



ANTIBACTERIAL CINNAMON ESSENTIAL OIL INCORPORATED POLY(ε-CAPROLACTONE) NANOFIBROUS MATS: NEW PLATFORM FOR BIOMEDICAL APPLICATION

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ABSTRACT

Wound healing is a complex process and prevention of wound infection is crucial for wound care as colonization of bacteria delays the healing process. For rapid healing, the wound dressing should have an antibacterial property and biocompatible. Herein, we proposed the use of cinnamon essential oil, a natural antimicrobial agents, incorporated electrospinning poly(ε-caprolactone) (PCL) fibrous dressings. The wound dressing scaffolds were successfully prepared by electrospinning of the blend solution of poly(ε-caprolactone) (PCL) with different concentrations of cinnamon essential oil. The mats were characterized by field emission scanning electron microscopy (FESEM), Fourier transform infra-red (FTIR), thermal gravimetric analysis (TGA), and differential scanning calorimetry (DSC) analysis. FESEM results revealed that the incorporation of cinnamon oil generated the membrane with fine fibers along with nanofibers compared to uniformly distribute the nanofibers for pristine PCL. Experimental results of cell viability assay and microscopy imaging showed that the as-fabricated composite wound dressing scaffold exhibited excellent cell viability for fibroblast (NIH-3T3) cells. The antimicrobial activity of the composite scaffold was assessed from the zone of inhibition against Gram-positive and Gram-negative bacteria. Results indicated that the fibrous mats inhibited the growth of Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria- *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* showing high antibacterial activity. Overall, our results concluded that the introduced scaffold might be an ideal biomaterial for wound dressing applications.

Keywords: Cinnamon essential oil, Electrospinning, Nanofiber, Polycaprolactone, Wound dressing.

INTRODUCTION

Skin is an intricate structure consisting of a high cellular epidermis and relatively cellular dermis of collagen-rich extracellular matrix (ECM) (Flanagan, 2013; Zhong *et al.*, 2010). The detriment of the skin integrity caused by physical, mechanical, thermal, or chemical factors, enfeebling the skin functions like protection, homeostasis, sensory detection, and self-healing is termed as wound (Zahedi *et al.*, 2010). Damage at the epidermal layer can be healed by re-epithelization whereas partial or complete damage of dermal layers cannot be adequately healed by the body or significantly delay the healing process. In such condition, proper dressing material with antibacterial properties is required (Chong *et al.*, 2007). Dressing plays an important role in protection against the possible contagion and amps up growth factors for rapid wound healing. Moreover, dressing material should provide a suitable environment by allowing gaseous exchange and diffusion of waste and nutrients. Thus, biocompatible,

biodegradable, non-toxic, non-allergic, non-sensitizing, non-adherent, and cost-effective dressing is preferred (Hilton *et al.*, 2004; Zahedi *et al.*, 2010). During ancient times, honey paste, plant fibers, animal fats were used to cover the wounds and protect them from infections (Dhivya *et al.*, 2015). However, in recent times many modern dressings use different natural and synthetic biopolymers with antibiotics and metal ions like zinc, copper, and silver incorporated (Maharjan *et al.*, 2017; Pant *et al.*, 2018). Furthermore, biodegradable synthetic polymers such as polylactic acid (PLA), poly lactic-co-glycolic acid (PLGA) and poly(ε-caprolactone) (PCL), loaded with natural agents like essential oils are under study for wound dressing properties (Unalan *et al.*, 2019; Zhang *et al.*, 2017). PCL is a member of aliphatic polyester having a low melting point and good compatibility with excellent processability (Tiwari *et al.*, 2019; Tiwari *et al.*, 2016). Being economic and having proper mechanical strength, PCL-based scaffolds has been widely studied for dressing applications (Estellés *et al.*,

2008). However, its poor hydrophilic nature limits cell adhesion, proliferation, and differentiation (Tiwari *et al.*, 2016). Most importantly, it does not show antibacterial properties; therefore PCL alone cannot be considered as an ideal material for wound dressing. Recently, an incorporation of essential oil in the wound dressing material has gained significant interest to impart the antimicrobial property and biocompatibility (Unnithan *et al.*, 2012).

