poly-lactic acid application in wound dressing

Poly (L-lactic acid) (PLLA) is a biocompatible synthetic polymer which was approved by the Food and Drug Administration for specific human clinical applications, and had eximious mechanical property. Its electrospinning product was increasingly used as an effective drug-delivery carriers [59]. PLA has been electrospun by Alsi et al. to act as a carrier matrix for controlled delivery of highly porous silver microparticles [60]. The author found that the highly porous silver microparticles (AgMPs) could be successfully incorporated in nanofibrous and that the PLA porous AgMPs-loaded nanofibers showed a steady silver ion release at a greater rate than that of AgNPs-loaded nanofibers. Also, the AgMPs-loaded nanofibers matrix exhibited comparable antimicrobial efficacy and cytotoxicity to the AgNPs-loaded nanofibers matrix.

In another study that utilized PLA as a carrier matrix, a crude extract of *Garcinia cowa Roxb.* (GC) were used as the bioactive drug. The fibers of both the neat and the GC-loaded PLLA fibers (up to 50% extract based on the weight of the polymer) were smooth, with average diameters of 0.80–1.13 μm [61]. The results showed that the release profile of the
GC extract depends on the release media used and temperature. The maximum cumulative amount of extract released from the GC-loaded PLLA fiber mats in the phosphate buffer medium (at 37 °C) was greater than that released in the acetate buffer medium (32 °C). The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, used to assess the antioxidant activity, showed that the GC-loaded PLLA fiber mats maintained its antioxidant potential even after being exposed to a high electrical potential like that applied during electropinning. Also, the prepared matrix showed antimicrobial effect against Staphylococcus aureus ATCC 25923 and Staphylococcus aureus DMST 20654. Lastly, almost all of the GC-loaded PLLA fiber mats were found to be nontoxic to normal human dermal fibroblasts except for those that contained 50 % extract.

In spite of the biocompatibility of PLA, further application of PLA fiber in biomedical materials is hindered by its hydrophobicity. Consequently, there is an attempt to increase the hydrophylicity of PLA to be applicable in wide range of tissue engineering applications including wound dressing. In this regard, number of modification techniques have been investigated including: copolymerization of lactide with other monomers or polymers such as malic acid, polyethylene glycol (PEG), polyglycolic acid (PGA), or dextran, as well as surface modification such as plasma treatment and surface coating [24,62-64]. However, blending polylactide with other polymers is a simple yet attractive method for modification[63]. Polymer blending can combine and optimize the polymeric physical properties of interest. One advantage of using polymer blends over the synthesis of new polymers is that the composition of the polymer blend is controlled easily and is subject to change according to applications requirements. Polymer blends can also provide effective control on the release profile for pharmaceuticals [64]. Thus, it can offer significant advantages over traditional drug delivery techniques. Optimizing and fine-tuning of the bio-functionality of PLA electrospun nanofibers through blending method was demonstrated in number of studies. Polyvinylpyrrolidone (PVP) is also a biocompatible synthetic polymer, and is a hydrophilic in nature. So, PVP is a good material to prepare composite material with PLLA for improving the hydrophilicity of obtained hybrid matrix. Xu, et al conducted a study to introduce PVP into PLLA nanofibrous structure. Different ratios by weight (PVP/PLLA: 1/1, 1/3, and 1/9) were prepared using trichloromethane as
solvent [65]. The water contact angle assay, used to test the degree of hydrophilicity of a membrane, indicated that the higher is the PVP ratios, the higher is the hydrophylicity of the matrix. Thus, the hydrophilicity of nanofiber matrix could be adjusted by controlling the content of PVP. The authors assumed that that the prepared spun ultrafine fibers can be used as a synthetic extracellular matrix scaffold for tissue engineering.

Another issue which limit the application of PLA in tissue engineering and wound dressing purposes is the poor cell affinity and lack of cellular interaction, thus they do not favor cellular adhesion and migration [62, 63, 66]. To enhance cell adherence on scaffold and improve cellular response, the surface of PLA nanofibrous scaffold was modified by gelatin. Gelatin, as explained before, is the widely used natural polymer for its low cost and high cellular response [63]. For electrospinning, PLA and gelatin were dissolved in hexafluoroisopropanol (HFIP) solvent at varying compositions (PLA: gelatin at 3:7 and 7:3). Cellular viability and proliferation results showed that the modified PLA/gelatin 7/3 scaffold is more suitable for fibroblasts attachment and viability than the PLA or gelatin nanofiber alone. Thus fibroblast cultured on PLA/gelatin scaffold could be an alternative way to improve skin wound healing. In another study, the incorporation of collagen type I within the PLLA electrospun nanofibers can effectively modulate certain aspects of cellular behavior even at very minor concentrations (<1% w) [66]. Such aspects includes cellular attachment. The study showed that cell attachment after 24 h were significantly higher on collagen/PLLA scaffolds when compared to pure PLA scaffolds. Also, the results showed a clear differences in cell penetration. Cells migrated through 32 and 85% of PLLA and collagen/ PLLA scaffolds after one week, respectively. Finally, mineralization of primary calvaria osteoblasts provided further evidence that collagen- containing electrospun PLLA scaffolds could sustain cell differentiation.
2.5 Thymoquinone: Medicinal properties and applications

Thymoquinone (TQ) has been investigated for their antibacterial properties. In a study that was conducted by Halawani, the bactericidal potential of thymoquinone (TQ) and its derivative, thymohydroquinone (THQ) [35]. The two main ingredients in Nigella sativa, were investigated against 6 bacterial strains: Escherichia coli, Pseudomonas aeruginosa, Shigella flexneri, Salmonella Typhimurium, Salmonella Enteritidis and Staphylococcus aureus. Both TQ and THQ exerted antibacterial activity against all the tested bacterial strains. Gram-positive bacteria were more susceptible to both TQ and THQ than the gram-negative bacteria, with minimum inhibitory concentration (MIC) ranged between 200 and 1600 μg/ml. Among all the tested bacterial strains, S. aureus showed to be highly susceptible to TQ than THQ. The MIC of TQ that is required to inhibit the growth of S. aureus was 3-6 μg/ml while that required in case of THQ was 400-800 μg/ml (100 times higher). Also the combination of TQ and THQ with antibiotics (ampicillin, cephalexin, chloramphenicol, tetracycline, gentamicin, and ciprofloxacin) exerted synergism in only in case of S. aureus. While for gram-negative bacteria, synergism, antagonism and indifferent effects were detected. This study demonstrated that TQ is more effective than THQ regarding its antibacterial effect, and that their activity could be potentiated by antibiotics especially in case of S. aureus. On the other hand, Hossain et al. presented that the minimum bactericidal and inhibitory concentration of TQ to Staphylococcus aureus was 31μg/mL(188.8 μM) [34]. Also, the authors presented that the the minimum inhibitory concentration of TQ for Bacillus anthracis was 3 μg/mL (18.3 μM).

TQ not only used as antibacterial agent but it can be also used as an anti-biofilm agent, inhibit the formation of bacterial bio-films on surfaces. The antibacterial activity of (TQ) and its biofilm inhibition potencies were investigated on 11 human pathogenic bacteria [67]. The development of the bacterial biofilm were evaluated using the crystal violet (CV). The results showed that TQ exhibited a significant antibacterial activity against the majority of the tested bacteria (MICs values ranged from 8 to 32 μg/ml) especially Gram positive cocci (Staphylococcus aureus ATCC 25923 and Staphylococcus epidermidis CIP 106510). This results actually correlate with the results obtained by other authors as
discussed before. Crystal violet assay showed that the minimum biofilm inhibition concentration (BIC50) was reached with 22 and 60 μg/ml for *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* CIP 106510 respectively. In addition, TQ prevented cell adhesion to glass slides surface, thus it prevent biofilm formation. Consequently, TQ can be used as bioactive substance with anti-biofilm potential. All this studies done on TQ proves that such phytochemical deserves further exploration as an effective alternative to customary medicines for pathogenic bacteria.

Because of the anti-bacterial, anti-inflammatory and antioxidant activities of TQ, Selcuk, et al hypothesized that TQ would reduce inflammation and oxidative stress and accelerate wound closure in a rat model of deep second-degree burns. The results proved that topical and/or systemic administration of TQ reduced inflammation and oxidative stress and accelerated the rate of wound closure or re-epithelialization.
Chapter 3
Materials and Methods
3 Materials and Methods

3.1 Materials
Poly (lactic acid) (PLA) pellets with a commercial name “Ingeo” grade 4043D were purchased from NatureWorks LLC, Minnetonka, USA. The purchased grade has a relative solution viscosity (RV) of 4.0 ± 0.10, and a level of D-isomer of 4.25 ± 0.55%. Cellulose acetate was purchased from Loba Chemie, Mumbai, India. The cellulose acetate has degree of acetylation~ 24%. Criterion™ nutrient broth (500 g), and Criterion™ nutrient agar (500 g) were purchased from Hardy Diagnostic, Thymoquinone (extra-pure), Dichloromethane (DCM) (≤99%), \(N,N\) dimethyl-formamide (≤99%), absolute ethanol anhydrous, sodium phosphate dibasic (extra-pure MW=141.96), sodium phosphate monobasic dehydrate (extra-pure, MW=156.01 ), sodium hydroxide (extra-pure), Sodium dodecyl sulfate (extra-pure, MW= 288.38), Dulbecco’s modified eagle’s medium, MTT cell proliferation kit, and glutaraldehyde solution (25%) were purchased from Sigma Aldrich, Germany. All chemicals were used directly without further purification.

