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Development of antimicrobial films based on poly(lactic acid) incorporated with *Thymus vulgaris* essential oil and ethanolic extract of Mediterranean propolis

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ABSTRACT

Antimicrobial films based on polylactic acid (PLA) were developed by incorporating *Thymus vulgaris* essential oil (TV-EOs) with different concentrations of ethanolic extract of Mediterranean propolis (EEP) (5 wt% and 10 wt% based on PLA). The antimicrobial activities of EEP were performed by the agar disc diffusion method. The EEP exhibited high antimicrobial properties with inhibition zone diameter of 12.1 and 11.58 mm against *Staphylococcus aureus* and *Penicillium* sp., respectively. The addition of TV-EOs to films containing 5 and 10 wt% of EEP decrease the elastic modulus from 1292 MPa to 1084 MPa and 911.1 MPa to 794 MPa compared with films containing 5 and 10% of EEP alone, respectively. However, the elongation at break increased by 64% after the addition of TV-EOs to the film containing 10 wt% of EEP. Thermal stability of films improvement by the addition of TV-EOs and EEP. Antimicrobial activity of the films showed that films containing 10 wt% EEP inhibited the growth of *Candida albicans* and the combination of EEP and TV-EOs in the PLA matrix showed a synergistic effect against *Escherichia coli*. The developed PLA-based films with antimicrobial activity have a potential application in food packaging to increase the shelf life of packaged food.

1. Introduction

For a long time, the use of polymeric materials in the packaging sector has increased interest in fundamental research and the chemical industry. However, the potential use of these materials has quickly proven to be a significant source of pollution due to their non-biodegradability. In this fact, conventional polymers need to be replaced with eco-friendly materials and natural polymers derived from renewable resources such as starch, chitosan [1], cellulose [2], alginate [3,4], PLA [5,6].

Polylactic acid (PLA) is synthesized from lactic acid monomer by catalytic ring-opening polymerization [7] or by condensation polymerization of the lactides, and these monomers are obtained from the fermentation of corn, beet-sugar, cane-sugar, *etc.* [8]. Polylactide is well known for its good processability, biocompatibility [9], and biodegradability (mainly by simple hydrolysis) [10,11]. However, some disadvantages of PLA render it inadequate for food packaging applications, such as weak thermal stability, low toughness (caused by its inherent brittleness), poor gas barrier properties, and low deformation at break [12] .

Recently, the addition of natural extracts and herbal essential oils has been investigated to improve the functional proprieties of the PLA film in order to develop active packaging material that can inhibit microbial growth on foods and controlled process while maintaining quality, freshness, and safety [13]. Moreover, the use of natural compounds agent, which can protect consumers from potential chemical contaminations, represents an interest [4,14]. In this context, propolis has received great attention as an antimicrobial and antioxidant agents in several polymer films like hydroxypropylmethylcellulose [15], gelatin [3], chitosan [16]. Propolis is a natural waxy substance collected by honeybees (*Apis mellifera*) from leaf buds of different tree species and exudates of the plants [17,18]. There are more than 300 compounds

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(polyphenols, terpenoids, steroids, sugars, amino acids, and others) have been detected in a different kind of propolis in the world [19]. Taking into account the importance of Mediterranean propolis as it was reported by Graikou et al. [19], it should be exciting to incorporate these natural products in order to enhance the functional proprieties of biopolymers, such as PLA. In addition, Mediterranean propolis could be considered as a new type of propolis group, which has shown high antioxidant and antimicrobial activities [20–22].

Essential oils (EOs) are other natural bioactive materials can be encapsulated in plastic films and edible or biodegradable coatings [23]. Among EOs, *Thymus vulgaris* that belongs to the *Lamiaceae* family, is widely used in folk medicine. Furthermore, this can be directly added to different types of meat and meat products because it is considered an effective antioxidant agent [23]. Generally, it rich in bioactive monoterpenes such as thymol, carvacrol, and linalool, which are responsible for their therapeutic properties [24] such as antimicrobial and antioxidant effect [25–27]. According to Khodayari et al. [28], the films incorporated with *ziziphora clinopodioides* EOs alone and in combination with EEP at different concentrations showed the shelf life extension of minced beef during storage in refrigerated conditions for at least 11 days.

As a matter of fact, there are several studies carried out on PLA-based films containing bioactive compounds. Uloa et al. [29] have shown that the addition of ethanolic extract of propolis in PLA film decreased the tensile strength and elasticity modulus of the active films; however, the films with 13% of ethanolic extract of propolis showed a reduction over 4 log-cycles against *E. coli*. Yahyaoui et al. [30] have developed PLA-based films with different concentrations of EOs and founded that the films containing 1.5% of commercial thyme and 5% of natural myrtle showed significant antimicrobial activity against *Aspergillus niger* sp. Moreover, they reported that the elongation at break of films containing commercial EOs of thyme, myrtle, and rosemary was improved compared with PLA films. Qin et al. [31] have also reported that the incorporation of 9 wt% of EOs from bergamot, lemongrass, rosemary, and clove has showed a similar inhibition against both *E. coli* and *B. subtilis* and a decreases of the glass transition temperature of PLA/EOs blends. In spite of the great potential of such bioactive compounds in antimicrobial packaging, there is no study published on the combination of EEP and *Thymus vulgaris* EOs in the PLA matrix.

