Original article

ANTIBACTERIAL EFFECTS OF *ZIZIPHORA CLINOPODIOIDES* AND *MENTHA SPICATA* ESSENTIAL OILS AGAINST COMMON FOOD-BORNE PATHOGEN BIOFILMS ON STAINLESS STEEL SURFACE

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Summary

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The aim of the current study was to assess the effects of *Ziziphora clinopodioides* independently and in combination with *Mentha spicata* essential oils against biofilms of six food-borne pathogens including *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus* developed on the surface of stainless steel coupons. The minimum inhibitory concentrations (MICs) of essential oils (EOs) were determined using broth micro-dilution method. The order of adhered cell counts in biofilms on the stainless steel surfaces was as followed: *S.* Typhimurium (5.75 log CFU/cm^2) > *E. coli* O157:H7 (5.31 log CFU/cm^2) > *S. aureus* (5.21 log CFU/cm^2) > *L. monocytogenes* (4.62 log CFU/cm^2) > *B. subtilis* (4.23 log CFU/cm^2) > *B. cereus* (4.17 log CFU/cm^2). There was significant difference between the population of all investigated pathogens on the stainless steel coupons treated with ZEO and MEO (P<0.05). The number of surface-adhered cells significantly was decreased after 1.5, 3 and 4.5 min of exposure to the disinfectant solutions based on ZEO separately and in combination with MEO (P<0.05). In all bacterial biofilms treated with the combination of ZEO and MEO logarithmic reductions higher than 2 log CFU/cm^2 were observed. The highest biofilm removal activity of disinfectant solutions based on ZEO and MEO was found for *B. cereus*, followed by *B. subtilis*, *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S.* Typhimurium, respectively. It can be concluded that the disinfectant solution based on the combination of ZEO and MEO can be used as an appropriate natural disinfectant to control biofilm formation in food processing facilities and utensils.

Key words: biofilm, *Mentha spicata,* stainless steel surface, *Ziziphora clinopodioides*

INTRODUCTION

The adhesion of bacterial pathogens to surfaces of food handling, storage and processing equipment leads to cross-contamination of foods, which can be re-

sponsible of food-borne diseases and important economic losses (Bae *et al.,* 2012; Kuda *et al.,* 2016). Stainless steel is the most preferred material for fabricating food processing facilities and utensils such as containers, pipelines and working surfaces due to its high mechanical strength, resistance to corrosion and longevity (Kusumaningrum *et al.,* 2003; Casarin *et al.,* 2014). Previous studies have shown that *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus* are capable of adhering and form biofilms on stainless steel surfaces (Schlisselberg & Yaron, 2013; Ouali *et al.,* 2014; Casarin *et al.,* 2014; Kim *et al.,* 2016; Kuda *et al.,* 2016). It has been suggested that commonly used disinfectants in food processing environments including sodium hypochlorite, sodium hydroxide and benzalkonium chloride are capable of eradicating biofilms developed by aforementioned pathogens on a stainless steel surface to varying extent (Nam *et al.,* 2014; Chen *et al.,* 2015). Therefore, further investigations for development of concepts and the discovery of novel strong antimicrobial compounds to decrease the risk of contamination in food sector is of unquestionable interest. On the other hand, due to recent increasing consumer concerns about the toxic residues of classical synthetic sanitisers these research efforts must be shifted on natural alternatives (Ryu *et al.,* 2004; Raffaella *et al.,* 2017). Related to this topic, plant essential oils (EOs) and extracts have attracted research interest to control bacterial biofilms in food industries (Bazargani & Rohloff, 2016; Raffaella *et al.,* 2017). Potential antimicrobial effects against common food-borne pathogens has been shown for cinnamon (de Oliveira *et al.,* 2012), lav-

ender (Budzynska *et al.,* 2011), coriander and anise (Bazargani & Rohloff, 2016). The genus *Ziziphora clinopodioides* has been reported to be an effective aromatic plant with a wide range of medicinal effects such as appetitive, carminative, sedative, stomach tonic, expectorant and antiseptic (Shahbazi & Shavisi, 2016). The phytochemical studies conducted in all parts of *Z. clinopodioides* characterise the presence of thymol, ɣ-terpinene, carvacrol, linalool and 1,8-cineole as commonly predominant EO compounds (Behravan *et al.,* 2007; Aghajani *et al.,* 2008; Shahbazi & Shavisi, 2016). The genus *Mentha*, of *Lamiaceae* family, comprises about 25–30 species originating in Iran, which present a number of biological effects. It has several biological uses e.g. antimicrobial, antioxidant and antispasmodics, in good correlation with the high contents of phenolic compounds (Sandasi *et al.,* 2011).

Our previous studies indicated that *Z. clinopodioides* and *M. spicata* essential oils have strong antibacterial activity against common food-borne pathogenic bacteria in food models and *in vitro* condition (Shahbazi, 2015, Shahbazi *et al.,* 2016a,b; Shahbazi & Shavisi, 2016). However, a comparative study on the antibiofilm activity of *Z. clinopodioides* and *M. spicata* essential oils against common food-borne pathogenic bacteria has not been carried out so far. Therefore, the aim of the current study was to assess the effects of *Z. clinopodioides* and *M. spicata* essential oils against biofilms of six food-borne pathogens including *L. monocytogenes, S.* Typhimurium, *E. coli* O157:H7, *B. subtilis, B. cereus* and *S. aureus* developed on the surface of stainless steel coupons.

