

Characterization, Antibacterial and Anti-Inflammatory Activities of Electrospun poly (vinyl alcohol) (PVA) Containing Aquilaria Malaccensis Leaf Extract (ALEX) Nanofibers

Najihah Mohd Noor, Yumi Zuhanis Has-Yun Hashim, Muhammad Shirwan Abdullah Sani, Wan Wardatul Amani Wan Salim

Abstract: Plant-based electrospun nanofibers are widely fabricated as wound dressing in recent years primarily due to the presence of bioactive compounds which can facilitate the wound healing effects. In this study, poly(vinyl alcohol) (PVA) fibre mats containing Aquilaria malaccensis leaf extract (ALEX) [5, 10 and 15 % (w/w)] were fabricated by electrospinning method as potential wound dressing material. The nanofibers were uniform, beadless and randomly oriented with average diameters ranged between 195.27 – 281.20 nm. The presence of ALEX in the PVA nanofibers were evaluated by Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and differential scanning calorimetry (DSC). The mechanical properties, swelling degree and weight loss of nanofiber mats were also determined. ALEX was rapidly released from the ALEX-loaded PVA nanofibers in the first 12 hours and increased gradually afterwards. The released rate of ALEX was dependent on the ALEX content in the PVA nanofibers. This result is also contributed by the swelling degree and porosity of the nanofibers where the results were found to be between 241.66 – 305.86% and 64.53 – 30.81%, respectively. Meanwhile, the tensile stress and maximum elongation at break for all electrospun nanofiber mats were in the range of 8.56 – 2.68 MPa and 205.94 – 166.31%, respectively. The nanofiber mats inhibited growth of Escherichia coli, Vibrio vulnificus, Bacillus subtilis and Staphylococcus aureus with zone of inhibition of 7.5 - 15.0 mm for gram positive bacteria and 6.1 - 11.7 mm for gram negative bacteria. ALEX-loaded PVA nanofibers also showed potent anti-inflammatory activity against lipoxygenase with percentage of inhibition between 80.887 – 86.977%. Taken together, the results of this study suggest that ALEX-loaded PVA nanofibers have the desired properties of bioactive wound dressing.

Keywords: Electrospinning, agarwood, polyvinyl alcohol, wound dressing

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I. INTRODUCTION

Wound management plays an important role in health care to maintain the integrity of skin. Selection of suitable material and design of functional wound bandage is essential in creating an ideal wound dressing that can provide suitable environment for the wound to rehabilitate underneath while preventing further infection and achieving highest rate of healing. Nanofibrous scaffold is a suitable candidate for wound dressing material as it able to promote the haemostatic phase of wound healing without the presence of haemostatic agent due to its highly porous structure and huge surface area of the fibre [1]. The nanofibrous wound dressing can be further functionalized as bioactive wound dressing through the incorporation of bioactive molecules such as antibiotics, antimicrobial agents and anti-inflammatory drugs. Recently, the use of plant in extract in nanofibrous mats via electrospinning technique for wound healing application has been widely demonstrated. There has been a surge of the number of scientific publications in recent years toward alternative therapies by using herbal medicine as probable candidates due to its less toxicological properties. Plant extracts have been traditionally used as herbal remedies in the treatment of wound by accelerating the wound healing process through its pharmacological activities. The phytochemical constituents found in plant has been reported to improve and accelerate the wound healing process by exhibiting antibacterial, anti-inflammatory, antioxidant, antifungal, analgesic and anticancer activities [2]. Agarwood or eaglewood (also known as *gaharu* in South East Asia and *karas* in Malay) is a dark resinous heartwood produced inside the tropical rainforest trees growing mainly in South and South-East Asia and belongs to the genera of *Aquilaria*, *Gonystylus* and *Gyrinops*. Agarwood plays an important role in many religious traditions all over the world since ancient times and it were also used in various activities [3]. *A. malaccensis* is a primary producer of agarwood in Malaysia. It is a species with highest recorded number that has been planted by karas tree farmers [4]. The leaves are endowed with myriad of bioactive compounds that supports the basis for its use as wound healing agent.



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Eissa et al., (2018) reported on the presence of phytol as major bioactive compound of *A. malaccensis* leaves Soxhlet extract which accounted for 34.1% in the hexane and 29.3% for ethanol at RT of 18.26 minutes [5]. Phytol is known to exert antimicrobial and anti-inflammatory activities that are able to enhance wound healing potency [6]. Anti-bacterial and anti-inflammatory activities are important in the treatment of acute and chronic wound by mitigating the wound healing process. The presence of anti-bacterial and anti-inflammatory agent helps in suppressing the production of inflammatory cytokines and inflammatory transduction cascades while preventing the growth of bacteria at the wound site [7].

However, there are no reports on the fabrication of electrospun *A. malaccensis* leaf extract (ALEX) loaded polyvinyl alcohol (PVA) nanofibers for wound dressing application. In this study, PVA-ALEX nanofiber mats were fabricated via electrospinning method for a potential use as wound dressings. The physicochemical properties, thermal and mechanical behaviour characterization of the nanofiber mats were evaluated along with the antibacterial study and anti-inflammatory study.

