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Emu oil-based electrospun nanofibrous scaffolds for wound skin tissue engineering

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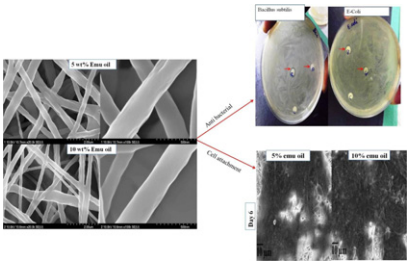
HIGHLIGHTS

- One-step preparation of emu oil-loaded polyurethane nanofibers is introduced.
- The introduced emu oil/PU electrospun mats are none cytotoxic and biocompatible.
- Broad-spectrum antibiotics effect was obtained.
- The introduced mats can be utilized in therapeutic and cosmetic applications.

GRAPHICAL ABSTRACT



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ABSTRACT

Emu oil blended nanofibrous membranes scaffolds were fabricated successfully via electrospinning with different composition ratios with polyurethane (PU). Emu oil is derived from the emu (*Dromaius novaehollandiae*), which originated in Australia, and has been reported to have anti-inflammatory properties. It has been observed that 10 wt.% is the optimum oil content in the electrospun solution to get good morphology ultrafine PU/emu oil blended nanofibers. The influences of emu oil content in nanofibrous morphology, and the viability and proliferation properties of cells (3T3-L1 fibroblasts) on blended nanofibers were analyzed. The composite material could support long-term cell growth, form three-dimensional networks of the nanofibrous structure, and provide good antibacterial activity. The antibacterial activity of this composite material was found to be active in both Gram positive and Gram negative species. So according to our results this composite material can be successfully applied in various biomedical fields, including wound dressing, skin disease treatments, etc.

1. Introduction

Many serious works have been done toward developing scaffolds for tissue engineering using biodegradable and biocompatible natural biological macromolecules or extracts of innate materials. An ideal scaffold should mimic the structure and biological function of native extracellular matrix (ECM) proteins, which provide mechanical support and regulate cellular activities. Furthermore

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the scaffold must support and define the three-dimensional organization of the tissue-engineered space and maintain the normal state of differentiation within the cellular compartment. To attain this objective, an engineered scaffold must be biocompatible and must not induce any adverse effects to surrounding tissues. The potential use of traditional and complementary medicines to be used in dermatology, particularly in the area of wound healing and burns [1,2] were well reported. Over the past decades, considerable efforts have been directed towards developing a range of plant extracts and other products (e.g. aloe vera, emu oil) that have been used traditionally as wound healing agents [3–6]. In these regards, emu oil has been used in a variety of applications.

The emu (*Dromaius no-vaehollandiae*) is a free-roving, large, flightless bird indigenous to Australia, now farmed in Canada, Europe, USA and other parts around the world. The native Aboriginals and early white settlers in Australia rubbed on the liquid fat to facilitate wound healing and to alleviate pain and disability from various musculoskeletal disorders. Emu oil has also been reported to possess analgesic properties. Topical application of emu oil to animals has been shown to reduce the levels of tumour necrosis factor-α, and other proinflammatory cytokines in a model of adjuvant-induced inflammation [7]. Emu oil is predominantly composed of fatty acids (FA), with a lipid content of 98.8% for the subcutaneous adipose tissue and 98.0% for the retroperitoneal adipose tissue [8]. Oleic acid constitutes approximately 43–46% of the fatty acid component, with linoleic acid (9.6%), palmitic acid (23.5%), stearic acid (9.1%) and linolenic acid (0.6%) also being present. The composition of the remaining 1–2% is yet to be conclusively defined, although natural antioxidants, such as carotenoids and flavones, and skin permeation-enhancing factors have been identified in oil preparations [9]. Emu oil naturally contains a high level of linolenic acid and oleic acid, which provides a local anti-inflammatory effect. Emu oil has been reported to have significant anti-inflammatory effects, and has been used both in cosmetics and therapeutic applications.