MATERIALS AND METHODS

Plant materials

The wild samples of ziziphore and spearmint were obtained from natural habitats in the Zagros region (Gilan-e-Gharb, Kermanshah, western Iran) during the full flowering period (March-July 2015). The plant materials were identified as *Z. clinopodioides* and *M. spicata* by Dr. Seyed Mohammad Masoumi, Faculty of Agriculture, Razi University, Kermanshah, Iran. The obtained plants were shade dried at room temperature for two weeks. The dried plants were exposed to hydro-distillation using a Clevenger-type apparatus for approximately 3.5 h. The isolated EOs were dried over anhydrous sodium sulfate (Na_2SO_4), transferred to sealed dark vials and stored under refrigerated condition (4 ± 1 ºC) until use.

GC–MS analysis of Ziziphora clinopodioides and Mentha spicata essential oils

Gas chromatography-mass spectrometry (GC-MS) analyses of EOs were performed on a Thermo Quest Finnigan apparatus fitted with HP-5MS 5% phenyl methylsiloxane capillary column (30 m length × 0.25 mm i.d. and 0.25 μm film thickness). Helium (purity: 99.99%; flow rate 1.2 mL/min and split ratio 1:20) was the carrier gas. The column temperature was initially programmed at 50 °C for 6 min and then gradually increased up to 265 °C at 2.5 °C/min. Finally, the temperature was increased to 280°C at 15 °C/min and held isothermally for 3 min. EOs analyses were also run on Thermo Quest Finningan coupled to mass spectrometer with the same analytical conditions indicated above. The MS was run using an ionisation energy of 70 eV. The volatile chemical compounds of the EO were identified by comparison among their retention indices, retention indices existed in database, standard mass spectral fragmentation pattern (Wiley/NBS Pak v.7, 2003), and US National Institute of Standards and Technology. The GC peak area normalisation of the three injections was expressed as mean percentage of the individual EO composition.

Bacterial strain, culture conditions and inoculum preparation

Six different pathogenic bacteria including *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), *B. cereus* (ATCC 11774), *L. monocytogenes* (ATCC 19118), *S.* Typhimurium (ATCC 14028) and *E. coli* O157:H7 (ATCC 10536) were purchased from the culture collection of the Iranian Research Organisation for Science and Technology (IROST), Tehran, Iran. All bacterial strains were cultured in Brain Heart Infusion broth (BHI; Merck, Germany) at 37 ºC for 24 h. The density of bacterial cultures needed for the antibacterial tests were examined using a spectrophotometer at 600 nm. The determination of inoculum dose (7 log CFU/mL) also was assessed using plate count on BHI agar.

Determination of minimum inhibitory concentrations (MICs) of essential oils

The minimum inhibitory concentrations (MICs) of EOs were determined according to the protocol of Shahbazi & Shavisi (2016) with minor modifications. Different concentrations of *Z. clinopodioides* essential oil (ZEO; 0.0001, 0.0002, 0.0003, 0.0004, 0.0005, 0.0006, 0.0007 and 0.0008, 0.0009, 0.001 μL/mL) and *M. spicata* essential oil (MEO; 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009 and 0.01 μl/mL) were constructed in BHI broth containing 1% (v/v) dimethyl sulfoxide (DMSO). The 96-well sterile micro-dilution plates with

U-bottom wells were prepared by pouring 180 µL of BHI broth containing different concentrations of ZEO and MEO, and 20 µL of the investigated microorganisms. The last row well containing 180 µL BHI broth without EOs and 20 µL of inoculum was used as parallel positive control. As a negative control, the same amount of BHI broth and 1% (v/v) dimethyl sulfoxide (DMSO) was also considered. Contents of each well were mixed on a plate shaker for 30 s and incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of the ZEO or MEO at which the microorganism did not demonstrate visible growth.

Stainless steel coupons

The flat stainless steel coupons (2×2×0.8 cm) as the experimental surface were prepared according to the method previously described by Raffaella *et al.* (2017) with slight modifications. Prior to use, the coupons were immersed in HCl 5N for 15 min, cleaned in a detergent solution and rinsed with distilled water. The washed stainless steel coupons were air dried, wrapped with aluminum foil and autoclaved at 121 °C for 15 min.

Biofilm development

Prepared sterile stainless steel coupons were individually immersed in 10 mL BHI broth containing each inoculum suspension targeting at a cell density of 7 log CFU/mL, placed in petri dishes and incubated at room temperature (25±1 °C) for 24 h. After incubation, the stainless coupons were aseptically removed with sterile forceps, washed with sterile phosphate buffered saline (PBS; a final concentration of 10 mM PO_4^{3-}, 137 mM NaCl and 2.7 mM KCl at pH 6.9) to remove unattached cells and dried for 120 min at room temperature in a bio safety cabinet. After

drying, each coupon was swabbed with sterile polyester-tipped swabs. Then, the swab was suspended into a tube containing 1 ml buffered peptone water and gently vortexed for 2 min to detach bacteria. After agitation, ten decimal dilutions of this suspension containing the de-attached bacteria of each treated coupon were prepared, spread on BHI agar and incubated at 37 ºC for 24 h (Poimenidou *et al.,* 2016; Raffaella *et al.,* 2017).

Moreover, biofilms of bacterial pathogens developed on stainless steel surfaces were analyzed for biomass production using crystal violet (CV) according to the previously method described by Raffaella *et al.* (2017). Briefly, each coupon with adherent bacteria was removed from petri dishes, washed with PBS and immersed in a new dish containing CV 0.1% (v/v) for 15 min. The coupons were washed with PBS again and air-dried. The remaining CV was dissolved in 85% ethanol for 15 min at room temperature and, finally, 200 µl from each sample was transferred to a 96-well plate for spectrophotometry at 570 nm. All experiments were performed in triplicate and each experiment were repeated three times.