The objective of this study was to use ethanolic extract of Mediterranean propolis in PLA based packaging materials and to investigate the effect of the incorporation of EEP and *Thymus vulgaris* EOs in PLA based films on the microstructural, thermal, mechanical, and antimicrobial properties of the films developed.

2. Materials and methods

2.1. Materials

Polylactic acid (PLA) (Nature works® PLA polymer 3051D) bought from Nature works. Chloroform (high-performance liquid chromatography grade). Ethanol 96%, PALCAM agar, and peptone water were obtained from Alpha Bioscience Inc. (Baltimore, MD, USA). *Thymus vulgaris* EOs, also known as common thyme, was purchased from Aliksir (Grondines, QC, Canada), propolis samples were manually collected from beehives located in the Bejaia region in the north of Algeria. The bacterial strains (*Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia 2042*, and *Enterococcus faecalis* wdcm 00009) and fungal strains (*Botrytis cinerea*, *Fusorium* sp., *Aspergillus niger*, and *Penicillium* sp.) were obtained from Renewable Energy Management Laboratory (LMER) of the microbiology department, University of Bejaia (Algeria). The bacterial strain *E. coli* 0157:117 and yeast strain *Candida albicans*10231 was obtained from Laboratories in Sciences Applied to Food from Institute Armand-Frappier (Canada).

2.2. Preparation of ethanolic extract of propolis EEP

The crude propolis was grounded with a mortar and was washed with distilled water. Then the product was dried at room temperature. A sample of 30 g of propolis powder was dissolved in 100 mL of 96% ethanol under continuous stirring at 50 °C for 40 min. The suspension was filtered with Whatman No. 3 filter paper and centrifuged at 600 ×*g* for 20 min. The supernatant was concentrated using a rotary evaporator under reduced pressure at 40 °C and stored at 4 °C until further use. The EEP was prepared according to the method of Khodayari et al. [28].

2.3. Preparation of composite films

The composite films were prepared according to Rezaeigolestani et al. [32] with some modification using a solvent casting method. One gram of PLA granules was dissolved in chloroform and vigorously stirred using a magnetic bar for 8 h until complete dissolution of PLA. Then, different concentrations of EEP (0, 5, and 10 wt%) and TV-EOs (0 and 2 wt%) based on PLA were added to the film solution, and the mixture was homogenized at 8000 rpm for 3 min using an IKA homogenizer (IKA T25-digital ultra turrax, Germany) equipped with S25N-25F probe. The resultant solution was poured into glass Petri dishes (diameter: 8 mm, deep: 15 mm). The chloroform was allowed to evaporate at room temperature for 48 h. The samples were divided into four groups: PLA corresponds to control film, F0 corresponds to PLA film containing EOs, F1and F2 corresponds to PLA film containing 5 wt% and 10 wt% of EEP, respectively, and F3 and F4 corresponds to PLA films containing 5 wt% and 10 wt% of (EEP) with TV-EOs, respectively, as it showed in Table 1.

2.4. Characterization

2.4.1. Antimicrobial activity of EEP

In order to evaluate the antimicrobial activity of the EEP, a paper disc diffusion method was employed according to Karabay-Yavasoglu et al. [33]. A Muller-Hinton agar (MHA) and Potato Dextrose Agar (PDA) were prepared for bacterial and fungal strains, respectively. A quantity of 100 μL of each microorganism was deposited on the surface of the nutrient agar and was uniformly distributed with a sterile swab. The sterile paper discs (6 mm in diameter) placed on the agar surface was impregnated with 20 μL of EEP (0.85 g/mL in DMSO). The plates were incubated at 37 °C for 24 h (for bacteria) and 25 °C for 72 h (for fungal strains).

2.4.2. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of raw propolis and EEP were recorded using a SHIMADZU FTIR-8400S spectrophotometer with a resolution of 4 cm^{-1}. Samples were ground finely with potassium bromide and mixed. The mixture was then compressed into pellet form. The FTIR analysis of films and TV-EOs was performed with ATR-FTIR spectrum one spectrometer (Perkin-Elmer, Woodbridge, Canada) in the wavenumber range of 400–4000 cm^{-1}. A total of 32 scans were taken in absorption mode with a resolution of 4 cm^{-1}.

Table 1
Composition of films.

Film	PLA (% w/v)	EEP (wt%)	TV-EOs (wt%)
PLA	1	0	0
F0	1	0	2
F1	1	5	0
F2	1	10	0
F3	1	5	2
F4	1	10	2

PLA: Poly(lactic acid); EEP: Ethanolic extract of Mediterranean propolis; TV-EOs: *Thymus vulgaris* essential oil.