Electrospinning is a simple and versatile technique producing non-woven membranes with individual fiber diameters ranging from a few nanometers to hundreds of nanometers [10]. Nanofiber possesses the characteristic features of high length-to-diameter ratio and specific surface areas, enabling it to be applied for protective clothing, filter, catalyst support, reinforced composite, and tissue engineering [11,12]. Recently, there is a growing interest in the design and preparation of novel composite nanofiber with improved properties, because it may combine the merits of each component. However, it is not easy to electrospin the emu oil. So for performing the electrospinning, we needed one electrospin driving polymer. That will enhance spinnability of the emu oil along with better mechanical stability. So in this study, we have adopted a technique of direct in situ electrospinning of emu oil with a suitable carrier (namely polyurethane [PU], an FDA approved polymer) to aid in its electrospinning. We demonstrate the process, stability, and characterization of the biological properties of the emu oil loaded nanofibrous scaffolds.

2. Experimental

2.1. Materials and methods

Emu oil was obtained from Sree Shakthi Farms, Coimbatore, Tamil Nadu, India and polyurethane (PU (Mw = 110,000) Cardio Tech. Intern., Japan,) were used in making the solution. PU (10 wt.%) solution with 5 and 10 wt.% concentration of emu oil solution to the PU was used to prepare the composite nanofiber mats. A mixed solvent, DMF:THF (1:1) was used to prepare the PU polymer solution. Emu oil was added dropwise to the polymer solutions carefully and kept for stirring for 1 h prior to electrospinning. A high voltage power supply (CPS-60 K02V1, Chungpa EMT, South Korea) of 16 kV to the syringe micro-tip was supplied to electrospin the nanofibers, whereas a ground iron drum covered by a polyethylene sheet served as the counter electrode. The solution was kept in the capillary by adjusting the inclination angle. The tip-to-collector distance was kept at 15 cm. Polymer solution was fed to the 5 ml syringe with a plastic micro-tip. Finally, the emu oil loaded nanofiber mats were vacuum dried in an oven at room temperature for 24 h to remove the residual solvent, and this sample was used for further characterizations.

2.2. Characterizations

The morphology of the electrospun PU and emu oil composite nanofibers were observed by using scanning electron microscopy (SEM, S-7400, Hitachi, Japan) and field-emission scanning electron microscopy (FE-SEM, Hitachi S-7400, Hitachi, Japan). The bonding configurations of the samples were characterized by means of Fourier-transform infrared (FT-IR) spectroscopy. Mechanical properties were measured with a universal testing machine (AG-5000G, Shimadzu, Japan), under a crosshead speed of 10 mm/min. The samples were prepared in the form of a standard dumbbell shape as according to ASTM Standard 638 via die cutting from the mat and tested in machine direction.

2.3. Cell culture

3T3-L1 fibroblasts (preadipocytes, Korean Cell Line Bank, Korea) were initially maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. The electrospun scaffolds on the cover slips were ultraviolet (UV) sterilized, rinsed in phosphate-buffered saline (PBS) and soaked in the cell culture medium overnight prior to cell seeding to facilitate protein adsorption and cell attachment. The fibroblasts were separated by trypsinization, centrifuged, counted using a hemocytometer and seeded on the scaffolds at a cell density of 2×10^4 cells/well and incubated at conditions suitable for cell growth. In order to observe the cell attachment manner on composite nanofibers, chemical fixation of cells was carried out in each sample. After 1, 3 and 6 days of incubation, the scaffolds were rinsed twice with PBS and subsequently fixed in 2.5% glutaraldehyde for 1 h. After that, a sample was rinsed with distilled water and then dehydrated with gradient concentration of ethanol, i.e., 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.

2.4. MTT test

The viability of cultured fibroblasts was monitored on the third, sixth and ninth day of culture using the colorimetric MTT assay (Sigma, USA). The scaffolds were washed twice with PBS and were then treated with approximately 50 μl of the MTT solution (DMEM); the scaffolds, after mixing of the contents by side-tapping, were incubated at 37 °C for 2 h. The scaffolds containing MTT-cell mixtures were gently rocked to deposit the cells. The supernatant MTT solution was pipetted out and then acid–isopropanol (95 mL isopropanol with 5 ml 3 N HCl) was added to the colored cell deposit. After gently mixing the acid–alcohol-treated scaffolds was then allowed to react for 5 min. One hundred microliters of the purple-blue colored supernatant that contained the solubilized formazan in each sample was added to a well in a 96-well plate for

