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Fabrication of Chitosan-Polyethylene Oxide Electrospun Nanofibrous Mats Containing Green Tea Extract

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ABSTRACT

Novel electrospun nanofibrous CS-PEO nerve conduits containing 0, 2.5, and 5 % of green tea methanolic extract were developed and characterized by FE-SEM, FT-IR, TGA/DSC as well as tensile strength analysis. The FE-SEM images revealed that all of the nanofibers had an average diameter of ~80nm. The swelling degree was decreased by increasing the GT amount from 2.5 to 5 %, and this might be attributed to the enhanced interactions of NH_2*,* $C(O)NH_2$*, and OH groups of chitosan and PEO polymers with the OH groups of GT leading to a less hydrophilic mat surface, thus reducing the attraction by the aqueous medium. Moreover, the swelling was at its highest in acidic medium; however, it was decreased in the neutral environment and had the least value within the alkaline medium. The CS-PEO-5 % GT exhibited the highest antibacterial activity among three samples examined against both S. aureus and E. coli microorganisms. The CS-PEO-5 % GT was proved to be a very suitable candidate to be used as a nerve conduit due to its improved tensile and antibacterial activities.*

1. Introduction

Peripheral nerves are commonly exposed to physical injuries usually caused by transportation and construction accidents, natural disaster and war damage, and other traumas, including iatrogenic side effects of surgery. Peripheral nerve injuries affect 2.8 % of trauma patients, frequently leading to life-long disability [1]. The incidence of nerve injuries is relatively high all over the world. In the US, over 200,000 peripheral nerve repair procedures are performed annually [2]. Around 5 % of wounds in the extremities can be associated with peripheral nerve injuries [3].

Many techniques have been developed to design fibrous scaffolds in order to be used as a tissue substitute. Electrospinning is a fiber spinning technique to produce nanofibers and microfibers under a high-voltage electrostatic field [4]. Scaffolds, prepared via electrospinning, using native and/or synthetic polymers with nanoscale structures similar to native skin extracellualr matrix (ECM), allow for preparation of aligned fibrous matrix with both adjustable porosity and mechanical properties. Recently, various techniques have been developed to fabricate nanofibrous

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scaffolds for biomedical applications such as phase separation [5], self-assembly [6], melt-blowing [7], and templating [8]. Nevertheless, the electrospinning technique is valuable to produce nanofibrous scaffolds for numerous biomedical purposes [9,10].

Nanofibers have played important roles in miscellaneous biomedical fields ranging from drug delivery, wound healing, and peripheral nerve repair because of having high surface areas and tunable properties due to different compositions and fabrication parameters [11]. The nanofibrous scaffolds obtained from natural and synthetic polymers are promising as peripheral nerve repair systems by electrospinning of blended polymers or through coaxial spinning of two different polymers along with drugs and active agents [12,13].

Chitosan (CS), a linear polysaccharide of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is a deacetylated product of chitin and is considered a bio-based environmentally friendly material [14,15]. It is well known that pure CS is difficult to electrospin, and the nanofiber creation is facilitated by its blending with other polymers [16-18]. Additionally, the combination of CS with other polymers may lead to higher analogy of the scaffold to natural ECM components and induces superior properties, which are essential in tissue regeneration. Combining CS with polyethylene oxide (PEO) might lead to novel materials suitable for diverse applications in the biomedical field. For instance, CS-PEO nanofibers can be utilized as 3D scaffolds for peripheral nerve repair due to their good adhesion and proliferation [19,20].

Green tea (GT) extract is a great source of flavonoids with the status of food additive. The flavonoids are of considerable interest due to their prospective favorable effects on human health. They have antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant activities [21-24]. The most important compounds responsible for the antioxidant activity are gallic acid and eight major catechins [25]. Indeed, the tea polyphenols are natural antioxidants [27-29]. Also, the antibacterial activities of green tea extract [26] and chitosan-based materials [30-32] have been proved. The strong antibacterial activity of tea extracts is mainly based on the presence of catechins. Important and active catechins are known as Epicatechin (EC), Epigallocatocin (EGC), Epigallocatechingallate (EGCG), and Epicatechinaglate (ECG) [33-35].

The aim of this work is to design and manufacture nerve guidance conduits based on chitosan-polyethylene oxide (CS-PEO) nanofibers containing 0, 2.5, and 5 % of green tea (GT) methanolic extract to product antimicrobial nerve conduits for regeneration of peripheral nerves injuries.