3.2 Experimental Procedures

3.2.1 Preparation of polymer solutions
Polymer solutions were prepared in DCM: DMF (7:3 V/V) at concentration of 6\% w ratio. PLA: CA solutions were prepared in two different weight ratios including, 9:1 and 7:3 (% w) to get polymer solution of final concentration 6\% w. To prepare the drug-loaded samples, 3\% w TQ (concentration based on the weight of the polymers) was added for each polymer solution. Two other drug concentration ratios have been also prepared (1 and 5 \% w) within the PLA NFs, to get the optimum morphology and biological activity. All the prepared samples were stirred vigorously for 4-6 h until a homogenous solution was obtained. TQ is known to be a light sensitive drug, thus all samples were covered with aluminum foil sheet and maintained at room temperature until electrospinning.
3.2.2 Electrospinning

After stirring for 4-6 h, each polymer solution was loaded into a 5-ml syringe attached to a 27G blunted stainless steel needle at a flow rate of 1.5-3 ml/h. A high voltage of 20-24 kV was applied to the tip of the needle. The fibers were deposited on a flat aluminum foil that is wrapped around a stationary collector. The distance between needle tip and the stationary collector was kept at 15 cm throughout the process. The electrospinning conditions for each polymeric solution has been adjusted, depending on trial and error, until the optimum electrospinning solutions are obtained (Table 3.1). This is reflected in the morphology of the fibers, examined by the SEM. All the electrospinning processes were carried out at approximately 28 °C and 30% humidity. Nanofibrous membranes were dried in a vacuum oven for 24 h to ensure the complete vaporization of all solvent residuals.

Table 3.1: Electrospinning parameters used for fabricating TQ-loaded PLA, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3.

<table>
<thead>
<tr>
<th>Polymer solution ratio</th>
<th>Voltage (KV)</th>
<th>Needle tip-collector distance (cm)</th>
<th>Nozzle</th>
<th>Flow rate (ml/h)</th>
<th>Concentration (% w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA 10 CA 0</td>
<td>20</td>
<td>15</td>
<td>25</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>PLA 9 CA 1</td>
<td>22</td>
<td>15</td>
<td>25</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td>PLA 7 CA 3</td>
<td>24.5</td>
<td>15</td>
<td>25</td>
<td>1.5</td>
<td>6</td>
</tr>
</tbody>
</table>
3.3 Characterization of Polymeric solution

3.3.1 Viscosity measurements
The viscosity of the polymeric solutions has been determined using the viscometer (VR 3000 model: L. Viscotech Hispania S.L., Spain). All measurements were carried out at room temperature 25 °C, 200 RPM and the probe used is L2.

3.4 Characterization of the prepared electrospun nanofibers

3.4.1 Scanning electron microscopy
The morphology of the prepared e-spun nanofibers were observed under scanning electron microscopy (SEM: FEI-QUANTA 200F, Netherland) at an accelerating voltage of 15 kV. Before SEM observations, the samples were sputter-coated with gold films of a thickness of 10 nm. (JEOL JFC-1600 Auto fine coater, Japan). The samples were gold-sputtered to obtain images of better quality through charge dissipation. SEM images were analyzed with image analysis software (Image J, National Institutes of Health, USA) to determine average fiber diameter. A total of 50 random NFs per image were used to calculate the mean and standard deviation of fiber diameter.

3.4.2 Fourier transform infrared spectroscopy
The chemical composition of the prepared samples was studied by Fourier transform infrared spectroscopy, using a Spectrum PerkinElmer (Wiesbaden, Germany). The infrared spectra of the drug–free PLA: CA 7:3, TQ-loaded PLA: CA 7:3 and TQ-loaded PLA electrospun nanofibers matrices were measured over a wavelength range of 4,000–550 cm⁻¹ at a resolution of 4 cm⁻¹.
3.4.3 Porosimetry
Pore diameter distribution, pore surface area distribution, and the porosity of the electrospun TQ-loaded PLA, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3 were measured using a mercury intrusion porosimeter (Poresizer Quantachrome Instruments, Florida, USA) in mercury intrusion under an increasing pressure from 100 kPa to 207MPa, which was carried out at the YJSTRC Research Center (AUC). The determination of porosity was based on the relationship between the applied pressure and the pore diameter into which mercury intrudes, according to the Washburn equation:

\[ D = \frac{-4\gamma \cos \theta}{P} \]  

(1)

Where \( P \) is the applied pressure, \( D \) is the pore diameter, \( \gamma \) is the surface tension of the mercury (484mNm\(^{-1}\)) and \( \theta \) is the contact angle between mercury and the pore wall, taken as 141.31\(^{\circ}\).

3.4.4 Swelling behavior and water retention capacity
The water retention and swelling behaviors of the medicated NFs scaffolds were measured in a phosphate buffer solution at the physiological temperature (37 °C) for 12, 24 and 48 h with four replicas. After immersion, fibrous mats were weighted after wiping the excess solution adhered on the ultrafine mat with filter paper. The percentage swelling or the water-up take capacity of the nanofibers was calculated using the following equation:

\[ \text{Degree of swelling (\% of water up take)} = \frac{Wt - Wd}{Wd} \times 100 \]  

(2)
Where \( W_d \) is the weight of each sample before submersion in the buffer solution and \( W_t \) is the weight of the sample after submersion for a certain time interval (t). Each sample was investigated in triplicates and the data was expressed as \( \pm \) mean standard deviation.

3.4.5 Water vapor permeability
The water vapor permeability of the developed medicated scaffolds was determined through the evaluation of the water vapor transmission rate (WVTR) through the membranes. The test was carried out by the ASTM standard E 96 method [68]. The scaffolds were cut into circular discs with a 4.5 cm diameter and a 0.25\( \pm \) 0.05 mm thickness. Then, scaffolds were mounted onto the mouth of autoclave bottles (100 ml). The bottle was filled with pre-dried desiccant (calcium chloride). The desiccant was first dried in a vacuum oven for 24 h at 40\( ^\circ \)C. About one-quarter of the total volume of each bottle was filled. The scaffolds were tightly fixed using paraffin tape along the edges of the bottle to prevent water vapor entrance across the periphery. The whole setup was maintained at ambient temperature with a 75% constant relative humidity in a dry cabinet (Digi-Cabi, AD-200) to ensure accurate control of humidity. The humidity was kept constant throughout the time of the experiment with the aid of saturated sodium chloride solution. At regular intervals, the samples were weighed, and the weight gain was determined. A graph was plotted for the weight gain versus time and from the graph slope, we calculated WVTR (g/m\(^2\)/day) from the following equation:

\[
WVTR = \frac{\text{Slope}}{A} \quad (3)
\]

Where \( A \) is the exposure area of the scaffolds (m\(^2\)). Four sets of samples were examined to obtain the replicable results.

3.5 In Vitro drug release study
3.5.1 Calculation of Total TQ content in TQ-loaded nanofibers

Prior to investigating the release profile of TQ from the prepared e-spin nanofibers, the actual content of drug was first calculated. This represents the amount of drug incorporated within the nanofibers, inside it or on the surface, compared to the initial amount of drug used in the solution. A pre-weighted mount of each sample (circular disc of 3-5 mg) was dissolved in 5 ml of 7:3 V/V DCM: DMF. The samples were dissolved under vortexing in order to destroy the fibrous structure, thus releasing the drug in the organic phase. After that, the Absorbance of TQ in the organic phase was determined by UV/VIS spectrophotometry (Cary 60 UV-VIS/Agilent technologies) at the characteristic wavelength for TQ (255 nm). The actual amount of TQ in the e-spun fibers was then back calculated from the obtained data against a predetermined calibration curve for TQ. Such linear calibration curve was obtained by measuring the absorption of different TQ solutions of known concentrations (see appendix). The obtained relationship is shown in the following equation:

\[
\text{Drug concentration (\(\mu g/ml\))} = 9.587 \times \text{Absorption (R2=0.9984)}
\]

The total drug content (entrapment efficiency) was then calculated using the following equation:

\[
\text{Total drug content} = \left( \frac{X_i}{X_f} \right) \times 100 \quad (4)
\]

Where \(X_i\) is the amount of drug successfully incorporated into the nanofibers and \(X_f\) is the initial amount of drug added in the electrospun solution. In order to examine the uniformity of drug distribution throughout the fibrous mats, the total drug content from random parts of the fibrous construct was determined as described above. Three replicate mats were analyzed each time. The average of the obtained values was used as the basis to determine the cumulative release of TQ from the TQ-loaded e-spun mats.
3.5.2 Release of TQ from TQ-loaded nanofibers

3.5.2.1 Preparation of release medium

For the intended application of the TQ-loaded nanofibers as wound dressing materials, phosphate buffer solution of the physiological pH (pH=7.4) was used as the release medium. 1000 ml of phosphate buffer was prepared by dissolving 6.177 g of anhydrous disodium hydrogen orthophosphate and 1.014 g of sodium dihydrogen orthophosphate in 100 ml distilled water. Then, 8.7 g of sodium chloride were added to the solution. Finally, distilled water was added to obtain the required volume. The pH was then adjusted to 7.4 by addition of few drops of 2 N sodium hydroxide. Due to the poor solubility of TQ in aqueous solutions, modification of the release media was necessary. In this regards, 1% w/v of sodium dodecyl sulfate (SDS) and 10% v/v of ethanol were added to the media to facilitate the dissolution of TQ in aqueous media.

Addition of emulsifiers and alcohols, as dissolution aids in aqueous media, for investigating the release of hydrophobic drugs is very common. Taepaiboon et al and Suwantong et al used methanol-containing acetate buffer as the release medium to study the release profile of Vitamin E and curcumin from Vit E-loaded and curcumin-loaded cellulose acetate nanofiber, respectively [69]. On the other hand, Konstantinos et al used 1% SLS within the acetate buffer solution to study the release of shikonin from CA nanofibers [55]. Again, Suwantong used both 0.5% v/v tween 80 and 3% v/v methanol to facilitate the dissolution of *Garcinia cowa* extract, loaded on PLLA nanofibers, into acetate buffer release medium [61].

3.5.2.2 Thymoquinone release assay

The total immersion method was used to investigate the release characteristics of TQ from the medicated nanofibers TQ-loaded neat PLA, TQ-loaded PLA: CA 9:1 and TQ-loaded...
PLA: CA 7:3. Since TQ is a hydrophobic drug that is readily insoluble in water, the release study was carried out under the sink conditions i.e. the drug concentration was kept below its solubility limit. The drug release experiment was carried out by incubating the samples in the aforementioned release medium. 30-35 mg of each sample were added in separate vials with 5 ml release medium. Samples were incubated at the physiological temperature, 37 °C. At specific immersion times, ranging from 1 h to 9 days, the 5 ml release medium were withdrawn from the vials and replaced with equal amount of fresh medium.