II. MATERIALS AND METHODOLOGY

A. Raw materials

Poly(vinyl alcohol) (PVA) (Mw 145,000, 99% hydrolysed) was purchased from Merck Chemicals (Darmstadt, FR Germany). Soybean Lipoxygenase (1.13.11.12) type I-B, linoleic acid, sodium phosphate buffer, sodium acetate buffer, sodium borate buffer and dimethyl sulfoxide (DMSO) were supplied by Sigma-Aldrich Chemicals (St. Louis, MO, USA). Ethanol absolute was obtained from HmbG Chemicals (Germany). All other chemicals were of analytical grades and used without further purification. Fresh leaves of healthy non-inoculated *A. malaccensis* were collected from a local farm in Bangi, Malaysia. The leaves were identified according to their morphology and voucher specimen was deposited at KAED Herbarium, International Islamic University Malaysia.

B. Experimental methods

1) Extraction of *A. malaccensis* leaf

A total of 25 g of leaf powder was put in a thimble and placed into the main chamber of Soxhlet extractor with 250 mL of ethanol as the solvent. The leaf powder was extracted simultaneously in another 5 chambers making a total of 6 set of extraction. The extraction was run for 18 hours according to Zainurin, (2020) [8]. The concentrated ethanolic extract was then obtained by removing the solvent using a rotary evaporator (Heidolph-instruments, Rotavapor, Germany) under reduced pressure at 40°C. The crude extracts were weighed and preserved in a petri dish sealed with aluminium foil for further analysis.

2) Preparation of PVA and PVA-ALEX solutions

For the preparation of electrospun polymer matrix fibres, the fully hydrolysed PVA powder was dissolved in distilled water to form 10% (w/v) aqueous PVA solution under constant stirring at 80°C for 2 hours. The solution was left to cool at room temperature. Next, ALEX (5 – 15 % (w/w) to weight of PVA) were loaded into the PVA solution. The mixture was stirred further for 2 hours to obtain a homogenous solution.

3) Electrospinning of PVA and PVA-ALEX solutions

The PVA and PVA-ALEX solutions were electrospun under applied voltage of 20 kV using a high voltage power supply (7xx30 series, Genvolt, United Kingdom) with 18 cm of collection distance. The flow rate of the solutions was adjusted to 0.7 mL/h using syringe pump (NE-1000, United States). The electrospinning process was done at constant ambient conditions (temperature: 25°C and relative humidity (RH): 40%). Meanwhile, the collection time for the rest of experiment were done in 6 h. Subsequently, the fabricated nanofibrous scaffold were vacuum-dried at 30°C for 24 h to completely remove any potential residual solvent. The dry samples were used for further characterization.

4) Scanning electron microscopy (SEM)

Scanning electron microscope (SEM) (JEOL, JSM-IT500HR InTouchScope™, USA) with an excitation voltage of 8 kV was used in this study to analyse the morphology appearance of both the PVA and ALEX-loaded PVA fibre mats. ImageJ software, National Institute of Mental Health, Bethesda, Maryland, USA was used to determine the average diameters of the nanofibers by measuring the diameters of 100 randomly selected strands of fibre obtained from the SEM images. Prior to testing, all samples were sputtered with a gold coating under vacuum.

5) Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

The existing functional groups of PVA nanofibers, ALEX solutions, and ALEX-PVA composite nanofibers were determined by using the ATR-FTIR analysis (Nicolet™ iS50 FTIR Spectrometer, USA). The wavenumbers ranged within 600 to 4000 cm⁻¹, in absorbance mode. The typical functional groups of each sample were determined by referring to their peak number. Prior to the scanning of samples, a background spectrum was collected to remove any interference. Next, the nanofibers were cut in a small piece and placed on the stage, compressed on ATR crystal and scanned against the background of the clean crystal. Hexane was used for crystal surface cleaning between the measurements.

6) Thermal analysis

Differential scanning calorimetry (DSC 822 Mettler Toledo, Switzerland) was used to analyse the crystallinity and thermal behaviour of the material after polymer processing. Samples (5 – 15 mg) were placed in alumina crucible and heated between 27°C to 200°C with heating rate of 10°C/min under nitrogen atmosphere. An empty crucible was used as a reference.

7) Mechanical testing

Tensile testing was carried out by universal testing machine (AGS-X, Shimadzu Corporation, Kyoto, Japan) with a crosshead speed of 5 mm/min at 25°C. The sample dimensions were based on American Society for Testing and Materials ASTM D-882 type V. At least 5 sample specimens for each set were tested to get the average value.

8) Swelling degree and weight loss

The water uptake and weight loss of both PVA and ALEX-loaded fibre mats were determined by immersing the samples in phosphate-buffered solution (PBS) (pH 7.4) at 37°C for 24 hours, as described by Vatankeh., (2018) [9].

After that, the samples were removed from PBS solution and the excess water on the surface of the fibre mats were wiped with filter paper prior to weighing (W_w).



The swelling degree and weight loss of the samples were calculated according to the following equation:

$$\text{Degree of swelling (\%)} = \frac{(W_w - W_d)}{W_d} \times 100$$

$$\text{Weight loss (\%)} = \frac{(W_o - W_d)}{W_d} \times 100$$

where W_w and W_d are the weight of wet and dry mats after 24 hours submersion in buffer solution, respectively. W_o is the initial weight of dry sample.