2. Experimental

2.1. Materials

The high molecular weight chitosan (>75 % deacetylation degree, viscosity=200-800 cps) and polyethylene oxide (PEO) (average M_v=400,000) polymeric materials were purchased from Sigma–Aldrich Company; other compounds including acetic acid (CH_3COOH), methanol (CH_3OH), NaCl, KCl, Na_2HPO_4, KH_2PO_4, and NH_4OH were received from Merck Company. The green tea used in this study was purchased from Golestan Tea Company.

2.2. Preparation of green tea methanolic extract

Ten grams of green tea were extracted using methanol. The extracts were filtered using filter paper. The collected filtrates were concentrated in a vacuum at a temperature below 40 °C using a rotary evaporator (Buchi, Switzerland) and placed in a vacuum oven for 5 hours at 40 °C to be completely dried.

2.3. Solution preparation

50 % (v/v) of aqueous acetic acid solution ($CH_3COOH:H_2O$) was used as the solvent. Total concentration of CS and PEO polymers in the solution was 4 % (w/w) with CS-to-PEO-weight ratio was 75:25. The solutions contained CS and PEO with 0, 2.5, and 5 % of green tea methanolic extract. CS-PEO-GT extract solution was stirred overnight and, then, was sonicated for 15 minutes to get the electrospinning solution.

2.4. Electrospinning process

Electrospinning was performed by the laboratory spinning unit (ANSTCO-N/VI, Tehran, Iran), which was designed in terms of a vertical working principle. Each solution of CS-PEO containing 0, 2.5, and 5 % of green tea (GT) methanolic extract was placed in a 10 mL syringe and sent to the drum collector (covered with aluminum foil) through a nozzle. The power supply (AC) was set for a positive voltage of 21 kV. The flow rate of the solution was also determined by setting up the syringe pump at 1 mL/h. The rotational speed of the drum collector was 2500 rpm and its distance was set to 10 cm (optimum distance based on preliminary tests) away from the nozzle. Finally, neural conduction channels were provided by folding the CS-PEO-GT nanofibrous mats.

2.5. Characterization techniques

To analyze the morphology of the CS-PEO-GT nerve conduits containing 0, 2.5, and 5 % GT, field–emission scanning electron microscopy (SEM) was performed using Philips instrument (XL30). The mechanical properties of biocomposites were evaluated by tensile test measurements conducted at room temperature by an INSTRON dynamometer (LLY-006, China) according to the ASTM D638 for plastics with the loading speed of 10 mm/min, distance of 10 mm, break limit of 50 % in ambient condition, and humidity of 65 %. At least three specimens were tested for each formulation. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra were achieved using Bruker FT-IR spectrometer in the 400-4000 cm^{-1} range at room temperature in transmission mode. In order to investigate the swelling behaviors of the CS-PEO nanofibrous mats containing 0, 2.5, and 5 % GT extract, they were immersed in 0.1 M phosphate buffer saline (PBS) (pH 7.4), acetic acid (pH 4), and alkaline (pH 12) solutions at 25 °C. The water uptake experiments continued for three days, during which the nerve conduits were weighted at 10 min time intervals for the first four hours and, then, every day after removing water from the nerve conduits surface using a filter paper. The water uptake or swelling percent was calculated according to Eq. 1, where W_0 is the initial weight of dry mat and W_s is swollen weight of mat at equilibrium.