2.4.3. Color measurement

The color of the films was assessed by measuring L* (lightness), a* (redness), and b* (yellowness) using a colorimeter (Minolta CR-10 plus, Konica Minolta Camera INC. Japan).

The color difference ΔE was calculated by the following equation:

$$\Delta E^* = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right]^{0.5} \quad (1)$$

where ΔL*, Δa*, and Δb* represent the difference of L*, a*, and b* values of the films with the same values of the pure PLA film, respectively.

2.4.4. Thermal analysis

Thermogravimetric analyses of EEP, the neat PLA film, and PLA films containing EEP alone or EEP with TV-EOs were performed using a Thermogravimetric Analyzer Instruction Manual, LISEINS STA PT1600. Film samples of approximately 5 mg were tested under a nitrogen atmosphere at a heating rate of 10 °C/min from 25 °C to 600 °C.

2.4.5. Mechanical properties

The tensile strength, elasticity modulus, and elongation at break of films were evaluated. Films were cut in a rectangular shape with a width of approximately 12 mm. The width was then measured using a Traceable Carbon Fiber Digital Caliper (resolution of 0.1 mm; Fisher Scientific, ON, Canada) at three random positions. Mechanical properties were carried out using a Universal Testing Machine (model H5KT; Tinius Olsen Testing Machine Co., Inc., Horsham, PA, USA), equipped with a 100 N-load cell (type FBB) and 1.5 kN-specimen grips. The mechanical parameters were automatically collected after the film break due to elongation, using Test Navigators 7 software.

2.4.6. Antimicrobial test of films

In order to evaluate the antimicrobial property of the PLA based-films containing EEP and TV-EOs against *E. coli* and *C. albicans*, a conventional liquid culture assay was used according to Arfat et al. [34] with some modifications. Firstly, 0.25 g of each active film was cut into small pieces and placed in separate sterile tubes containing 10 mL of the culture suspension (10^4 CFU/mL) with TSB obtaining with serial dilution. After 6 days of incubation at 25 °C, 100 μL of bacterial suspension with films in each tube was spread plated onto the PDA or TSA nutrient agar, incubated at 37 °C for 24 h for the enumeration of the microorganisms. The Neat PLA films served as control.

2.5. Statistical analysis

The experiment was done in triplicate. For each replicate, three samples were analyzed. The data were reported as mean ± standard deviation. Data were subjected to one-way analysis of variance (ANOVA) by SPSS 22.0 software (IBM, NY, USA). Differences among means values were examined by Duncan's multiple comparison tests at a $P \leq 0.05$.

3. Results and discussion

3.1. Antimicrobial activity of EEP

The results of antimicrobial activities of EEP are shown in Fig. 1. EEP showed antimicrobial activity against all the assessed microorganisms evaluated. The best results were obtained against *Staphylococcus aureus* for bacterial strains and *Penicillium* sp. for fungal strains with a diameter of inhibition zone of 12.1 mm and 11.58 mm, respectively. Seibert et al. [35] similarly reported that their propolis extract was more effective against *Staphylococcus aureus*.

In addition, The EEP showed the same diameter inhibition ($P > 0.05$) against *Bacillus cereus* and *Enterococcus faecalis* with 10, 8 mm and 10, 5 mm, respectively. However, *K. pneumoniae* was less susceptible to EEP. It