The amount of TQ released at the different time intervals was determined using UV-vis spectrophotometer at 255 nm, with the aid of the following calibration curve of the TQ in the same release medium:

\[
Drug\ concentration\ (\mu g/ml) = 9.023 \times Absorption\ (R^2=0.9991)
\]

The cumulative percentage of the drug released was calculated using the equation below and then plotted versus time.

\[
\%\ Cumulative\ drug\ release = \left(\frac{Drug\ released\ at\ time\ (t)}{total\ entrapped\ drug}\right) \times 100 \quad (5)
\]

3.6 In vitro antibacterial assessment

3.6.1 Microbial strains and culturing conditions

The following micro-organisms were used for antibacterial assessment study: *Escherichia coli* and *Staphylococcus aureus* ATCC25923. The bacterial strains were supplied by the VACSERA center. Preparation of bacterial inoculum occurs as follows: the gram-negative *E. coli*, and gram-positive bacteria *S. aureus* were pre-cultured in Criterion™ nutrient broth (NB) overnight in a rotary shaker at 37°C, centrifuged at 13,000 rpm for 2 min, then pellet was suspended in sterile water and the cell density was standardized.
spectrophotometrically (Absorbance: 570 nm) to be $10^8$cells/ml. A melted agar/bacterial suspension is then prepared by the agar overlay technique. Briefly, 100 ul of diluted bacterial suspension was added to a soft top agar (0.75%, as opposed to the usual 1.5% for agar plates) which has been melted at 100°C and cooled to 45°C. This is warm enough so the agar remains liquid, but cool enough so that the bacteria are not killed. Suspension of microorganisms in the agar melt is mixed and then spread evenly as a thin layer across the top of Petri dishes. The melted agar/bacterial suspension is then allowed to solidify. The Criterion™ Mueller–Hinton (MH) nutrient agar was used for both S. aureus and for E. coli.

3.6.2 Antibacterial activity measurement
The antibacterial efficiency of the medicated samples, containing TQ, was tested by the disk diffusion method or the Modified Kirby-Bauer antibiotic test [70]. The nanofiberous mats, TQ-loaded PLA: CA 7:3, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA, were cut into circular discs of diameter around 1.5 cm each and denoted as A, B and C, respectively. The discs were first sterilized under UV light for 2 h then placed on the top of the smeared agar plates. The antibacterial activity plates were placed in the refrigerator for 8 h to allow for the release of the drug. Then, the plates were incubated at 37°C for 24 h. Upon reaching the inhibitory concentration, microbial growth stops, which can be seen as a clear inhibition zones around the disc samples. The diameters of the inhibition zones were measured with transparent ruler. The experiments were performed in triplicate. All results were expressed as the mean ± standard deviation of the mean. The antibacterial plates were photographed for further evaluation.

3.7 Cytotoxicity, Cell proliferation and cell attachment assessment:

3.7.1 Cell line and maintenance
Cell culture studies were carried out using mouse fibroblast cells (3T3-L1). The 3T3-L1 fibroblasts (VACSERA center, Cairo) were initially maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The medium was replaced every 3 days and cultures were maintained in an incubator with 5% CO₂ at 37°C. (incubator, ASTEC, Japan).

The cells were sub-cultured in a 25 cm² tissue culture flasks with modified DMEM. The fibroblasts were grown to confluency. Before each assay, confluent cells were detached by adding 1ml of 0.25% of trypsin containing 0.1% EDTA. Detached cells were centrifuged at 1500 r/min for 5 min, counted by trypan blue using a hemocytometer and seeded on the desired substrate (prepared nanofibrous scaffolds or the tissue culture polystyrene plate (TCP) as control ) at a certain cell density (mentioned below).

The electrospun nanofibrous mats were cut into circular discs of diameter 1.5 cm. The scaffolds were first sterilized by being exposed to UV radiation for 1 h, before using in any cell culture assay. Each sample underwent each treatment in triplicate. The results were expressed as the mean ± standard error of the mean.

3.7.2 Cytotoxicity assay
In this study, the release of TQ from TQ-loaded nanofibers in a cell culture medium was investigated in order to test the effect of TQ concentration on cell growth. The indirect cytotoxicity evaluations of the medicated NFs mats were carried out by the ISO10993-5 standard test method as a reference [71]. ISO 10993–5 specifies the incubation procedure of cultured cells in contact with a device or with extracts of a device either directly or through diffusion. Briefly, the assay was performed in a 96-well tissue culture polystyrene plate (TCPS; Costar®, Corning, NY, USA) using mouse fibroblast. The UV-sterilized circular discs (1.5 cm) were immersed in serum-free medium (SFM), containing DMEM, 1 % L-glutamine, and 1 % antibiotic and antimycotic formulation for 24 h of incubation to produce extraction media at various extraction ratios (i.e., 100, 50, 25, 12.5 and 0 %). The extraction media was then filtered using a 0.20 μm filter (DISMICR -25AS). Fibroblast cells were separately cultured in 96 well cell culture plate of at 10⁴ cells/well in serum-
containing DMEM for 24 h. After that, the medium was replaced with the extraction medium (100μl) of every fibrous scaffold, and the cells were re-incubated for 24 h. The viability of the cells cultured by each extraction medium was finally determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by the action of dehydrogenase enzymes, secreted from the mitochondria of metabolically active cells [58]. The amount of purple formazan crystals formed is proportional to the number of viable cells. After the 24 h incubation, the extraction medium in each plate was aspirated and replaced with 25 μl/well of MTT solution (5 mg/ml in PBS). The plate was incubated at 37 °C for 4 hours, the medium was then aspirated and 150μl/well of DMSO was added to dissolve the formazan crystals. The optical absorbance was measured at 595 nm using an ELISA reader (Turner Biosystems CE, Promega Corporation, USA). The viability of the cells cultured with the fresh SFM was used as a control. Cell viability (%) was calculated based on the following equation:

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

### 3.7.3 Cell proliferation and cell growth viability

3T3-L1 fibroblast were cultured on medicated e-spun membranes to evaluate the direct biocompatibility of the wound dressing scaffold, and the results were compared to cell cultures on tissue culture polystyrene as the control (TCPS). The proliferation of cultured fibroblasts was monitored on the first, third and seventh day of culture using the colorimetric MTT assay and the viable cell count technique.
3.7.3.1 MTT assay

In the MTT cell proliferation assay, the control (tissue culture polystyrene only), the TQ-loaded PLA, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3 scaffolds (circular disc 1cm in diameter) were placed inside the 24-well plate. The pre-sterilized electrospun scaffolds were first rinsed twice with phosphate-buffered saline (PBS) and soaked in cell culture medium overnight prior to cell seeding to facilitate protein adsorption and cell attachment. Mouse fibroblast cells were grown to confluency and then the cells were detached by adding 1 ml of 0.25% trypsin containing 0.1% EDTA. The pre-prepared cells were seeded on the scaffolds at a cell density of approximately 2×10⁴ cells/well and incubated at conditions suitable for cell growth. The scaffolds were transferred to a new plate (24 well-plate) after incubation and rinsed with PBS for twice. Then, 200μl of the MTT solution was added to the scaffolds and incubated at 37 °C for 4 h. The scaffolds containing MTT-cell mixtures were gently rocked to deposit the cells. After incubation, the supernatant MTT solution was discarded and 1ml of DMSO was added to the colored cell deposit to dissolve the formazan crystals. Then, 150 μl of the purple-blue solution containing the solubilized formazan in each sample, was transferred to a 96-well plate. Again, optical absorbance was measured at 595 nm using an ELISA reader. The cell viability was obtained by comparing the absorbance of cells cultured on the nanofibers scaffold to that of control well containing cells.

3.7.3.2 Determination of viable cell suspension concentration

Cell viability and proliferation were also assessed using the viable cell count technique, using haemocytometer. The fibroblast cells were cultured on each scaffold for 1, 3 and 7 days as previously mentioned. The cell monolayer was trypsinized using 1 ml of Trypsin / EDTA solution (0.25%) for the detachment of cells. The detached cells were then re-suspended in appropriate amount of fresh medium containing 10% fetal bovine serum. 100 μl of cell suspension was taken and mixed with an equal volume of trypan blue and mixed by gentle pipetting (dilution factor = 2). The haemocytometer and cover slip was cleaned with 70% ethanol then the cover slip was slide over the chambers. Both sides of the
chamber were filled with 10 µl of cell suspension and the cell viability was examined under the inverted microscope. The bright non-stained cells (viable cells) and stained (non-viable cells) were counted and viable cell concentration were determined using the following equation:

\[
\text{Concentration of viable cells (cells/ml) = } A \times C \times D
\]

(7)

Where: A: The mean number of viable cell counted; C: The correction factor (this is provided by the haemocytometer manufacturer) and D: The dilution factor

### 3.7.4 Cell attachment assay

In order to evaluate the cell adhesion capacity and attachment manner on the composite scaffolds, chemical fixation of cells on each scaffolds was carried out. Briefly, the samples were harvested after 3 and 5 days of culturing, rinsed twice with PBS to remove non-adherent cells, fixed with 2.5% glutaraldehyde for 1 h and washed with PBS twice (15 min per time). The samples were then dehydrated using a series of ethanol solutions, 20, 30, 50, 70 and 100% ethanol for 10 min each. Finally, the samples were kept in a vacuum oven and then sputter coated with gold for the cell morphology observation by using SEM. All samples were air-dried overnight. The dried cells were sputtered with a 10 nm thick gold film for the cell morphology observation using the MED020 instrument (Bal-Tech system, USA). Cell attachment was then observed at each time point using SEM (JEOL JSM-5600LV) with an accelerating voltage of 10 kV.