$$\text{Swelling percent} = [(W_s - W_0)/W_0] \times 100 \quad (1)$$

2.6. Antibacterial activities

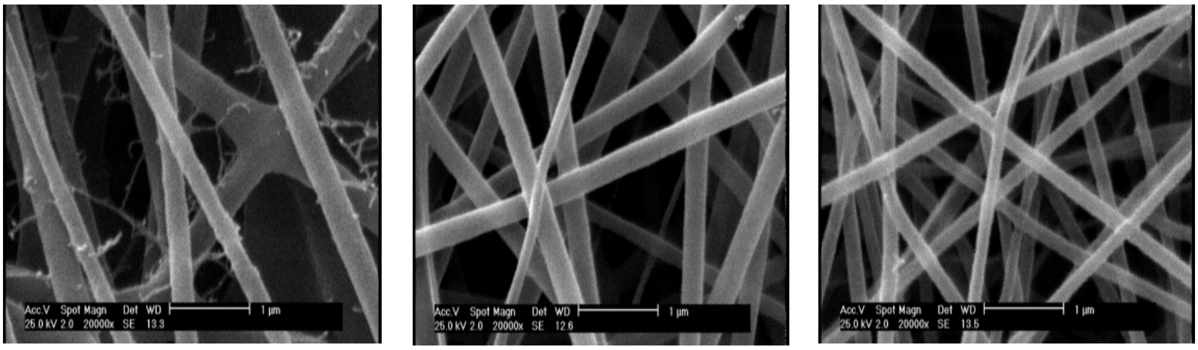
Two referenced bacterial strains, including Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC 25923) and Gram-negative *Escherchia coli* (*E. coli*, ATCC 25922) bacteria (prepared from Pasteur Institute of Iran), were used for the antibacterial experiments according to a slightly modified version of AATCC test. Briefly, test bacteria were grown overnight in Muller Hinton agar. Initial inoculations were prepared by washing cells two times in 85 % sterile saline solution. 5 cm^2 swatches of each nanofiber were prepared in aseptic conditions and delivered in sterile plastic falcon tubes. Each nanofiber was than saturated with 100 μL suspension of test microorganisms. One series of samples were immediately diluted and plated at zero contact time, and another series of the samples were incubated at 37 °C for 18 h. Afterwards, the colonies were counted and concentration of bacteria in each sample was determined. All experiments were accomplished in triplicate and mean values were employed for statistical evaluation. The reduction rate in the number of test microorganisms was calculated using the formula R (%)= [(B–A)/B]×100, where R is the reduction rate percentage, A and B are the numbers of microorganisms recovered from the inoculated nanofibers over 1 h and at zero contact time, respectively.

3. Results and discussuon

3.1. Characterization of CS-PEO-GT nerve conduits

3.1.1. FE-SEM micrographs

In this study, CS-PEO nanofibrous mats containing 0, 2.5, and 5 % of green tea (GT) methanolic extract were fabricated using electrospinning method for application as nerve regeneration conduits. The FE-SEM images of the mats are shown in Fig. 1. It is observed that, in all cases containing 0-5 % GT, very uniform fibers free of any nanobeads and droplets are formed that are about 80 nm in diameter. The digital photographs of the neural conduction channels are shown in Fig. 2.



(a) **(b)** **(c)**

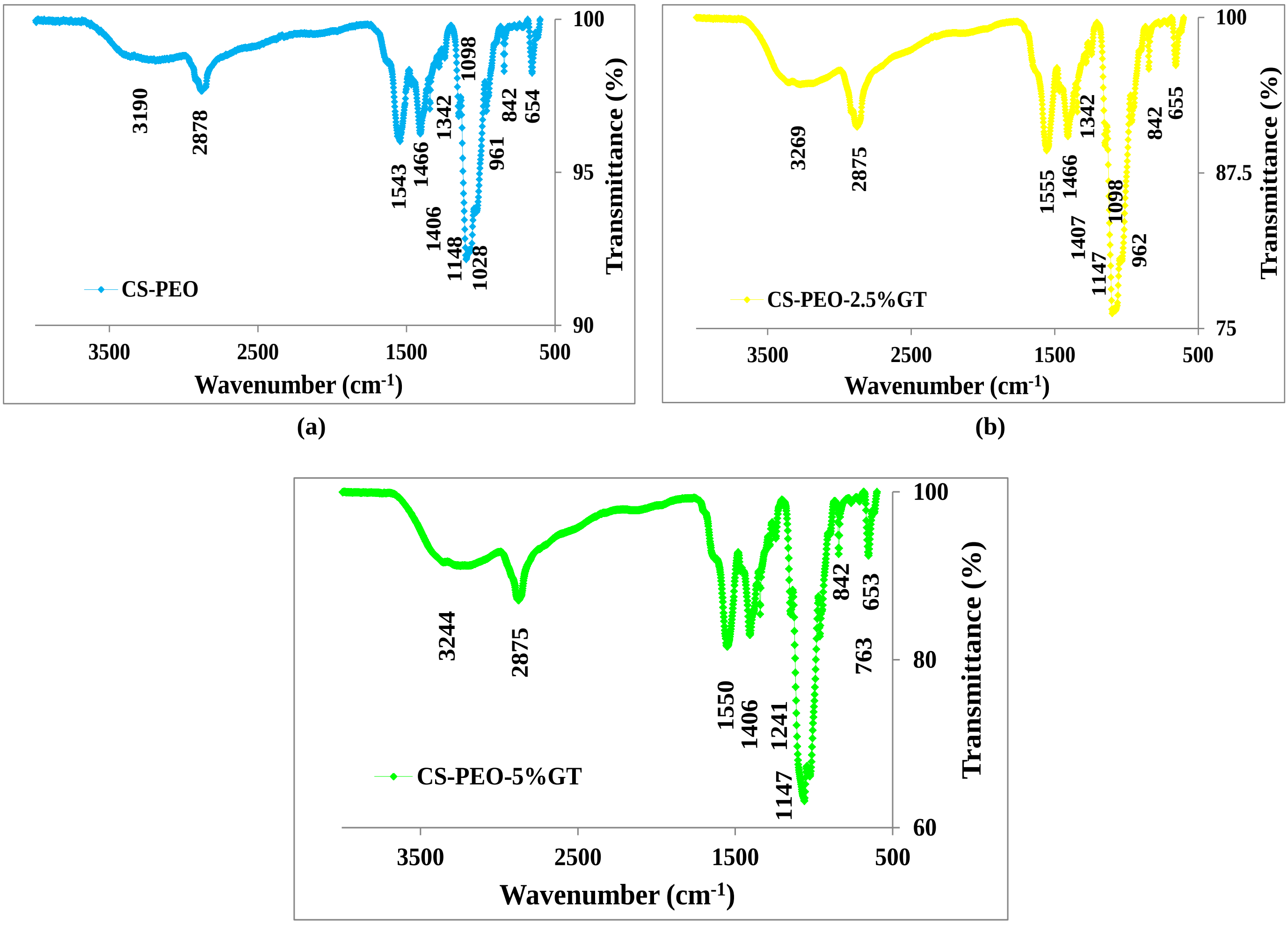
Figure 1. The FE-SEM images of CS-PEO mats containing (a) 0 %, (b) 2.5 %, and (c) 5 % of green tea (GT) extract.