Treatments of biofilms with Ziziphora clinopodioides and Mentha spicata essential oils

To determine the bactericidal effects of ZEO separately and in combination with MEO, the dried stainless steel coupons were dipped in 10 mL BHI broth containing each EO or a mixture of both at MIC concentration at room temperature (25 ± 1 ºC) for 1.5, 3 and 4.5 min. At each time point, the coupons were transferred into 5 mL of neutralising solution (3% polysorbate 80 v/v, 3% saponin w/v, 0.3% lecithin w/v) for 2 min and dried for 120 min at room temperature in a bio safety

cabinet. The logarithmic reduction (LR) was estimated from the following equation:

$$\log DP = \log (N/N_0) = (\log N) - (\log N_0);$$

where N and N_0 are initial adhered cells and remaining viable adhered cells following disinfection (CFU/mL) at times t and zero, respectively. Moreover, biomass removal activity of the antimicrobials against the bacterial biofilms developed on stainless steel coupons was measured as previously described.

Statistical analysis

All experiments were done in triplicate. The analysis was performed using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package. All data were subjected to one-way analysis of variance to determine the differences of samples. Significance level was considered $P < 0.05$ for all experimental data.

RESULTS

GC–MS analysis of Ziziphora clinopodioides and Mentha spicata essential oils

The chemical compositions of ZEO and MEO are presented in Tables 1 and 2, respectively.

Twenty-four compounds in the ZEO were identified, representing 99.65% of the total oil. The major compounds de-

Table 1. Composition of essential oil of *Ziziphora clinopodioides* identified by GC-MS

No.	Compound name	Composition %	Retention time (min.)
1	α-Thujene	0.26	11.33
2	α-Pinene	0.27	11.71
3	Camphene	0.13	12.61
4	β-Pinene	0.06	14.06
5	1-Octen-3-ol	0.08	14.32
6	Myrcene	0.51	14.62
7	α-Phellandrene	0.13	15.58
8	α-Terpinene	0.79	16.11
9	p-Cymene	4.86	16.62
10	Limonene	0.1	16.77
11	β-Phellandrene	0.11	16.89
12	γ-Terpinene	4.63	18.31
13	cis-Sabinene hydrate	0.07	19.02
14	Terpinolene	0.08	19.69
15	Linalool	0.13	20.5
16	Borneol	0.61	24.36
17	Terpinene-4-ol	0.48	24.7
18	α-Terpineol	0.08	25.49
19	Carvacrol, methyl ether	0.04	27.38
20	Thymol	19.51	29.61
21	Carvacrol	65.22	30.57
22	E-Caryophyllene	1.07	35.47
23	Spathulenol	0.12	42.10
24	Caryophyllene oxide	0.31	42.30
	Other	0.08	
	Total	99.65	

Table 2. Composition of essential oil of *Mentha spicata* identified by GC-MS

No.	Compound name	Composition %	Retention time (min.)
1	*β*-Myrcene	0.25	450
2	Limonene	11.50	509
3	γ-Terpinene	0.16	553
4	Menthone	1.01	703
5	Menthol	1	713
6	Terpinen-4-ol	0.99	720
7	*α*-Terpinol	0.31	737
8	Dihydrocarveol	0.22	742
9	*cis*-Dihydrocarveol	1.43	746
10	Dihydrocarvone	0.43	756
11	*trans*-Carveol	0.3	773
12	Carvone	78.76	819
13	Dihydrocarvyl acetate	0.57	906
14	L-carveol	0.32	946
15	*β*-Bourbonene	11.23	981
16	*trans*-Caryophyllene	1.04	1021
17	γ -Amorphene	0.21	1048
18	*α*-Amorphene	0.16	1058
	Others	0.11	–
	Total	100	

tected in the ZEO were carvacrol (65.22%), thymol (19.51%), *p*-cymene (4.86%) and *γ*-terpinene (4.63%).

As it can be seen in Table 2, the GC-MS analysis of MEO indicated the presence of eighteen compounds representing 99.89% of the total oil. The MEO was mainly composed from carvone (78.76%), limonene (11.50%) and β-bourbonene (11.23%).

Antibacterial activity of Ziziphora clinopodioides and Mentha spicata essential oils

The first part of the current study was conducted to investigate the *in vitro* antibacterial activity of ZEO and MEO against planktonic cells of common foodborne pathogenic bacteria including *S. aureus*, *B. subtilis*, *B. cereus*, *L. monocy-*

togenes, *S.* Typhimurium and *E. coli* O157:H7. Based on the results of the present study (Table 3), the antibacterial activity differed significantly between the two EOs (P<0.05). As it can be seen, the MIC values of ZEO and MEO against investigated microorganisms were 0.0003–0.0004 and 0.005 µL/mL, respectively.

Biofilm formation

The ability of *S.* Typhimurium, *E. coli* O157:H7, *L. monocytogenes, S. aureus, B. subtilis* and *B. cereus* to adhere and form biofilms on stainless steel surfaces was determined by CFU/cm^2 enumeration and biomass analysis (Table 4). The rank order of biofilm production were as follow: *S.* Typhimurium > *E. coli* O157:H7 > *S. aureus* > *L. monocytogenes* > *B. subtilis* > *B. cereus*.