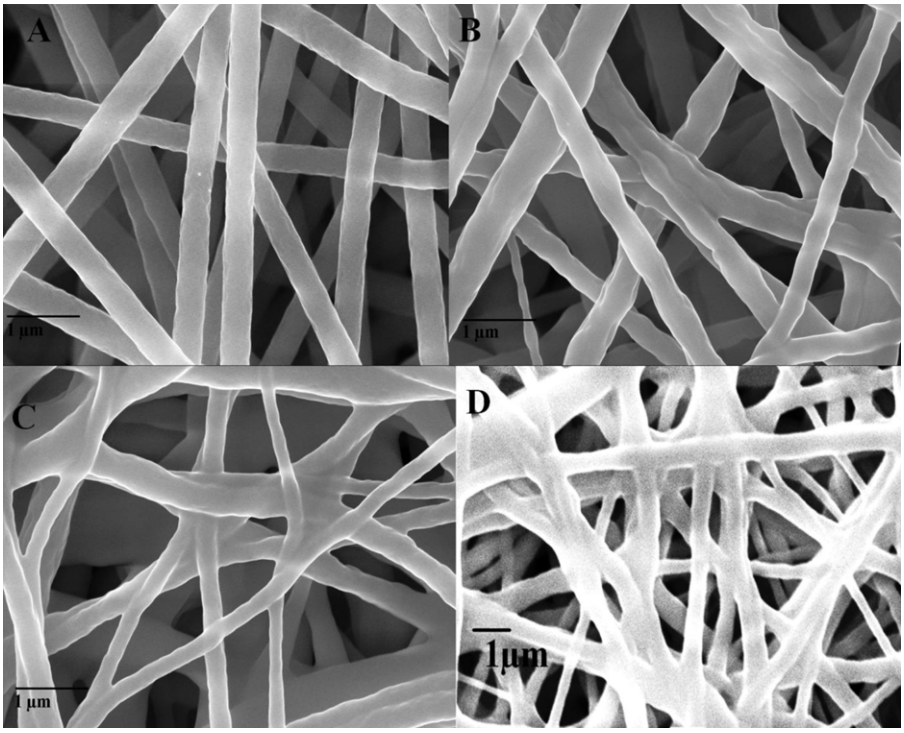

Fig. 1. FE-SEM images of electrospun (A) PU, (B) PU/emu oil (5 wt.%) and (C) PU/emu oil (10 wt.%) nanofibrous mat. (D) SEM image of electrospun PU/emu oil (10 wt.%) nanofibrous mat.

spectrophotometric analysis at 580 nm in an ELISA reader. The cytotoxicity of emu oil loaded nanofibers were evaluated in comparison with pristine PU nanofibers. The cell viability was obtained by comparing the absorbance of cells cultured on the nanofibers scaffold to that of control well containing cells. The tissue culture coverslips were used as control for the study. The results were expressed as the mean ± standard error of the mean. The data were analyzed via the Student's *t*-test and repeated measures of analyses of variance (ANOVA) test. A probability of less than 0.01 was considered to be statistically significant.

2.5. Antibacterial assessment

2.5.1. Microbial strains and culturing conditions

The Gram-negative *Escherichia coli* K12-MG1655, and Gram-positive *Bacillus subtilis* were used for antibacterial assessment study. These *E. coli* and *B. subtilis* were precultured in Luria–Bertani Broth (LB) overnight in a rotary shaker at 37 °C, and centrifuged at 13,000 rpm for 2 min. The pellet was suspended in sterile water, and the cell density was standardized spectrophotometrically (A570 nm). The 100 μl of diluted bacterial suspension (10^8 cells/ml) was seeded into the respective medium in duplicate by spread plate method. The nutrient agar was used for *B. substilis* and MacConkey agar used for *E. coli*.

2.5.2. Antibacterial activity measurement

An inhibitory test of nanofibers containing 5% and 10% emu oil and pristine PU were tested by the disc diffusion method [13]. The nanofibrous mats, 5% and 10% emu oil contained PU fibers and pristine PU, were cut into small circular discs of diameter around 5 mm each and denoted as A, B and C respectively. These discs were put on the surface of the Petri dishes. These plates were incubated at 37 °C. The inhibition zones were estimated after 0–240 min. The diameters of the inhibition zones were measured with a transparent ruler.