(a) **(b)** **(c)**

Figure 2. Digital photographs indicating the CS-PEO nerve conduction channels containing (a) 0 %, (b) 2.5 %, and (c) 5 % of green tea (GT) methanolic extract.

3.1.2. FT-IR spectra

The ATR spectra of CS-PEO mats, containing 0, 2.5, and 5 % GT, are given in Figs. 3a-3c, respectively. It is obvious that pure CS-PEO mat displays a broad band near 3400 cm^{-1} that corresponds to the stretching vibration of amine and hydroxyl groups. This band becomes weak for the CS-PEO mat loaded by 2.5 and 5 % GT, which is possibly due to the strong interaction of GT with both polymers. The spectra in Figs. 3b and 3c reveal a band at 3200 cm^{-1} which is related to OH groups of its constituents and also to the OH groups of water which is possibly adsorbed by the GT powder. The bands around 2900 cm^{-1} are assigned to the CH stretching vibration, whereas bands at 1550 and 1466 cm^{-1} are characteristics of amides. The bands at about 1400 and 1250 cm^{-1} are attributed to CH_3 symmetrical deformation modes, and those near 1150 and 1100 cm^{-1} are indicative of the C-O and C-C stretching vibrations. The small bands at around 650 and 600 cm^{-1} correspond to the wagging of CS saccharide structure. The ATR spectrum of the mat containing GT shows sharper peaks compared with that of pure CS-PEO mat, which is due to the presence of more amounts of organic molecules in the GT extract.



(a) (b)

(c)

Figure 3. The ATR spectra of CS-PEO mats containing (a) 0 %, (b) 2.5 % and (c) 5 % of green tea (GT) methanolic extract.

3.1.3. Swelling and water uptake

The water uptakes of the CS-PEO mats containing 0, 2.5, and 5 % of GT were performed at 25 °C in PBS (pH 7.4), acetic acid (pH 4) and alkaline (pH 12) solutions. The experiments were carried out for three days, and the weights of the films were measured. The plots of swelling percentages against time are displayed in Fig. 4. It is observed that the addition of 2.5 % GT to the CS-PEO mat leads to an increase in the swelling percent reflecting enhanced hydrophilicity of the mat. However, it is obvious that increasing the GT percentage to 5 % results in decreasing the water uptake. The reason for the smaller swelling of this mat by the addition of 5 % GT can be described as the enhanced interactions of the NH_2, $C(O)NH_2$, and OH groups of the chitosan and OH groups of PEO polymer with the OH groups of GT leading to decreased hydrophilicity of the mat, thus decreasing its interactions with the aqueous medium. Furthermore, the swelling is the highest in acidic medium; however, it decreases in the neutral environment and has the least value within the alkaline medium.