Table 3. Minimum inhibitory concentrations (MICs) of *Ziziphora clinopodioides* and *Mentha spicata* essential oils against selected bacterial pathogens

Bacteria	MIC (μL/mL)		
	Z. clinopodioides	*M. spicata*	*Z. clinopodioides + M. spicata*
Staphylococcus aureus	0.0003	0.005	0.0002
Bacillus subtilis	0.0003	0.005	0.0002
Bacillus cereus	0.0003	0.005	0.0002
Listeria monocytogenes	0.0003	0.005	0.0002
Salmonella Typhimurium	0.0004	0.005	0.0003
Escherichia coli O157:H7	0.0004	0.005	0.0003

Table 4. Colony forming unit counts and biomass of bacterial biofilms formed on stainless steel coupons

| Bacteria	Plate count agar (CFU/cm^2)	Biomass analysis (OD)
Staphylococcus aureus	5.21±0.01[c]	1.61±0.01[c]
Bacillus subtilis	4.23±0.02[e]	1.38±0.01[e]
Bacillus cereus	4.17±0.01[f]	1.21±0.02[f]
Listeria monocytogenes	4.62±0.05[d]	1.41±0.03[d]
Salmonella Typhimurium	5.75±0.04[a]	1.86±0.01[a]
Escherichia coli O157:H7	5.31±0.01[b]	1.72±0.02[b]

Data represent the mean values obtained in three independent experiments performed in triplicate; [a-b] Different letters indicate significant differences among the different bacterial pathogens (P<0.05).

Effectiveness of Ziziphora clinopodioides and Mentha spicata essential oils on bacterial pathogen biofilms

The anti-biofilm effects of disinfectant solutions based on ZEO separately and in combination with MEO against *S. aureus*, *B. subtilis*, *B. cereus*, *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 on the stainless steel coupons, with contact times of 1.5, 3 and 4.5 min, are presented in Fig. 1A-1F. Based on our findings (Fig. 1A-1F), there was significant difference between the population of all investigated pathogens on the stainless steel coupons treated with ZEO and MEO (P<0.05). The anti-biofilm activity of the disinfectant solutions based on ZEO and MEO can be expressed as logarithmic reduction (LR) between initial adhered cells and remaining viable adhered cells following disinfection. Therefore, in all bacterial biofilms treated with the combination of ZEO and MEO LRs higher than 2 log CFU/cm^2 were observed.

The percentages of biofilm removal activities of ZEO and MEO on the stainless steel coupons with contact times of 1.5, 3 and 4.5 min are presented in Table 5. Based on our findings, ZEO showed significantly higher biofilm removal activity compared to MEO (P<0.05). Indeed, regarding the disinfectant solution based on ZEO, the highest biofilm removal activity was found for *B. cereus*, followed by *B. subtilis*, *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S.* Typhimurium, respectively. The order of bio-
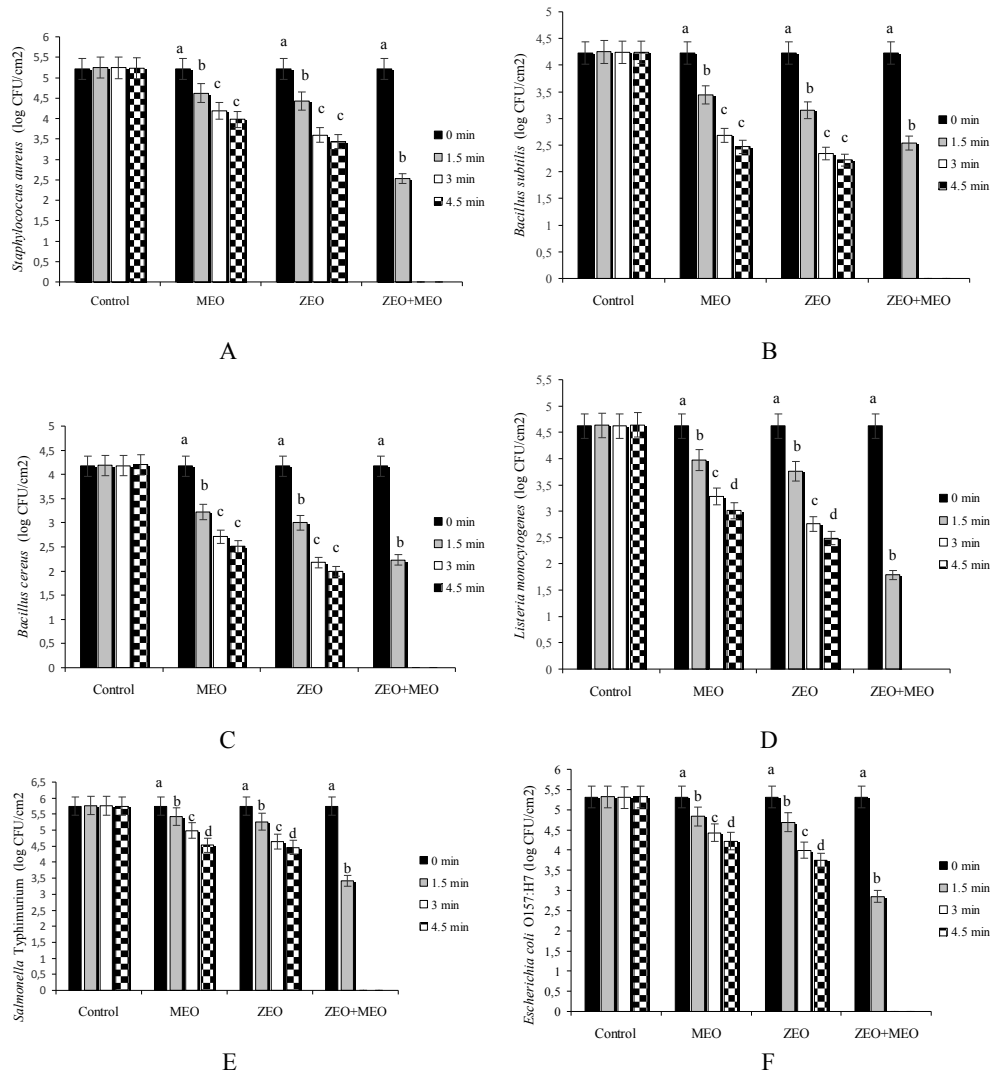
Fig. 1. Antibacterial activity of *Ziziphora clinopodioides* essential oil (ZEO) separately and in combination with *Mentha spicata* essential oil (MEO) against biofilms of *Staphylococcus aureus* (A), *Bacillus subtilis* (B), *Bacillus cereus* (C), *Listeria monocytogenes* (D), *Salmonella* Typhimurium (E) and *Escherichia coli* O157:H7 (F) developed on stainless steel coupons. Data represent the mean values obtained in three independent experiments performed in triplicate; [a-d] for each treatment, different letters indicate significant differences in the percentages of biofilm removal activities on the stainless steel coupons among contact times of 1.5, 3 and 4.5 min (P<0.05).