9) In-vitro release study

The release characteristics of ALEX from ALEX-loaded PVA fibre mats were studied by total immersion method as described previously by Suwanton et al., (2013) [10], with minor modifications based on Li et al., (2013) [11]. Each sample was immersed in 20 mL of sodium acetate buffer at pH 5.5. 1.0 mL of the sample solution was withdrawn and replaced with fresh medium at specified immersion time period (0 - 120 h). The amount of ALEX in the sample solutions were measured by Thermo Scientific™ Multiskan™ GO UV/Vis microplate spectrophotometer at the wavelength of 450 nm. The cumulative amounts of the released ALEX were determined from the obtained data.

Prior to the total immersion method, the actual amounts of ALEX in ALEX-loaded PVA fibre mats were determined by dissolving each sample in 10 mL of sodium acetate buffer at pH 5.5. After that, 1.0 mL of the solution was measured spectrophotometrically at the wavelength of 450 nm. The actual amounts of ALEX in ALEX-loaded PVA fibre mats were then back-calculated from the obtained data against a redetermined calibration curve for ALEX.

10) Antibacterial activity

The assessment on the antibacterial activity of the nanofibers were determined by using modified Kirby Bauer method against *Staphylococcus aureus* (ATCC 12600), *Bacillus subtilis* (ATCC 11774), *Vibrio vulnificus* (ATCC 27562), and *Escherichia coli* (ATCC 11229) [12]. The bacterial suspensions were prepared by growing a single colony in a sterile tryptone soy broths (TSB, Oxoid, England). The broth was incubated between 4 - 16 h at 37°C to achieve inoculum containing 10^6 CFU/mL. The suspension culture was dipped with a sterilized cotton swab and the bacterial cells were spread homogeneously over sterile Mueller Hinton agar (MHA, Oxoid, England). Next, the sterilized PVA-ALEX composite nanofibers (6 mm diameter) were placed onto an inoculated agar plate. PVA neat and tetracycline were used as negative and positive controls, respectively. The agar plates containing samples were incubated for 24 h at 37°C. The zone of inhibition (ZoI) was reported as the total diameter (mm).

11) Anti-inflammatory activity

The evaluation of anti-inflammatory of the extracts and electrospun nanofibers were determined by Lipoxygenase inhibitory assay (LOX) [13]. Initially, ALEX concentrations providing 50% inhibition (IC_{50}) were determined by using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). Nordihydroguaiaretic acid (NDGA) was used as the positive control in this assay. Next, nanofibers test solutions were prepared based on Vatankhah, (2018) [9]. Sodium acetate buffer solution (2 mL, pH 5.5) was used to immerse 5 mg of each nanofibrous sample. The solutions were placed in an incubator shaker at 37 °C and 50 rpm (4 h to 5 h). At predetermined time points, the nanofibrous samples were removed from the buffer and the buffer was used as the test solution. A mixture of a solution of sodium

phosphate buffer (160 μ L, 100 mM, pH 8.8) and soybean LOX (20 μ L, 5000 U/mL) were incubated with test solution of nanofibers (30 μ L) in 96 Well UV-Star® Microplate (Greiner Bio-One GmbH, Germany) at room temperature for 5 min. The reaction was initiated by adding 10 μ l (900 μ M) of linoleic acid which acts as a substrate. The change in absorbance at 234 nm was followed for 6 min. The percentage of inhibition of linoleic acid conversion was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{OD_{\text{control}} - OD_{\text{sample}}}{OD_{\text{control}}} \times 100$$

12) Statistical analysis

All quantitative results were obtained from three independent experiments and are presented as mean \pm SD. A one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparisons among group. Differences of $p < 0.05$ were considered statistically significant.

III. RESULTS AND DISCUSSION

A. Morphology of electrospun PVA and ALEX-loaded PVA nanofibers

In this study, PVA and ALEX-loaded PVA fibre mats with various concentrations [5, 10 and 15% (w/w), namely PVA-ALEX5, PVA-ALEX10 and PVA-ALEX15] were fabricated by electrospinning method. The nanofibrous mats were fabricated under optimized condition to minimize the presence of beads on the fibre which indicates poor incorporation of the loaded material. The average fibre diameter of the prepared mats is shown in Table 1.

TABLE 1
AVERAGE FIBRE DIAMETER OF ELECTROSPUN FIBRE MATS ACCORDING TO SAMPLE COMPOSITION

Sample code	PVA (w/v %)	ALEX (w/w %) (Based on weight of PVA)	Average fibre diameter (nm)*
PVA	10	-	195.27 \pm 67.08 ^d
PVA-ALEX 5	10	5	258.90 \pm 56.50 ^c
PVA-ALEX 10	10	10	275.41 \pm 40.32 ^b
PVA-ALEX 15	10	15	281.70 \pm 58.50 ^a

*Means with different superscript letter are significantly difference ($p < 0.05$); mean \pm SD; n=100.

Based on Table 1, it shows that the increase in concentration of ALEX slightly affect the fibre diameter. The concentration of the electrospinning solution is one the parameters that significantly affects fibre diameters due to the entanglement of polymer chains which make the viscous solution dominates the electrospun jet, thus leads to the thickening of fibre. However, the incorporation of ALEX in the PVA solution did not affect the morphological appearance of the obtained fibres although the fibre diameter of ALEX-loaded PVA nanofibers was enlarged with the increase of ALEX concentration. Furthermore, the SEM images show smooth surface of nanofibers with minimal presence of beads or any extract aggregates on the PVA and ALEX-loaded PVA nanofibers (Fig. 1).