### 3.8 Wound healing assay

Six C57BL/6 mice (14–16 g) were used in this study, divided into two groups (1 and 2). The mice were housed under standard conditions with controlled temperature (23 °C)
and a light/dark cycle (12/12 h). After anesthetization, the dorsal hairs of the mice were completely shaved. To create a wound on each animal, a biopsy puncher was used to create a wound along the dorsal side of the skin. Two circular wounds with a surface area of 1 cm$^2$ were created on the back of each mouse, one wound is treated with the NFs scaffold, while the second wound is treated with gauze, as control. In this assay, medicated PLA: CA (7:3) and medicated PLA scaffolds were used. The wound areas were sterilized with povidone iodide. After 1 day, the dead skin around the wound was excised to the full thickness with scissors and tweezers. For Group 1 mice represent wounds treated with TQ-loaded PLA: CA (7:3) scaffolds vs. control. On the other hand, Group 2 mice were treated with TQ-loaded PLA scaffolds vs. control. The changes in the wound areas were measured at 0, 7, 10 and 14 days after initial wounding. The percentage healing is defined by equation (8):

\[
Wound\ Closure\ rate\ (%) = \left[ \frac{IA - MA}{IA} \right] \times 100 \tag{8}
\]

In which IA is the initial wound area and MA is the measured area after certain time period of treatment.
Chapter 4

Results and Discussion
4 Results and Discussion

4.1 Viscosity measurements
The viscosity of the prepared polymer solutions are presented in table 4.1. It is clear that PLA: CA polymer solutions showed much lower viscosity compared the PLA solutions. The reason for decreasing solution viscosity, associated with CA incorporation, may be attributed to the interaction between the CA and PLA polymer chains. The CA polymer chain may also have interfered with the secondary interaction, occurring between PLA chains. Thus reducing the viscosity of the solution.

4.2 Morphological characterization
SEM micrographs of the drug free PLA, PLA: CA 9:1 and PLA: CA 7:3 NFs are presented in Figure 4.1. Figure 4.2 shows the morphology of the drug-loaded PLA, PLA: CA 9:1 and PLA: CA 7:3 NFs at drug ratio 3 % w. SEM micrographs of the drug-loaded PLA NFs at the drug ratio 1 and 5 % w are represented in the appendix.

The results shows that under the optimized spinning conditions, the fibers obtained were randomly oriented round-shaped with smooth surface and almost uniform diameters along their lengths. Thus, one can claim that the obtained randomly oriented nanofibers are beads-free. The diameter of electrospun drug-free PLA: CA 7:3 and PLA: CA 9:1 was significantly lower compared to that of drug-free PLA nanofibers (Table 4.1). These results indicate that blending CA with PLA has resulted in shifting the fiber diameter to the lower values. This can be attributed to the decrease in solution viscosity associated with the incorporation of CA in the prepared polymer solution.

Solution viscosity influences the morphological structure and average size of the resulting fibers. By increasing the viscosity, the diameter of the NFs increases. This is probably attributed to the higher polymeric chain entanglement. During electrospinning, such chain entanglement results in greater resistance to the stretching of the polymer jet by the effect of electrostatic repulsive force [22, 72] In a study done by Bai et al to determine the effect of solution viscosity on the diameter of polymeric nanofibers, phenolic-resin solution was investigated [72]. The viscosity of the prepared solution was changed by changing
concentration. The results of this study showed that more viscous solutions tend to form nanofibers of bigger diameter size.

The resulting nanofibers also showed that the incorporation of CA into the PLA nanofibers not only dramatically decreased their average diameter but also reduced the diameter distribution of electrospun nanofibers (see Table 4.1).

Figure 4.1 and 4.2, show the SEM results of the drug-free and drug-loaded NFs at concentration ratio 3% w, respectively. SEM results for 1% and 5% drug ratios, incorporated in PLA NFs, are shown in the appendix. Both drug-free (Sample A, B, and C) and the drug-loaded PLA and PLA: CA (D, E, and F) nanofibers appeared smooth and no drug crystals were detected on the surface or outside the polymer. This suggested that the drug was homogeneously dispersed within the prepared fibers and reflected a good compatibility of the drug–polymer–solvent system. Furthermore it was noticed that incorporation of the drug in the polymer solutions did not significantly affect the viscosity of the polymer solution, or decrease the mean diameter size of the resulting fibers (see Table 4.1).

It was interesting to observe that all the composite electrospun nanofibers mats prepared in this study were desirably smooth and flexible. Such flexibility can provides easy handling and comfortable texture while using in medical application.
Table 4.1: Mean diameter size of the prepared nanofibers.

<table>
<thead>
<tr>
<th></th>
<th>Range of diameter size (nm)</th>
<th>Mean diameter size (nm)</th>
<th>Viscosity of electrospun solution (mpas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug-free PLA: CA (7:3) NFs</td>
<td>250-520</td>
<td>401±90</td>
<td>37</td>
</tr>
<tr>
<td>Drug-free PLA: CA (9:1) NFs</td>
<td>500-800</td>
<td>640±56</td>
<td>50</td>
</tr>
<tr>
<td>Drug free PLA NFs</td>
<td>500-1200</td>
<td>850±91</td>
<td>61</td>
</tr>
<tr>
<td>TQ-loaded PLA: CA (7:3) NFs</td>
<td>200-520</td>
<td>390±45</td>
<td>35</td>
</tr>
<tr>
<td>TQ-loaded PLA: CA (9:1) NFs</td>
<td>520-800</td>
<td>623±101</td>
<td>48</td>
</tr>
<tr>
<td>TQ-loaded PLA NFs</td>
<td>550-1000</td>
<td>830±82</td>
<td>59</td>
</tr>
</tbody>
</table>
Figure 4.1: Representative SEM images of the corresponding electrospun fiber mats, including histogram for the diameters of fibers (n≈100). (A-A'): drug free PLA: CA 7:3 NFs, (B-B'): drug-free PLA: CA 9:1 NFs, (C-C'): drug-free PLA NFs
Figure 4.2: Representative SEM images of the corresponding electrospun fiber mats, including histogram for the diameters of fibers (n=100). (D-D'): TQ-loaded PLA: CA 7:3 NFs, (E-E'): TQ-loaded PLA: CA 9:1 NFs, (F-F'): TQ-loaded PLA NFs.
4.3 FT-IR characterization

The chemical structure of the prepared films were investigated with the aid of FT-IR. The samples investigated were, PLA: CA blend NFs (with ratio 7:3), TQ-loaded PLA: CA blend NFs and TQ-loaded PLA NFs. Such materials were chosen to show the chemical interaction between the two polymers and between the drug and polymers within the composite matrix. The chemical structure of the TQ, CA and PLA were also investigated (results are shown in the appendix).

Figure 4.3 shows the FT-IR chart of the drug-free PLA: CA (7:3) scaffolds. Most of the characteristic peaks for both PLA and CA were observed. The broad peak at 3443 cm\(^{-1}\) refers to the characteristic CA band for \(-\text{OH}\) stretching. It has been observed that the intensity of this characteristic peak for CA has been reduced, compared to that in pure CA chart (see appendix). This can be attributed to the existence of hydrogen bonding between the \(-\text{OH}\) of CA and the ester \(-\text{C}=\text{O}\) of PLA, resulting in the disappearance of the corresponding IR peak. Such results demonstrate the existence of secondary interactions between CA chains and PLA chains, which again could be a reason for the decrease in viscosity associated with CA incorporation, as stated earlier. The peak at 1217 cm\(^{-1}\) refers to the asymmetric stretching of the ester bond (C-O-C) in the glycosidic linkage of CA [58]. On the other hand, the two peaks at 1361 and 1457 cm\(^{-1}\) and the sharp peaks at 2992 cm\(^{-1}\) correspond to the symmetric and asymmetric valence vibrations of \(-\text{C-H}\) from \(-\text{CH}_3\), respectively. The mountainous triplet peak at 1046, 1080, and 1132 cm\(^{-1}\) and peak at 1181 cm\(^{-1}\) correspond to \(-\text{C-O}\) stretching vibration of the PLA. Some of the peaks for both PLA and CA overlapped including the sharp, intense peak at 1755 cm\(^{-1}\) which is assigned to \(-\text{C}=\text{O}\) stretching for both polymers. However, the value of this peak is somewhat shifted from the regular values assigned for the \(-\text{C}=\text{O}\) stretching of both PLA and CA (1749 cm\(^{-1}\) for PLA and 1731 for CA) (see appendix) [26, 29,58].

Figure 4.4 shows the FT-IR chart of the TQ-loaded PLA: CA (7:3) scaffolds. It is clear that the characteristic \(-\text{OH}\) peak, observed within the matrix, has been totally diminished after
incorporation of TQ. This could be due to the formation of hydrogen-bonding between the –OH of the polymeric matrix and the –C=O of the drug, TQ. The characteristic peak for TQ has been observed at 1615 cm$^{-1}$. This refers to the –C=C stretching in the cyclohexadiene, existing in the TQ structure. Also, broadening of some of the peaks has been observed including the peaks for –C=O at ( 1748 cm$^{-1}$) [29,58]. The broad peak obtained includes the range of the characteristic –C=O of cyclohexadiene. Such observation could be due to the overlapping of different peaks together. Consequently as an IR spectrum is acquired, IR absorptions will occur at different frequencies for each of these bonds. The end result is that the IR peak appears broadened, as if it is an average of all these slightly different absorptions.

For the TQ-loaded PLA NFs, all the characteristic peaks for PLA have been observed as discussed previously. The only characteristic feature for TQ-observed is the minor peak at 1650 cm$^{-1}$ which refers to the –C=C stretching. This can reflect that TQ has been highly intrapped within the NFs and hidden by the PLA features (Figure 4.5).

Figure 4.3: FT-IR chart of PLA: CA (7:3) NFs.
Figure 4.4: FT-IR chart of TQ-loaded PLA: CA (7:3) composite NFs.