film removal activity of disinfectant solution based on MEO was: *B. cereus > B. subtilis > L. monocytogenes > S. aureus >*

E. coli O157:H7 > *S.* Typhimurium. Data analysis relative to the biofilm removal activity of the disinfectant solu-

Table 5. Percentage of bacterial biofilm removal by *Ziziphora clinopodioides* (ZEO) and *Mentha spicata* (MEO) essential oils alone and in combinations

Bacteria	Contact time (min)	Biofilm removal (%) of the essential oils (mean ± sd)		
		Z. clinopodioides	*M. spicata*	*Z. clinopodioides + M. spicata*
Staphylococcus aureus	1.5	14.97 (±0.3)[Bc]	11.32 (±0.2)[Cc]	51.43 (±0.1)[Ab]
	3.0	31.09 (±0.3)[Bb]	19.57 (±0.3)[Cb]	100 (±0.0)[Aa]
	4.5	33.97 (±0.1)[Ba]	23.60 (±0.6)[Ca]	100 (±0.0)[Aa]
Bacillus subtilis	1.5	25.29 (±0.2)[Bc]	18.67 (±0.1)[Cc]	39.95 (±0.1)[Ab]
	3.0	44.68 (±0.1)[Bb]	36.64 (±0.8)[Cb]	100 (±0.0)[Aa]
	4.5	47.51 (±0.4)[Ba]	41.60 (±0.2)[Ca]	100 (±0.0)[Aa]
Bacillus cereus	1.5	28.05 (±0.3)[Bc]	22.78 (±0.3)[Cc]	46.52 (±0.2)[Ab]
	3.0	47.72 (±0.4)[Bb]	35.01 (±0.5)[Cb]	100 (±0.0)[Aa]
	4.5	52.03 (±0.1)[Ba]	42.80 (±0.1)[Ca]	100 (±0.0)[Aa]
Listeria monocytogenes	1.5	18.61 (±0.5)[Bc]	14.06 (±0.2)[Cc]	61.25 (±0.7)[Ab]
	3.0	40.25 (±0.1)[Bb]	29.00 (±0.5)[Cb]	100 (±0.0)[Aa]
	4.5	46.10 (±0.2)[Ba]	34.84 (±0.6)[Ca]	100 (±0.0)[Aa]
Salmonella Typhimurium	1.5	8.52 (±0.2)[Bc]	5.73 (±0.2)[Cc]	40.52 (±0.4)[Ab]
	3.0	19.13 (±0.1)[Bb]	13.21 (±0.1)[Cb]	100 (±0.0)[Aa]
	4.5	22.43 (±0.1)[Ba]	21.21 (±0.2)[Ca]	100 (±0.0)[Aa]
Escherichia coli O157:H7	1.5	11.67 (±0.1)[Bc]	9.03 (±0.2)[Cc]	46.32 (±0.1)[Ab]
	3.0	24.85 (±0.1)[Bb]	16.57 (±0.5)[Cb]	100 (±0.0)[Aa]
	4.5	29.56 (±0.4)[Ba]	20.51 (±0.4)[Ca]	100 (±0.0)[Aa]

Data represent the mean values obtained in three independent experiments performed in triplicate. [A-C] Means with different capital letters in the same row are significantly different ($P < 0.05$). [a-c] For each bacterium, means with different lowercase letters in the same column are significantly different ($P < 0.05$).

tion based on the combination of ZEO and MEO showed significant biofilm removal ability of the disinfectant solution against all bacteria, with percentages higher than those of obtained with each single EOs (P<0.05).

With respect to the contact times utilized, the number of surface-adhered cells was significantly decreased after 1.5, 3 and 4.5 min of exposure to the disinfectant solutions based on ZEO separately and in combination with MEO (P<0.05). The remained bacterial population after 3 and 4.5 min of contact were significantly lower than those that remained after 1.5 min of treatment (P<0.05). Moreover, the most drastic reduction of bacterial population was observed after 3 and 4.5 min treatment with the disinfectant solutions based on EOs (P<0.05).

DISCUSSION

The high percentages of carvacrol and thymol identified *Z. clinopodioides* as a good source of these valuable compounds. These two compounds together constitute 84.73% of the total oil composition of *Z. clinopodioides.* Our results agreed with another study which reported carvacrol and thymol as the major compounds (72.5%) of ZEO (Aghajani *et al.,* 2008). However, ZEO from different cultivars and locations were found to contain pulegone, terpineol, methyl acetate, isoneomenthol and 1, 8-cineole (Morteza-Semnani *et al.,* 2005; Behravan *et al.,* 2007; Sonboli *et al.,* 2010; Ma *et al.,* 2016). The composition contents of MEO in the current study is in good agreement with previous reports (Mahboubi & Haghi, 2008; Govindarajan *et al.,* 2012). Generally, the chemical compositions differences of EOs may be explained with variability of plant species, geographical,

climate and seasonal conditions (Shahbazi & Shavisi, 2016).

