



***In Vitro* Antibacterial Activity of Polylactic Acid Film Incorporated with Ethanolic Propolis Extract and *Ziziphora clinopodioides* Essential Oil**

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HIGHLIGHTS

- *Ziziphora* Essential Oil (ZEO) and Ethanolic Propolis Extract (EPE) revealed synergistic antibacterial effect.
- Polylactic acid films enriched with ZEO showed better antibacterial activity than EPE-incorporated films.
- Polylactic acid films enriched with ZEO showed higher antibacterial effect on Gram-positives than Gram-negatives.

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Acronyms and abbreviations

PLA=Polylactic Acid
ZEO=*Ziziphora clinopodioides* Essential Oil
EPE=Ethanolic Propolis Extract
EO=Essential Oil
GC/MS=Gas Chromatography/Mass Spectrometry

ABSTRACT

Background: Innovative bioactive films incorporated with antimicrobial agents have been recently developed for food preservation. The aim of this study was to evaluate antibacterial activity of Polylactic Acid (PLA) films incorporated with *Ziziphora clinopodioides* Essential Oil (ZEO) and Ethanolic Propolis Extract (EPE) against some common food-borne pathogens.

Methods: The volatile chemical compounds of ZEO were identified by analytical gas chromatography. Different PLA film groups were prepared using ZEO (1% and 2%) and EPE (1% and 2%), separately and in combination. Disk diffusion method was used in order to evaluate antibacterial activity of experimental groups. The analysis was performed using SPSS 16.0.

Results: The PLA films enriched with the ZEO possess better antibacterial activity compared to the films incorporated with EPE ($p<0.05$). The descending order antibacterial effects were as follows: *Staphylococcus aureus*>*Listeria monocytogenes*>*Bacillus subtilis*>*B. cereus*>*Salmonella enteritidis* serovar Typhimurium>*Escherichia coli* O157:H7. The combination of ZEO and EPE showed significantly higher antibacterial effects against all bacteria than those of obtained with each single ZEO or EPE ($p<0.05$).

Conclusion: The PLA films incorporated with ZEO and EPE had considerable antibacterial activity, indicating potential of these films for application as active packaging in food industry.

Introduction

In the last years, innovative bioactive films such as cellulose, starch, pectin, chitosan, gelatin, carboxymethyl cellulose, and Polylactic Acid (PLA) incorporated with

antimicrobial agents have been developed (Kuorwel et al., 2011). PLA, a biodegradable plastic produced from the ring-opening polymerization of lactide, has increas-

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ingly used for food packaging applications (Woraprayote et al., 2013). It could be produced from inexpensive natural resources such as corn, sugar beet, and biomass residues and also it has been approved for intended use in the fabricated materials for food-contact applications (Samsudin et al., 2014). Indeed, due to its remarkable intrinsic characteristics especially excellent film-forming capability, eco-friendly nature, biocompatibility, non-toxicity, biodegradability, and serving as a compound carrier, PLA can be used as an active packaging material for food preservation (Shavisi et al., 2017). Previous researches reported that the combination of this material with antimicrobial agents such as pediocin could be used as suitable active packaging for meat and meat products in order to inhibit spoilage and pathogenic microorganisms resulting from the post-process contamination (Woraprayote et al., 2013).

Among the natural antimicrobial agents, Essential Oils (EOs) and extracts have strong potential inhibitory effects against some common food-borne pathogens (Azizkhani et al., 2013; Ekhtiarzadeh et al., 2012; Shahbazi et al., 2015). Genus *Ziziphora*, belongs to the family of Lamiaceae, consists of four species including, *Z. clinopodioides* Lam, *Z. capitata* L., *Z. persica*, and *Z. tenuior* L. which are widespread worldwide especially in Turkey and Western Iran. *Z. clinopodioides* is the most abundant species which have been reported from Iran (Shahbazi et al., 2016a, 2016b). Previous studies revealed that the EO of this plant had potential activity as antibacterial agent in different food models like raw beef patty, commercial barely soup, and doogh dairy product (Shahbazi, 2015a; Shahbazi et al., 2016a, 2016b). On the other hand, propolis is a resinous material collected by bees and well known for plenty of biological as well as pharmacological properties, such as immunomodulatory, antitumor, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, and anti-parasitic activities (Sforcin and Bankova, 2011).

To demonstrate the usefulness of PLA films enriched with the EOs and extracts as potential active packaging material in food industries, the antibacterial efficacy of them must be evaluated in a laboratory and food models. However, based on our knowledge, no report is available on *in vitro* antibacterial effect of PLA films incorporated with *Z. clinopodioides* EO (ZEO) and Ethanolic Propolis Extract (EPE). Hence, the aim of the present study was to evaluate antibacterial activity of PLA films incorporated with ZEO and EPE against some common food-borne pathogens including, *Staphylococcus aureus*, *Bacillus subtilis*, *B. cereus*, *Listeria monocytogenes*, *Salmonella enteritidis* serovar Typhimurium, and *Escherichia coli* O157:H7.