Figure 4.5: FT-IR chart for TQ-loaded PLA NFs.
4.4 Porosity

Figure 4.6 shows the relation between pore diameter and the corresponding pore surface area. This can provide an overview for the pore surface area distribution among the prepared scaffolds; TQ-loaded PLA, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3. As shown in the figure, all the scaffolds featured mesopores (4-20 μm) and macropores (50-150 μm). However, the results indicated that the small pores (4-20 μm) have the largest differential surface areas within the three prepared samples. This reflects that their numbers must be significant in the scaffolds. On the other hand, pores with large size possess differential surface areas of the least values, indicating their limited numbers. In conclusion, the smaller pores (4-20 μm) accounted for most of the observed surface area in all the prepared scaffolds.

*Figure 4.6: Differential pore area vs. pore width for TQ-loaded PLA, PLA: CA 9:1 and PLA: CA 7:3 NFs*
Figure 4.7 represents the pore size and pore size distribution of the prepared samples. The results of mean pore size diameter and porosity (%) are also summarized in Table 4.2. The results showed an increased trend in porosity with increasing CA concentrations. Furthermore, the pore size significantly decreased within the medicated PLA: CA 7:3 composite scaffolds. Such pore size reduction can be attributed to the decrease in the fiber diameter after incorporation of CA into the matrix. As it was shown in the SEM results, CA-containing NFs showed smaller fiber diameters compared to that of PLA (Table 4.1). When the fiber diameter is small, more layers of fibers can overlap with each other. This can result in smaller pore diameter. Decreasing the pore size is considered an advantage when it comes to tissue engineering applications because it results in increasing the surface area. Consequently, it is expected that fibroblasts cells might attach and proliferate more effectively on PLA: CA 7:3 scaffolds compared to the medicated PLA scaffolds.

![Graph showing incremental intrusion volume of mercury versus pore diameter for TQ-loaded PLA, PLA: CA 9:1 and PLA: CA 7:3.](image)

*Figure 4.7: Incremental intrusion volume of mercury versus pore diameter for TQ-loaded PLA, PLA: CA 9:1 and PLA: CA 7:3.*
Table 4.2: Mean pore size and mean fiber diameter of medicated PLA, PLA: CA 9:1 and PLA: CA 7:3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean pore size (μm)</th>
<th>Measured Porosity (%)</th>
<th>Mean fiber diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TQ-loaded PLA scaffolds</td>
<td>4.857±0.01</td>
<td>54%</td>
<td>830±82</td>
</tr>
<tr>
<td>TQ-loaded PLA: CA (9:1) scaffolds</td>
<td>4.651±0.04</td>
<td>63%</td>
<td>623±101</td>
</tr>
<tr>
<td>TQ-loaded PLA: CA (7:3) scaffolds</td>
<td>4.201±0.02</td>
<td>79%</td>
<td>390±45</td>
</tr>
</tbody>
</table>

4.5 Swelling behavior and water retention
The percentages of water uptake of the medicated PLA, PLA: CA 9:1 and PLA: CA 7:3 scaffolds are given in Table 4.3. It is clear from the results that the three fibrous scaffolds show significant capability for water uptake. The medicated PLA matrices showed a water-absorbing capacity of 379% after 24 h, whereas in medicated PLA: CA 9:1 and PLA: CA 7:3 samples, the water absorbing capacity was 851.51% and 1060% respectively. Both medicated PLA: CA 9:1 and PLA: CA 7:3 mats absorbed much higher amount of water than the pure medicated PLA mats.
Table 4.3: Percentage of water uptake capacity of TQ-loaded PLA, PLA: CA 9:1 and PLA: CA 7:3 for 12, 24 and 48 hours.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>TQ-loaded PLA scaffolds</th>
<th>TQ-loaded PLA: CA (9:1) scaffolds</th>
<th>TQ-loaded PLA: CA (7:3) scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>247 ± 20.8 (%)</td>
<td>555 ± 35.5 (%)</td>
<td>911 ± 18.9 (%)</td>
</tr>
<tr>
<td>24</td>
<td>379 ± 12.3 (%)</td>
<td>851 ± 27.8 (%)</td>
<td>1060 ± 11.8 (%)</td>
</tr>
<tr>
<td>48</td>
<td>1933 ± 45.9 (%)</td>
<td>2703 ± 19.2 (%)</td>
<td>3145 ± 10.4 (%)</td>
</tr>
</tbody>
</table>

The results showed that the water uptake increased with increasing CA content in the blend. This implies that the scaffolds became more hydrophilic upon incorporating CA. The prepared medicated PLA scaffolds is hydrophobic, because of the poor solubility of its components (PLA and TQ), thus it shows the lowest water uptake during a 48-h test period. On the other hand, CA, within scaffolds structure of the two other samples, (medicated PLA: CA 9:1 and TQ-loaded PLA: CA 7:3 became slightly swollen upon contacting with PBS solution (Figure 4.8), because of its high solubility in water. This will leave tiny pores in the nanofibers within the fibrous mats, which is considered a desirable properties to the wound healing.
4.6 Water vapor permeability

As it is shown in Table 4.4, the WVP values of all the developed medicated scaffolds were found to be in the specified range (16-19 g/m²/h). Figure 4.9 shows the change of weight gained (in grams) with time (in h) for the three prepared scaffolds. It is also noticed that by increasing the percentage of CA in the e-spun membranes, the permeability also increases (Figure 4.10). This indicates that the presence of CA has further improved the water vapor permeability of the membranes.
Table 4.4: Water vapor permeability of TQ-loaded PLA: PLA: CA 9:1 and PLA: CA 7:3, correlated to fiber diameter.

<table>
<thead>
<tr>
<th>Sample</th>
<th>WVP (g/m²/h)</th>
<th>Measured Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medicated PLA NFs</td>
<td>16.3±0.9</td>
<td>54%</td>
</tr>
<tr>
<td>Medicated PLA: CA (9:1) NFs</td>
<td>17.5±1.5</td>
<td>63%</td>
</tr>
<tr>
<td>Medicated PLA: CA (7:3) NFs</td>
<td>18.75±2.1</td>
<td>79%</td>
</tr>
</tbody>
</table>

Such increase in WVP associated with CA incorporation can be attributed to two factors: First, is the hydrophilic nature of the CA. For polymer films, the water vapor permeability, (P), is the product of the solubility of polymer in the water, (S), and the diffusion coefficient of the water into the polymer, (D).

\[ P = SD \]

The value of S is higher when the solubility parameter of the polymer and water are similar. Values of S, calculated by the group contribution method, are given in Table 4.5. As it is shown in the table, more hydrophilic polymers, such as CA, have higher values of S, compared to more hydrophobic polymers, such as PLA. CA has solubility parameter 25.7 (J/Cm³), while amorphous and crystalline PLA have 21.5 (J/Cm³) [73]. This, in turn, will provide CA with higher water vapor transmission rate compared to PLA. Such phenomenon can be related to the significant hydrogen bonding interaction with water.
Table 4-5: Solubility parameter values for different polymer films [73].

<table>
<thead>
<tr>
<th>Film</th>
<th>Solubility parameter (J/Cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA (Crystalline)</td>
<td>21.7</td>
</tr>
<tr>
<td>PLA (Amorphous)</td>
<td>21.7</td>
</tr>
<tr>
<td>CA</td>
<td>25.7</td>
</tr>
<tr>
<td>PCL</td>
<td>20.8</td>
</tr>
</tbody>
</table>

Another reason for such increase in WVP associated with CA is the porosity and diameter size of the prepared scaffolds. Water vapor diffusion is highly dependent on air permeability of the membrane. Air permeability increases as the porosity of the membrane increases, which results in higher moisture transfer through the air voids [56]. It was clear from the SEM observation and porosity results that presence of CA has further decreased the fiber diameter, resulting in better permeability for water vapor (Table 4.4).
Figure 4.9: Water vapor gain vs. time for medicated PLA, PLA: CA 9:1 and PLA: CA 7:3 NFs

Figure 4.10: Comparison between the medicated PLA, PLA: CA samples, showing the effect of CA on WVP.
4.7 In Vitro drug release study

4.7.1 Calculation of total TQ content in the TQ-loaded NFs
The drug loading efficiency within the prepared fiber mats was determined. Table 4.6 summarizes the actual amount of TQ in each of the three samples (TQ-loaded PLA. TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3 nanofibers mats). As shown in the table, high drug loading efficiencies were successfully achieved. This can be attributed to the lipophilic nature of the drug, TQ. During electrospinning, the solution jet rapidly elongates and the solvents evaporate quickly. If the drug is highly compatible with the electrospinning matrix/solvent system, phase separation would be difficult to take place and the drug tends to remain within the fiberous structure, where sufficient solvent is left. Thus when the nanofibers finally dry, the drug will be successfully encapsulated. Due to its lipohylicity and high solubility in all solvents used in the preparation of polymer solution (DCM: DMF), TQ appears to have high compatibility with the electrospinning system. The same conclusion has been achieved by Zeng et al during the fabrication of paclitaxel-loaded PLLA nanofibers [74]. Paclitaxel, a lipophilic drug, was highly incorporated in the nanofibers using PLLA/chloroform/acetone as the polymer/solvent system.

It should be mentioned also that the prepared drug-loaded PLA and PLA: CA blend nanofibers displayed no significant differences in entrapment efficiencies among different regions alongside their constructs.
Table 4.6: Actual drug content and drug release % of TQ from the prepared nanofibers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total drug content %</th>
<th>Cumulative drug release % (24 hours)</th>
<th>Cumulative drug release % (9 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TQ-loaded PLA NFs</td>
<td>90.49 ± 2.33</td>
<td>17.7</td>
<td>59</td>
</tr>
<tr>
<td>TQ-loaded PLA: CA (9:1) NFs</td>
<td>82.63 ± 6.89</td>
<td>32.1</td>
<td>69</td>
</tr>
<tr>
<td>TQ-loaded PLA: CA (7:3) NFs</td>
<td>79.51 ± 5.78</td>
<td>55.9</td>
<td>79</td>
</tr>
</tbody>
</table>

4.7.2 Release profile of TQ from the TQ-loaded NFs

For the prepared medicated scaffolds to exert their therapeutic functionality, the loaded TQ has to be released. The cumulative amount of TQ released from the medicated nanofibers is depicted in figure 4.12. The amount of drug finally released varied from one sample to another. Figure 4.11 indicated that the percentage of TQ released depends on the polymer composition in the fibrous matrix. The TQ released from medicated PLA NFs, medicated PLA: CA 9:1 and medicated PLA: CA 7:3 after 24 h were 17.7%, 32.1% and 55.9%, respectively. Afterwards, a gradual increase in the cumulative release followed over the next days to reach 59%, 69% and 79% for the medicated PLA, medicated PLA: CA (9:1) and medicated PLA: CA (7:3), respectively, after 9 days. Thus, it is clear that the addition of CA to PLA resulted in higher drug release.