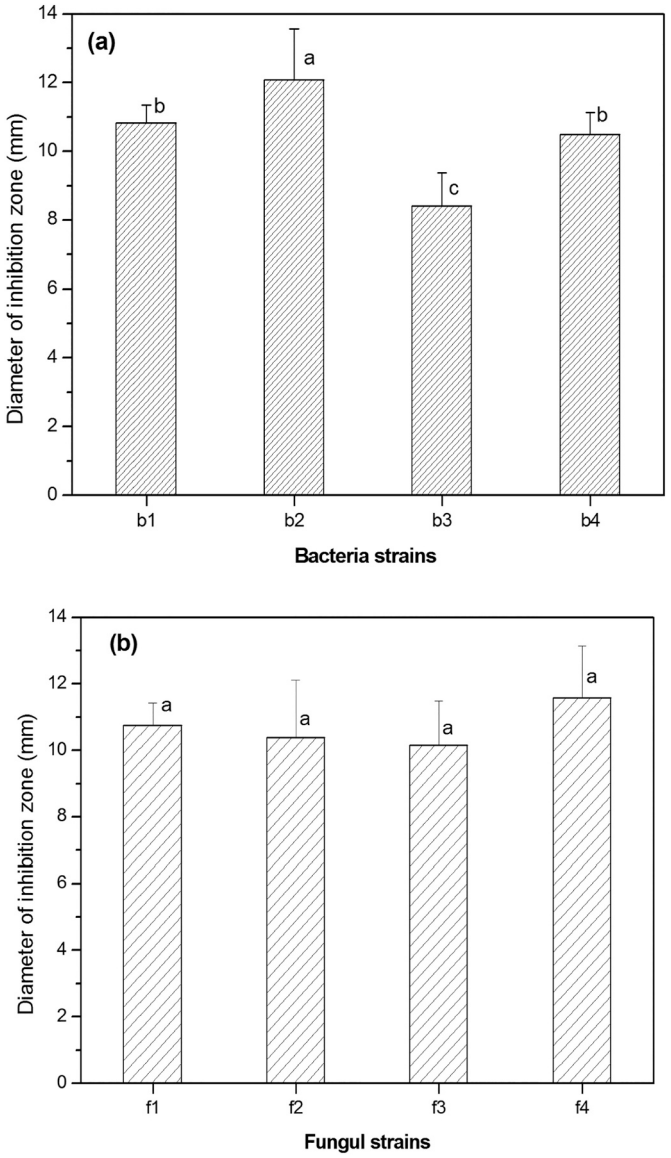

Fig. 1. (a) Antimicrobial activities of EEP against bacteria (b1: *Bacillus cereus*; b2: *Staphylococcus aureus*; b3: *Klebsiella pneumonia*; b4: *Enterococcus faecalis*) and (b) against fungus (f1: *Botrytis cinerea*; f2: *Fusorium* sp.; f3: *Aspergillus niger*; f4: *Penicillium* sp.). The bars with same alphabet are not significantly different ($P \leq 0.05$).

has been reported previously by many studies that Gram-positive bacteria are more susceptible to several types of propolis than Gram-negative bacteria [36,37]. This is due to the structural differences between their bacterial cell wall. The out layer of Gram-negative bacteria containing phospholipids, proteins, and lipopolysaccharides are impermeable to some molecules. The major presence of flavonoids and phenolic compounds in the chemical composition in propolis may be related to the promising antimicrobial activities observed [19,20]. It can also be noted that the biological effect of EEP was related to the action of several aromatic compounds that existed in the propolis such as caffeic acid, benzyl cinnamate, cinnamyl cinnamate [38]. The finding showed the ability of EEP to inhibit bacterial growth and confirming the use of EEP as an antimicrobial agent in active food packaging.

3.2. FTIR spectra of raw propolis, EEP, TV-EOs and composite films

FTIR analysis was realized in order to find out the structural modification of raw propolis after being extracted with ethanol. FTIR spectra

of Mediterranean raw propolis and their ethanolic extract are shown in Fig. 2(a). As shown in Fig. 2(a), the same absorbance bands are observed in raw propolis and EEP. The spectrum of EEP showed a board band at 3358 cm^{-1}, which corresponding to (O—H) stretching vibration of hydroxyl groups from alcohols and phenols [39,40]. The absorbance peaks at 2925 cm^{-1} and 2849 cm^{-1} related to stretching vibration of C—H and N—H, respectively [41]. It can be noted that sharp peaks found in 1689–1600 cm^{-1} region correspond to stretching vibration of C═O and C═C bonds and asymmetric bending vibration of N—H due to the presence of flavonoids and amino acids [42]. Furthermore, it can be observed that the peaks at 1278 cm^{-1} might be due to the vibration of the C—O group of polyols, such as hydroxyflavonoids [43], and at 1443 cm^{-1} corresponds to the C—C stretching vibration of aromatics rings [41]. On the other side, the indicating peaks at 1088 and 1030 cm^{-1} can be assigned to the C—O stretching of ester group, while the band at 879 cm^{-1} attributed to stretching vibration of C—H from aromatic ring [40]. The vibrational intensities of peaks spectrum increased in the EEP spectrum comparing with the spectrum of raw propolis, and this is suggested that more aromatic compounds are present in EEP.