Materials and methods

Plant material and isolation of the EO

The fresh leave part of *Z. clinopodioides* plant was collected from Gilan Gharb city, Kermanshah province, West of Iran during full flowering phase from March to July 2014. Authentication of the plant was conducted in Faculty of Agriculture, Razi University, Kermanshah, Iran. In order to extraction of the EO, 100 g air-dried leave part of *Z. clinopodioides* plant was subjected to hydro-distillation for 3.5 h by Clevenger-type apparatus according to standard technique. Then, the oily layer on top of the aqueous distillate was collected and dried by adding 0.5 g anhydrous sodium sulfate (Na_2SO_4 ; Merck, Darmstadt, Germany). The obtained ZEO was kept in dark glass bottle at refrigerated condition before further use.

Preparation of EPE

Fresh propolis was collected from a honeybee husbandry located in Kermanshah province, West of Iran during March-July 2014. Air dried propolis was mixed with ethanol (30:100 g/ml) and extracted with shaker at 10 °C for 24 h. The extract was filtered through a Whatman No. 3 filter paper to remove solid material, concentrated by a rotary evaporator and maintained in a dark place at 4 ± 1 °C before antibacterial analysis.

Gas Chromatography/Mass Spectrometry (GC/MS) analysis of EO

The volatile chemical compounds of ZEO were identified by analytical GC (Thermo Quest 2000, UK). The GC apparatus was equipped with a HP-5ms 5% phenyl methyl silicone capillary column (30 m length \times 0.25 mm ID, 0.25 μm film thickness). Temperature program for the column included: the initial oven temperature was kept at 50 °C for 3 min and then temperature was raised from 50 °C to 265 °C, at program ramp rate 2.5 °C per min. The ultimate temperature was 280 °C and maintained for 6 min. The temperature of the injector was 250 °C. Helium (purity: 99.99%) was the carrier gas (at constant flow rate 1.2 ml/min and split ratio 1:20). Then, 1 μl ZEO was injected manually and identification of EO was performed by analytical GC coupled with MS detector (Thermo Quest Finningan, UK). The capillary column and temperature condition of MS detector was similar with GC as described above. The MS was carried out in the electron ionization mode (Khanjari et al., 2013) using electron impact ionization that was set on 70 eV and completed scans from 30 to 550 amu (atomic mass units).

The volatile chemical compounds of the EO were identified by comparison among their retention indices, retention indices existed in database (Behravan et al., 2007; Morteza-Semnani et al., 2005; Ozturk and Ercisli, 2007; Sonboli et al., 2010), standard mass spectral fragmentation pattern (Wiley/NBS Pak v.7, 2003), and US national institute of standards and technology. The GC peak area normalization of the three injections was expressed as mean percentage of the individual EO composition.

Preparation of PLA films

The PLA-based film was prepared by dissolving 1 g PLA powder (Sigma-Aldrich, UK) in 50 ml chloroform to a concentration of 2% (w/v) while stirring on a magnetic stirrer/hot plate. The solution was stirred at room temperature which typically required 8 h stirring. After that, tween 80 to a level of 0.25 ml/100 ml PLA emulsion was added as an emulsifier to assist EO dissolution in film forming solution. Then, the EO (1% and 2%) and EPE (1% and 2%), separately and in combination, were added to the mixture and homogenized at 12000 rpm for 1 min. The film forming solutions were casted on the center of glass plates and then dried for 24 h at room temperature (Bie et al., 2013).

Preparation of test microorganisms

The antibacterial activity of PLA films incorporated with ZEO and EPE was evaluated against common food-borne pathogenic bacteria including, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *B. cereus* ATCC 11774, *L. monocytogenes* ATCC 19118, *S. enterica* serovar Typhimurium ATCC 14028, and *E. coli* O157:H7 ATCC 10536. All bacteria were obtained as a lyophilized culture from the culture collection of the Iranian Research Organization for Science and Technology, Tehran, Iran.

Before start of the antibacterial analysis, all the microorganisms were sub-cultured in Brain Heart Infusion broth (BHI; Merck, Darmstadt, Germany), incubated at 37 °C for 18 h. Optical density of the cultured microorganisms in BHI broth was determined using a spectrophotometer at 600 nm. After that, the enumeration of bacterial cells was carried out on BHI medium as described previously by Shahbazi and Shavisi (2016).