In our study, the dynamic of drug release from the scaffolds is controlled by their structure, which is represented by the ratio of polymer components in the matrix. It is concluded from the above data that the higher amounts of CA in PLA: CA 7:3 matrix resulted in a burst
release of TQ during the first 24 h, followed by more sustained release of the drug for 9 successive days

\[ \text{Figure 4.11: Cumulative release of TQ from the prepared nanofibers matrices.} \]

This can be attributed to the hydrophilic nature of CA. Incorporation of CA into PLA nanofibers further improved the hydrophilicity of the matrix. This was proved by the higher swelling behavior and water retention capacity of CA-containing scaffolds. The higher the amount of CA, the higher is the swelling behavior of the scaffold. Such swelling increases fiber size and thus increases water content within the matrices, which enables more drug to diffuse through the network. These results correlate with the results obtained by Liu et al. In their study, the effect of addition of CA on the release of PHMB from polyester urethane (PEU): CA fiber mats was investigated. The 2:1 blend matrix showed higher release rate than their corresponding lower CA matrix [57]. In another study, the addition of PVP proved to have a tuning effect on the release of bovine serum albumin (BSA) from
PVP: PLLA nanofibers. After the first hour of release, more BSA was released from PVP-containing e-spun fibers than that from the pure PLLA nanofibers [65].

Another reason for such increase in drug release, associated with incorporation of CA is the size of fiber diameter. The morphological characterization of samples shows that CA incorporation resulted in significant decrease in the average fiber diameter. A reduction in the average diameter of the nanofibers resulted in an increase in the surface-area-to-volume ratio of the nanofibers. Therefore, the tendency of the drug molecules absorbed on the surface to be released increased remarkably.

The effectiveness of a certain drug is highly dependent on the rate of drug release from the NFs. For the delivery of antibiotic drugs, a large initial burst is considered an advantage because it is important to eliminate the intruding bacteria before they begin to proliferate. However, for the few organisms that may survive the initial burst, a continued release of antibiotic is also required to prevent their further population. Thus, it can be concluded that the electrospun PLA: CA 7:3 scaffold having 3% w of TQ exhibits an ideal release profile for potentially preventing infections associated with chronic and surgical wounds.

Also, it is very interesting to notice the increase of drug release from the medicated PLA scaffolds after around 5-6 days. This can be attributed to the hydrolytic degradation of PLA (as discussed in the introduction, Section 5.1.4). Hydrolytic degradation of poly-lactide family proceeds through four stages (Figure 4.12): First, is the stage of water diffusion, followed by the second stage in which oligomers, with acidic group terminals, autocatalyze the hydrolysis reaction. In the third stage, oligomers start to diffuse out from the polymer while water molecules start to diffuse more into to the cavities created by the removal of the oligomers. This, in turn, encourage further oligomers diffusion. In this stage, marked decrease in polymer molecular weight occurs and a sharp increase in the drug release rate occur as the drug diffuses from the porous regions. Such decrease in polymer mass is associated with a sharp increase in the drug release rate, as the drug diffuses from the porous regions created in the matrix. In the fourth stage, polymeric matrix become highly porous and degradation proceeds homogeneously and more slowly [75, 76].
Figure 4.12: Schematic representation of hydrolytic degradation of poly-lactide polymers

In general, the degradation rate of drug-loaded PLA matrix is highly dependent on the molecular weight of polymer and on the interaction between the polymer and drug. The impacts of these parameters that increase the degradation rate are not exactly clear [75, 76]. It could be the high molecular weight of the polymer used in our study and the way of physical interaction between the TQ and the PLA that resulted in such degradation pattern which further altered the release profile.

4.8 In Vitro antibacterial assessment

The drug under investigation, TQ, is known for its antibacterial properties against both Gram-negative and Gram-positive bacteria. In this work, the antibacterial activity of TQ-loaded PLA and TQ-loaded PLA: CA composite nanofibers was investigated using the modified Kirby–Bauer method (disc diffusion method). *S. aureus* and *E.coli* were used as a model for Gram positive and gram negative bacteria, respectively.
The medicated NFs discs were pasted onto the agar plate, seeded with the bacteria. The three prepared scaffolds, TQ-loaded PLA: CA (7:3), TQ-loaded PLA: CA (9:1) and TQ-loaded PLA NFs discs and denoted as A, B, and C, respectively. Figure 4.13 and 4.14 show the inhibitory zone formation around the medicated NFs sample discs in agar plates of S. aureus and E. coli, respectively. It is clear that after incubation for 24 h, the agar regions with the medicated PLA: CA 9:1 and PLA: CA 7:3 scaffolds display visible bacterial inhibition rings. In contrast, medicated pure PLA mats did not show a significant bacterial inhibition effect. This could be attributed to the slow cumulative release of TQ from the medicated PLA scaffolds for the first 8 h (13.3 %), compared to that of PLA: CA 9:1 and PLA: CA 7:3 (24.5% and 44.8 % respectively). The inhibition zone of medicated PLA: CA 7:3 scaffold is the biggest, which should be attributed to the faster drug release rate and more TQ released from PLA: CA 7:3 than that from PLA: CA 9:1 nanofibers (Table 4.7).

<table>
<thead>
<tr>
<th></th>
<th>Medicated PLA: CA (7:3) scaffolds</th>
<th>Medicated PLA: CA (9:1) scaffolds</th>
<th>Medicated PLA scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td>3.3 ±0.1 cm</td>
<td>2.8 ±0.2 cm</td>
<td>0</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>2.1 ±0.0 cm</td>
<td>1.7 ±0.1 cm</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.7: Diameter of inhibition zones for the medicated nanofibrous mats.
It should be noted also that the inhibitory zones against *S. aureus* is larger than that against *E. coli* (Table 4.7). The main reason for this observation is that the activity of TQ against Gram-positive bacteria is more significant than that against Gram-negative ones. The minimum inhibitory concentration (MIC) of TQ for *S. aureus* and *E. coli* are 3 µg/ml and 800 µg/ml, respectively. These results indicate that the encapsulated TQ drug within the PLA: CA scaffolds (at the two ratios used) is able to be released from the medicated nanofibrous mats and diffused onto the agar, thereby effectively inhibiting the bacterial growth. Consequently, medicated PLA: CA composite nanofibers could be effective in inhibiting the growth of bacteria and have the potential to be used in functional wound dressing and tissue engineering.
Figure 4.13: Antibacterial activities of the TQ-loaded NFs mats against S.aureus. Sample A is TQ-loaded PLA: CA 7:3, Sample B is TQ-loaded PLA: CA 9:1 and Sample C is TQ-loaded PLA.
Figure 4.14: Antibacterial activities of the TQ-loaded NFs discs against E-coli. Sample A is TQ-loaded PLA: CA 7:3, Sample B is TQ-loaded PLA: CA 9:1 and Sample C is TQ-loaded PLA.
4.9 Assessment of Cytotoxicity, cell proliferation and cell attachment

4.9.1 Cytotoxicity assay:
Figure 4.16 shows the cytotoxicities of the TQ-loaded PLA: CA 7:3, TQ-loaded PLA: CA 9:1 and the TQ-pure PLA membranes as assessed by the MTT assay. The percentage of drug loaded is 3% w. Cytotoxicity results of another two drug loading percentage (1% and 5% w) is included in the appendix. It was found that the 5% drug loading resulted in a cytotoxic effect of mouse fibroblast, while the 1 and 3% did not show cytotoxic effect. However, 1% drug-loaded scaffolds did not show significant antibacterial activity (data not shown in the results). Thus, the 3% w drug loading was chosen for the preparation the scaffolds in this study.

In the cytotoxicity examination, the extraction medium of the prepared scaffolds were used to evaluate their cytotoxicities against mouse fibroblast cells. The results indicated that no significant difference in absorption of pure culture medium and the extraction medium for all the prepared scaffolds. The extraction medium enabled cell viability of more than 97%. Also, the cell viability (%) for the 50%, 25% and 12.5% extracted solutions were similar to those for the 100% extracted solution. According to ISO 10993–5, these results indicate that the co-components of the prepared scaffolds are non-toxic and have excellent biocompatibility. Thus, the amount of TQ released at this point time from the three scaffolds are below the cytotoxic dose of TQ.
4.9.2 Cell proliferation and cell growth viability assay

4.9.2.1 MTT assay
The proliferation capacity of mouse dermal fibroblast (MDF) on the prepared electrospun scaffolds, medicated-PLA, medicated PLA: CA 9:1 and medicated PLA: CA 7:3, was determined. Figure 4.16 shows the proliferation results for 1, 3 and 7 days of cell culture, assessed by MTT assay. It was observed that the optical density of MDF on all the electrospun substrates increased during the 7 days of cell culture, which indicates that the HDF were metabolically active on the TQ-loaded scaffolds. These results correlated with the results obtained from the cytotoxicity analysis, which ensure the lack of cytotoxicity effect of the prepared scaffolds. Remarkable difference in cell proliferation was observed between days 3 and 7. The percentage increase in the rate of proliferation from day 3 to
day 7 on TQ-loaded PLA NFs, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3 nanofibrous scaffolds was found to be 23%, 41%, and 60%, respectively.