It is observed that the highest amount of swelling is achieved in the acidic environment (pH 4). In acidic pH, NH_2 groups of chitosan are protonated and converted to NH_3^+. As a result, a polyaction will be produced, which will cause more nano-fibers to swell in acidic environment through the formation of very strong hydrogen bonds with water molecules. In neutral medium (pH 7.4), NH_2 groups of chitosan are not protonated; in comparison with the acidic medium, they form weaker hydrogen bonds with water molecules and other chains of chitosan polymer. Thus, the hydrogen bond strength in the buffer is much less than that of acidic pH. The reason for this behavior can be attributed to the presence of H^+ ions created due to the ionic separation of acids. Since the ion separation is greater, the number of hydrogen ions that tend to form bonds with chitosan NH_2 groups will increase, resulting in more protonation. In the alkaline environment (pH 12), swelling is the least among others which is due to the least interactions of OH^- ions in the basic environment with the nano-fibers.

It was found that CS–PEO-0.50 % F. vulgaris-Ag NPs had the greatest water uptake among three mats containing 0, 0.25, and 0.50 % of F. vulgaris-Ag NPs [37]. Comparing the swelling percentages of CS–PEO mats containing 0 % and 0.25 % F. vulgaris-Ag NPs revealed that the results were very comparable; however, the values were slightly greater for the mat incorporated with 0.25 % F. vulgaris-Ag NPs indicating higher hydrophilic nature of this mat [37]. In another work, it was exhibited that the swelling percentages of all CS–PEO, CS-PEO-ZnO, and CS-PEO-hyd-ZnO(Im) nanofibrous mats were the highest in acidic medium; however, they decreased in the buffer and the least swellings were obtained in the alkaline environment [38]. Similar results were observed for the CS-PEO, CS-PEO-1 % F. silica and CS-PEO-1 % F. silica-0.5 % cefazolin nanofibrous mats [39].

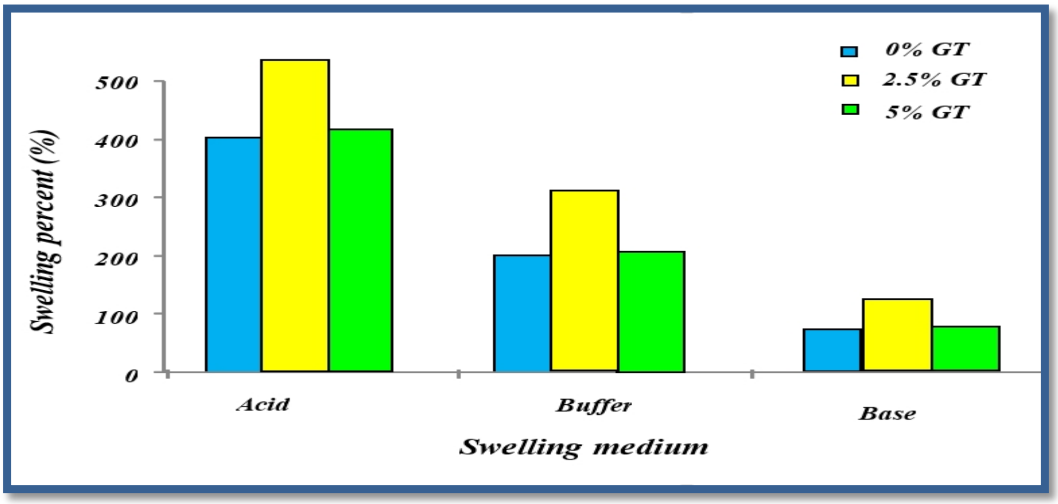


Figure 4. The swelling diagrams of CS-PEO mats containing 0, 2.5 and 5 % of green tea (GT) methanolic extract.

3.1.4. Tensile strength

The tensile strength of a material reveals that how much stress the material will bear before suffering from permanent deformation/tearing. It is well known that CS is a rigid and brittle natural polymer; however, PEO chains are flexible; hence, it is expected that the CS-PEO blended mats will show increased flexibility. The tensile strength diagrams of CS-PEO mats containing 0, 2.5, and 5 % of green tea (GT) methanolic extract are presented in Fig. 5. It is clear that a slightly higher elongation at break (maximum tensile strain on horizontal axis) is measured for the mat loaded with 2.5 % GT indicating higher flexibility of this mat. Fig. 5 also displays that the tensile strength (the maximum value of vertical axis) of the fibers is highly increased by the addition of 5 % GT so that the tensile strength is smaller for CS-PEO mat loaded by 2.5 % GT. Green tea contains phenolic groups. Moreover, chitosan polymer has OH and NH_2 groups. Establishing a hydrogen bond between the OH group of phenols from the green tea and OH and NH_2 groups of chitosan makes the channel structure harder. With increasing hydrogen bonds and hardening of the channel, the tensile and tensile strain is reduced; however, tensile strength rises. The Young's Modulus, which is the slope of the graphs, displays a similar increasing trend to that of tensile strength with a much greater Young's Modulus obtained for the CS-PEO mat including 5 % GT.

