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Edible films based on chitosan-flaxseed mucilage: *in vitro* antimicrobial and antioxidant properties and their application on survival of food-borne pathogenic bacteria in raw minced trout fillets

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ABSTRACT

Various natural oils/extracts and their constituents incorporated into biopolymer-based edible films as a promising technology with the knowledge that these compounds have been able to reduce microbial growth and chemical changes of packed foodstuffs. The objective of this study was to evaluate the effect of incorporation of *Ziziphora clinopodioides* essential oil (ZEO; 0, 0.25 and 0.5%) and sesame oil (SO; 0, 0.5 and 0.75%) into chitosan-flaxseed mucilage (CH-FM) film against *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 *in vitro* condition and raw minced trout fillets during refrigerated condition. The *in vitro* antibacterial and antioxidant properties of CH-FM films were evaluated using agar disk diffusion method and free radical scavenging activity assay, respectively. The most important constituents of ZEO were found to be carvacrol (65.22%), thymol (19.51%), ɣ-terpinene (4.63%) and *p*-cymene (4.86%). The lowest and highest antimicrobial effect against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium* were found for CH-FM films enriched with SO 0.5% (0.98-1.24 mm) and ZEO 0.5% + SO 0.75% (5.01-6.25 mm), respectively. The antioxidant property of CH-FM based films were found to be ranged 5.45% ± 0.04-37% ± 0.45. In treated trout fillets, the counts of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. typhimurium* were 1.54-4.18, 0.34-3.35, 0.29-1.45 and 0.19-1.27 log CFU/g significantly lower than control groups after two weeks of refrigerated storage, respectively. The designated films had good antibacterial effect against some food borne pathogenic bacteria including *L. monocytogenes*, *S. aureus*, *S. typhimurium* and *E. coli* O157:H7 in raw rainbow trout fillets.

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Introduction

Raw fish and fishery products are perishable to microbial and chemical spoilage during long period of refrigerated/frozen storage condition (1). Because fish fillets have high water activity, abundant nutrients (e.g. nitrogen compounds, unsaturated fatty acids, fermentable carbohydrates and minerals) and appropriate pH (2), it can be considered as an ideal culture medium for the growth of many spoilage and food-borne pathogenic microorganisms such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Bacillus subtilis*, *B. cereus*, Enterobacteriaceae family, *Pseudomonas* spp., *P. fluorescens* and *Shewanella putrefaciens* (3). It was reported that the growth of microbial spoilage on the surface of the fresh products change the main organoleptic properties including odor, color, taste and other sensory characteristics (4). In developed countries, 30-40% of people annually suffer from food-borne diseases especially *S. aureus*, *E. coli* O157:H7 and *S. typhimurium* (3). Nowadays, shelf life quality and safety of the wide variety of raw foodstuffs such as peeled shrimp, beef meat and rainbow trout fillets are two important concerns for the food industry (5).

In the last decades, biopolymer-based edible films and coatings are considered as an efficient and eco-friendly way to extend the shelf-life and safety of vulnerable food products such as minced trout fish fillets (6). Natural polysaccharides are excellent candidates as base ingredients of both edible films and coatings (7). Among them, chitosan (CH) is one of the most abundant renewable polymers used in the medical, food, agricultural and chemical industries mainly due to its unique properties including intrinsic wound-healing, antioxidant and antimicrobial properties, hemostatic activity, film-forming ability, biodegradability, biocompatibility, availability and non-toxicity (8). Of the biodegradable polymers, flaxseed mucilage (FM) is also one of the most promising materials due to its ready availability, low cost and food compatibility (9). It occurs mainly at the outermost layer of flaxseed hulls and composed around 8-12% of seed dry mass and therefore it is water-soluble and easily produce when seed is soaked in water (10).