In Figure 4.16, it can be seen that cells grown on each of the prepared scaffolds had equal or better cell proliferation than did cells grown on the two-dimensional control (two-dimensional tissue culture polystyrene (PS) on days 3 and 7. This indicates that the cells on the nanofibers could migrate into the scaffolds thus allowing for more proliferation. This is attributed to the fact that cells have more area to grow in a 3-dimensional environment compared to the 2-dimensional one (Figure 4.17) [5]. Such 3-dimensional

![Figure 4.16: Optical absorbance of mouse fibroblast cells seeded on TQ-loaded PLA, TQ-loaded PLA: CA 9:1 and TQ-loaded PLA: CA 7:3 scaffolds after 1, 3 and 7 days of incubation.](image-url)
environment resembles that of the natural extracellular matrix (ECM). On day 7, TQ-loaded PLA: CA 7:3 scaffold showed a significantly higher proliferation and viability than other scaffolds and the two-dimensional tissue culture polystyrene. The same conclusion has been achieved by Sisson, et al. In their study, the results revealed that human osteosarcoma cell (MG63 cells) can grow and proliferate more on cross-linked gelatin NFs than the polystyrene tissue culture plate, used as a control [77].

Figure 4.17: 3D structure of the NFs enhances cell binding and spreading [5].
By comparing the cellular proliferation between the three prepared scaffolds, it is obvious that there is a negligible difference in cell viability after 1 day incubation. However, after 3 days, the proliferation of MDF on the medicated PLA: CA 9:1 and PLA: CA 7:3 nanofibers was found significantly higher than that on medicated pure PLA. We observed 58% higher cell proliferation on TQ-loaded PLA: CA 7:3, compared to TQ-loaded PLA NFs after 7 days of incubation. This can be due to the hydrophobicity of the PLA nanofibers surface and its TQ component.

Thus, it can be concluded that the inclusion of CA within PLA scaffolds provides the scaffold with higher bioactivity and affinity for cell proliferation. This can be attributed to the fact that CA improves the hydrophilicity of the scaffolds, thus provide better cellular interaction. The same effect was also achieved by blending PLA with gelatin. As in a study done by Hoveizi1 et al, where modified PLA/gelatin 7/3 scaffold was proved to be more suitable for fibroblasts proliferation and that the cellular growth was significantly higher on the gelatin-blended nanofibers than the PLA, compared to PLA or gelatin NFs alone [66].

4.9.2.2 Determination of viable cell suspension concentration
The cell proliferation results were confirmed by the total cell count technique. Table 4.8 represents the results of viable cells attached to the three different scaffolds after 1, 3 and 7 days. After 3 and 7 days of cell culture, the number of fibroblasts attached on medicated PLA: CA 7:3 nanofibers was much higher than the cell attached to medicated PLA nanofibers. These results also confirmed the better adhesion and proliferation of fibroblasts on CA-containing scaffolds. From this data, one can clearly confirm the cell attachment and cell spreading in the NFs matrix.
Table 4.8: the total cell count of the mouse fibroblast on the medicated PLA, PLA: CA 9:1 and PLA: CA 7:3 NFs as reflection to the cell proliferation capability.

<table>
<thead>
<tr>
<th></th>
<th>TQ-loaded PLA scaffolds</th>
<th>TQ-loaded PLA: CA (9:1) scaffolds</th>
<th>TQ-loaded PLA: CA (7:3) scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>5×10³ ± 6.36</td>
<td>6×10³± 11.23</td>
<td>6×10³± 8.98</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td>5×10³± 10.12</td>
<td>8×10³± 7.90</td>
<td>10×10³± 7.61</td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
<td>7×10³± 8.01</td>
<td>12×10³± 5.96</td>
<td>15×10³± 10.43</td>
</tr>
</tbody>
</table>

4.9.3 Assessment of cell attachment:
To confirm the cellular regeneration, the morphological appearances of cells on composite NFs mats were investigated. Figure 4-18 and 4-19 shows the SEM images of cell attachment manner of the mouse fibroblast cells under static seeding on the medicated PLA, PLA: CA 9:1 and PLA: CA 7:3 after 3 and 7 days of culture, respectively. The SEM micrographs of fibroblasts showed a normal spindle shape morphology of cell growth on all the prepared NFs. After 3 days of cell culture, the number of fibroblasts attached on the medicated PLA: CA 9:1 and PLA: CA 7:3 nanofibers (Figure 4.18. A, 4.18. B, respectively) was significantly higher than the cell attached on PLA nanofibers (Fig. 4.18. C). After 7 days of cell culture, cells covered the available surface area on the PLA: CA 9:1 and PLA: CA 7:3 nanofibers (Figure 4.19. H, 4.19. K, respectively).

The cells spread over the scaffold fibers, linked with fibers by cytoplasmic extensions. The presence of lamellapodia and an interlaced fibrous network indicated good cell adhesion. On the other hand, more empty nanofibers surfaces were observed for mediated PLA nanofibers with no lamellapodia formation. Thus, it was concluded that the cells were
well incorporated into the medicated PLA: CA composite nanofibers compared to the medicated PLA nanofibers. These results are in agreement with the results obtained from cell viability and proliferation, and confirmed that blending CA with PLA provides the scaffold higher cell affinity for skin tissue regeneration.

The hydrophilic nature of the PLA: CA scaffolds is a reason for the observed better adhesion and proliferation of fibroblasts. Such conclusion was also reached by Unnithana et al. In this study, the authors blended dextran, hydrophilic polysaccharide, with polyurethane to prepare biocompatible scaffolds. The results indicated that the cells interacted favorably with the PU-dextran scaffolds better than the pure PU scaffolds. The higher bioactivity and cell affinity was attributed to the hydrophilic nature of the PU-dextran scaffolds [78]. Another reason for such behavior is the smaller fiber diameter and pore size associated with the CA-containing scaffolds. As it was explained previously in section 4.1 and 4.3, medicated PLA: CA scaffolds showed smaller fiber diameters and pore size as compared to medicated PLA scaffolds. Sun et al. studied the influence of fiber diameter, and pore-size on the behavior of human dermal fibroblasts. They assumed that smaller values of fiber diameter and inter-fiber space is necessary for cell adhesion, migration and for cell aggregate formation [3]. This can be attributed to the fact that the smaller the pore size and fiber diameter, the higher is the surface area available for cellular adhesion and proliferation.

It is not only the hydrophilic nature of CA which resulted in such improved cellular affinity, also the biocompatible nature of CA played an important role. The extracellular matrix of cells (ECM), described earlier in the introduction, is consists of a nanosized network of biomolecules, such as proteins and glycosaminoglycans (polysaccharides) [5]. CA is a polysaccharide derivatives, thus it is similar in structure and composition to the glycosaminoglycans components of the ECM. Consequently, it can further allow the scaffolds to mimic the ECM, not only in the 3-D architecture, but also in chemical composition.
Another observation which is very interesting, is the lack of cellular infiltration. It is clearly noticed that the fibroblast cells migrate and adhered to the surface of the three prepared scaffolds, but did not penetrate inside the scaffold. This observation actually correlates with the data obtained from the pore size analysis (section 4.3). The results of pore size analysis indicated that the mean pore size for the three prepared scaffolds are $\sim 4 \mu m$ (4.857, 4.651, 4.201 for medicated PLA, PLA: CA 7:3 and PLA: CA 9:1 NFs, respectively). The average size of fibroblast cells is (15-20 $\mu m$) [3]. So, the pores within the scaffolds structure will not allow for cellular infiltration within the fibrous structure. Another reason for such poor cellular infiltration is the orientation of the NFs. It has been proved that aligned nanofibers significantly enhanced cell infiltration into the nanofibrous matrices in vitro, compared to the randomly oriented NFs.

Such lack of cellular infiltration is considered an advantage when it comes to wound dressing materials. The ideal wound dressing should be able to promote fibroblast migration and proliferation within the wound bed, while preventing tissue ingrowth within the fibrous structure, in order to avoid tissue damage after dressing removal.
Figure 4.18. Morphology of the mouse fibroblast on the prepared NFs; after 3 days of cell culture (a) TQ-loaded PLA: CA 7:3, (b) TQ-loaded PLA: CA 9:1, (c) TQ-loaded PLA.
Figure 4.19. Morphology of mouse fibroblast on prepared NFs; after 7 days of cell culture (D) TQ-loaded PLA: CA 7:3 (E) TQ-loaded PLA: CA 9:1 (F) TQ-loaded PLA.
4.10 In Vivo wound healing test

Wound healing is a special biological process that comprises four consecutive phases: (1) hemostasis and inflammation, (2) migration, (3) proliferation, and (4) tissue remodeling. It mainly requires more than 2 weeks between the time of injury and the complete fibroblast coverage on the surface of wound as a new skin layer. Owing to its antibacterial activity, and proliferation performance, TQ-loaded LA: CA (7:3) has been chosen for the in vivo study, in comparison to the TQ-loaded PLA scaffolds, to ensure the effect of incorporation of CA into the PLA scaffolds on the wound healing performance. The wound-healing properties of the prepared nanofibers mats were examined via an in vivo wound-closure assay on the dorsal wounds of mice. TQ-loaded scaffolds were used, together with gauze, as the control. The gross appearance of each wound was observed at 0, 7, 10 and 14 days of treatment. Figure 4.20 and 4.21 shows the representative macroscopic observation of each wound at the different healing times for the medicated PLA: CA (7:3), and medicated PLA scaffolds respectively. Also, the rate of wound closure as a function of time for both scaffolds is shown in figure 4.22 and 4.23, respectively.