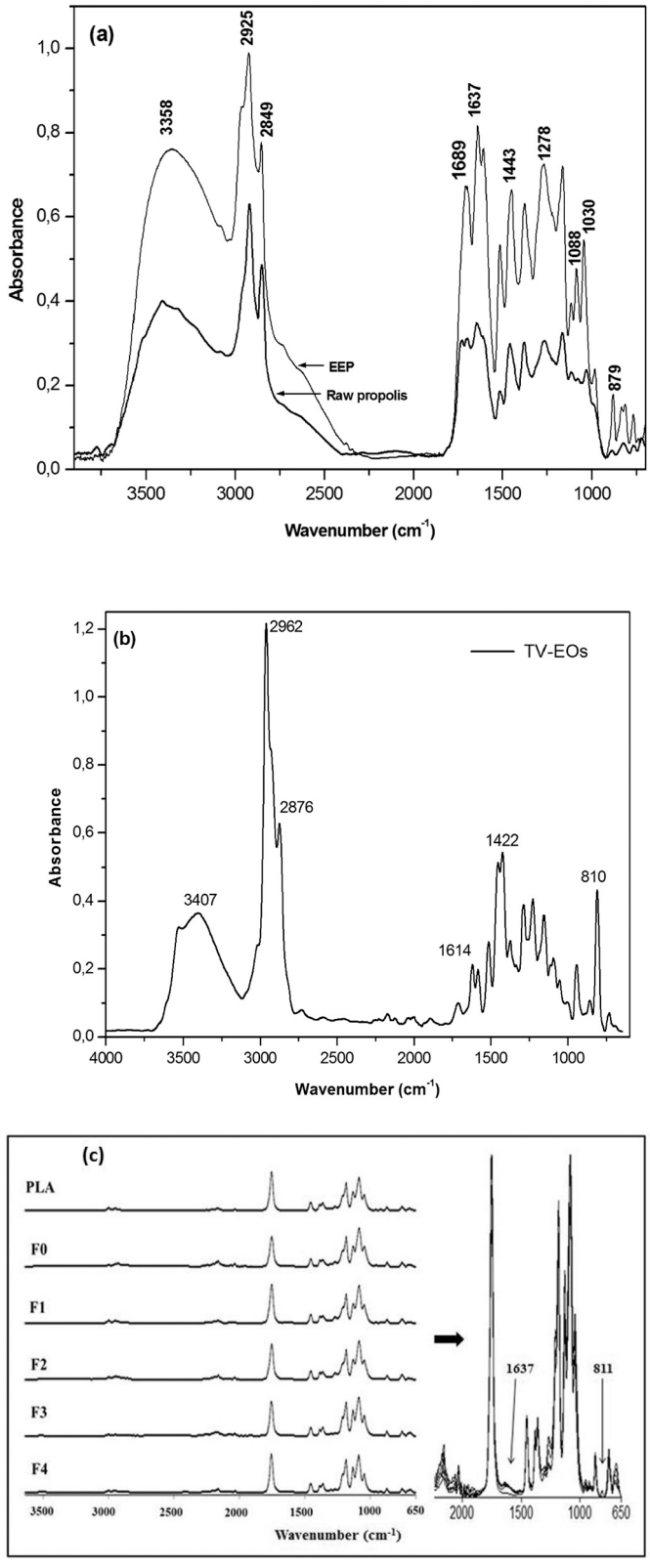

Fig. 2. FTIR spectra of (a) raw propolis and EEP, (b) *Thymus vulgaris* EOs and (c) elaborated films.

Fig. 2(b) shows FTIR spectra of TV-EOs. A wide absorbance band at 3407 cm^{-1} was observed, which corresponding to stretching vibrations of OH groups of phenol [44].

The characteristic peaks at 2962 and 2876 cm^{-1} correspond to symmetric and asymmetric stretching of (C—H). The peaks in the region between 1614 and 1422 cm^{-1} are assigned to phenol ring present in thymol. Also, it can be seen that a peak at 811 cm^{-1} assigned to out of plane aromatic C—H wagging vibrations [45].

FTIR analyses were used in order to investigate the incorporation of EEP and TV-EOs in the PLA films, The FTIR spectra of all elaborated films are shown in Fig. 2(c). According to Fig. 2(c), it can be seen that the characteristic bands of PLA at 2950 cm^{-1} (-CH stretching bands), 1760 cm^{-1} (-C═O carbonyl group), 1453 and 1368 cm^{-1} (-C-H deformation) 865 cm^{-1} (-C-C- stretching) are displayed in all samples [30,31]. The FTIR spectrum of PLA films containing 5% and10% of EEP showed a large absorption band at 1637 cm^{-1} attributed to stretching vibration of (-C═C) aromatic ring due to flavonoids compounds present in EEP [16]. Therefore, when TV-EOs was combined with EEP, the band at 1637 cm^{-1} shifted slightly from 1620 cm^{-1}, which assigned to (-C═C) stretching of the phenolic group present in TV-EOs [46].

Furthermore, the films containing TV-EOs showed an increase in the intensity of the peak at 3300 cm^{-1}, which is associated with the presence of O—H stretching vibrations of a hydroxyl group of thymol present in TV-EOs [30,45]. Additionally, a new band appeared for these film spectrums between 800 cm^{-1} and 817 cm^{-1} attributed to aromatic ring vibration (C—H) belonged to the major compounds of essential oils [45,47–49]. Moreover, the spectrum of the composite film did not indicate apparition of a new peak, suggesting that interaction between the PLA matrix and bioactive compounds were physical adsorption, as was reported by Bodini et al. [3], where they investigated the effect of EEP in gelatin films. Similarly, Zancanela et al. [50] were observed the same effect in FTIR spectra of a natural rubber latex membrane incorporated with EEP. These results can confirm the good dispersion of EEP and TV-EOs into PLA films.

3.3. Color parameters of composite films

The color of food packaging materials is very important because it can straightly blight food appearance [3,30]. The color parameters of composite films are summarized in Table 2. Results of the color parameters showed that the L* values of the active films decreased significantly ($P \leq 0.05$) as the concentrations of EEP increased. However, the addition of TV-EOs did not have a considerable influence on the L* values of the films. Yahyaoui et al. [30] have stated that the addition of different essential oils in PLA films exhibited a slight change in the color of the composite. The composite films containing EEP and TV-EOs also showed a greener and yellower color than PLA-based film without additive. These observations correlate with a decrease of a* value and an increase of the b* value. Uloa et al. [29] had obtained the same results when EEP was added to the PLA matrix, and they stated that the films containing 8.5 and 13 wt% of EEP were darker.