Interestingly, various natural oils/extracts and their constituents incorporated into biopolymer-based edible films as a promising technology with the knowledge that these compounds has been able to reduce microbial

growth and chemical changes of packed foodstuffs (1,2,5,6). *Ziziphora clinopodioides* is one of the most common used spice in dairy and meat products in western provinces of Iran. Several studies have reported the antibacterial, antifungal and antioxidant effects of *Z. clinopodioides* essential oil (ZEO) in *vitro* and food models (minced beef meat, peeled shrimp, minced trout fillets and silver carp fillets) (1,11-14). Sesame (*Sesamum indicum* L.) is a nutritive edible oil crop for humans and has a long history of cultivation throughout the world. Sesame oil (SO) has abundant, pleasant and significant aroma and it has been considered as an effective antimicrobial agent against some foodborne pathogens (15,16). Based on our knowledge, there was no published data in literature review about antimicrobial properties of CH-FM films containing ZEO and SO in food models and *in vitro* condition. Therefore, the objective of this study was to evaluate the effect of incorporation of ZEO and SO into CH-FM film against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium in vitro* condition and raw minced trout fillets during refrigerated condition.

Materials and methods

Isolation of essential oil

The *Z. clinopodioides* plant was obtained from the Gilan-e-Gharb, Kermanshah, Iran (17). Authentications of the plant was conducted by Dr. Seyed Mohammad Masoumi (Faculty of Agriculture, Razi University, Kermanshah, Iran). Representative vouchers specimen (No. 6816) deposited in the herbarium of the Research Center of Natural Resources of Tehran, Iran. The isolation of the essential oil was performed using Clevenger-type apparatus according to the standard technique (18). For this purpose, 100 g of the fine powdered *Z. clinopodioides* plant was subjected to the hydro-distillation for until full recovery essential oil (3 h). The essential oil on top of the distillate water was collected using a micro-pipette without adding any solvent in a sealed bottle. The collected volatile oil was dehydrated with anhydrous sodium sulfate (0.5 g) (Merck, Darmstadt, Germany) and then filtered through a 0.22 µm filter (Millipore™, Bedford MA, USA) and stored at 4 ± 1˚C for further analysis. Fresh SO was obtained from a specialized local market, Kermanshah, Iran. All analytical materials and culture media were obtained from Sigma-Aldrich and Merck companies, Germany. The FM was purchased from a specialized local market (Kermanshah, Iran) and authenticated by Dr. Seyed Mohammad Masoumi.

Gas chromatography-mass spectrometry (GC–MS) analysis of Ziziphora clinopodioides essential oil

Gas chromatography-mass spectrometry (GC-MS) analysis of ZEO was performed on a Thermo Quest Finningan 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. ZEO analysis was also run on Thermo Quest Finningan, UK coupled to mass spectrometer with the same analytical conditions indicated above. The MS was run in the electron ionization mode, using an ionization energy of 70 eV.

Extraction of flaxseed mucilage

The FG was extracted from whole raw flaxseed using the previously published method by Kaushik et al., (9) with slight modification. Accordingly, the flaxseeds were washed, soaked in distilled water at ratio of 1:25 w/v and heated at 55 ± 1 ºC with continuous and gentle stirring over a magnetic hot plate for 3 h. The soaked seeds were removed, filtered via triplet layers of cheese cloth and the water containing the dissolved mucilage was centrifuged using lab homogenizer (HG-15D, Wise Tis, Korea) at 12000 rpm for 25 min and treated with hexane to precipitate the mucilage. The precipitated mucilage was collected by centrifuging (Sigma, Osterode am Harz, Germany) at 12000 rpm for 25 min and freeze dried for further use.

Preparation of films based on flaxseed mucilage-chitosan

2 g CH was dissolved in 1% acetic acid (100 ml) at room temperature for approximately 3 h. 2 g FM was also gradually added into 100 ml distilled water under stirring at room temperature for 2 h. After that, two solutions were mixed at ratio of 50:50 and stirred for another 1 h. Glycerol as a plasticizer was added at 0.75 ml/g (with respect to the amount of polysaccharide) into the solution. Tween 80 as an emulsifier was incorporated to a level of 0.25 ml/100 ml of the emulsion. ZEO (0, 0.25 and 0.5%) and SO (0, 0.5 and 0.75%) were incorporated into the viscose CH-FM solution and the mixture was homogenized at 1300 × g for 1 min. All prepared solutions were poured into 12 cm petri dish and dried at room temperature for 48 h (9,19).

