Fabrication, Characterization, *In Vitro* Release, and Some Biological Activities of Eucalyptus Essential Oil Loaded Poly (Lactic Acid) Nanofibers

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Abstract

The objective of this work was to evaluate antibacterial and antibiofilm activity of *Eucalyptus camaldulensis* EO-loaded PLA nanofibers. Different concentrations of *E. camaldulensis* EO (7.5, 10 and 15%) were incorporated into PLA nanofibers via an electrospinning technique. The nanofibers are characterized by Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and dynamic mechanical analysis (DMA). Antibacterial activity of essential oil-loaded PLA nanofibers against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* were investigated by disc diffusion method. The antibiofilm activity of essential oil-loaded PLA nanofibers was studied against *Pseudomonas aeruginosa*. The results obtained from the biofilm formation test revealed that PLA essential oil inhibited the *Pseudomonas aeruginosa* biofilm by 9.3%, 12.8% and 12.6%, respectively. Drug releasing behavior of essential oil-loaded PLA nanofibers was investigated by UV-Visible spectroscopy. According to releasing results, as the amounts of *Eucalyptus camaldulensis* essential oil in PLA nanofibers increased, the releasing decreased. The essential oil-loaded PLA nanofiber successfully delivered about 160 h.

Keywords: *Eucalyptus camaldulensis* Dehn; Electrospinning; Nanofibers; Drug Delivery; Antibacterial Activity; Antibiofilm Activity

Abbreviations

EO: Essential Oil; PLA: Poly (Lactic acid); PLA-EUC Nanofibers: *E. camaldulensis* EO Loaded PLA Nanofibers; FT-IR: Fourier Trans-

form Infrared Spectrometer; SEM: Scanning Electron Microscopy; DMA: Dynamic Mechanical Analysis.

Introduction

The electrospinning method produces nanofibers by electrostatic spinning. It is based on stretching a jet of polymer solution through an electric area to produce synthetic fibres with diameters extending a few nanometres to a few micrometres [1-4], which is arousing the interest of the scientific community [5]. In recent years, the synthetic additives used in many fields (pharmaceutical, cosmetics, food and agriculture) raised many health concerns, associated to their side effects [6]. Hence, natural alternatives such as EOs, which are classified as safe (GRAS) by the United States Food and Drug Administration [7], are needed to replace them. EOs have been the subject of extensive research with the aim of demonstrating biological properties. Today, different antibacterial compounds such as EOs [8] have been incorporated into the PLA matrix to produce new films with antibacterial properties. In addition, research on the development of PLA-based materials having antibacterial properties experienced a great boom in order to extend the shelf-life and increase the quality of food [9]. PLA has received a lot of attention thanks to its renewable resources, its biocompatibility, its biodegradation, good mechanical performance, and the transparency of the [10]. The research cited below reports that natural extracts from bioactive plants could potentially be used as plasticizers for PLA, which reflects the mobility of the polymer chains and minimizes the rate of interactions between the chains, as has also been observed in the case of EO loaded PLA films [8]. *Eucalyptus camaldulensis* Dehn. is an aromatic plant and medicinal widely used in traditional medicine; their antimicrobial power and chemical compositions have been demonstrated by several studies [11,12,41-43]. The main objective of this work is to produce and develop materials based on PLA and to study the applicability of PLA (a biodegradable polymer made from renewable resources such as corn) containing natural agents, as biofilm inhibitor agents. For this, we have chosen the essential oil of *E. camaldulensis* EO to produce EO-containing PLA nanofibers. First, the mode of inclusion of these active agents in the PLA matrices and their effects on various thermal and mechanical properties were studied. Secondly, a study of the release of active agents was investigated. An evaluation of the antibacterial and anti-biofilm activity of these nanofibers was carried out.

Materials and Methods

Materials

PLA was purchased from Sigma-Aldrich. Dichloromethane

(DCM), N, N-dimethylformamide (DMF) and tetrahydrofuran (THF) were used as a solvent. The raw material used for the extraction of EO was *E. camaldulensis* Dehn leaves and twigs, harvested in March 2019 from trees of *E. camaldulensis* Dehn, located in northern Tunisia (Latitude 37° 17'48"N/Longitude 10° 0' 2"E).

Extraction of *Eucalyptus camaldulensis* EO

Fresh leaves and twigs of *E. camaldulensis* Dehn (12 kg) was extracted by steam entrainment method [13] for 3 h (yield, 0.095% (w/w)). The EO obtained was dried over anhydrous sodium sulphate and stored at 4°C.

Preparation of the sample

Figure 1: Preparation of EOs extracted from the aerial part of *Eucalyptus camaldulensis* Dehn.