Essential oils are the odorous liquids (sometimes semi-solid or solid) occurring in the most parts of the plant body such as buds, flowers, stems, seeds, fruits, roots, leaves, woods, and barks (Bakkali *et al.*, 2008). Among various essential oils, the cinnamon essential oil possess antihyperglycemic, antidiarrheal, antihyperlipidemic, antioxygenic, anti-inflammatory, acaricidal, hepatoprotective, gastroprotective, antioxidant, antibacterial, α -amylase inhibitory, and immunomodulatory properties (Kumar *et al.*, 2012). The leaves of *Cinnamomum tamala* yield pale yellow essential oil with a clove-like peppery odor (Mir *et al.*, 2004). *Cinnamomum tamala* is a tropical and subtropical evergreen tree or shrubs growing between 450-2100 m elevation in Nepal (Kumar *et al.*, 2012). The components of cinnamon leaf oil constitute of linalool (54.66 %), α -pinene (9.67 %), p-cymene (6.43 %), B-pinene (4.45 %), limonene (2.64 %) and sixteen minor components (< 2 %) (Sharma & Rao, 2014). The leaf oil is a rich source of eugenol. The essential oil from the bark contains 70-85 % cinnamic aldehyde (Lamichhane & Karna, 2009). The aqueous and alcoholic extracts of *Cinnamomum tamala* inhibited the growth of *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, and *Salmonella typhimurium* (Parekh, 2007). Therefore, fabrication of cinnamon essential oil incorporated antimicrobial PCL nanofibrous composite with tunable physicochemical and biological properties can benefit in the wound healing process.

Electrospinning has been a simple, straightforward, cheap, and versatile technique to generate functional nanofibrous scaffolds from different polymers for diverse applications, especially for biomedical applications (Joshi *et al.*, 2015a). Electrospinning employs a high electric field to the droplet of the fluid leading to deformation of the droplet with the formation of continuous fibers having high porosity and a large surface to volume ratio (Tae *et al.*, 2020). Recently, fabrication of functional electrospun nanofibrous mat containing active components such as drugs, inorganic nanoparticles, and plant extracts have attracted significant attention for various biomedical applications (Tae In Hwang *et al.*, 2020; Joshi *et al.*, 2020; Maharjan *et al.*, 2017). The non-woven electrospun polymeric fibers with identical diameters are analogous to the native collagen-rich ECM mimicking its structure and functions (Joshi *et al.*, 2015a; Joshi *et al.*, 2015b). A high

surface area and porous nature of fabricated mats help to promote homeostasis at the wound site providing a suitable structure for cell differentiation and attachment (Tiwari *et al.*, 2016). Extensive studies have been conducted to develop biocompatible electrospun nanofibrous scaffolds for wound dressing applications (Liao *et al.*, 2015). An electrospun nanofiber membrane containing antibiotic has been used as a barrier to prevent the post wound infections. Therefore, herein we prepared composite PCL nanofibrous membranes with different concentrations of cinnamon essential oil via electrospinning process. As-fabricated composite nanofibrous membranes were analyzed for cell proliferation, growth, and antibacterial activity to determine the efficacy of nanofibrous scaffolds.

MATERIALS AND METHODS

Materials

PCL pellets (molecular weight 70,000-90,000 D) were purchased from Sigma-Aldrich, USA. Similarly, N, N-dimethylformamide (DMF) and tetrahydrofuran (THF) were purchased from Qualigens Fine Chemicals and Hi-Media laboratories, India respectively. Cinnamon essential oil was purchased from the Jadibuti Association of Nepal. All the reagents were used as received without further purification.

Preparation of solutions and electrospinning

Pristine PCL solution (12 wt %) was prepared by dissolving in the solvent system of THF and DMF (1:1) under magnetic stirring for about 20 hours at room temperature. Subsequently, cinnamon oil blended electrospinning solutions were prepared by adding different amounts of cinnamon essential oil (10 and 20 wt % of PCL) into each PCL solution and stirred for 12 h to make a homogeneous solution.