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B. Fourier transform infrared spectroscopy (ATR-FTIR)

Fig. 2 shows the Fourier transform infrared spectra for the PVA, ALEX and ALEX-loaded PVA nanofibers. The FTIR spectrum of PVA nanofibers shows a wide band in the range of 3000-3500 cm^{-1} with the peak at 3311.20 cm^{-1} and an additional peak at 2943.16 cm^{-1} due to O-H stretching (polyhydroxy group). It also gave multiple peaks at 1330.14 and 846.92 cm^{-1} representing C-H rocking medium as the alkanes group, 1142.85 and 1093.46 cm^{-1} for C-O strong stretching, and 1662.09 cm^{-1} due to C=O stretching as aldehydes group. The characteristic peaks of ALEX were assigned as follow: 1710.44 cm^{-1} (aliphatic ketone), 1736.14 cm^{-1} (aldehyde), 1499.55 cm^{-1} (methyl group). The peak at 1162.54 cm^{-1} representing C-O strong stretching as aliphatic ether and medium C-H stretching of alkane at 2918.21 cm^{-1} . For the ALEX-loaded PVA nanofibers, the three peaks show only slight shift from PVA nanofiber due to the presence of aliphatic ketone and methyl group of ALEX. This suggested that the ALEX were physically entrapped in the scaffold by intermolecular forces.

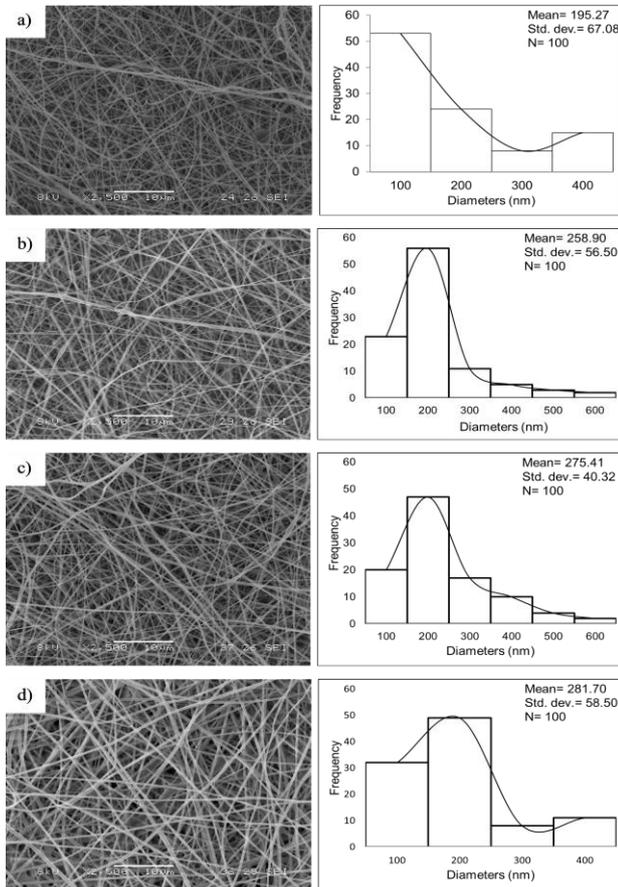


Fig. 1 The morphology and fibre diameter distribution of (a) PVA (B) PVA-ALEX5 (C) PVA-ALEX10 and (d) PVA-ALEX15 electrospun fibre mats, respectively.

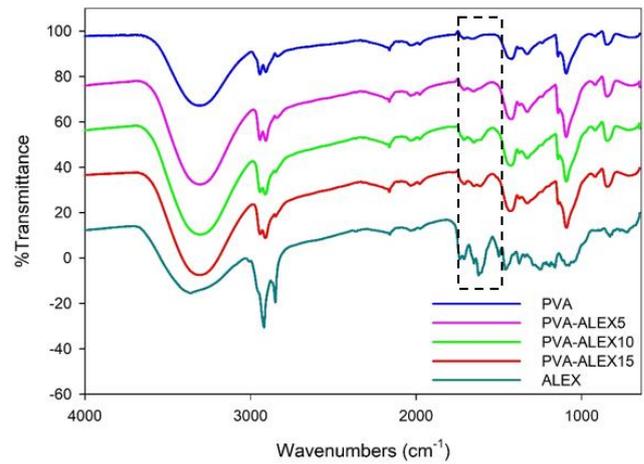


Fig. 2 ATR-FTIR spectra of ALEX, PVA nanofibers, PVA-ALEX5 nanofibers, PVA-ALEX10 nanofibers and PVA-ALEX15 nanofibers.