Chemical composition of *E. camaldulensis* Dehn. EO

GC-MS analyses were performed on an Agilent 7890B GC system coupled to a mass selective detector 5977B (Agilent, Santa Clara, CA, USA). HP-5MS capillary column (5% phenyl-95% methyl siloxane, 30 m x 0.25 mm, x 0.25 µm) was used. Helium was used as a carrier gas (the flow rate was 1 mL/min). One µl EO in hexane was injected in split-less mode. The programming of the oven temperature was maintained at 50°C (0 min), and then increased at a rate of 5°C/min up to 260°C (5 min). The source and quadrupole temperatures were fixed at 230°C and 150°C, respectively. Mass range was scanned from 40 to 400 m/z with ionizing voltage 70 eV. Data were analyzed using Mass Hunter Workstation Software, Qualitative Analysis Navigator and Qualitative Analysis Workflows (Version B.08.00, Agilent Technologies, Inc. 2016). The identifica-

tion of the EO components was performed based on chromatographic comparison of the recorded retention time with computed mass-spectrum data libraries (Pal600K).

Antibacterial activities of the *E. camaldulensis* EO

Spot viability assay

For the assays, the bacterial community was exposed to (1.55, 0.775 and 0.3875) (v/v) of *E. camaldulensis* Dehn EO. After treatment, the cell viabilities were evaluated using a spot viability plate [14]. For this, the exposed cultures were serially diluted into LB media and 10 µL were spotted on LB agar in a Petri dish and incubated for 24 hours at 35°C. The extent of growth was used as an indication of viability.

Production of PLA film and *E. camaldulensis* EO-loaded PLA nanofibers

PLA nanofibers and *E. camaldulensis* EO-loaded PLA nanofibers were produced according to the procedure described by [15-17], with some modifications. *E. camaldulensis* EO-loaded PLA films were prepared by adding different concentrations (5%, 10% and 15%) of *E. camaldulensis* EOs to 0.4 g of PLA dissolved in a mixture of three solvents (3 mL of DCM/1 mL of DMF and 1 mL of THF) and stirred for 24 hours at room temperature. The final solutions were stirred at room temperature for 30 minutes before electrospinning. The polymer solutions were introduced in a 5 mL syringe. The injection of this solution was done with a constant flow of 1.5 ml/h. To obtain the electrospinning of the solutions, a voltage of 15 kV and a separation distance of 15 cm were used. *E. camaldulensis* EO loaded PLA nanofibers with 7.5%, 10% and 15%, and 50% were ticketed PLA-EUC-7.5, PLA-EUC-10 and PLA-EUC-15, respectively.

Nanofibers characterization

The surface morphology of the samples was analyzed using scanning electron microscopy (SEM; JEOL JSM7100-F) with 15 kV working voltage. Fourier transform infrared spectrometer (FT-IR) spectra of PLA control nanofibers and PLA-EUC nanofibers were recorded from wave number 600-4000 cm⁻¹ with a resolution of 2 cm⁻¹, using a Perkin Elmer spectrum. TGA analysis of the samples were carried out with a TGA 8000 Perkin Elmer. The approximately 3 mg samples were tested under a nitrogen atmosphere at a heating rate of 10°C/min with a temperature profile of 30°C to 550°C. Dynamic mechanical analysis of PLA-EUC nanofibers was performed with a DMA 8000 Perkin Elmer. Samples were heated at

30°C to 100°C; tension film geometry was used under frequency 1 Hz and heating rate 3°C/min.

In vitro release studies

In a microtube, 20 mg of PLA-EUC nanofibers were dissolved in 5 mL of 60% phosphate buffer saline (pH = 7.4) + 40% ethanol [18] and incubated at room temperature. At definite time intervals, a specific volume of supernatant was withdrawn for analysis, and was replaced with an equivalent volume of fresh buffer. The amount of released *E. camaldulensis* EO was measured using UV-Visible spectrophotometer at a wavelength of 222 nm. Cumulative percentage of *E. camaldulensis* EO released was determined using the following equation:

$$\text{Cumulative release percentage} = \sum_{t=0}^t \frac{M_t}{M_0} \times 100 \quad (1)$$

Where:

M_t : Cumulative amount of *E. camaldulensis* EO released at each sampling point.

M_0 : The initial weight of the *E. camaldulensis* EO- loaded in the sample.