Agar disk diffusion assay

In order to evaluate antibacterial activity of PLA films incorporated with ZEO and EPE agar disk diffusion method was used according to Kakaei and Shahbazi (2016) with some minor modification. Firstly, the initial inoculum (10^8 CFU/ml) was cultured on Mueller-Hinton

agar by surface method and sterile cotton swab. Then, the PLA film (6 mm) impregnated with ZEO separately and in combination with EPE was placed on the surface of Mueller-Hinton agar. Afterwards, the plates were incubated at 37 °C for 24 h. In order to evaluate antibacterial activity, the diameter of inhibition zone was measured.

Statistical analysis

All the experiments of this study were repeated in triplicate. The analysis was performed using SPSS 16.0 (Chicago, IL, USA) software package. Significance level was considered at $p < 0.05$.

Results

The GC/MS analysis of ZEO leads to the identification of twenty four components, representing 99.65% of the total EO (Table 1). Oxygenated monoterpenes, carvacrol, and thymol were determined as the major abundant compounds.

The results of antibacterial activity of PLA films incorporated with ZEO separately and in combination with EPE are exhibited in Table 2. Based on our findings, PLA film as a control did not show any antibacterial activity against all test microorganisms. Moreover, the films enriched with the ZEO possess better antibacterial activity against both Gram-positive and Gram-negative bacteria compared to the films incorporated with EPE ($p < 0.05$). According to the results of the present study, the descending order antibacterial effects were as follows: *S. aureus* > *L. monocytogenes* > *B. subtilis* > *B. cereus* > *S. enteritidis* serovar Typhimurium > *E. coli* O157:H7. As shown in Table 2, our findings indicated that the antibacterial activities of films were significantly improved with increasing concentration of ZEO and EPE. The combination of ZEO and EPE showed significantly higher antibacterial effects against all bacteria, with higher inhibition zone than those of obtained with each single ZEO or EPE ($p < 0.05$).

Discussion

Based on the results of the present study, carvacrol and thymol were the most abundant constituents of ZEO. In agreement with our findings, Aghajani et al. (2008) reported that carvacrol and thymol were the main components of ZEO obtained from the Lorestan province, West of Iran. However, other previous studies announced pulegone, 1,8-cineole and limonene as the major chemical compounds of ZEO (Behravan et al., 2007; Morteza-Semnani et al., 2005; Ozturk and Ercisli, 2007; Sonboli et al., 2010). It is stated that volatile chemical constituents of the EOs can be varied depending upon several

factors such as genetic and growth stage, age of the plant, part of the plant, seasonal and environmental condition,

geographical location, the method used in extraction of the EO, etc. (Burt, 2004; Shahbazi and Shavisi, 2016).

Table 1: EO composition of *Z. clinopodioides* identified by GC/MS

No.	Compound name	Composition%	Retention time (min)	KI ^a
1	α -Thujene	0.26	11.33	927
2	α -Pinene	0.27	11.71	934
3	Camphene	0.13	12.61	952
4	β -Pinene	0.06	14.06	981
5	1-Octen-3-ol	0.08	14.32	986
6	Myrcene	0.51	14.62	992
7	α -Phellandrene	0.13	15.58	1010
8	α -Terpinene	0.79	16.11	1021
9	<i>p</i> -Cymene	4.86	16.62	1030
10	Limonene	0.1	16.77	1033
11	β -Phellandrene	0.11	16.89	1036
12	γ -Terpinene	4.63	18.31	1063
13	<i>cis</i> -Sabinene hydrate	0.07	19.02	1077
14	Terpinolene	0.08	19.69	1089
15	Linalool	0.13	20.5	1105
16	Borneol	0.61	24.36	1183
17	Terpinene-4-ol	0.48	24.7	1190
18	α -Terpineol	0.08	25.49	1206
19	Carvacrol, methyl ether	0.04	27.38	1246
20	Thymol	19.51	29.61	1293
21	Carvacrol	65.22	30.57	1315
22	<i>E</i> -Caryophyllene	1.07	35.47	1427
23	Spathulenol	0.12	42.10	1590
24	Caryophyllene oxide	0.31	42.30	1595
	Total	99.65		