3. Results and discussion

Fig. 1B and C shows the FE-SEM images of electrospun nanofibers of 5% and 10% emu oil to PU solution respectively. Fig. 1D represents the SEM image of electrospun nanofibers 10% emu oil to PU solution. It can be observed that these randomly oriented as-spun nanofibers exhibited bead-free, smooth surface with almost uniform diameters along their lengths. The diameters of these composite nanofibers were determined to be in the range of 400–600 nm as shown in Fig. 2. The diameter distribution diagrams of the nanofibrous mats were shown in Table 1. For pristine PU electrospun mat (Fig. 1A), the fibers appear well-defined without any interconnection among the fibers. The hybrid mats containing different amounts of emu oil showed some changes in fibrous morphology (Fig. 1B and C). As the concentration of emu oil was increased, a slightly fused morphology (connection of fibers) was noticeable, and became even more visible while the amount of emu oil was 10% to PU solution. This change in morphology can be explained by the low volatility of the emu oil. So it becomes increasingly difficult for the electrospun material to completely dry before it hits the collector due to the presence of less volatile emu oil. Moreover, the adhesive property of emu oil could provide the connection of two fibers at the points where they were crossing with each other. Non woven mats were characterized by three different types of bonding structures named as segmented, agglomerated, and point-bonded [14]. In our case, we found the point-bonded fiber structure caused by emu oil during electrospinning. Fig. 1A clearly

Table 1
Fiber diameter values of pristine PU mat and different percentages of emu oil/PU mat (values are expressed in mean ± SD).

	201–400 nm	401–600 nm	601–800 nm	801–1000 nm
PU	0 ± 0.00	516.65 ± 38.48	666.94 ± 47.57	897.46 ± 86.12
5% oil	347.51 ± 22.79	455.74 ± 27.41	0 ± 0.00	0 ± 0.00
10% oil	278.08 ± 65.34	425.20 ± 20.85	0 ± 0.00	0 ± 0.00