C. Thermal analysis

The differential scanning calorimetry thermogram for PVA and ALEX-loaded PVA nanofiber mats are depicted in Fig. 3. The PVA nanofiber showed a relatively sharp endothermic curve with a peak at 142.25°C without the presence of ALEX. Meanwhile, PVA-ALEX5 showed a shift of peak towards lower temperature which leads to the formation of broad endothermic curve at 122.30°C. However, the peaks of PVA-ALEX5 and PVA-ALEX10 endothermic curve shifted to higher temperature with the increasing amount of the extract loaded in the nanofibers. The endothermic curves of PVA-ALEX5 and PVA-ALEX10 nanofibers became less prominent and began to disappear with the incorporation of ALEX. These indicated that there was strong interaction occurred between the functional groups of PVA and ALEX which constrained the presence of PVA in the blend fibres. The peaks were observed at 144.24°C and 154.85°C, respectively.

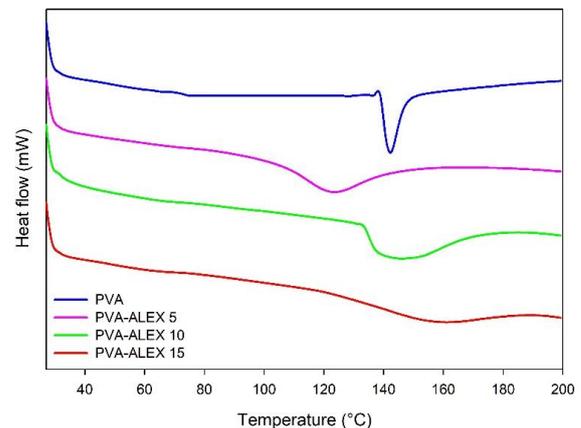


Fig. 3 DSC thermogram of PVA and ALEX-loaded PVA nanofibers.

D. Mechanical testing

Mechanical properties of electrospun PVA and ALEX-loaded PVA nanofiber mats were evaluated based on ASTM D-882 type V (n=5). Based on Table 2, it shows that the tensile stress and maximum elongation at break for all electrospun nanofiber mats were in the range of 8.56 – 2.68 MPa and 205.94 – 166.31%, respectively.



The tensile stress and maximum elongation at break of the nanofiber scaffolds were reduced with the addition of ALEX ($p < 0.05$). The results indicated that ALEX exerts low mechanical effect on the nanofiber mats, probably due to poor hydrogen bonding with the polymer solution.

Fig. 4 shows non-linear stress-strain curves of PVA and extract-loaded PVA nanofibers containing 5-15 (w/w)% ALEX. The mechanical properties of PVA and ALEX-loaded PVA nanofibers were sufficient and durable enough to provide good elasticity and high bending flexibility. The mechanical strength of human skin is reported to be in the range of 1 – 40 MPa [14]. The elongation at break shown in Fig. 5 also indicates high elongation at break of PVA compared to ALEX-loaded PVA nanofibers. According to Adeli et al., (2018), the elongation at break for human skin varies in range of 17 – 207% [15]. This range are contributed by the heterogeneity on human skin and other several factors such as age, skin colour and genetics [16]. In this study, the tensile strength and maximum elongation at break for PVA and ALEX-loaded PVA nanofibers were in the reported range, thus indicates the resemblance in the mechanical properties of human skin.

TABLE 2
MECHANICAL PROPERTIES OF PVA AND ALEX-LOADED PVA NANOFIBERS

ALEX content (w/w %)	Tensile strength (MPa)*	Maximum elongation at break (%)*
0	8.56 ± 4.53 ^a	205.94 ± 7.81 ^a
5	7.01 ± 1.23 ^b	203.27 ± 13.98 ^a
10	3.65 ± 1.44 ^c	189.94 ± 8.06 ^b
15	2.68 ± 0.18 ^d	166.31 ± 18.19 ^{ab}

*Means±SD are from triplicate measurements; n = 5; means with different superscript letter are significantly difference ($p < 0.05$)

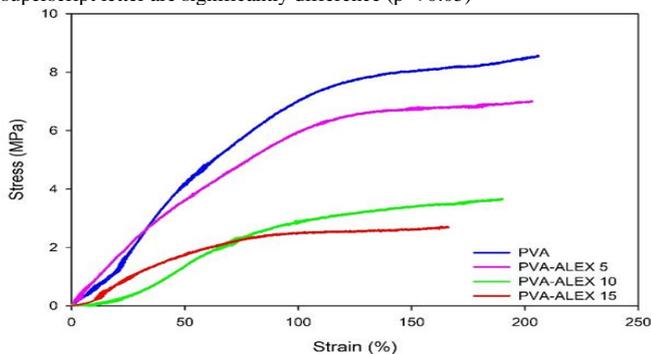


Fig. 4 Tensile stress-strain curve of PVA and ALEX-loaded PVA nanofibers

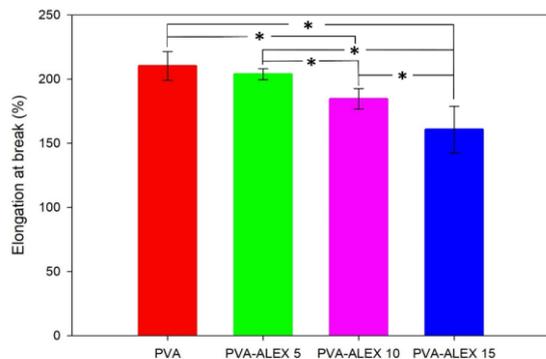


Fig. 5 Percentage of elongation at break of electrospun PVA and ALEX-loaded PVA nanofibers