^a: Kovats index

Table 2: The antibacterial activities of PLA film incorporated with EPE and ZEO

Experimental groups	Inhibition zone (mm)					
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>B. cereus</i>	<i>L. monocytogenes</i>	<i>S. enteritidis</i> serovar Typhimurium	<i>E. coli</i> O157:H7
PLA (control)	-	-	-	-	-	-
PE 1%	4.46±0.11 ^{ba}	4.26±0.00 ^{ba}	4.68±0.00 ^{ba}	4.24±0.01 ^{ba}	2.22±0.02 ^{ba}	2.24±0.12 ^{ba}
PE 2%	6.22±0.22 ^{ba}	4.90±0.27 ^{ba}	4.84±0.27 ^{ba}	4.48±0.14 ^{ba}	4.20±0.27 ^{ba}	4.26±0.06 ^{ba}
ZEO 1%	13.32±0.12 ^{aA}	10.24±0.12 ^{aAB}	10.58±0.24 ^{aAB}	10.32±0.43 ^{aAB}	8.28±0.13 ^{aB}	8.24±0.13 ^{aB}
ZEO 2%	14.84±0.36 ^{aA}	13.52±0.19 ^{aAB}	12.28±0.18 ^{aAB}	14.64±0.15 ^{aA}	10.24±0.36 ^{aB}	10.32±0.17 ^{aB}
PE 1%+ZEO 1%	16.20±0.21 ^{aA}	10.24±0.13 ^{aB}	10.90±0.21 ^{aB}	12.30±0.22 ^{aB}	9.50±0.27 ^{aB}	8.20±0.14 ^{aB}
PE 1%+ZEO 2%	16.46±0.12 ^{aA}	13.34±0.00 ^{aAB}	13.44±0.34 ^{aAB}	14.28±0.37 ^{aA}	10.24±0.25 ^{aB}	10.42±0.21 ^{aB}
PE 2%+ZEO 1%	16.24±0.44 ^{aA}	10.30±0.41 ^{aB}	10.92±0.16 ^{aB}	12.84±0.24 ^{aA}	10.46±0.31 ^{aB}	8.34±0.19 ^{aB}
PE 2%+ZEO 2%	16.88±0.12 ^{aA}	14.84±0.22 ^{aA}	13.52±0.44 ^{aA}	15.76±0.12 ^{aA}	10.90±0.16 ^{aB}	10.64±0.34 ^{aB}

- Means with different lowercase letter in the same column are significantly different between the groups ($p < 0.05$)

- Means with different capital letters in the same row are significantly different between the bacteria ($p < 0.05$)

According to the current work, PLA films incorporated with ZEO and EPE had appropriate antibacterial activity against both Gram-positive and Gram-negative bacteria. Shavisi et al. (2017) found that PLA films containing EPE, cellulose nanoparticle, and ZEO reduced significantly total mesophilic and psychrotrophic bacteria, *Pseudomonas* spp., and Enterobacteriaceae family in minced beef. Besides, Woraprayote et al. (2013) showed that PLA/sawdust particle biocomposite film impregnated with pediocin PA-1/AcH decreased *L. monocytogenes* count in raw pork. Indeed, the most important possible reason of the antibacterial activities of carvacrol and thymol as the major compounds of ZEO could be at-

tributed to the acidic nature of their hydroxyl group and involvement in the formation of hydrogen bonds with compounds of outer membrane structure of bacteria (Čavar et al., 2008). Similar to our results, ZEO obtained from North-East of Iran (Khorasan Razavi province) and Turkey (Erzurum province) showed strong antibacterial activity against *S. epidermidis*, *S. aureus*, *E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, and *B. subtilis* but its efficacy varied (Behravan et al., 2007; Ozturk and Ercisli, 2007). Moreover, the possible mechanism for antibacterial effect of EPE could be due to its flavonoids compounds such as flavanols, procyanidins, and phenolic acid (Mascheroni et al., 2010; Sforzin and Bankova,

2011). It has been indicated that EPE has antibacterial effect against food-borne pathogenic bacteria which is in agreement with our findings (Mascheroni et al., 2010). In our research, regarding to the inhibition zone, *S. enteritidis* serovar Typhimurium and *E. coli* O157:H7 were more resistant to EO than the other bacteria. This phenomenon could be attributed to intrinsic tolerance and compounds of microorganisms especially the presence of hydrophobic lipopolysaccharide outer membrane structure of Gram-negative bacteria which is especially impermeable to EO and extract molecules and the nature and combinations of phytochemicals present in the EO (Shahbazi, 2015b).

The combination of ZEO and EPE studied in this work, revealed higher antibacterial effects against all bacteria, with higher inhibition zone than those of obtained with each single ZEO or EPE, showing their synergistic effect. The mechanism of combination effects of antimicrobial materials such as various EOs is not fully studied. It is stated that EOs cause important morphological damages, disturb the phospholipid bilayer membrane structure and increase the permeability of cell membrane that lead to leakage of intracellular proteins and electrolytes (Tajkarimi et al., 2010). Also, it has been demonstrated that the mechanisms of antimicrobial interaction for synergy includes inhibition of the common biochemical pathway and enzymatic systems and also increasing of the number of pores in the bacterial cell membrane and the size of the pores formed (Lv et al., 2011).

Conclusion

The oxygenated monoterpenes afforded a main portion of ZEO with carvacrol and thymol as the major abundant constituents. Moreover, the PLA films incorporated with ZEO and EPE had considerable antibacterial activity against both Gram-positive and Gram-negative bacteria, indicating potential of these films for application as active packaging in food industry.

Conflicts of interest

There is no conflict of interest in this study.

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