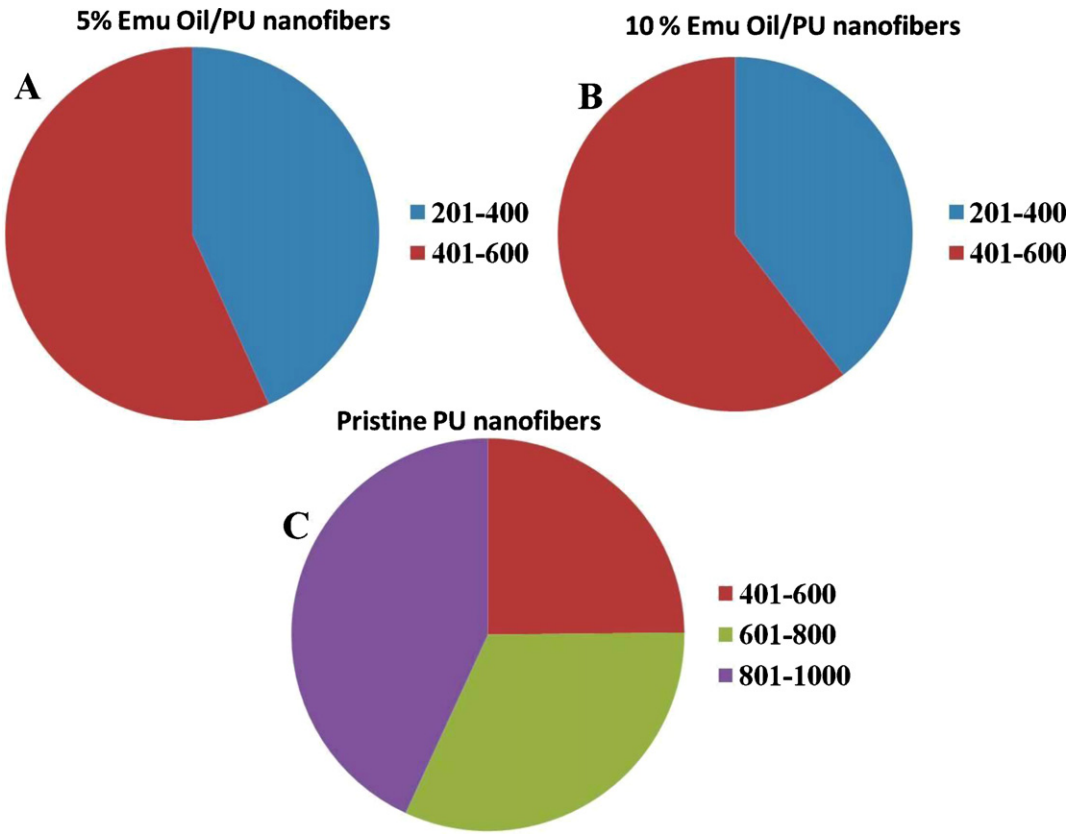

Fig. 2. Diameter distribution of (A) PU/emu oil (5 wt.%), (B) PU/emu oil (10 wt.%) and (C) pristine PU nanofiber. (Values, are expressed in nm.)

shows that the pristine PU mat has non-bonded fibers whereas the increasing amounts of emu oil led the transformation from the non-bonded to the point-bonded fibrous structure.

FT-IR spectroscopy was used to investigate the changes of the functional groups that occur during the blending of emu oil with PU nanofibers. Fig. 3 illustrates the interaction between the PU and emu oil. The characteristic transmittance peaks of the PU/emu oil nanofibers can be assigned as shown in Fig. 3. The FTIR spectrum of pure PU shows typical bands corresponding to the hard segments: N—H (1532 cm^{-1}, 3330 cm^{-1}), C=O (1714 cm^{-1}), C—O—C (1242 cm^{-1}) groups and to soft segments, C—H (1374, 1460, and 2870 to 2970 cm^{-1}) groups. The spectrum of the hybrid compound

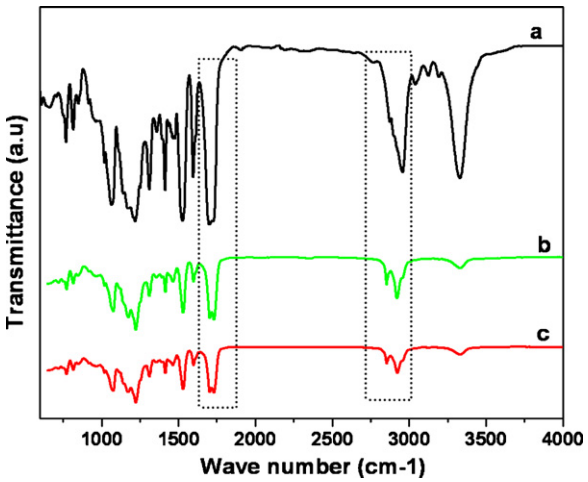

Fig. 3. FTIR spectra of electrospun (a) PU nanofiber, (B) PU/emu oil (10 wt.%) and (C) PU/emu oil (5 wt.%) nanofibrous mat.

showed additional bands, mainly in the 1700 to 1900 cm^{-1} region and 2800 to 3000 cm^{-1} region. It may be due to the interaction of emu oil with PU. On the other hand, the characteristic peaks of PU were observed to be decreased with increasing emu oil content. This may be due to the formation of hydrogen bonding. It is already reported that the inter-hydrogen bonds formed between two different macromolecules are stronger than those formed between the molecules of the same polymer [15]. Therefore, the inter-hydrogen bonds between PU and emu oil were prone to formation, and they appeared as new peaks in the FTIR spectrum. The broad peak between 3000 and 3500 cm^{-1} corresponds to a stretching of —OH and to physisorbed moisture on the surface in several modes. However, the intensity of this band was found to be decreased with increasing emu oil concentration in PU/emu oil blended nanofibers.

Fig. 4 shows the stress strain curves of pristine PU and composite electrospun mats. We found that the mechanical strength of PU/emu oil blended composite mat was greater than that of pristine PU mat. Mechanical strength of composite mat was found to increase with the amounts of emu oil. This increased mechanical strength can be explained by considering the adhesive property of emu oil. At the point where polymeric nanofibers cross with each other, emu oil can provide the attachment of fibers by means of point bonding. This attachment in molecular level may be due to the formation of hydrogen bond between PU and emu oil molecules. Our FE-SEM images (Fig. 1) showed that increasing emu oil amounts can provide more point-bonded sites in the mat and therefore can increase the mechanical strength of PU mats (Fig. 4).