The electrospinning solution was drawn into a plastic syringe. The flow rate was maintained at 0.5 mL/h. Electrospinning was performed at the ambient conditions with the parameters applied as; voltage of 12 kV, tip collector distance of 25 cm, and collector drum rotation of around 620 rpm. The electrospun fibers were collected on the surface of the rotating drum using aluminum foil. Fiber mats were dried for 12 h in a vacuum oven at 35 °C. The resulting electrospun membranes were named as PCL, PEO10, and PEO20 for pristine PCL mat, 10 wt % cinnamon oil incorporated PCL mat, and 20 wt % cinnamon oil incorporated PCL mat, respectively.

Characterization

The morphology of the as-prepared nanofibrous scaffolds was observed using Field Emission Scanning Electron Microscopy (FESEM, JSM-5900, Japan). The as-prepared mats were sputter-coated with osmium, before imaging. The fiber diameter distribution was determined by ImageJ

(NIH, USA) software. The ABB Bomen MB100 Spectrometer (Bomen, Canada) was used to record the Fourier transform infrared (FT-IR) spectrum of the samples. The thermal stability of different samples was investigated utilizing thermal gravimetric analysis (TGA, Q50 TA Instruments), heating from 30 to 800 °C at a rate of 10°/min. Mechanical properties measurements were carried out using a universal testing machine (AG-5000G, Shimadzu, Japan) at room temperature.

Antibacterial tests

The antimicrobial activity of composite fibers was assessed from the zone of inhibition. Gram-positive bacteria, *Staphylococcus aureus* and Gram-negative bacteria- *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* were used for this study. The samples were cut into circular discs of 0.6 cm diameter under sterile condition. The samples were placed in the Mueller Hinton agar (MHA) plate swabbed with the suspension of the organism (1×10^7 CFU/mL) then incubated for 24 hours at 37 °C. The zone of inhibition was determined by measuring the clear area around each disc sample. The photographs were taken by a digital camera (Samsung galaxy A30).

Biocompatibility test

Fibroblast cells (NIH-3T3) cells were cultured in DMEM (Gibco) medium supplemented with 10 % FBS (Gibco) and 1 % penicillin/streptomycin (Gibco) incubated 37 °C in a humidified atmosphere of 5 % CO₂ according to previous reports (Joshi *et al.*, 2020; Tiwari *et al.*, 2019). The mat of 12 mm diameter was placed into the wells of 48 well-plates and immersed for 2 h into 70 % ethanol for sterilization purpose and subsequently washed with phosphate buffer saline (PBS). The scaffolds were further incubated in complete culture medium for 2 h at 37 °C to customize the environment for cells before seeding. Later, 500 µL of cell suspension at a cell density of 5×10^4 was seeded in each well containing different mats and cultured for 3 and 7 days. The culture medium was replenished every two days.

At the designated time points, the Dojindo's cell counting kit-8 (CCK-8) assay was performed to check the indication of viability according to the manufacturer's instructions (CCK-8, Dojindo). Briefly, 50 µL of CCK-8 solution was added to each well, and the cells were incubated for 2 h under dark conditions. 200 µL of suspension was later transferred into the 96 well plates, and absorbance was taken at 450 nm by a microplate reader (Sunrise™ Tecan, Austria). Besides, the cell attachment after 3 days of cell seeding was examined by scanning electron microscopy from the cells fixed with 4 % formaldehyde. Tissue culture plate (TCP) was used as control, and the data were presented as mean ± standard error (n = 3).