Table 2
Color parameters of films.

Films	L	a*	b*	ΔE
PLA	93.5 ± 0.1[a]	−1.4 ± 0.1[a]	0.1 ± 0.1[e]	–
F0	93.1 ± 0.1[a]	−1.9 ± 0.2[a]	3.6 ± 1.9[d]	3.56 ± 1.9[c]
F1	91.7 ± 0.2[b]	−2.7 ± 0.5[b]	11.1 ± 1.7[c]	11.1 ± 1.5[b]
F2	89.3 ± 0.4[c]	−3.0 ± 0.6[b]	21.1 ± 2.7[b]	21.4 ± 2.7[a]
F3	91.4 ± 0.3[b]	−3.2 ± 0.1[b]	12.2 ± 1.3[c]	12.4 ± 1.2[b]
F4	89.1 ± 0.1[c]	−3.1 ± 0.4[b]	24.0 ± 1.9[a]	24.5 ± 2.1[a]

[a] Each values are the mean of three replicates with the standard deviation. Any values in the same column followed by the same letter are not significantly (P > 0.05) different by Duncan's multiple range tests.

Siripatrawan et al. [16] were observed that the incorporation of EEP in chitosan films significantly increased the a* and b* parameters of composite films. Rezaeigolestani et al. [32] have reported that the combination of EEP and EOs (Zataria Multiflora Bioss) in PLA films showed a significant change in the optical parameters comparing with the films containing EEP and EOs alone.

The variation of a* and b* values had a direct effect on the color variation of the films. An increase was observed in the total color difference (ΔE*) with the incorporation of EEP and TV-EOs. According to Khodayari et al. [28] and Liu et al. [51], the addition of complex substances as a natural extract in polymer films could distort their transparency and visual quality.

3.4. Thermal properties of composite films

The thermograms (TG) and derative of thermograms (DTG) obtained for EEP and films containing EEP alone at a different concentration or with TV-EOs are presented in Fig. 3. The thermogravimetric analysis of EEP showed slow thermal degradation sub-divided into two steps. The first degradation between 75 and 120 °C corresponds to the evaporation of solvent traces (Ethanol) and other volatile compounds. However, the second degradation between 180 and 400 °C could be associated with the degradation of bioactive compounds present in EEP. The thermal degradation shape of EEP is related to their chemical composition. According to Pola et al. [52], the simplest volatiles compounds that do not contain many aromatic rings in their structures were decomposed at low temperatures due to their heat-sensitive, but the degradation of complex aromatic compounds present in EEP takes place generally around 380 °C. The thermogravimetric analysis of all films exhibited mainly two weight loss stages. The first stage between 80 and 190 °C was associated with the water removal and the degradation of volatile bioactive compounds present in EEP and TV-EOs. The second stage between 290 and 340 °C was related to the degradation of aromatic ring present in the major compounds of EEP and TV-EOs and the decomposition of PLA.

According to DTG thermograms, it can be drawn that the addition of EEP and TV-EOs into PLA films displayed a decrease in the onset degradation temperature of films compared with the neat PLA films without additives. However, when TV-EOs was added in films containing 5% of EEP, the onset degradation temperature increased from 140 to 190 °C. Nevertheless, the addition of TV-EOs in films containing 10 wt% of EEP did not show a significant difference in the onset temperature degradation comparing with PLA films containing 10 wt% of EEP alone. As observed, the maximum decomposition temperature (T_{dmax}) of the neat PLA films is centered at 328 °C. However, when 10 wt% of EEP was added to PLA based films, the maximum decomposition of films was shifted to higher temperatures (348 °C). In addition, the same result was observed when the TV-EOs was added into film containing 5 wt% of EEP comparing to films containing 5 wt% of EEP alone. Peres et al. [53] have reported that the addition of propolis extract in silicone matrix increased the thermal stability of the composite. Qin et al. [31] have observed that the incorporation of essential oils into PLA films displayed a slight increase in (T_{dmax}) from 361 °C to 363 °C comparing with PLA alone. Similarly, Estevez-Areco et al. [54] have also stated that with the addition of rosemary extract as a natural agent in the PVA matrix, the thermal stability of composites was improved.