Preparation of bacterial strains

S. aureus (ATCC 6538), *L. monocytogenes* (ATCC 19118), *E. coli* O157:H7 (ATCC 10536) and *S. typhimurium* (ATCC 14028) were supplied from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The bacterial strains were cultured in Brain Heart Infusion broth (BHI) at 37 ºC overnight and immediately used to make appropriate inoculum dose (7 log CFU/ml) in BHI broth for further experiment (14).

In vitro antibacterial and antioxidant activities of films based on flaxseed mucilage-chitosan

The *in-vitro* antibacterial property of FM-CH films was evaluated using agar disk diffusion (8). Briefly, amount of

15 ml molted BHI agar was cast into 9 cm petri dishes and thereafter 0.1 ml of each bacterial suspension individually was cultured. The designated films (7 mm diameter) were put on the surface of inoculated BHI agar. After incubation overnight at 37 ˚C, the diameter of the inhibition zones was determined.

The antioxidant property of films was determined based on the previously published method (free radical scavenging activity assay (DPPH method)) by Siripatrawan *et al.,* (20). Briefly, 25 mg of each film was added into 3 ml of distilled water, and then a 2.8 ml of film extract solution was mixed with 0.2 ml of 1 mM methanolic solution of DPPH. The mixture was shaken and maintained at room temperature in the dark for 30 min. The absorbance was measured at 517 nm. Then, the percentage of DPPH radical scavenging activity was calculated as follows: DPPH scavenging effect (%) = $((Abs_{DPPH} - Abs_{film\ extract})/Abs_{DPPH}) \times 100$.

Preparation of raw minced trout fillets

Raw rainbow trout fillets were obtained from a local market in Kermanshah, Iran and immediately transferred to the laboratory under refrigerated condition within 45 min. The fillets were minced with a sterile steel meat grinder. It was divided into portions of 100 g, 7 log CFU/g bacterial suspensions were inoculated and then upper and bottom surfaces of the samples were covered with the prepared films. All packed samples were put in sterile stomacher bags and stored at refrigerated condition for two weeks. In this experiment, minced trout fillets without CH-FM film was considered as a control group.

Bacterial enumeration

For each day, 25 g of each designated group was homogenized with 225 ml of 0.1% peptone water for 1 min in a stomacher at room temperature, diluted using ten-fold serial dilution for plating onto Palcam *listeria* selective agar (*L. monocytogenes*), Eosin methylene blue agar (*E. coli* O157:H7), *Salmonella Shigella* agar (*S. typhimurium*) and Baird Parker agar (*S. aureus*) (3).

Statistical analysis

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

Results

GC–MS analysis of Ziziphora clinopodioides essential oil

The results of GC-MS analysis of ZEO are presented in Table 1 and Fig. 1. It was found that the oxygenated monoterpenes and monoterpene hydrocarbons were the major groups of chemical compositions in ZEO. The most important constituents of ZEO were found to be Carvacrol (65.22%), thymol (19.51%), *γ*-terpinene (4.63%) and *p*-cymene (4.86%).

Table 1 Essential oil 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	*p*-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	*cis*-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	*E*-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

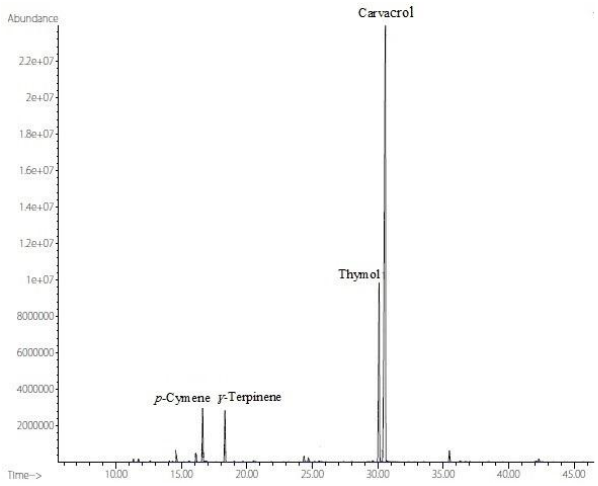

Figure 1 GC-MS profile of Z. clinopodioides essential oil

In vitro antimicrobial and antioxidant properties of films based on flaxseed mucilage-chitosan

Our findings indicated that the straight CH-FM film had inhibitory effect against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium*, while growth inhibitory zone was not determined (Table 2). The lowest and highest antimicrobial effect against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium* were found for CH-FM films enriched with SO 0.5% (0.98-1.24 mm) and ZEO 0.5% + SO 0.75% (5.01-6.25 mm), respectively.