RESULTS AND DISCUSSION

Physicochemical characterization

The FESEM images of pristine PCL and essential oil loaded PCL mats under the magnification are shown in Fig. 1. The pristine PCL mat, as given in Fig. 1(a) showed uniformly distributed smooth nanofibers. The essential oil loaded PCL mats showed smooth nanofibers along with fine fibers without any beadings demonstrating a good compatibility of essential-polymer-solvent, as shown in Figs. 1(c) and 1(e). The nanofibers were more twisted and grouped in essential oil incorporated composite mats and the effect was pronounced with increased concentration of essential oil in the blend solution. The average fiber diameter gradually decreased with increasing concentration of the essential oil in the blend solutions with diameter, as shown in Fig. 1. The gradual decrease in fiber diameter can be ascribed to the decrease in the viscosity of electrospinning solution with increase in the concentration of essential oil. Solution viscosity influences the morphological structure and average size of resulting fibers (Liao *et al.*, 2015). Furthermore, the resulting nanofibers revealed that the incorporation of the essential oil into the nanofibers not only dramatically decreased their average diameter but also reduced the diameter distribution of electrospun nanofibers.

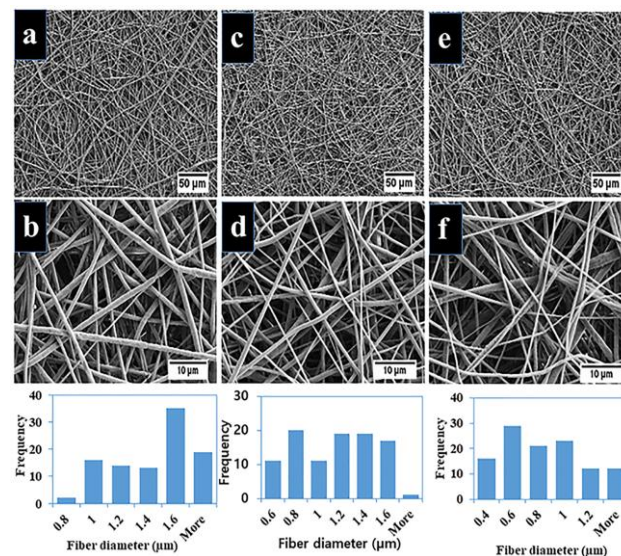


Fig. 1. FESEM images of pristine PCL (a, b), composite PEO10 membrane (c, d), and composite PEO20 membrane (e, f) in different magnifications and corresponding fiber diameter distribution

Wound dressing must provide and retain sufficient mechanical support during cell proliferation and tissue regeneration without new-tissue deformation. A typical strain–stress curve of the electrospun pristine PCL and the essential oil incorporated PCL membranes are shown in Fig. 2.

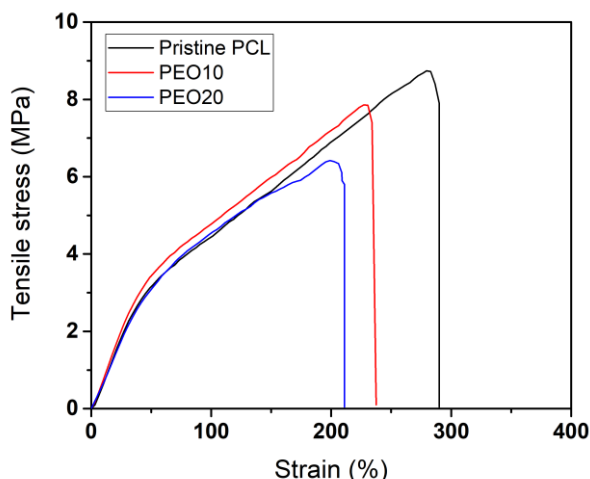


Fig. 2. Strain stress curves for different samples

The corresponding tensile stress, tensile strain, and Young's Modulus are presented in Table 1. The Young's modulus slightly increased with the incorporation of essential while tensile stress and percentage strain gradually decreased with increasing the essential oil content into the blend solution (Table 1). Miscibility and crystallization played crucial roles in the interfacial interaction and mechanical performance of polymer blends. PCL is a semicrystalline polymer, and the presence of a foreign body in the fiber matrix can alter its crystallinity thereby decreasing tensile stress and strain (Joshi *et al.*, 2015a). The electrospinning process rapidly removes solvent, preventing the preferred packing of polymer chains. Furthermore, the presence of essential oil in the blend solution might have retarded the crystallization of PCL during the electrospinning process. However, the oil incorporated mat (PEO10) still showed Young's Modulus 14.23 ± 0.27 the tensile stress of 7.61 ± 0.54 MPa and 230 ± 8 percentage strain. These values are considered good enough to support soft tissue regeneration (Tiwari *et al.*, Kim, 2018)