Recently, it has been indicated that the CS-PEO mat loaded with 0.25 % F. vulgaris-AgNPs has had the highest elongation at break (16.25 %) among the mats containing 0, 0.25, and 0.50 % F. vulgaris-AgNPs approving its highest flexibility; however, the least elongation at break belongs to CS–PEO-0.50 % F. vulgaris-AgNPs (11.58 %) which was related to the greater amount of silver NPs in this mat that prevented its high flexibility [37]. It was also illustrated that the tensile strength of the mat was increased by the addition of silver NPs so that the tensile strength was the lowest for pure CS–PEO mat; however, it was the highest for the mat with 0.50 % silver content [37].

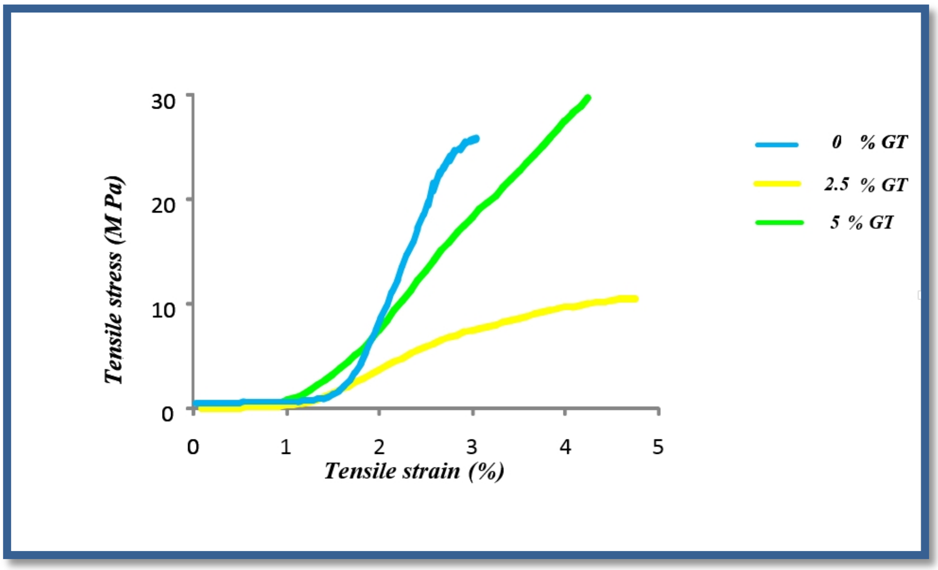
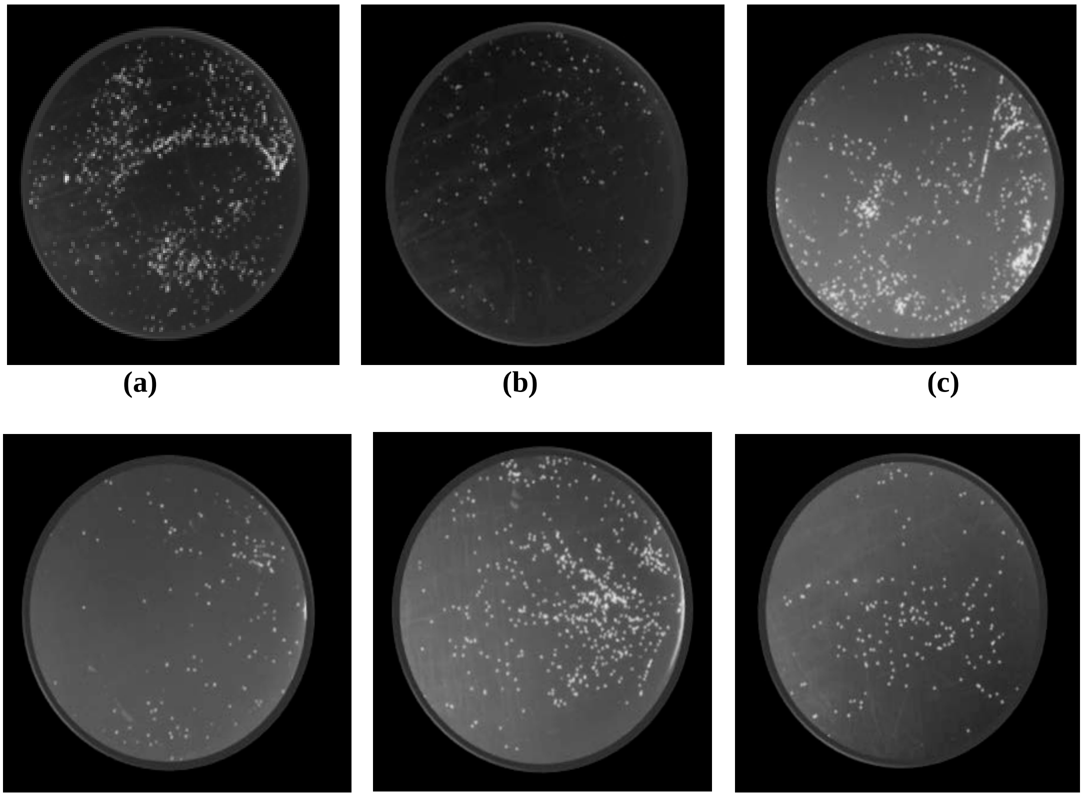
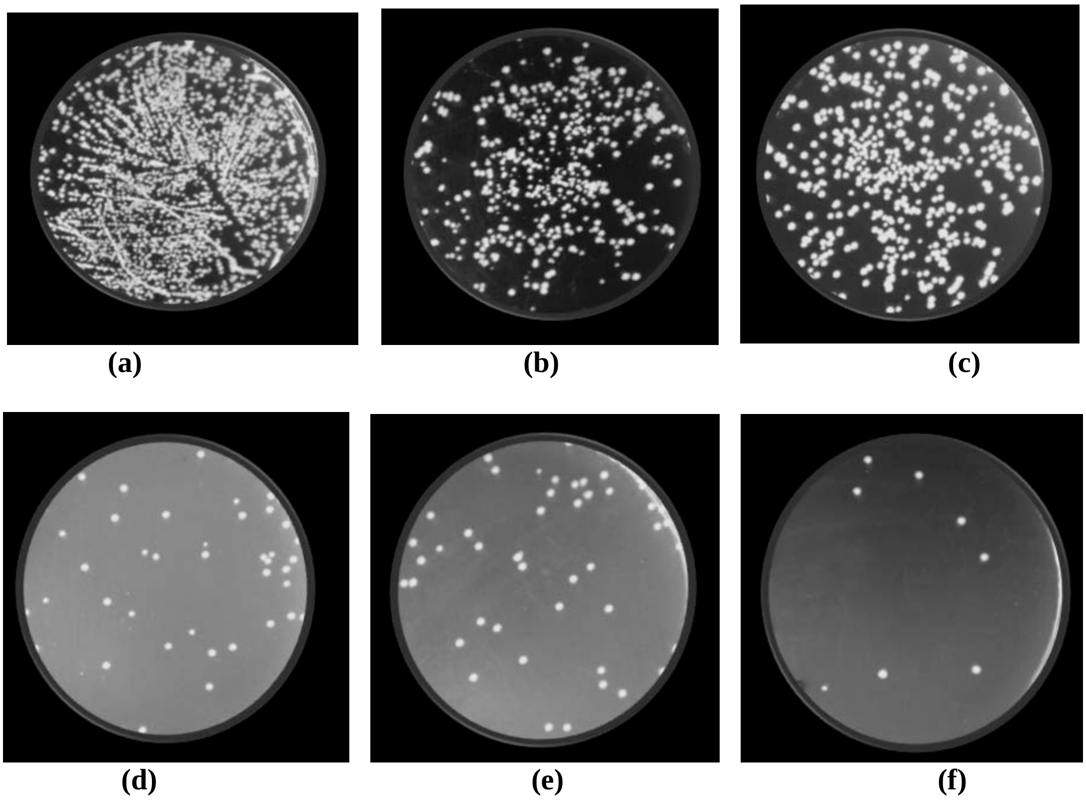