Antibacterial activities of EO loaded PLA nanofibers

The antibacterial activity of the nanofibers was qualitatively tested by the disc diffusion method against Gram (+) strains; *S. aureus* ATCC 25923 and, *E. faecalis* ATCC 29212, and Gram (-) strains *P. aeruginosa* PA01 and, *E. coli* ATCC 25922. Initially, 100 µL of the bacterial suspension (0.5 McFarland turbidity) was spread on the Luria Bertani Agar (LBA) plate. And then, the nanofibers (diameter = 15 mm) were placed on LBA plate and incubated at 37°C for 24 h. Finally, the diameters of inhibition zones around the nanofibers were measured [19].

Anti-Biofilm properties of EO loaded PLA nanofibers

P. aeruginosa PA01 cells were inoculated into LB medium to an OD_{600nm} of 0.02. One milliliter of the diluted cultures was mixed with the PLA-EUC nanofibers and incubated at 35°C. Non adherent cells were removed by washing with distilled water. The biofilms were stained for 10 min with 0.1% crystal violet and the cells were washed with distilled water to remove excess dye. For quantification of attached cells, crystal violet was extracted with 95% etha-

nol, and the absorbance was measured spectrophotometrically at OD570nm [20].

Results and Discussion

Chemical compounds of *E. camaldulensis* Dehn. EO

GC–MS analysis of *E. camaldulensis* Dehn. EO had detected 28 constituents (Table 1). The main components of the EO was p-cymene (26.62%) followed by eucalyptol (19.04%), spathulenol (13.62%), cryptone (7.34%), β-Phellandrene (5.63%) and (-)-Terpinen-4-ol (4.52%) (Figure 2). The chemical composition of *E. camaldulensis* EO has been studied by other research teams. For example, Dogan., *et al.* (2017) studied the chemical composition of *E. camaldulensis* Dehn. EO from different parts of the plant (leaf and fruit) collected from natural habitats in Mersin (Turkey) [21]. They reported that the composition of the EO extracted from eucalyptus leaves was characterized by high proportions of p-Cymene of around 42.1%. While 1, 8-cineol (34.5%) was the major component of EO extracted from fruits. Sliti., *et al.* (2010), identified 26 components representing 92.82% of the total *E. camaldulensis* oil collected in the Korbos region, including Spathulenol (20.2%), p-Cymene (14.83%), 1,8-cineole (12.16%) and phellandral (6.6%) [22]. However, the EO of *E. camaldulensis* from Malaysia was rich mainly in γ-terpinene (71.36%) then o-cymene (17.63%) and terpinen-4-ol (7.01%) [23]. The EOs of *E. camaldulensis* originating from Taiwan have two chemotypes: the first is characterized by the predominance of 1,8-cineole (29.6%) [24], while the second is marked by the co-dominance of α-pinene (22.52%), p-cymene (21.69%) and α-phellandrene (20.08%) [25]. The variations in the chemical components of *E. camaldulensis* Dehn. EOs might be expected to occur according to plant organ, geographical origin, season and extraction procedures [21,26,27].

Figure 2: A typical GC/MS chromatogram showing the chemical analysis of EO from fresh aerial part of *E. camaldulensis* Dehn.

No	Compounds	N° CAS	T _R	%
1	α-Thujene	2867-05-2	5.728	0.62
2	α-pinene	80-56-8	5.889	3.43
3	(-)-β-Pinene	18172-67-3	6.903	2.66
4	β-Myrcene	123-35-3	7.222	0.29
5	α-Phellandrene		7.576	0.35
6	p-Cymene	99-87-6	8.162	26.62
7	β-Phellandrene		8.24	5.63
8	1,8 Cineole/ Eucalyptol	470-82-6	8.347	19.04
9	γ-Terpinene	99-85-4	9.011	0.95
10	Trace	-	9.83	0.41
11	Linalool	78-70-6	10.102	0.4
12	2-Cyclohexen-1-ol, 1-methyl-4 -(1-methylethl)- ,trans	29803-81-4	10.72	0.43
13	1-Terpineol	586-82-3	11.213	0.62
14	1-Butylcyclohexene	3282-53-9	11.507	0.47
15	(-)-Terpinen-4-ol	20126-76-5	12.29	4.52
16	Cryptone	500-02-7	12.581	7.34
17	α-Terpineol	98-55-5	12.648	0.73
18	Myrtenal	564-94-3	12.81	0.27
19	Cuminaldehyde	122-03-2	14	3.23
20	Phellandral	21391-98-0	14.956	3.24
21	Carvacrol	499-75-2	15.62	0.54
22	Caryophyllene	87-44-5	18.744	0.39
23	Aromadendrene	72747-25-2	19.232	0.27
24	Alloaromadendrene	25246-27-9	19.784	1.68
25	Trace	-	20.452	0.62
26	Spathulenol	6750-60-3	22.661	13.62
27	(+) Spathulenol	77171-55-2	22.754	1.32
28	Ledol	577-27-5	23.186	0.3

Table 1: The chemical composition of EO extracted from aerial part of *E. camaldulensis* Denh.