Table 1. Tensile properties of different samples

Sample	Young's Modulus	Tensile strength	% strain at maximum
Pristine PCL	12.89 ± 0.61	8.74 ± 0.9	289 ± 13
PEO10	14.23 ± 0.27	7.61 ± 0.54	230 ± 8
PEO20	13.44 ± 0.78	6.39 ± 43	210 ± 9

Incorporation of cinnamon essential oil in the PCL matrix was further evaluated using DSC analysis. The DSC curves for different samples are shown in Fig. 3(a). Pure PCL showed an endothermic melting peak at 59.26 °C. On increasing the essential oil concentration in blend solution, the melting point increased to 60.70 °C and 60.54 °C for PEO10 and PEO20, respectively. The melting peaks for different composite mats were detected

close to the pure PCL mat indicating the good miscibility of essential oil in the PCL. This indicated that the essential oil was molecularly dispersed in the polymeric matrix (Hwang *et al.*, 2019).

Thermal gravimetric analysis is the method to measure the thermal stability of the prepared samples by detecting the mass of a sample over time as a function of temperature. Fig. 3(b) shows the TGA curve for pristine PCL mat and essential incorporated PCL (PEO10). For pure PCL the initial decomposition temperature was 379.22 °C which slightly decreased to 372.85 °C for PEO10. The weight loss below 200 °C for composite membrane was attributed to the evaporation of essential oil and moisture absorbed. A proper incorporation of essential oil through the polymer matrix may affect the orientation of the polymer chain and decrease the crystallinity (Joshi *et al.*, 2015b). Therefore, there was a slight decrease in thermal stability upon the incorporation of essential oil in composite fiber. This result revealed that the essential cinnamon oil incorporated composite mat was thermally stable enough to be used in wound dressing.

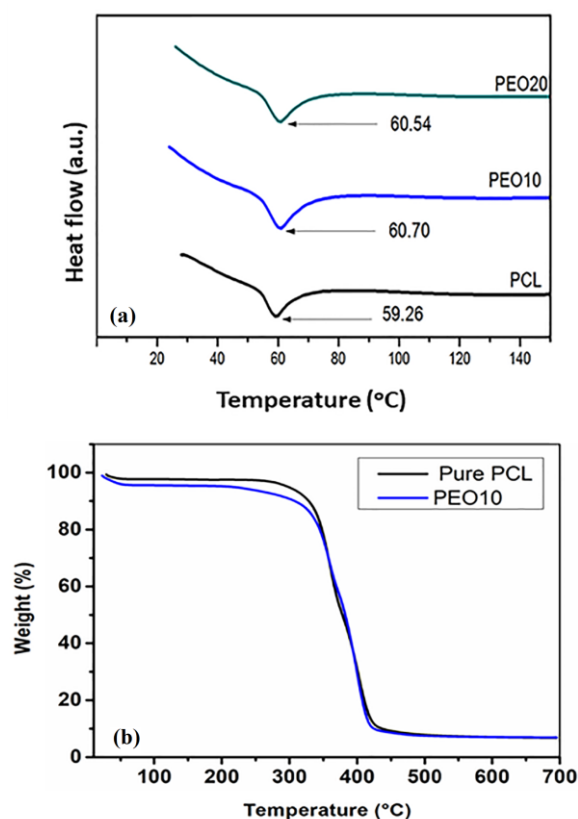


Fig. 3. DSC (a) and TGA (b) curves for different samples

Antibacterial activities

Cinnamon oil consists of active compounds like eugenol and cinnamaldehyde which showed an effective antibacterial activity. The cinnamon essential oil showed a clear zone of inhibition against *S. aureus*, *E. coli*, *P.*

aeruginosa, and *S. typhimurium* as shown in Fig. 4, suggesting that the cinnamon essential oil possesses potential antibacterial properties against a wide range of bacteria.