Based on the results of the present study (Table 3), the antibacterial activity differed significantly between the two EOs (P<0.05). The significant difference in the antimicrobial action of EOs is related to the variability in the nature and concentration of main groups of chemical compositions (Kakaei & Shahbazi, 2016). The antibacterial mechanism of ZEO was suggested to be due to the -OH groups located at the *meta* and *ortho* positions in thymol and carvacrol, respectively, which are able to interact with cytoplasmic membrane of bacterial cells. It is believed that this phenomenon can disrupt the bacterial phospholipid membrane resulting in inhibition of electron transport, protein translocation, phosphorylation and other enzymatic activity and thus leading to cell death (Gyawali & Ibrahim, 2014). Mahboubi & Haghi (2008) have indicated that carvone as the major compounds of MEO caused extensive damage of phospholipid bilayer structure, reduced the pH gradient across the bacterial membrane and subsequently inhibited the bacterial barrier function. However, it has been reported that a number of compounds in relatively low concentrations such as *p*-cymene, *ɣ*-terpinene, *α*-pinene, *β*-bourbonene, *α*-terpinol and *α*-terpinene could also be expected to enhance the antimicrobial activity of the EOs (Lv *et al.,* 2011). As shown in Table 3, it is clear that Gram-positive bacteria (*S. aureus*, *B. subtilis, B. cereus* and *L. monocytogenes*) were more sensitive than Gram-negative bacteria (*S.* Typhimurium and *E. coli* O157:H7). The most probable reason for the differences in microorganism's susceptibility to EOs might be attributed to the outer cytoplasmic membrane surrounding the thin peptidoglycan structure

of Gram-negative bacteria, which restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering (Bazargani & Rohloff, 2016).

In this study, the rank order of biofilm production was as followed: *S.* Typhimurium > *E. coli* O157:H7 > *S. aureus* > *L. monocytogenes* > *B. subtilis* > *B. cereus*. The probable reason behind considerable differences in biofilm formation by investigated bacteria can be attributed to differences in mobility, inherent cell surface characteristics and production of capsular polysaccharide/adhesion among the microbial species (Rivas *et al.,* 2010). According to the literature review performed by Kusumaningrum *et al.* (2003), the presence of extracellular appendages such as flagella and pili, interactions involved in cell–cell communication and production of extracellular polymeric substances are the most important factors which affect the behaviour of bacterial cells on stainless steel surfaces. Most of findings reported from other studies are not comparable since different methodology and environmental conditions such as room temperature and type of bacterial strains can remarkably influence the recovery ratios of microorganisms on stainless steel surfaces (Perez-Rodriguez *et al.,* 2013). However, Ryu *et al.* (2004) reported that approximately 5.30 log CFU/cm^2 of *E. coli* O157:H7 were found adherent on a stainless steel after 24 h incubation at room temperature with 7 log CFU/mL bacterial suspension, which is in good agreement with our findings. In another study, Bae *et al.* (2012) demonstrated that *S.* Typhimurium could adhere to a stainless steel surface at a rate of 3 log CFU/cm^2, considering an initial contamination of 5 log CFU/mL. de Oliveira *et al.* (2010) inoculated 8 log CFU/mL of *E. coli* and *L. monocytogenes* onto stain-

less steel surfaces and formed biofilms, followed by incubation at room temperature for 24 h. According to their results, 4.86 and 5.64 log CFU/cm^2 of aforementioned bacteria in biofilms were recovered.

Based on our findings (Fig. 1A-1F), there was significant difference between the population of all investigated pathogens on the stainless steel coupons treated with ZEO and MEO (P<0.05). Differences in the antibacterial effects between ZEO and MEO can be attributed to the nature and concentration of their active chemical compositions (Vázquez-Sánchez *et al.,* 2015).

Despite the lack of any conclusive data about the anti-biofilm activity of ZEO, some studies have showed its powerful antibacterial activity against several important food-borne pathogens including *B. subtilis*, *B. cereus*, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 (Aghajani *et al.,* 2008; Kakaei & Shahbazi, 2016; Shahbazi & Shavisi, 2016; Mohebi & Shahbazi, 2017). Moreover, the disinfectant solution based on MEO could moderately inhibit the growth of sessile cells of all tested strains (Fig. 1A-1F). Valeriano *et al.* (2012) examined the anti-biofilm activity of MEO against *S. enterica* serotype Enteritidis on stainless steel surface after 10, 20 and 40 min. They reported that ten minutes of MEO disinfectant solution contact significantly reduced adhered bacterial population (P<0.05). The same results were also reported in the study of Sandasi *et al.* (2011) who investigated the effect of MEO against biofilm formation by *Pseudomonas aeruginosa* and *Candida albicans*.

As presented in our findings, the most drastic reduction of bacterial population was observed after 3 and 4.5 min treat-

ment with the disinfectant solutions based on EOs (P<0.05). Since active groups of EOs such as -OH can interact with the bacterial cell membrane and subsequently cause the leakage of cellular components, it can be concluded that the long contact time between the microorganism and the disinfectant solutions based on EOs lead to extensive loss of cell contents or critical molecules and ions (Valeriano *et al.,* 2012; Gyawali & Ibrahim, 2014). Despite the fact that some loss in the amount of cell content is tolerated by the bacteria without loss of their viability, extensive loss of cell content or essential molecules and ions will lead to bacterial cell death (de Oliveira *et al.,* 2010). The results of the present study demonstrated that treatments of stainless steel surfaces for 3 and 4.5 min with the disinfectant solution based on the combination of ZEO and MEO was sufficient for total removal biofilm formed by all tested pathogens (Table 5). Our *in vitro* previous study has proved that the combination of ZEO and MEO displayed a synergistic activity against *B. subtilis*, *B. cereus*, *S. aureus*, *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 (Shahbazi & Shavisi, 2016). It has been shown that synergistic effects of EOs affect microbial cells by various antimicrobial mechanisms, including sequential inhibition of a common biochemical pathway, disrupting of protective enzymes and increasing the number and size of pores created in phospholipid bilayer of the cell membrane (Mohebi & Shahbazi, 2017). In accordance with our findings, de Oliveira *et al.* (2010) reported that the disinfectant solutions based on combination of *Cymbopogon citratus* and *Cymbopogon nardus* EOs could completely remove biofilm of *L. monocytogenes* on the stainless steel surface. The American Public Health Association