E. Swelling degree and weight loss of the PVA and ALEX-loaded PVA fibre mats

One of the significant parameters in the fabrication of nanofibrous mats for wound healing application is absorptive capability. Wound dressing is considered ideal when it able to remove excess wound exudates while preventing dehydration. The exudation can result in maceration and excoriation to the periwound region of healthy tissues. According to Adeli et al., (2018), the range for fluid uptake of an ideal wound dressing should be between 100 to 900 % [15]. The swelling degree and weight loss for PVA and ALEX-loaded PVA electrospun nanofibers after 24 hours immersion in phosphate buffer solution (pH 7.4, 37°C) are shown in Fig. 6. The electrospun PVA nanofibers showed the highest swelling degree among all. The highly porous and hydrophilic nature of electrospun PVA fibre mats contributed to the highest percentage of swelling degree compared to ALEX-loaded PVA fibre mats. Such porous swelling fibre mats are important in absorbing large amounts of body fluids, therefore removing excess exudates from the wound bed.

Meanwhile, PVA-ALEX15 showed the highest weight loss followed by PVA-ALEX10, PVA and PVA-ALEX5. The weight loss of nanofibers occurs due to degradation mechanisms such as depolymerization and solvation. Based on the Fig. 6, the percentage of weight loss increased by increasing the concentration of ALEX loaded in the matrix material. It could be resulted from high density of cross-linking efficiency between PVA and ALEX. The electrospun PVA and the corresponding ALEX-loaded PVA nanofiber mats shows statistically significant difference ($p < 0.05$) in the swelling degree and weight loss measured. These are good merits for an ideal wound dressing whereby it should have high swelling degree and little weight loss to maintain a moist environment while preventing degradation.

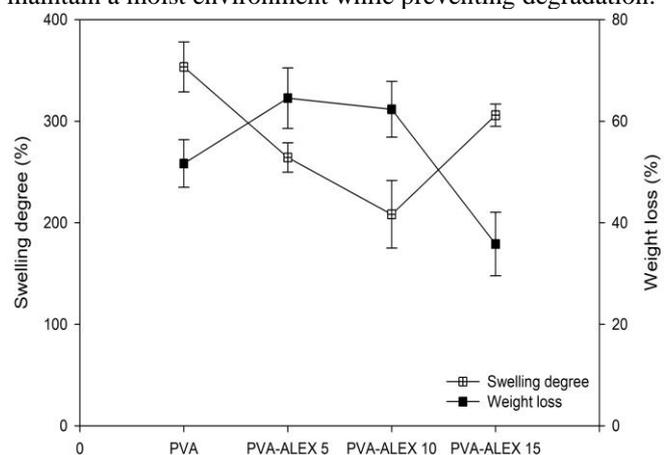


Fig. 6 Percentage of swelling degree and weight loss of PVA and ALEX-loaded PVA nanofibers.

F. In-vitro release study

The actual amount of ALEX incorporated in the PVA nanofibers were investigated prior to the release study of ALEX in ALEX-loaded PVA nanofibers. It was found that the actual amount of ALEX in PVA-ALEX5, PVA-ALEX10 and PVA-ALEX15 were 84.9 ± 2.1 , 82.1 ± 1.8 and 88.2 ± 2.6 % (based on the initial amount of ALEX loaded within the spinning solutions), respectively.



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Next, the release study of ALEX from ALEX-loaded PVA nanofibers were carried out by total immersion method. The cumulative amount of ALEX released from PVA electrospun nanofibers are depicted in Fig. 7. The results showed that the electrospun ALEX-loaded nanofibers underwent rapid release during the first 12 hours with gradual increment afterwards. PVA-ALEX15 showed the highest cumulative release of ALEX compared to others. This result correlates to the swelling degree as discussed previously. As the nanofibers begin to swell, ALEX are solvated and leached out from the fibrous matrix. Another factor contributing to the release of ALEX is due to the erosion of electrospun nanofibers within the medium (Li et al., 2013) [11].

Fig. 8 shows the morphology of ALEX-loaded nanofibers after immersion in the medium for 120 hours, whereby the nanofibers swollen to a size greater than before. However, the electrospun fibre mats still retain their fibrous structure upon immersion in the releasing medium for 120 hours which indicates the degradation stability of the fibre mats [17]. The rapid and constant release of the extract could contribute to the effectiveness of antimicrobial activity at the wound site. Bayat et al., (2019) and Vakilian et al., (2018) reported on the release of Bromelain from chitosan nanofibers and *L.inermis* from PLLA-gelatine nanofibers [18,19]. Both nanofibers also showed rapid release during initial hours and gradually increased in the cumulative release over the next hours. The initial rapid release is essential in suppressing the wound infection immediately.

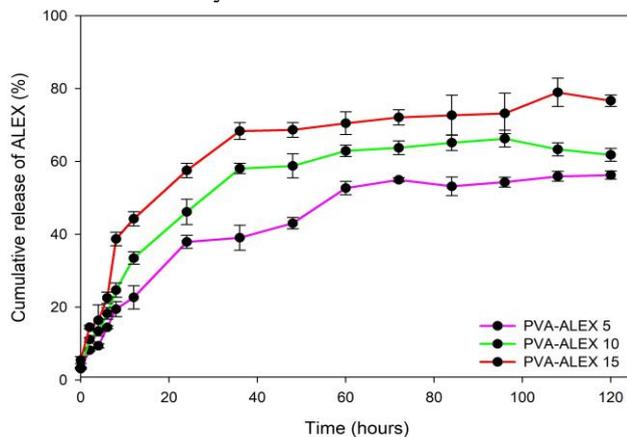


Fig. 7 In-vitro ALEX release profiles from PVA and ALEX-loaded PVA nanofibers.