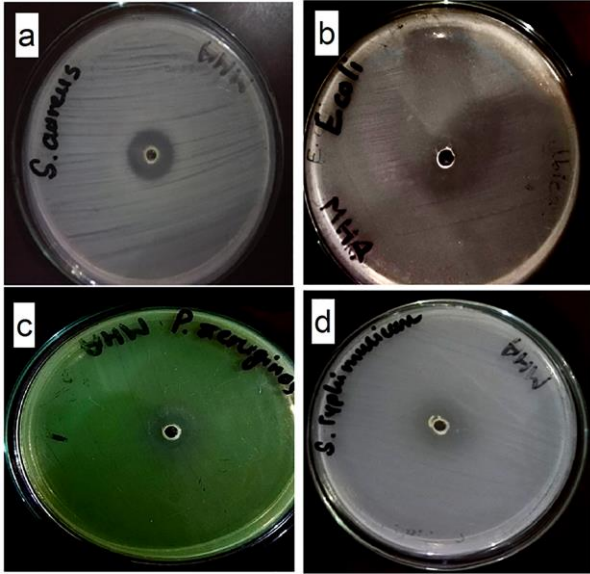


Fig. 4. Antimicrobial activities shown by cinnamon essential oil against (a) *Staphylococcus aureus*, (b) *Escherichia coli*, (c) *Pseudomonas aeruginosa* and (d) *Salmonella typhimurium*

A comparative antibacterial activity from the evaluation of ZOI between the pristine PCL and essential oil loaded composite mat is shown in Fig. 5. “A” in each plate represents the pristine PCL mat whereas “B” represents the essential oil incorporated PCL nanofibrous mats. The PCL mat (A) did not show a distinct clear zone of inhibition in any of the plates. Thus, we concluded that the pristine PCL does not inhibit bacterial growth or have

any antibacterial property. But, a clear small zone was seen around the essential oil blended PCL mats (B) in each plate indicating the oil embedded PCL mats hindered the bacterial growth.

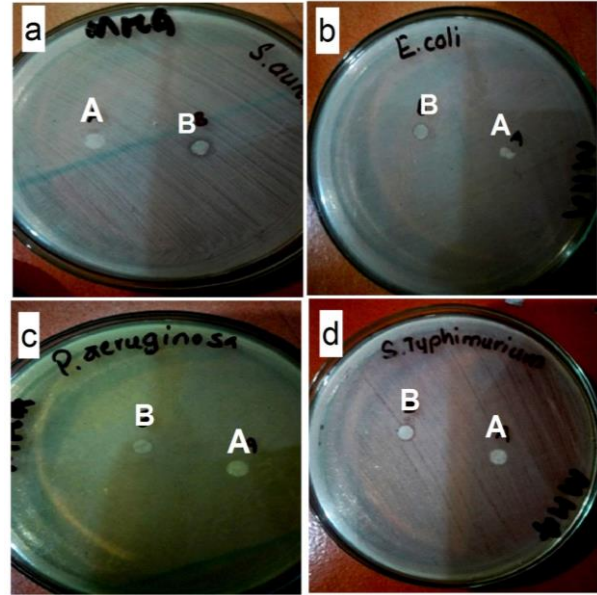


Fig. 5. Antimicrobial activity of and pristine PCL (A) and essential oil blended PCL nanofibrous mats (B) against (a) *Staphylococcus aureus*, (b) *Escherichia coli*, (c) *Pseudomonas aeruginosa*, and (d) *Salmonella typhimurium*

Earlier studies suggested that the antibacterial activity of cinnamon oil was probably due to the major component, cinnamaldehyde and their properties could be multiple (Prabuseenivasan *et al.*, 2006). The measurement of the zone of inhibition by pristine PCL and essential oil loaded PCL is shown in Table 2.