Figure 5. The tensile diagrams of CS-PEO mats containing 0, 2.5, and 5 % of green tea (GT) methanolic extract.


(a) (b) (c)

(d) (e) (f)

Figure 6. Antibacterial activities against *E. coli* bacterium using laboratory controls with (a) 1:50000 CFU/mL and (b) 1:5000 CFU/mL, (c) 1:500 dilutions, (d) CS-PEO, (e) CS-PEO-2.5 %GT and (f) CS-PEO-5 %GT nanofibrous mats.


(a) (b) (c)

(d) (e) (f)

Figure 7. Antibacterial activities against *S. aureus* bacterium using laboratory controls with (a) 1:50000 CFU/mL and (b) 1:5000 CFU/mL, (c) 1:500 dilutions, (d) CS-PEO, (e) CS-PEO-2.5 %GT and (f) CS-PEO-5 %GT nanofibrous mats.

3.1.5. Antibacterial activity

Antibacterial properties of CS-PEO nanofibrous mats containing 0, 2.5, and 5 % green tea (GT) methanolic extract are evaluated on both Gram negative *E. coli* and Gram-positive *S. aureus* bacteria. Antibacterial properties of CS-PEO nanofibrous mats containing 0, 2.5, and 5 % green tea (GT) extract are exhibited in Figs. 6 and 7. Three different dilutions of each bacterial sample are used as laboratory controls which are 1:50000, 1:5000, and 1:500 CFU/mL. The CS-PEO mat containing 5 % GT extract reveals the highest antibacterial activity among those of CS-PEO and CS-PEO-2.5 % GT nanofibrous mats. It is apparent that the CS-PEO-2.5 % GT sample also presents appropriate bactericidal performance.

Interestingly, the CS-PEO mat containing 5 % displays 98 % antibacterial activity against *S. aureus* and 91 % against *E. coli* bacteria which is due to the presence of green tea (GT) methanolic extract and its release from the fibers to the medium. It is well known that although the Gram-positive bacteria have a thicker peptidoglycan layer in their cell walls, they are more susceptible to chemicals than Gram-negative ones which is due to their impermeable lipid-based bacterial outer membrane [36]. Consequently, the CS-PEO and CS-PEO-GT mats reveal smaller antibacterial activities against *E. coli*.

It was shown that the CS–PEO mats containing 0.25 and 0.50 % F. vulgaris-AgNPs displayed nearly 100 % antibacterial activities against both *S. aureus* and *E. coli* bacteria which were related to the presence of silver nanoparticles and their release from the fibers to the medium [37]. In another work, it was found that the CS-PEO nanofibrous mat containing 3 % ZIF-8NPs revealed 100 % antibacterial efficacy against both *S. aureus* and *E. coli* microorganisms ascribed to the presence of ZIF-8 nanoparticles and their release from the fibers [40].

It is noteworthy that the antibacterial tests were performed in petri dishes with the same size using identical concentrations of bacteria by the counting method. Thus, the results are comparable. In fact, in the count method, a bacterial concentration is used in the presence of an antibacterial agent, and the mortality rate of the bacteria is calculated considering the bacterial concentration at the beginning of the work. These figures are due to differences in the concentrations of bacteria at the end of antibacterial test relative to its beginning. By comparing the disks of the Gram positive and negative bacteria, it can be proved that the penetration into the Gram-negative bacteria is more severe than into the Gram positive ones. This is because (as it has been shown) the reduction in the number of *Staphylococcus aureus* bacteria is more than *E. coli*. The overall antibacterial efficiency for killing the Gram positive bacteria is 98 %, and it is 91 % for the Gram-negative bacteria. These percentages indicate that in disks containing gram-negative bacteria, 91 % of the bacteria were killed and only 9 % of them remained. Also, 98 % of the bacteria in the disks containing Gram-positive bacteria were killed and only 2 % of them were viable.

4. Conclusions

In this research, three electrospun CS-PEO nanofibrous mats containing 0, 2.5, and 5 % of green tea (GT) methanolic extract were produced and characterized by FE-SEM, tensile strength analysis and ATR spectroscopy. The FE-SEM images of the mats revealed that very uniform fibers free of any nanobeads and droplets were formed that were about 80 nm in diameter. The tensile strength analysis revealed that the tensile strain of the mat including 2.5 % GT increased; however, its tensile strength was significantly decreased. The swelling experiments confirmed that the addition of 2.5 % GT to the CS-PEO mat led to a great increase in the swelling percent reflecting much enhanced hydrophilicity of the mat. The ATR spectrum of the mat containing 5 % GT showed sharper peaks was compared with that of CS-PEO mat loaded by 2.5 % GT, and this can be due to the presence of higher amounts of organic molecules in the GT extract. The use of green tea extract as a herbal remedy in CS-PEO nanofibers increases the inherent antibacterial properties of chitosan. In addition to antibacterial green tea, its antioxidant properties also contribute to the superiority of these types of canals to non-drug neural conduction channels. Also, the GT extract increases the uptake water property by the nanofibers. By studying the tensile strength test results, it was found that the most flexibility was related to the sample containing 2.5 % GT extract. Therefore, it can be concluded that by increasing the weight percent of GT extract, the number of hydrogen bonds formed between OH groups of catechins with CS-PEO enhanced. The CS-PEO-5 % GT displayed the highest antibacterial efficacy against both *S. aureus* and *E. coli* microorganisms. The CS-PEO-5 % GT was selected to be the most appropriate sample as a nerve conduit as a result of its enhanced tensile and antibacterial properties.

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