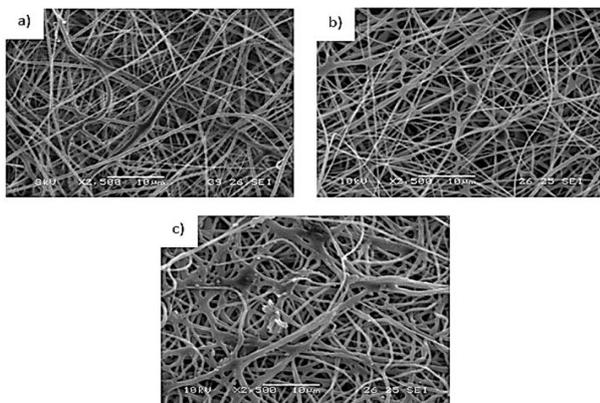


Fig. 8 SEM images of electrospun nanofibers of (a) PVA-ALEX5, (b) PVA-ALEX10 and (c) PVA-ALEX15; after 120 hours immersion in sodium acetate buffer at pH 5.5

G. Antibacterial activity

Wound dressings with antibacterial activity are important in preventing and managing bacterial infection. In this study, the antibacterial properties of ALEX-loaded PVA nanofibers against *Escherichia coli*, *Vibrio vulnificus*, *Bacillus subtilis* and *Staphylococcus aureus* were determined. These bacteria have been reported to be responsible in necrotizing skin and soft tissue infections besides interrupting wound healing process.

As shown in Fig. 9, the effectiveness of the fibre mats in inhibiting bacteria were indicated by the formation of halo areas without the bacterial growth around the mats. There were no zones of inhibition observed for PVA nanofibers on each bacterium. Meanwhile, PVA-ALEX15 showed the highest inhibition zone followed by PVA-ALEX10 and PVA-ALEX5. Table 3 shows the diameters of inhibition zone of ALEX-loaded PVA nanofibers on tested pathogens.

The pharmacological activity of the ALEX remained unchanged after experiencing high electric potential during electrospinning process, since it able to inhibit the growth of bacteria. This result is also proven by numbers of electrospun fibre mats encapsulated with plant extract such as *Gymnema sylvestre* leaf extract, *Tecomella undulata* crude bark extract, *Centella asiatica* extract and *Aloe vera* extract [20,21,22,23].

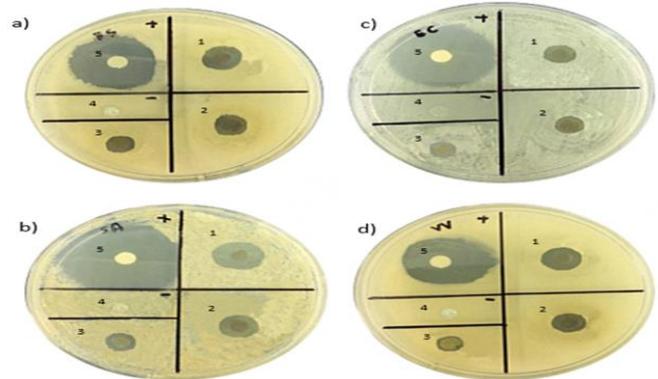


Fig. 9 Antibacterial activity of electrospun ALEX-loaded PVA fibre mats (¹PVA-ALEX15, ²PVA-ALEX10, ³PVA-ALEX5) and control [⁴PVA neat (negative) and ⁵Tetracycline disc (positive)] against: (a) *B. subtilis*, (b) *S. aureus*, (c) *E. coli* and (d) *V. vulnificus*

The increase of ALEX concentration loaded in the nanofibers enhance the antibacterial activities due to the increase of bioactive compound in present in the extract ($p < 0.05$). Therefore, the highest inhibition zones were presented by PVA-ALEX15 (Table 3).

TABLE 3
INHIBITION ZONE OF ALEX-LOADED PVA NANOFIBERS ON *S. AUREUS*, *B. SUBTILIS*, *V. VULNIFICUS* AND *E. COLI*

Bacteria	Average zone of inhibition with disc ^{1,2} ± S.D (mm)			
	Tetracycline	PVA-ALEX5	PVA-ALEX10	PVA-ALEX15
<i>S. aureus</i>	33.0±2.6 ^a	8.3±1.5 ^d	11.5±1.4 ^c	15.0±2.2 ^b
<i>B. subtilis</i>	20.5±2.1 ^a	7.5±1.7 ^d	8.3±1.5 ^c	10.2±1.9 ^b
<i>V. vulnificus</i>	23.7±2.8 ^a	6.9±1.4 ^d	9.5±1.7 ^c	11.7±2.0 ^b
<i>E. coli</i>	28.6±2.6 ^a	6.1±2.2 ^{ab}	8.4±0.8 ^b	9.0±2.1 ^b