Table 2. Zone of inhibition of different bacteria in pristine PCL and PEO10

Name of Bacteria	Bacteria type	Zone of Inhibition (mm)		
		Essential oil	Pristine PCL	PEO10
<i>Staphylococcus aureus</i>	Gram positive	17	0	8
<i>Escherichia coli</i>	Gram negative	16	0	7
<i>Pseudomonas aeruginosa</i>	Gram negative	12	0	7
<i>Salmonella typhimurium</i>	Gram negative	17	0	7

Biocompatibility study

For this study, the NIH-3T3 cells were seeded on tissue culture plate (TCP), PCL and essential oil incorporated composite scaffolds (PEO10) for 3 and 7 days. The CCK-8 assay was used to evaluate the cell viability of the scaffolds and the corresponding result is shown in Fig. 6A. Although, the control group showed higher OD values than the pure PCL and PEO10 scaffolds, the

increase in cell viability in response to increased culture duration suggests that pure PCL and PEO10 are cytocompatible.

Moreover, the test also revealed that the PEO10 scaffold exhibited higher OD values compared to pure PCL from day 3. Given that OD values correlates directly with the cell viability, we can assume that the incorporation of essential oil into the PCL nanofiber provides more

desirable conditions for cell survival. This indicates that oil incorporation increases the suitability of the PCL scaffolds which can be attributed to the presence of bioactive molecules corresponding to the oil on the composite nanofiber as discussed in a previous study (Liakos *et al.*, 2015). Further, the morphology of cell growth on the scaffold observed by scanning electron

microscopy, is depicted in Figs. 6B and 6C. Cells were found well spread on to the scaffold of 3 days culture. Moreover, the number of cells increased when the culture was extended to 7 days. This study confirmed the biocompatibility of the composite membranes and be used in wound healing applications.

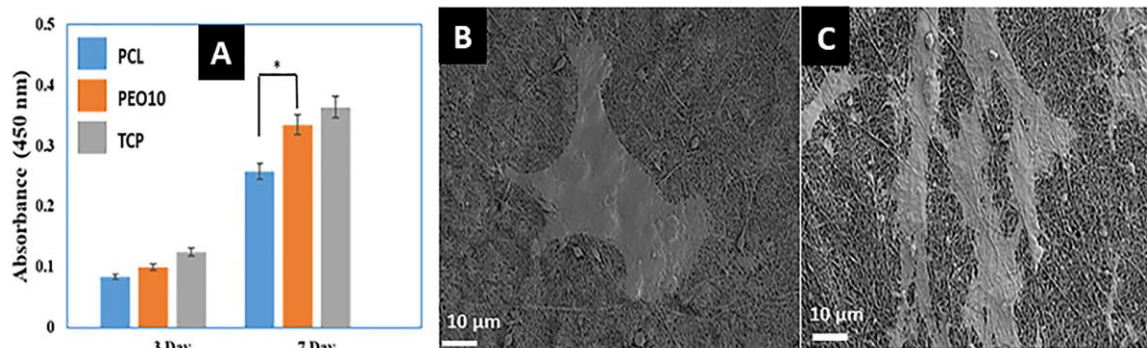


Fig. 6. (A) Graph showing the NIH-3T3 cell viability indices for pure PCL, PEO10, and tissue culture plate (TCP) on the 3-days and 7-days of cell culture. Significant differences in cell viability between PCL and PEO10 of same-day incubation were indicated by * $p < 0.05$. SEM images of the cells on the PEO10 scaffolds after 3-days (B) and 7-days (C) of culture

CONCLUSION

In this study, a potential wound dressing material, cinnamon oil incorporated PCL composite nanofibrous scaffold were fabricated using a single nozzle electrospinning technique. The varying mass composition of essential oil in blend solution showed a pronounced effect on the fiber morphology and physicochemical properties of the composite fibers. Essential oil incorporated scaffolds were found to have better cell support compared to neat PCL mat. Incorporation of cinnamon essential oil onto the composite fiber showed the antibacterial behavior against both Gram-positive and Gram-negative bacteria, which is an important criterion for an effective wound dressing. Therefore, cinnamon oil incorporated PCL nanofibrous scaffold was depicted as promising candidates for bactericidal applications and presents a reasonable alternative for the development of new biomaterial to be used for wound dressing.

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