Antioxidant property of prepared films based on CH-FM are presented in Table 3. As it can be seen, the straight CH-FM film showed a little antioxidant activity (5.45% ± 0.04). The antioxidant property of CH-FM based films containing ZEO increased up to 37% ± 0.45.

Survival of food-borne pathogenic bacteria in raw minced trout fillets

The results of survival of inoculated *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. typhimurium* in raw minced trout fillets are presented in Fig. 2a-d, respectively. In control group, the initial count of 5 log CFU/g of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S. typhimurium* reached 6.63, 3.35, 4.28 and 4.42 log CFU/g at the end of monitored study, respectively. In treated groups, the counts of aforementioned pathogens were 1.54-4.18, 0.34- 3.35, 0.29-1.45 and 0.19-1.27 log CFU/g significantly lower than control groups after two weeks of refrigerated storage, respectively ($P < 0.05$). Intrinsically, the growth of *S. aureus* in ZEO 0.25% + SO 0.5%, ZEO 0.25% + SO 0.75%, ZEO 0.5% + SO 0.5% and ZEO 0.5% + SO 0.75% completely inhibited after 12, 12, 14 and 14 days of refrigerated storage (Fig. 2b). There were no significant differences among groups containing SO 0.5 and 0.75% combined with ZEO 0.25 and 0.5%.

Discussion

In the present study, the most important constituents of ZEO were found to be carvacrol (65.22%), thymol (19.51%), *ɣ*-terpinene (4.63%) and *p*-cymene (4.86%). Aghajani et al., (11) and Shahbazi et al., (21) also found that the main chemical composition of ZEO were carvacrol and thymol, evidenced by 53.6-74.29% and 7.18-18.55%, respectively. Ma et al., (17) reported that ZEO comprised pulegone (53.5%), isomenthone (10.4%) and carvone (5.7%). Moreover, the previous studies on ZEO growing in Turkey and Iran were shown to contain pulegone as the main constituent of the oils (12,22-24).

Our findings indicated that the straight CH-FM film had inhibitory activity. Based on previous studies, the antimicrobial activity of CH is mainly due to interaction between NH_3^+ and OH groups of bacterial cell phospholipids, resulted in depletion of essential intracellular compounds and cell death (2). The lack of observation of inhibition zone in pure CH-FM film is likely attributed to the inability of dried film in diffusing to BHI agar (8). Similar findings have been reported by Peng *et al.,* (25), Siripatrawan *et al.,* (20) and Akyuz *et al.,* (15) for straight CH and FM. As presented in Table 2, the lowest and highest antimicrobial effect against *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7 and *S. typhimurium* were found for CH-FM films enriched with SO 0.5% (0.98-1.24 mm) and ZEO 0.5% + SO 0.75% (5.01-6.25 mm), respectively. The antibacterial mechanism of plant essential oils/extracts was generally suggested to be due to its major compounds especially polyphenolic compounds which is able to interact with cytoplasmic membrane of bacterial cells and subsequently cause the leakage of cellular components (26). Sesame seed lignans, such as sesamin, episesamin, sesamolin, sesaminol as well as sesamol, are known to inhibit the growth of bacterial

Table 2 *In vitro* antibacterial activity (inhibition zone diameter; mm) Of essential and edible oils against some food-borne pathogens