¹Values are expressed as mean Inhibition±SD (n=3)

²Means with different superscript letter are significantly difference (p < 0.05)

PVA nanofibers were used as negative control

H. Anti-inflammatory activity

In this study, anti-inflammatory activity of ALEX-loaded PVA nanofibers was carried out by in-vitro lipoxygenase assay. Lipoxygenase (LOX) is non-heme iron-containing dioxygenase that is commonly found in both plants and animals. LOX is involved in arachidonic acid metabolism which produces several biologically active leukotrienes that significantly contribute in inflammatory responses. It catalyses the oxidation of unsaturated fatty acids, such as linoleic acid to form hydroperoxides. According to Wisastra et al., (2012), 13-hydroperoxy-9(Z),11(E)- octadecadienoic acid (13-HPOD) is generated through oxygenation of linoleic acid [24]. LOX plays an important role in causing various disorders such as inflammation, cancer, bronchial asthma and autoimmune diseases [13]. Hence, LOX inhibitory assay is a suitable assay to evaluate the anti-inflammatory properties of compound in vitro.

Prior to the assessment of anti-inflammatory effect of ALEX-loaded PVA nanofibers, IC₅₀ value of ALEX was found to be between 6.25 to 200 µg/mL. The LOX inhibition by ALEX and NDGA is shown in Table 4. NDGA was used as positive control as it is known as LOX inhibitor and exhibit strong anti-oxidant [25].

TABLE 4

LIPOXYGENASE INHIBITION ASSAY OF ALEX AND NDGA

Sample	Concentration (µg/ml)	% Inhibition ¹	IC ₅₀ (µg/ml)
NDGA	200	99.591±0.208 ^a	6.383±0.218
	100	98.304±0.725 ^b	
	50	89.453±1.368 ^c	
	25	86.467±1.199 ^d	
	12.5	83.053±0.396 ^e	
	6.25	77.523±1.945 ^f	
ALEX	200	97.319±0.453 ^a	21.365±2.146
	100	75.121±3.653 ^b	
	50	60.368±1.950 ^c	
	25	55.508±2.394 ^d	
	12.5	43.799±2.767 ^e	
	6.25	34.236±3.778 ^f	

^aValues are expressed as mean Inhibition (%) ± SD (n=3); means with different superscript letter are significantly difference (p < 0.05); NDGA: nordihydroguaiaretic acid

The anti-inflammatory activity of NDGA and ALEX increased with increasing concentration (p < 0.05). It can be seen from Table 4 that percentage of inhibition of ALEX is almost comparable to NDGA. This result indicates that ALEX possess strong anti-inflammatory activity. This activity may be due to the presence of flavonoids and terpenoids which serves as primary oxidant, thereby inhibiting inflammation [26].

Next, the anti-inflammatory activity of ALEX-loaded PVA nanofibers were evaluated and compared to the free ALEX. The ALEX-loaded PVA nanofibers demonstrated potent inhibitory activity against LOX which has close similarity of results between the free ALEX. This result indicates that electrospinning process did not adversely affect ALEX bioactivity. Therefore, the incorporation of ALEX within the PVA nanofibrous dressing can open new horizons for the development of an effective bioactive dressings with

remarkable characteristics. Table 5 shows the LOX inhibition percentage of ALEX-loaded PVA nanofibers.

TABLE 5
LIPOXYGENASE INHIBITION BY ALEX-LOADED PVA NANOFIBERS

Sample	Absorbance (234 nm)*	% Inhibition*
PVA-ALEX5	0.044 ± 0.008 ^a	86.977 ± 2.783 ^a
PVA-ALEX10	0.052 ± 0.011 ^a	83.213 ± 3.910 ^b
PVA-ALEX15	0.059 ± 0.004 ^a	80.887 ± 0.898 ^c

*Means±SD are from triplicate measurements; n = 5; means with different superscript letter are significantly difference (p < 0.05)

The increase of ALEX content in PVA nanofibers significantly affect the inhibitory activity of the fibre mats (p < 0.05). PVA – ALEX nanofibers also showed slightly lower LOX inhibition in comparison to the highest concentration tested for free ALEX. This might occur due to high voltage during electrospinning process or cross-linking of the PVA with ALEX, which reduce the effectiveness of compound that is responsible for the anti-inflammatory activity. In comparison to the free ALEX, ALEX-loaded PVA nanofibers displayed lower inhibition zone due to the loading concentration of ALEX in the nanofibers.

IV. CONCLUSION

In the present study, electrospun PVA nanofibers with different ALEX concentrations produced a uniform, beadless and randomly oriented nanofibers with average diameters in nanometre range. The presence of ALEX in the PVA nanofibers was confirmed via Attenuated total reflectance-Fourier transform infrared spectroscopy and differential scanning calorimetry. The ALEX-loaded PVA nanofiber mats displayed sustained-release characteristics and high swelling capacity with increasing ALEX concentration. In addition, the obtained mats exhibited good flexibility and tensile strength which can simulate the mechanical properties of human skin. The mats were effective in inhibiting the growth of *Vibrio vulnificus*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. ALEX-loaded PVA nanofibers also displayed strong anti-inflammatory activity against LOX. To this end, the results suggest that ALEX-loaded PVA nanofiber mats have great potential for use as wound dressings.

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