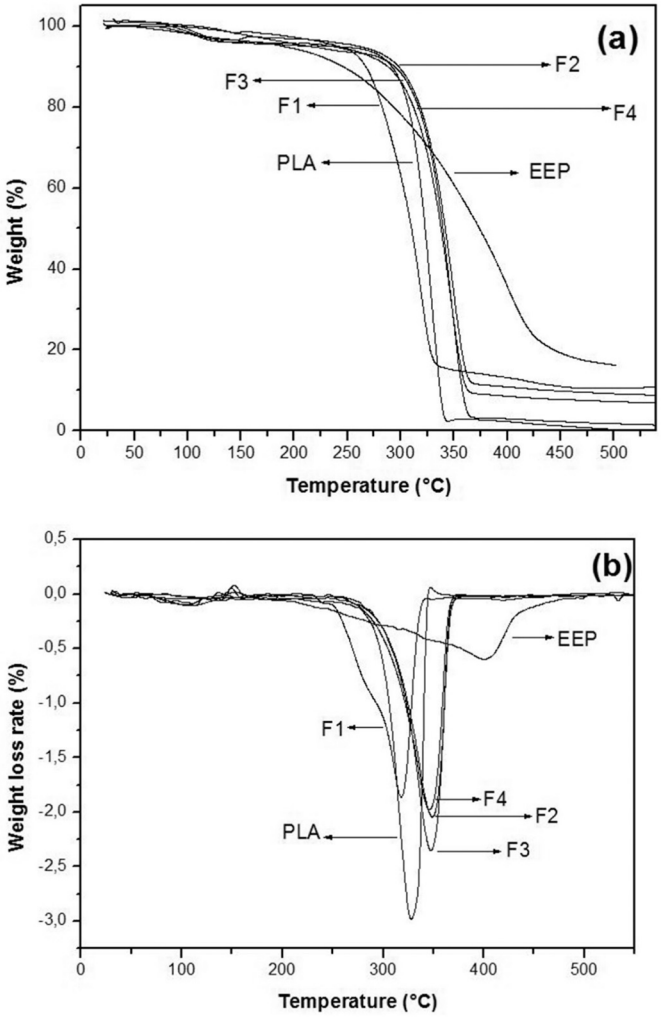

Fig. 3. (a) TGA and (b) DTG curves of EEP and films.

3.5. Thickness and mechanical properties of composite films

The results of the thickness of the films are presented in Table 3. The results show that incorporating EEP and TV-EOs into PLA films did not significantly (P > 0.05) affect the thickness values of films, which are arranged between 52.3 and 63.7 μm. The obtained effect is in accordance with de Araújo et al. [17] when they studied the effect of EEP in starch-based film. The results of the tensile strength (TS), the elasticity modulus (EM), and the percentage of elongation (EAB) of different films are also presented in Table 3. As expected, the addition of EEP and TV-

Table 3
Mechanical properties of films.

Films	Thickness (μm)	TS (MPa)	EM (MPa)	EAB (%)
PLA	55 ± 3[b]	36.8 ± 2.0[a]	1471.5 ± 37.9[a]	2.9 ± 0.12[d]
F0	58 ± 5[ab]	23.2 ± 1.0[b]	862.2 ± 207.1[c,d]	6.2 ± 2.9[d]
F1	58 ± 2[ab]	26.2 ± 5.5[b]	1292.3 ± 97.1[b]	3.0 ± 0.2[d]
F2	61 ± 5[ab]	23.0 ± 1.3[b]	911.1 ± 128.2[d,e]	11.6 ± 4.5[c]
F3	61 ± 2[ab]	23.3 ± 1.3[b]	1084.5 ± 29.3[c]	18.9 ± 5.9[b]
F4	63 ±6[a]	11.2 ± 1.5[c]	794.7 ± 40.2[e]	76.2 ± 4.1[a]

[a] Each values are the mean of three replicates with the standard deviation. Any values in the same column followed by the same letter are not significantly (P > 0.05) different by Duncan's multiple range tests.

EOs to the PLA based film changed the mechanical proprieties compared with the neat PLA.

The elasticity modulus and the tensile strength of the films containing EEP or TV-EOs decreased significantly ($P < 0.05$) compare to the value obtained for the neat PLA. The addition of TV-EOs to films containing 5 wt% and 10 wt% of EEP decreases the elasticity modulus from 51% and 13%, respectively. Moreover, the addition of EEP and TV-EOs decreased the tensile strength comparing with the control films, and it can be observed that the combination between 10 wt% of EEP and TV-EOs reduced the tensile strength from 23 to 11 MPa. Additionally, an increase ($P \leq 0.05$) in elongation at break was observed for all active films. The PLA based-film containing 10 wt% of EEP with TV-EOs exhibited the most important value of elongation at break. Thus, the combination of EEP and TV-EOs improves the flexibility of the polymer matrix. Similar results were observed by Khodayari et al. [28] using PLA polymer with *Tanacetum balsamita* EOs and EEP, and they reported that lower tensile strength and elasticity modulus of 9.15 and 490 MPa, respectively. Whereas, the higher values for the elongation at break (74.5%) were obtained for the film containing the high amount of EEP and EOs. Eskandarinia et al. [55] stated that increasing EEP concentration in corn starch films showed a decrease in mechanical properties of active films comparing with the control. Similar effects have been reported for gelatin films incorporated with EEP [3]. Moreover, Javidi et al. [56] reported that the addition of *Ligustrum vulgare* EOs in PLA films caused a decrease in elasticity modulus and tensile strength by 58% and 67%, respectively; compared with PLA films without EOs. According to Rezaeigolestani et al. [32], the addition of EOs or EEP resulted in lower compactness of the polymer and caused the formation of areas discontinuity in the film matrix, which reduces the traction resistance and leading to decreased elasticity modulus and tensile strength. However, the dispersion of EEP or EOs in polymer induces the mobility of the polymer chains, which improves the flexibility and the ductility of the active films. This was demonstrated by increasing the elongation at break values of the film.