(APHA) has indicated that a standard physical or chemical disinfectant must be capable of eradicating pathogenic bacteria and decreasing the number of deteriorating mesophilic aerobic microorganisms to acceptable levels of 0.3 log CFU/cm^2 or 2 log CFU/utensil at the end of the disinfection process (Anonymous, 1992). Therefore, the disinfectant solution based on the combination of ZEO and MEO, that fulfilled the proposed recommendation, can be used as an appropriate natural disinfectant to control biofilm formation in food processing facilities and utensils.

CONCLUSION

The results of the present study indicated that ZEO and MEO presented a good source of bioactive compounds such as carvacrol, thymol, carvone, limonene and β-bourbonene. These substances were mostly found in the plant species, but further research is required to identify the phytochemical compounds of the MEO and ZEO responsible for their antibiofilm effects. To our knowledge, this is the first time the antibiofilm activity of ZEO separately and in combination with MEO has been reported against biofilms formed by aforementioned pathogens. Based on our findings, ZEO and MEO are new alternative compounds to sanitise industrial stainless steel surfaces contaminated with *S. aureus*, *B. subtilis*, *B. cereus*, *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7.

REFERENCES

Aghajani, Z., F. Assadian, S. Masoudi, F. Chalabian, A. Esmaeili, M. Tabatabaei-Anaraki & A. Rustaiyan, 2008. Chemical composition and *in vitro* antibacterial activities of the oil of *Ziziphora clinopodioides* and *Z. capitata* subsp. *capitata* from Iran. *Chemistry of Natural Compounds,* **44**, 387–389.

Anonymous, 1992. Compendium of methods for the microbiological examination of foods. Hanover: EPS Group.

Bae, Y. M., S. Y. Baek & S. Y. Lee, 2012. Resistance of pathogenic bacteria on the surface of stainless steel depending on attachment form and efficacy of chemical sanitizers. *International Journal of Food Microbiology,* **153**, 465–473.

Bazargani, M. M. & J. Rohloff, 2016. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. *Food Control,* **61**, 156–164.

Behravan, J., M. Ramezani, M. Hassanzadeh, M. Eskandari, J. Kasaian & Z. Sabeti, 2007. Composition, antimycotic and antibacterial activity of *Ziziphora clinopodioides* Lam. essential oil from Iran. *Journal of Essential Oil Bearing Plants,* **10**, 339–345.

Budzynska, A., M. Wieckowska-Szakiel, B. Sadowska, D. Kalemba & B. Rózalska, 2011. Antibiofilm activity of selected plant essential oils and their major components. *Polish Journal of Microbiology,* **60**, 35–41.

Casarin, L. S., A. Brandelli, F. de Oliveira Casarin, P. A. Soave, C. H. Wanke & E. C. Tondo, 2014. Adhesion of *Salmonella Enteritidis* and *Listeria monocytogenes* on stainless steel welds. *International Journal of Food Microbiology,* **191**, 103–108.

Chen, D., T. Zhao & M. P. Doyle, 2015. Control of pathogens in biofilms on the surface of stainless steel by levulinic acid plus sodium dodecyl sulfate. *International Journal of Food Microbiology,* **207**, 1–7.

de Oliveira, M. M. M., D. F. Brugnera, M. das Graças Cardoso, E. Alves & R. H. Piccoli, 2010. Disinfectant action of *Cymbopogon* sp. essential oils in different phases of biofilm formation by *Listeria monocytogenes* on stainless steel surface. *Food Control,* **21**, 549–553.

de Oliveira, M. M. M., D. F. Brugnera, J. A. do Nascimento, N. N. Batista & R. H. Piccoli, 2012. Cinnamon essential oil and cinnamaldehyde in the control of bacterial biofilms formed on stainless steel surfaces. *European Food Research and Technology,* **234**, 821–832.

Govindarajan, M., R. Sivakumar, M. Rajeswari & K. Yogalakshmi, 2012. Chemical composition and larvicidal activity of essential oil from *Mentha spicata* (Linn.) against three mosquito species. *Parasitology Research,* **110**, 2023–2032.

Gyawali, R. & S. A. Ibrahim, 2014. Natural products as antimicrobial agents. *Food Control,* **46**, 412–429.

Kakaei, S. & Y. Shahbazi, 2016. Effect of chitosan-gelatin film incorporated with ethanolic red grape seed extract and *Ziziphora clinopodioides* essential oil on survival of *Listeria monocytogenes* and chemical, microbial and sensory properties of minced trout fillet. *LWT-Food Science and Technology,* **72**, 432–438.

Kim, M., S. Y. Park & S. D. Ha, 2016. Synergistic effect of a combination of ultraviolet–C irradiation and sodium hypo-chlorite to reduce *Listeria monocytogenes* biofilms on stainless steel and eggshell surfaces. *Food Control,* **70**, 103–109.

Kuda, T., T. Koyanagi, G. Shibata, H. Takahashi & B. Kimura, 2016. Effect of carrot residue on the desiccation and disinfectant resistances of food related pathogens adhered to a stainless steel surfaces. *LWT-Food Science and Techno-logy,* **74**, 251–254.

Kusumaningrum, H., G. Riboldi, W. Hazeleger & R. Beumer, 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *Interna-*

tional Journal of Food Microbiology, **85**, 227–236.

Lv, F., H. Liang, Q. Yuan & C. Li, 2011. *In vitro* antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International,* **44**, 3057–3064.