The antibacterial activity of emu oil is already well-known and has been applied for centuries. Fig. 5 shows the antibacterial activity of the electrospun emu oil loaded nanofibrous. It is well known that when an antibacterial material is in contact with bacterial strain, a clear area around the antibacterial material forms, and is referred to as zone of inhibition [16]. The strains susceptible to antibacterial

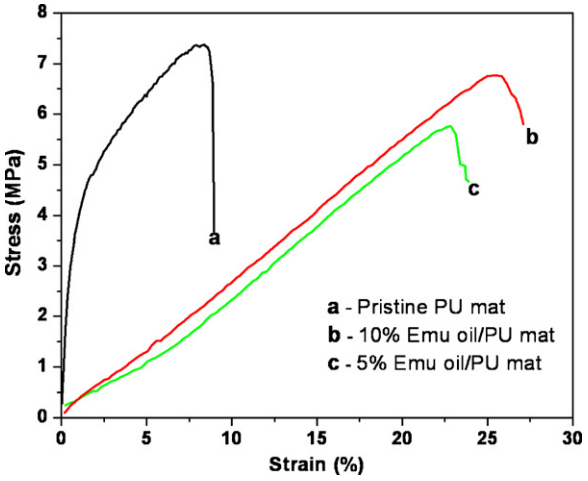

Fig. 4. Representative stress–strain curves of (A) PU nanofiber, (B) PU/emu oil (10 wt.%) and (C) PU/emu oil (5 wt.%) nanofibrous mat.

agents exhibit a larger diameter of the inhibition zone, while resistant strains exhibit smaller inhibition zone. In this study, *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) were used as model organisms. Circular nanofibrous mats ($d = 5$ mm) were placed in an agar plate inoculated with bacteria and were incubated overnight. Pristine PU nanofibrous mat sample served as the control. From Fig. 5 a and b it is clear that, pristine PU samples showed no zones of inhibition, suggesting the absence of any antibacterial activity for both bacterial strains, mainly due to the lack of emu oil content. On the other hand, the PU/emu oil composite mats clearly showed increasing area of zones of inhibition with the increase of emu oil content for both bacterial strains. The zones of inhibition were 8 mm and 10 mm for *E. coli* and *B. subtilis* respectively for 10 wt.% of emu oil concentration as shown in Table 2. 10 wt.% emu oil loaded nanofibers showed more antibacterial activity than 5 wt.% emu oil composite nanofibers. Emu oil's bacteriostatic activity along with nanofibrous structure offers great promises to its application. The results showed that our composite mat is a good antibacterial membrane, and it will be useful in both cosmetic and pharmaceutical industries.

To confirm the cell viability in the emu oil, the morphological appearances of cells on composite nanofiber mats were observed after 3 and 6 days of culture. Fig. 6 shows the SEM images of the cell attachment manners on PU and PU–emu oil composite nanofibers. The SEM micrographs showed a normal morphology of cell growth on the nanofibers. During the first three days of incubation, cells cultured on emu oil nanofibers had been already spread although they were still round on neat PU nanofibers. Due to rapid proliferation of cells in the presence of emu oil, after 6 days of culture, the nanofiber matrices were completely covered with cells and ECM proteins, secreted by cells. Cell growth was higher on emu oil loaded composite nanofibrous scaffolds than on PU nanofibers.

Blending emu oil with PU provides the scaffold with improved bioactivity and cell affinity for tissue regeneration. Emu oil is already used as a natural moisturizer, and it supports the tissue regeneration. Emu oil is a deep penetrating, natural moisturizer that helps eliminate scars, stretch marks, treat acne and skin rash, moisturize dry skin and eliminate skin irritation, and redness. So the emu oil loaded electrospun nanofiber mats can be successfully used as patches or masks or as bandages for the above mentioned applications. Our study confirmed the cell attachment and cell spreading in the emu oil nanofiber matrix.