Study of the antibacterial activity of *E. camaldulensis* Dehn EO

The antibacterial property of *E. camaldulensis* Dehn. EO against four different strains, including two Gram-positive (*S. aureus*, *E. faecalis*) and two Gram-negative strains (*E. coli*, *P. aeruginosa*) was tested using the spot viability assay. This property is shown in table 2.

Figure 3: Spot viability assay of the EO of *E. camaldulensis*.

In the present study *E. camaldulensis* EO showed a strong antibacterial activity for *S. aureus* (4 log reduction) at a concentration of 0.7% (v/v), while a limited antibacterial activity for *E. faecalis* (1 log reduction) and no antibacterial activity for *E. coli* and *P. aeruginosa*.

At a concentration of 1.55%, the *E. camaldulensis* EO exhibited very strong antibacterial activity for *S. aureus* (7 log reduction) and *E. faecalis* (3 log reduction), while it demonstrated limited antibacterial activity for *E. coli* (1log reduction). The result demonstrated that *E. camaldulensis* EO did not show any antibacterial activity against *P. aeruginosa* PA01 for any of the concentrations tested. As far as we know, this is the first report on antibacterial activity of the *E. camaldulensis* EO for *E. faecalis*.

Similarly, other previous studies [28-30] had also supported the antibacterial behavior of *E. camaldulensis* EO.

SEM analysis

The analysis of the structure of EOs loaded nanofibers (10 %) was carried out by SEM. The working voltage is 15 kV.

<i>E. camaldulensis</i>	Viability cfu/mL			
	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
1.55%	1	5	7	8
0.775%	4	7	8	8
0.3875%	8	8	8	8
Control (initial)	8	8	8	8
DMSO	8	8	8	8

Table 2

In this case, the polymer polylactic acid (PLA) was used to prepare nanofibers. Using SEM observations, an examination, of the modifications in the morphology of PLA films, is performed after including antibacterial agents. Usually, the surface morphology of a neat PLA is smooth and homogeneous and does not contain any pores and cracks [31].

SEM images of PLA pure and PLA-EUC nanofibers as depicted in figure 4. As can be seen in figure 4, G and I, diameters of PLA-EUC nanofiber were between 116-387nm. According the table 3, the average diameter of the neat PLA was 607 ± 197 and PLA-EUC was 377 ± 194 . From these results, the diameters of neat PLA are larger than the EO loaded PLA [32].

Essential Oils Loaded Nanofibers	Diameters (nm)
Neat PLA	607 ± 197
PLA-EUC	377 ± 194

Table 3: The average diameters of the PLA-EUC nanofibers and neat PLA nanofiber.

FT-IR

According the FT-IR spectra of PLA nanofiber (Figure 5), the peaks at 2990 cm^{-1} and 2940 cm^{-1} were attributed to $-\text{CH}-$ stretching, the peak at 1760 cm^{-1} was assigned to $\text{C}=\text{O}$ carbonyl group, the peaks at 1180 cm^{-1} and 1080 cm^{-1} were belong to $-\text{C}-\text{O}-$ stretching and the peak at 860 cm^{-1} ascribed to $-\text{C}-\text{COO}$ stretching [33].

The figure 5 shows the FTIR spectrum of PLA-EUC. It is characterized by the presence of the functional groups' characteristic of this type of nanofiber. We note indeed the appearance of a new absorption around 3460 cm^{-1} attributable to the vibrator OH. This spectrum shows the existence of the absorption bands attributed

Figure 4: SEM images of the nanofibers. A. Neat PLA., G, H, I, J, K. PLA-EUC nanofibers.

Figure 5: FT-IR Spectrum of neat PLA and PLA-EUC nanofibers.

to the two vibration modes of valence symmetrical around 2980 cm⁻¹ and asymmetrical around 2922 cm⁻¹ of the CH₃ motif. We also note the appearance of a new characteristic band of amide I group in 1681 cm⁻¹. The bending vibration in 1460 cm⁻¹ and 1380 cm⁻¹ was clearly come up [34]. The existence of the peak at 760 cm⁻¹ was assigned to C=C alkene. The appearance of a new band characteristic of a band relating to alkene C = C around 760 cm⁻¹.

Thermal properties of PLA-EUC (%15) nanofibers

Figure 6: TGA curves of neat PLA and PLA-EUC (%15) nanofibers.