Ma, B.X., X. Q. Ban, J. S. He, B. Huang, H. Zeng, J. Tian, Y. X. Chen & Y. W. Wang, 2016. Antifungal activity of *Ziziphora clinopodioides* Lam. essential oil against *Sclerotinia sclerotiorum* on rapeseed plants (*Brassica campestris* L.). *Crop Protection,* **89**, 289–295.

Mahboubi, M. & G. Haghi, 2008. Antimicrobial activity and chemical composition of *Mentha pulegium* L. essential oil. *Journal of Ethnopharmacology,* **119**, 325–327.

Mohebi, E. & Y. Shahbazi, 2017. Application of chitosan and gelatin based active packaging films for peeled shrimp preservation: A novel functional wrapping design. *LWT-Food Science and Technology,* **76, Part A**, 108–116.

Morteza-Semnani, K., M. Saeedi & G. Eslami, 2005. Essential oil composition of *Ziziphora clinopodioides* Lam. from Iran. *Journal of Essential Oil Bearing Plants,* **8**, 208–212.

Nam, H., H. S. Seo, J. Bang, H. Kim, L. R. Beuchat & J. H. Ryu, 2014. Efficacy of gaseous chlorine dioxide in inactivating *Bacillus cereus* spores attached to and in a biofilm on stainless steel. *International Journal of Food Microbiology,* **188**, 122–127.

Ouali, F. A., I. Al Kassaa, B. Cudennec, M. Abdallah, F. Bendali, D. Sadoun, N. E. Chihib & D. Drider, 2014. Identification of lactobacilli with inhibitory effect on biofilm formation by pathogenic bacteria on stainless steel surfaces. *International Journal of Food Microbiology,* **191**, 116–124.

Perez-Rodriguez, F., G. Posada-Izquierdo, A. Valero, R. García-Gimeno & G. Zurera, 2013. Modelling survival kinetics of *Staphylococcus aureus* and *Escherichia coli* O157:H7 on stainless steel surfaces soiled with different substrates under static conditions of temperature and relative humidity. *Food Microbiology,* **33**, 197–204.

Poimenidou, S. V., M. Chrysadakou, A. Tzakoniati, V. C. Bikouli, G. J. Nychas & P. Skandamis, N. 2016. Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and poly-styrene materials and resistance to peracetic acid and quaternary ammonium compounds. *International Journal of Food Microbiology,* **237**, 164–171.

Raffaella, C., L. Casettari, L. Fagioli, M. Cespi, G. Bonacucina & W. Baffone, 2017. Activity of essential oil-based mic-roemulsions against *Staphylococcus aureus* biofilms developed on stainless steel surface in different culture media and growth conditions. *International Journal of Food Microbiology,* **241**, 132–140.

Rivas, L., M. J. McDonnell, C. M. Burgess, M. O'Brien, A. Navarro-Villa, S. Fanning & G. Duffy, 2010. Inhibition of verocyto-toxigenic *Escherichia coli* in model broth and rumen systems by carvacrol and thymol. *International Journal of Food Microbiology*, **139**, 70–78.

Ryu, J. H., H. Kim, J. Frank & L. Beuchat, 2004. Attachment and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. *Letters in Applied Microbiology,* **39**, 359–362.

Sandasi, M., C. Leonard, S. Van Vuuren & A. Viljoen, 2011. Peppermint (*Mentha piperita*) inhibits microbial biofilms *in vitro*. *South African Journal of Botany,* **77**, 80–85.

Schlisselberg, D. B. & S. Yaron, 2013. The effects of stainless steel finish on *Salmonella* Typhimurium attachment, bio-film formation and sensitivity to chlorine. *Food Microbiology,* **35**, 65–72.

Shahbazi, Y., 2015. Chemical composition and *in vitro* antibacterial activity of *Mentha spicata* essential oil against common food-borne pathogenic bacteria. *Journal of Pathogens,* 2015, 916305, http://doi.org/10.1155/2015/916305**.**

Shahbazi, Y. & N. Shavisi, 2016. Interactions of *Ziziphora clinopodioides* and *Mentha spicata* essential oils with chitosan and ciprofloxacin against common food-related pathogens. *LWT-Food Science and Technology,* **71**, 364–369.

Shahbazi, Y., N. Shavisi & E. Mohebi, 2016a. Effects of *Ziziphora clinopodioides* essential oil and nisin, both separately and in combination, to extend shelf life and control *Escherichia coli* O157:H7 and *Staphylococcus aureus* in raw beef patty during refrigerated storage. *Journal of Food Safety,* **36**, 227–236.

Shahbazi, Y., N. Shavisi & E. Mohebi, 2016b. Potential application of *Ziziphora clinopodioides* essential oil and nisin as natural preservatives against *Bacillus cereus* and *Escherichia coli* O157:H7 in commercial barley soup. *Journal of Food Safety,* **36**, 435–441.

Sonboli, A., M. Atri, & S. Shafiei, 2010. Intraspecific variability of the essential oil of *Ziziphora clinopodioides* ssp. *rigida* from Iran. *Chemistry & Biodiversity,* **7**, 1784–1789.

Valeriano, C., T. L. C. De Oliveira, S. M. De Carvalho, M. das Graças Cardoso, E. Alves & R. H. Piccoli, 2012. The sanitizing action of essential oil-based solutions against *Salmonella enterica* serotype *Enteritidis* S64 biofilm formation on AISI 304 stainless steel. *Food Control,* **25**, 673–677.

Vázquez-Sánchez, D., M. L. Cabo & J. J. Rodríguez-Herrera, 2015. Antimicrobial activity of essential oils against *Staphylococcus aureus* biofilms. *Food Science and Technology International,* **21**, 559–570.

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