3.6. Antimicrobial test of composite films

The main purpose of the antimicrobial test was to evaluate the potential use of EEP and the combination with TV-EOs in active food packaging. The results of *in vitro* antimicrobial activity of active films against *C. albicans* and *E. coli* are shown in Fig. 4. The results showed that after 6 days of incubation, the neat PLA films did not inhibit the tested bacteria. The lack of antimicrobial activity of PLA films are being reported by many studies [30,31]. The PLA based-film with TV-EOs and 10 wt% of EEP alone or PLA films containing different concentrations of EEP in combination with TV-EOs showed a total inhibition growth against *C. albicans*. However, this study exhibited that films containing 10 wt% EEP alone were more effective against *C. albicans*. Abdulkhani et al. [38] reported that coating of nanofibers/Polylactic composites with different concentrations of EEP showed inhibition growth against *C. albicans*. Therefore, it has been suggested that the antimicrobial potential of propolis is related to the presence of flavonoids (isoflavones) in their chemical composition.

On the other hand, the PLA films containing EEP at different concentrations did not show any inhibition against *E. coli*. This result agrees with the results of Abdulkhani et al. [38] and Rezaeigolestani et al. [32]. However, the combination of EEP with TV-EOs into PLA matrix showed a total inhibition against *E. coli*. The same behavior was previously reported by Rezaeigolestani et al. [32], who found that the PLA-based films containing EEP and *Zataria multiflora* EOs showed synergistic effect against the all tested bacteria. Moreover, it has been demonstrated by Probst et al. [57] that the combination of EEP with ginger and peppermint essentials oils showed a synergistic effect against Gram-positive and Gram-negative microorganisms. The antimicrobial activity of TV-EOs against bacteria can be attributed to the presence of monoterpene phenol compounds as thymol [58], which can also interact

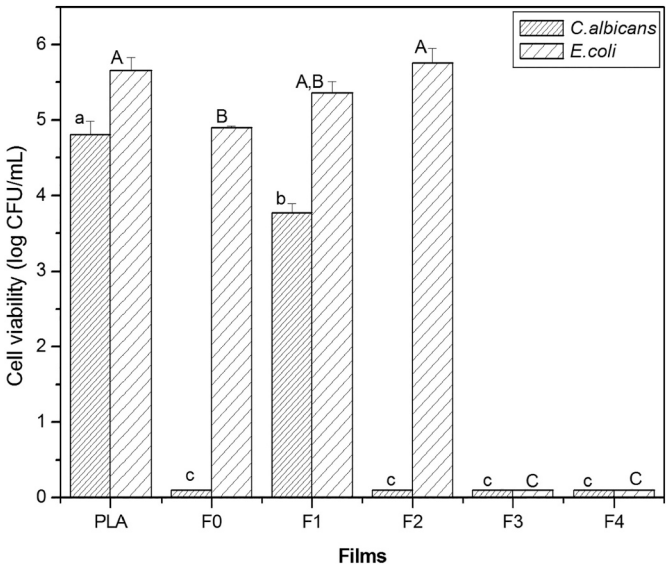

Fig. 4. Antimicrobial activity of films. The bars with same alphabet are not significantly different ($P \leq 0.05$).

with the lipids present in the microbial cell membrane, and this induces a deterioration of the cell membrane [59]. As shown in Fig. 1, the antibacterial activity of the pure EEP is more effective than EEP incorporated in PLA-based films [32]. It was previously reported that the diffusivity of EEP polyphenols encapsulated into PLA was very low [60]. However, this study exhibited that films containing 10 wt% EEP alone were more effective against *C. albicans* than *E. coli*. The difference in antimicrobial activity of propolis againt yeast strains and bacterial strains might be due to the structural differences between them [16].

4. Conclusion

Although the use of EEP and *Thymus vulgaris* EOs as an antimicrobial agent in packaging materials was gradually interesting, this study shows the effect of the combination between them in the characteristics of the PLA films. The antimicrobial test results showed that a combination between EEP and TV-EOs in PLA based-films has promising antimicrobial effects. Moreover, the finding suggests that PLA based films with EEP alone show a potential inhibition growth against yeast strains. Additionally, the incorporation of both antimicrobial substances induces the plasticizing effect and improving the thermal stability of films, which should be considered these films more suitable for active food packaging application. The results show that the developed PLA-based films incorporated with EEP and TV-EOs has potent application in antimicrobial food packaging to increase the shelf life of packaged food.

CRediT authorship contribution statement

NA, SS, and YBF executed the experiments. ML, NC, and HD designed the experiment. ML supervised the experiments. NA and SS wrote the manuscript. SS and ML edited and revised the manuscript.

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