According to figure 6, PLA showed single-step process decomposition. The polymer matrix contains 15% *Eucalyptus camaldulensis* EO resulted in a two-step degradation process, where the first degradation step, between 80-180°C, corresponds to the degradation or vaporization of certain compounds molecular weight present in the polymer as reported by Muller., *et al.* (2017) [35]. While the second step is around 300°C which corresponds to the degradation of the PLA polymer as reported by Martino., *et al.* (2006) [36]. Thermal stability of PLA matrixes decreased with adding essential oils in polymer matrix [37].

DMA

The addition of plasticizers leads to decrease of Tg and the increase of the elasticity [37]. As could be seen from figure 7, EO containing nanofiber mats exhibited a lower Tg for both samples. The Tg value PLA nanofiber is 53.74°C, for Eucalyptus containing (15% percent) nanofiber mat is 42.28°C. The changing range of Tg for

PLA matrixes have been reported between 17 and 35°C with low molecular weight molecules, such as citrates, terpenes, and oil [38-40]. Fillers act as a plasticizers, polymer chain mobility and polymer free volume increase and, these resulted the decreasing of Tg of [38,41,42].

Figure 7: (a) Tan Delta, (b) loss, (c) Storage of the neat PLA and PLA-EUC (%15) nanofibers.

It can be also seen that the elasticity of PLA nanofiber mats increased by adding EOs. The EOs behave as a plasticizer and, this resulted to increasing of free volume, decreasing of interactions between the polymer chains. These changing's lead to improving the chain mobility and flexibility [42]. Zhang, *et al.* (2017) [40] reported that the EOs work as plasticizers for PLA, lowering the glass transition temperature of the resulting composite fibers up to 60% and increasing elongation-at-break and tensile strength up to 12 times.

Release profile

Release behavior of PLA-EUC nanofibers was shown in figure 8. The PLA-EUC nanofibers were released about 160h. In 6 hours burst release were observed for PLA-EUC. According the results lowest percentage of EO (7.5%) in nanofibers was showed the best releasing behavior and the amount of the releasing decreased as the rate increased. In 10% of PLA-EUC nanofibers were released about 70 percent and nearly 50 percent were released from 15% of PLA-EUC [43].

Figure 8: Releasing behavior of the PLA-EUC nanofibers.

The antibacterial activity of nanofibers

The antibacterial *property* of *E. camaldulensis* EO loaded PLA nanofibers at different concentrations (7.5, 10 and 15%), against the *S. aureus* and *E. coli* was tested using the agar diffusion method. *E. camaldulensis* EO loaded nanofibers do not show such behavior (Table 4).

Study of the anti-Biofilm Activity of *E. camaldulensis* EO loaded PLA nanofiber

The biofilm production was determined in the presence of varying amounts of *Eucalyptus camaldulensis* EO (ranging from 2.5 to 0.15625%) against *P. aeruginosa* PA01, significant inhibition of biofilm formation was not observed.

(Nanofibers 1 cm ²)	Inhibition zon diameters (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
<i>E. camaldulensis</i> 7.5%	*	*
<i>E. camaldulensis</i> 10%	*	*
<i>E. camaldulensis</i> 15%	*	*
Kanamisin	10 ± 1.1	11 ± 1.1
*: No Inhibition		

Table 4: The antibacterial activity of *E. camaldulensis* EO loaded PLA nanofiber.

After 24 h in the presence of *E. camaldulensis* EO loaded PLA nanofiber samples, biofilm formation of *P. aeruginosa* PA01 decreased with approximately 9.3%, 12.8% and 12.6% (Figure 9), compared to biofilm formation in the absence of nanofibers.

Figure 9: Anti-biofilm activity of *Eucalyptus camaldulensis* Dehn. EO loaded PLA nanofibers against *P. aeruginosa*.

Conclusion

Chemical analysis of *E. camaldulensis* EO by GC-MS reveals its richness in p-cymene (26.62%) followed by eucalyptol (19.04%), spathulenol (13.62%) and cryptone (7.34%). The *E. camaldulensis* EO exhibited significant antibacterial activity against *S. aureus* and *E. faecalis*. This antibacterial activity could be attributed to p-cymene, eucalyptol and spathulenol. Two-step decomposition process was observed for EO containing PLA matrixes and the decreasing on thermal stability of the matrixes was observed by adding EOs. The adding of EOs in polymer matrixes was resulted the decreasing of Tg and increasing on elasticity of final matrixes.

In our case, it can be revealed that *E. camaldulensis* Dehn. EOs can serve as bio plasticizers when they are incorporated into the PLA matrix.

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