	S. aureus	*L. monocytogenes*	*S. typhimurium*	*E. coli* O157:H7
Film	ND	ND	ND	ND
ZEO 0.25%	2.15 ± 0.03	2.10 ± 0.01	1.78 ± 0.02	1.62 ± 0.05
ZEO 0.5%	3.94 ± 0.05	3.65 ± 0.03	2.15 ± 0.03	2.01 ± 0.01
SO 0.5%	1.12 ± 0.01	0.98 ± 0.04	ND	ND
SO 0.75%	1.24 ± 0.03	1.13 ± 0.02	ND	ND
ZEO 0.25% + SO 0.5%	4.23 ± 0.04	4.18 ± 0.05	3.25 ± 0.04	3.03 ± 0.06
ZEO 0.25% + SO 0.75%	4.76 ± 0.02	4.56 ± 0.01	3.82 ± 0.05	3.34 ± 0.03
ZEO 0.5% + SO 0.5%	5.43 ± 0.06	5.21 ± 0.04	4.68 ± 0.02	4.23 ± 0.04
ZEO 0.5% + SO 0.75%	6.25 ± 0.01	6.09 ± 0.03	5.34 ± 0.01	5.01 ± 0.06
Ampicillin	2.61 ± 0.00	3.45 ± 0.05	ND	ND
Trimethoprim	ND	ND	4.87 ± 0.02	3.56 ± 0.00

ND: Not determined

Table 3 DPPH scavenging activity (%) of films based on chitosan-flaxseed mucilage containing Ziziphora clinopodioides essential oil (ZEO) and sesame oil (SO).

	DPPH (%)
Film	5.45 ± 0.04[b]
ZEO 0.25%	15.07 ± 1.43[b]
ZEO 0.5%	21.04 ± 3.62[a]
SO 0.5%	8.90 ± 0.41[b]
SO 0.75%	9.75 ± 0.71[b]
ZEO 0.25% + SO 0.5%	25.00 ± 0.44[a]
ZEO 0.25% + SO 0.75%	28.11 ± 4.12[a]
ZEO 0.5% + SO 0.5%	36.03 ± 2.76[a]
ZEO 0.5% + SO 0.75%	37.00 ± 0.45[a]

[a,b] Different lowercase letters are significantly different (P<0.05).