The proliferation of fibroblasts on pristine PU and PU/emu oil matrices (5% and 10%) were evaluated by MTT assay on days 1, 3 and 6 (Fig. 7). The relatively high optical densities on the first day of incubation indicated that emu oil highly supported cell adhesion on nanofibrous matrices. After 6 days of incubation, metabolic activities of cells on emu oil immobilized PU matrices were comparatively higher than that of pristine PU nanofibers. Results clearly indicated that emu oil highly accelerates fibroblast proliferation on PU/emu oil nanofibrous matrices. Acceleration of fibroblast proliferation during wound closure is crucial for an effective skin healing. It is well known that emu oil greatly stimulates proliferation and migration of skin tissues and thereby enhancing the early wound closure. During the recovery of serious wounds, proliferation of fibroblasts around lesions widely occurs, which stimulates deformation of the original skin. Since emu oil posses all these properties it lightens towards the application of emu oil loaded PU nanofiber matrix as an ideal wound dressing material. Taking into account all these data, emu oil immobilization on nanofibrous matrices was vital in terms of tissue regeneration

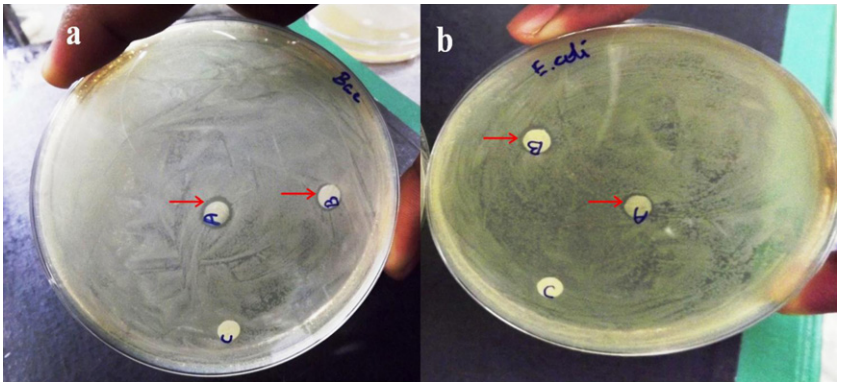

Fig. 5. Bactericidal activity of PU/emu oil nanofibrous mat with Gram-positive *Bacillus subtilis* (a) and Gram-negative *Escherichia coli* (b), respectively. PU/emu oil (10 wt.%), PU/emu oil (5 wt.%) and pristine PU discs were denoted as A, B and C respectively in the Petri plates (arrow denotes the zone of inhibition).

Table 2
Antibacterial data showing the diameter of zone of inhibition.

Bacterial strain	Pristine PU (zone of inhibition)	5 wt.% emu oil loaded nanofibers (zone of inhibition)	10 wt.% emu oil loaded nanofibers (zone of inhibition)
E. coli	–	7 mm	8 mm
Bacillus subtilis	–	8 mm	10 mm