After 7 days of treatment, more regenerated epidermis were observed for TQ-loaded PLA: CA scaffolds (Figure 4.20). Also, it is evident that re-epithelialization of the wound treated with TQ-loaded PLA: CA nanofibers was almost complete on day 10 while this stage took more than 14 days for the control wound. On day 14, a new layer of skin with almost no scarring was present on wounds treated with TQ-loaded PLA nanofibers. Starting from day 7 to day 14, TQ-loaded PLA: CA (7:3) composite scaffold did show faster contraction compared to the wound control, indicating the effect of the fibrous scaffold for promoting ECM formation and the delivery of TQ for preventing infection. Wound contraction can make the re-epithelialization easier [79]. On the other hand, figure 4.21 shows that there was no significant difference in wound appearance for both the wounds treated with medicated PLA scaffolds and the control wound for the first 7 days. However, after 10 days of treatment, wounds treated with PLA NFs mat showed a significant increase in the rate of wound closure compared to the control.
**Figure 4.20:** Digital photograph assessment of healing progression from day 0 to day 14 for wounds treated with TQ-loaded PLA: CA (7:3) scaffolds vs. the control. (b) Representative mice with two wounds on its back for control and treated wounds after 7 days.
Figure 4.21: Digital photograph assessment of healing progression from day 0 to day 14 for wounds treated with TQ-loaded PLA scaffolds vs. the control. (b) Representative mice with two wounds on its back for control and treated wounds after 7 days.
The rate of wound closure as function of time is represented in figure 4.22 and 4.23 for wounds treated with medicated PLA: CA (7:3) and medicated PLA scaffolds, respectively. The results showed that the wounds treated with the medicated PLA: CA (7:3) NFs mat showed a significant increase in the rate of wound closure compared with the control starting from day 7. On day 7, the closure rate of PLA: CA (7:3) treated wounds was almost 51%, while only approximately 33.3% wound closure was observed in the control samples (Figure 4.22). The wounds treated with TQ-loaded PLA: CA NFs mats healed much faster. It showed approximately 89% and 99% wound closure on days 10 and 14, respectively.

On the other hand, wounds treated with a PLA NFs mat did not show a significant increase in the rate of wound closure compared to the control after 7 days of treatment (Figure 4.23). On day 10, the closure rate of wounds treated with PLA NFs mats started to show better performance compared to the control. It showed almost 67% wound closure after 10 days, compared to the control which showed 50% wound closure. This might be attributed to the slow release of TQ from the fibrous mats which can play a role in controlling the bacterial infection and invasion, thus allowing for better wound healing. Also, it could be attributed to the biocompatibility of PLA as well as the porous nature of NFs mats. The high surface area to volume ratio and the porous structure of NFs mats could maintain an ideal environment at the wound interface via absorption of exudates, gas and fluid exchanges, and bacterial protection.

In a study done by Karami et al, the wound closure percentage of the electrospun PCL/PLA hybrid nanofibrous mats containing thymol, herbal drug, was more significant compared with those of the other dressings. The wound-closure percentages of the wounds treated with the electrospun PCL/PLA nanofibrous mats, and gauze bandages were 92.4%, and 68%, respectively, after 14 days of treatment [80].

It is clear that the medicated PLA: CA (7:3) NFs mats enabled better wound healing than the control (gauze), while the medicated PLA NFs mats produced results similar to those for the control. In conclusion, TQ-loaded PLA: CA 7:3 scaffolds exhibited a faster
healing with more regenerated skin after 10 days, compared to the medicated PLA scaffolds. Such results actually correlate with the data obtained from the cellular proliferation and attachment assays, where the medicated PLA: CA (7:3) NFs composite showed better cellular response compared to the medicated PLA scaffolds. Another reason for such advanced performance in wound healing is the controlled drug release of TQ from the PLA: CA (7:3) scaffolds. The principal aim of treating wound infections is to reduce the bacterial load in the wound to a level at which wound-healing processes can take place. If the drug is released too quickly, the entire drug amount could be released before the infection is stopped. On the other hand, if the release of the drug is delayed, infection may set in further, thus making it impossible to manage the healing of the wound. The release of TQ from the PLA: CA (7:3) scaffolds showed highly controlled profile. It started with a sort of burst release of TQ, followed by the sustained release for almost 9 days. Such controlled release profile is similar to that of local antibiotics. For all local antibiotic release profiles should exhibit a high initial release rate in order to respond to the elevated risk of infection from bacteria introduced during the initial shock, followed by a sustained release at an effective level for inhibiting the occurrence of latent infection [37, 78, 79].
Figure 4.22: Quantitative measurement for wound closure rate for wounds treated with PLA: CA (7:3) scaffolds vs. control.

Figure 4.23: Quantitative measurement for wound closure rate for wounds treated with PLA scaffolds vs. control.
Conclusion and Future Prospective
5. Conclusion and Future prospective

5.1 Conclusion
Many biocompatible polymers have been used to generate nanofibers. Biopolymers proved to be the most biocompatible polymers, thus they are widely used in tissue engineering and wound dressing applications. PLA is considered a very promising biopolymer, owing to its renewability, bioavailability and inexpensive price as compared to petroleum based polymers. However, one of the main issues which limit the wide application of PLA in tissue engineering purposes is the high hydrophobicity and lack of cellular interaction. Thus, by modifying its properties, poly-lactic acid (PLA) may prove to be a very promising biomaterial.

In this study, modification of PLA was achieved by blending it with cellulose acetate (CA). CA is a polysaccharide with high hydrophilicity and better cellular response than PLA. PLA: CA nanocomposite scaffolds was prepared by the electrospinning technique. Polymer solutions of both PLA and CA, at the ratios PLA: CA 9:1 and 7:3 was electrospun to prepare NFs scaffolds that can be further used as a wound dressing material. For the aim of achieving an interactive wound dressing scaffolds, an antibacterial agent was also introduced in the scaffolds to avoid bacterial infection. The antibacterial agents used for this study was Thymoquinone, an active ingredient of *Nigella sativa*. TQ is widely used due to its antibacterial properties. The drug was used in the ratio 3% w based on the weight of polymer. The obtained scaffolds were characterized for its morphological, chemical, physical (Porosity, swelling behavior & water vapor permeability) and biological (in vitro drug release, antibacterial activity, cytotoxicity and cellular proliferation) properties.

The results showed that the incorporation of CA within the PLA matrix altered the chemical and physical properties of the scaffolds. It resulted in scaffolds of smaller fiber diameters and smaller pore size, compared to that of PLA NFs. As well as, the incorporation of CA resulting in higher water vapor permeability and swelling behavior for
the scaffolds. Such alteration can be justified by the hydrophilicity of the CA and its ability to form hydrogen bonding. It’s the hydrogen bonding between CA and PLA, as shown by FT-IR analysis that decreased the viscosity of the polymer solution, which further resulted in fibers with smaller diameters. The hydrogen bonding of CA with water resulted into improving the water uptake capacity of the membranes and increased its WVP. Furthermore, hydrophilicity of CA has altered the release profile of the TQ from the prepared scaffolds. TQ-loaded PLA: CA 7:3 scaffolds showed a kind of burst release, when compared to the TQ-loaded PLA scaffolds, followed by a stable sustained release for almost 9 successive days. After 24 hours of incubation in modified PBS medium, approximately 56% of TQ was released from the TQ-loaded PLA: CA (7:3) scaffolds, compared to 18% released from the TQ-loaded PLA scaffolds. Such burst release is considered an advantage for controlled delivery of an antibacterial agent, because it is expected to prohibit the initial bacterial growth while the subsequent sustained release will prevent further proliferation of bacteria. This was further proved by the antibacterial assessment of the scaffolds. Medicated PLA: CA scaffolds showed a significant antibacterial activity against *S. aureus* and *E.coli*, assessed through the agar diffusion technique. On the other hand, medicated PLA scaffolds failed to show any antibacterial inhibition zone, attributed to the delayed drug release from the medicated PLA scaffolds. Incorporation of CA into the PLA matrix has further improved the biological performance of the scaffolds. Medicated PLA: CA scaffolds showed better cellular response, regarding biocompatibility and cellular proliferation when compared to medicated PLA scaffolds. This can be attributed not only to the improved hydrophilicity of the prepared scaffolds, but also to the biocompatible nature of CA, as one of the polysaccharides derivatives. Such conclusion was also confirmed via in vivo wound healing evaluation assay. In the in vivo study, medicated PLA: CA (7:3) scaffolds showed faster wound healing progress in 14 days (approximately 100% wound closure rate) compared to the control.

In this study, blending technique provided a simple, while effective method to modify the properties of PLA to be more suitable for biomedical applications. Thus, we can claim that the prepared TQ-loaded PLA: CA scaffolds are very promising antibacterial scaffolds that can be used effectively as an interactive wound dressing material.
5.2 Future prospective

A future prospective for the e-spun TQ-loaded PLA: CA NFs in wound care includes:

- Introducing the scaffold in a clinical trial to ensure its functionality

- Utilizing the scaffolds in the development of smart devices. A smart device refers to a multi-component system that can deliver multiple therapeutic benefits in a single product. In this regard, TQ, as an antibacterial agent, can be used in junction with a vascular endothelial growth factor (VEGF). Such multi-component scaffolds typically accelerate the wound healing process by promoting angiogenesis and further inhibit bacterial infection.

- Utilizing prepared matrix as a bioactive indicator for monitoring the progression of healing and/or bacterial load. This can be achieved by incorporating a sensor within the electrospun scaffolds, thus allowing the real-time detection of specific parameters from the wound bed. The sensor generates a visible output for the patient or the doctor to provide continuous monitoring of the wound status. There is a range of potential markers and parameters associated with wound healing and infections that can be detected. The pH is considered the most reliable marker for the healing process. Chronic non-healing wounds have an elevated alkaline pH. However, healing occurs more readily in an acid environment. Consequently, monitoring wound surface pH may provide a method of ‘measuring’ the condition of the wound bed and eventually aid in determining the wound’s response to treatment. pH biosensor TQ-loaded PLA: CA wound dressing can be prepared by immobilizing pH sensitive dyes into the NFs.
4 References


Biomater., 100B, pp. 1556–1565.


7 Appendix

Calibration curves:

*TQ Calibration curve in DCM: DMF*
TQ calibration curve in PBS

$y = 0.1235x$

$R^2 = 0.9991$
SEM results

*SEM image: TQ-loaded PLA NFs at drug loading ratio 1 wt. %*
TQ-loaded PLA NFs at drug loading ratio 5 wt. %
FT-IR charts

FT-IR chart for CA powder

FT-IR chart for TQ powder
**FT-IR chart for neat PLA NFs**

![FT-IR Chart for Neat PLA NFs]
Cytotoxicity

Cytotoxicity results of TQ-loaded PLA NFs at the drug ratios 1, 3 and 5 wt. %