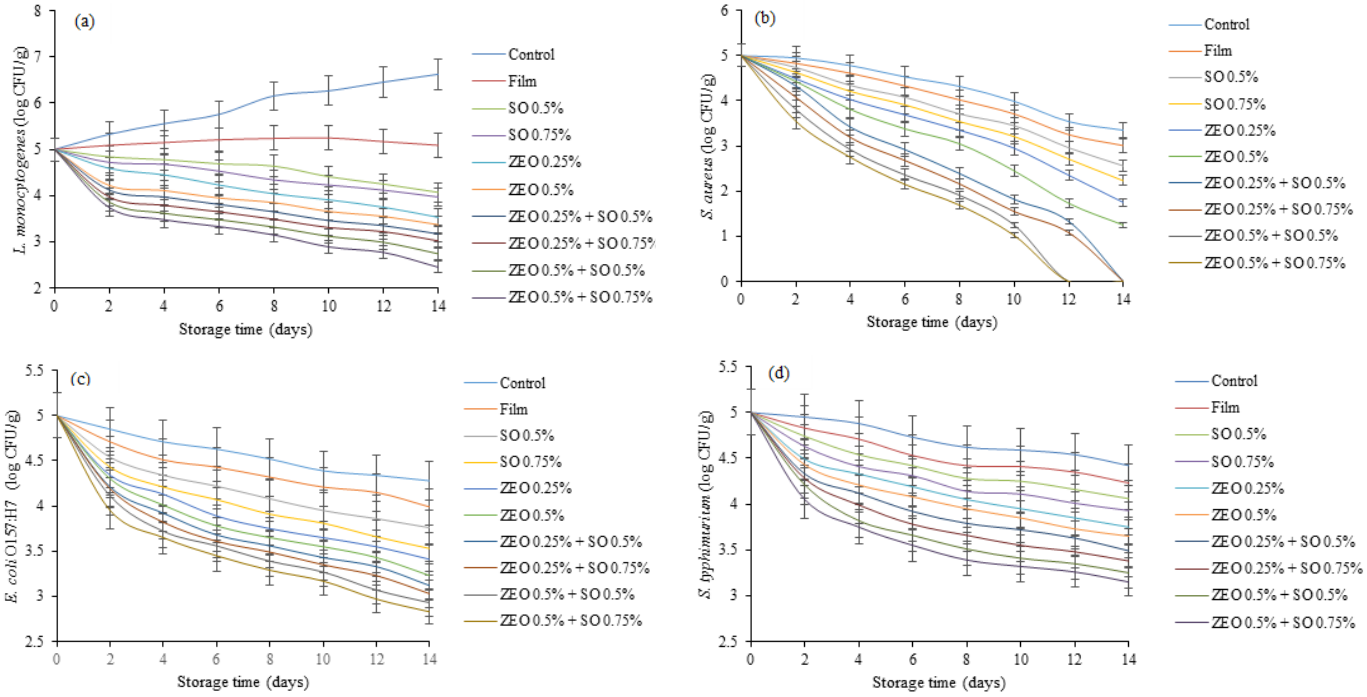

Figure 2 Changes in *L. monocytogenes* (a), *S. aureus* (b), *E. coli* O157:H7 (c) and *S. typhimurium* (d) inoculated into minced trout fillets packed in chitosan-flaxseed mucilage films enriched with *Ziziphora clinopodioides* essential oil (ZEO) and sesame oil (SO). Each number is the mean of three samples taken from different experiments. Each sample was analyzed in triplicate.

and fungal pathogens (27). Sonboli *et al.,* (23) and Behravan *et al.,* (22) indicated that ZEO collected from Hamedan and Khorasan Razavi provinces had good antibacterial activity against *Staphylococcus epidermidis*, *S. aureus*, *E. coli, B. subtilis*, *B. cereus* and *L. monocytogenes,* which are general in accordance with our findings. Several studies found that SO had antimicrobial effect against *B. subtilis*, *S. aureus*, *E. coli*, *Salmonella typhi*, *Proteus vulgaris*, *Cornebacterium diphtheria*, *Streptomyces, Streptococcus mutans* and *Lactobacilli acidophi* (16,28,29). Lv *et al.,* (30) also concluded that the mechanisms of combined antimicrobial interaction includes inhibition of the various biochemical pathway and critical enzymes and development of pores and cavities in bacterial cell membrane. In the current study, antimicrobial property of ZEO and SO against *S. aureus* and *L. monocytogenes* was higher than *E. coli* O157:H7 and *S. typhimurium*, which is mainly due to the lack of lipopolysaccharide layer in Gram-positive bacteria (31).

The straight CH-FM film showed a little antioxidant activity (5.45% ± 0.04). These results might be attributed to the reaction between the free radicals and the free residual amino groups to form ammonium groups (20). Similar antioxidant activity were also found in some previous studies for CH film (32,33). The antioxidant property of CH-FM based films containing ZEO increased up to 37% ± 0.45, which is mainly due to its major phenolic compounds especially thymol and carvacrol. Gutiérrez *et al.,* (34) indicated that incorporation of murta (*Ugni molinae* Turcz) extract into carboxymethylcellulose film increased its antioxidant property 18-folds compared to control group. The antioxidant property of CH film also significantly improved after addition of carvacrol (35).

Growth of *L. monocytogenes* in food models during prolonged storage condition was reported by Tosun et al., (36) in fresh salmon, which is general accordance with our findings. *S. aureus*, *E. coli* O157:H7 and *S. typhimurium* would not grow at refrigerated temperature due to their mesophilc nature (3). Previous study indicated the effects of essential and edible oils against *L. monocytogenes* (1,37,38), *S. aureus* (39), *S. typhimurium* (40,41) and *E. coli* O157:H7 (42) in refrigerated foodstuffs. Recent studies indicated that ZEO could significantly reduce the growth of *L. monocytogenes* up to 1-4 log CFU/g compared to control groups in raw minced trout fillets (1), refrigerated chicken meatballs (14) and minced camel meat (19). Moreover, ZEO separately and in combination with other antimicrobial compounds completely inhibited the growth of *S. aureus* and *E. coli* O157:H7 in raw beef patty (43) and commercial barely soup (44).

Conclusion

Based on our findings, application of ZEO and SO considered as good alternative compounds to control the safety of minced trout fillets during refrigerated condition for 14 days. The designated films had good antibacterial effect against some food borne pathogenic bacteria including *L. monocytogenes*, *S. aureus*, *S.*

typhimurium and *E. coli* O157:H7 in raw rainbow trout fillets.

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Conflicts of interest
The authors declare no conflict of interest.

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