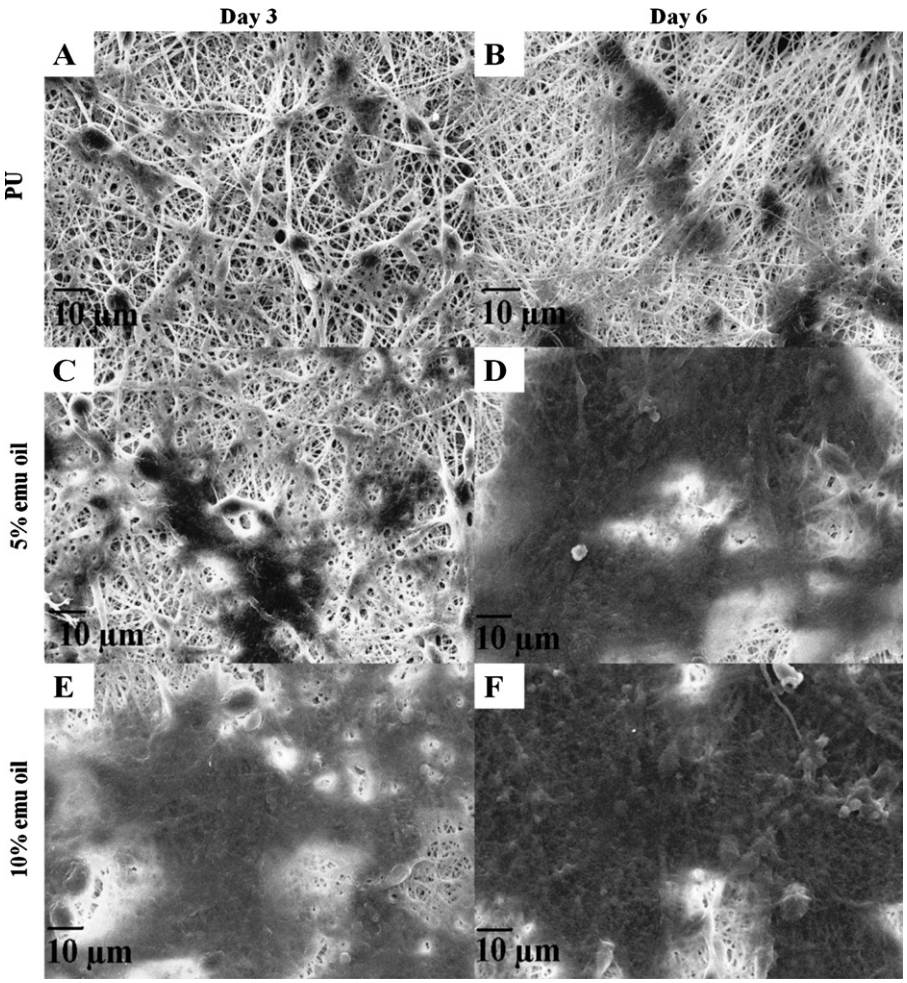

Fig. 6. SEM images showing the cell attachment (3T3-L1 fibroblasts) on PU (A and B), PU/emu oil (5 wt.%) (C and D) and PU/emu oil (10 wt.%) (E and F) after day 3 and day 6 respectively.

and these bioactive matrices shall be a potential wound healing substrate in skin tissue engineering applications with regard to acceleration of wound healing processes with in situ antibacterial activity.

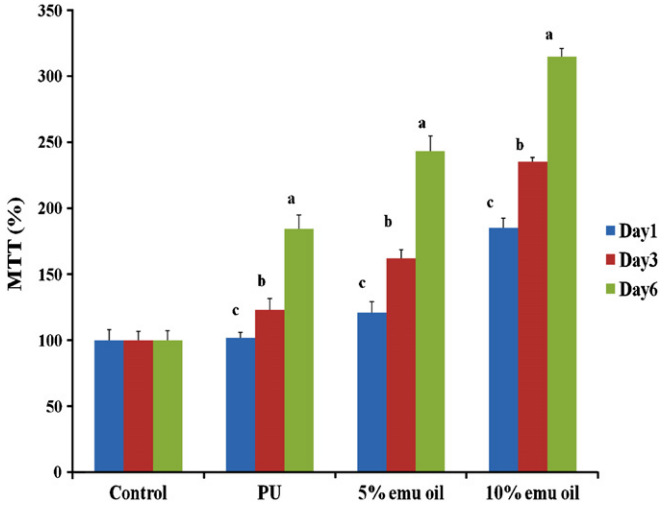

Fig. 7. MTT cell growth measurement assay. Values are mean ± SD. Values with different letters (a, b, c) are significantly different by ANOVA with Duncan's multiple range test at $p < 0.05$.

4. Conclusion

In summary, we successfully obtained PU/emu oil blended composite nanofibers by using the electrospinning process. The pure emu oil as such could not directly be electrospun to yield continuous and uniform nanofibers. Thus, to overcome the poor electrospinnability of emu oil solution, synthetic polymers such as PU has to be blended with the emu oil solutions to improve its spinnability. The intermolecular interaction of PU with emu oil through hydrogen bonding improves substantially the spinnability of each blended solutions and consequently uniform and continuous nanofibers are electrospun. It was observed that the best synthesis conditions for the ultrafine PU/emu oil blend nanofibers by the electrospinning technique were PU with 10 wt.% emu oil solution. Furthermore, a significantly improved cell proliferation was observed from PU/emu oil blend nanofibers. Such an enhanced cytocompatibility effect of emu oil, especially at 10 wt.% content, would be very preferable for tissue engineering and other cosmetic and therapeutic applications.

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