

# Antimicrobial effects of *Mentha spicata* essential oil and methanolic carrot extract against *Staphylococcus aureus* and *Listeria monocytogenes* in fish soup

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## ABSTRACT

Plant essential oils and natural extracts have been attracted research interest to control bacterial contamination of food products. *Staphylococcus aureus* and *Listeria monocytogenes* are considered as public health bacterial hazards which survive in various types of food. The aim of the current study was to assess the effects of *Mentha spicata* essential oil (MSO: 0, 0.1 and 0.2%) alone and in combination with methanolic carrot extract (MCE) (0, 0.25 and 0.5%) against *S. aureus* and *L. monocytogenes* in fish soup. Untreated and treated homemade fish soups with different concentrations of MSO and MCE were inoculated with 5 log CFU/ml of *S. aureus* and *L. monocytogenes*, and then stored at 4 ± 1 °C (refrigerated temperature), 9 ± 1 °C (abused temperature) and 25 ± 1 °C (room temperature) during 15 days. Based on these findings, carvone (78.76%) and limonene (11.50%) were the major compounds of the MSO. The following sequence inhibition effect on *S. aureus* and *L. monocytogenes* was observed in treated soups: MSO 0.2% + MCE 0.5% > MSO 0.2% + MCE 0.25% > MSO 0.2% > MSO 0.1% + MCE 0.5% > MSO 0.1% + MCE 0.25% > MSO 0.1% > MCE 0.5% > MCE 0.25%. The results of the present study demonstrated that antibacterial effects of different concentrations of MSO separately and in combination with MCE were higher at 4 and 9 °C than 25 °C.

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## Introduction

Food-borne pathogens are causing a great number of diseases with remarkable effects on human health. The global burden of food-borne diseases is significant and it is estimated that, only in the United States of America, each year roughly 48 million people in the USA gets sick, 128,000 are hospitalized, and 3,000 die from food-borne diseases (1). *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* have been isolated frequently from various foodstuffs. (2,3). The presence of *S. aureus* in foodstuffs is considered as one of the public health hazard owing to its ability to produce enterotoxin and the risk of subsequent food poisoning (1). Bakery products, chicken meat, salads and different types of soups are considered as the most preferred sources for staphylococcal food poisoning (1). *L. monocytogenes* is capable of growing over widespread temperature (1-45 °C), pH (4-9) and low water activity (0.91), and therefore it may be expected to survive in various types of food (e.g. traditional cheese, red meat, chicken and turkey products and vegetable salads) for long periods of time (4,5). Commercial/homemade fish soup is one of the most popular and appreciated types of soup throughout the world, due to its ready-to-use, low cost, delicious taste and desirable flavor (5). It has been suggested that the growth of *S. aureus* may become accelerated and consequently lead to the spread of

enterotoxin in the case of post processing contamination of homemade and commercial types of soup (1). Previous studies indicated that traditional methods such as mild heat treatment and the addition of chemical preservatives cannot fully prevent and control microbial growth of food products during prolonged post storage (6,7). Therefore, further investigations for development of concepts and the discovery of novel strong antimicrobial compounds to decrease the risk of microbial contamination in food products is of an unquestionable interest.

Plant essential oils (EOs) and extracts have been attracted research interest to control bacterial contamination of food products (2,8). *Mentha spicata*, belongs to the Labiatae family, which comprises about 20-30 species originating in Europe, North Africa and in Asia Minor and near East (9). It has several biological uses e.g. antimicrobial, antioxidant and antispasmodics, in good correlation with the high contents of phenolic compounds (10). Carrot (*Daucus carota* L.) is also a valuable source of β-carotene, minerals (calcium, potassium, phosphorus, sodium and iron) and some phenolic antioxidants (11). It can be widely used in the food industries to inhibit growth of spoilage microorganisms and subsequently extend the shelf life of foods due to its good antibacterial and antioxidant activities (12,13). Potential antimicrobial effects against common food-borne pathogens in different types of soup has been shown for *Ziziphora clinopodioides* (14),

*Rhus coriaria* (15), *Cuminum cyminum* (16) and chitosan film (5). Based on our knowledge, a comparative study on the antibacterial effects of *M. spicata* essential oil (MSO) separately and in combination with methanolic carrot extract (MCE) against common food-borne pathogenic bacteria such as *S. aureus* and *L. monocytogenes* in food models has not been carried out so far. Therefore, the aim of the current study was to assess the effects of MSO (0, 0.1 and 0.2%) alone and in combination with MCE (0, 0.25 and 0.5%) against *S. aureus* and *L. monocytogenes* in homemade fish soup stored at  $4 \pm 1$  °C (refrigerated temperature),  $9 \pm 1$  °C (abused temperature) and  $25 \pm 1$  °C (room temperature) during 15 days.

## Materials and methods

### Materials

The wild samples of *M. spicata* was obtained from the natural habitat in Gilane-e-Gharb city, Kermanshah, Iran. Authentication of the plant was conducted by Dr. Seyed Mohammad Masoumi (Faculty of Agriculture, Razi University, Kermanshah, Iran). The plant was carefully washed with distilled water and then air-dried indoor in a shady place at room temperature for 7 days. A portion of 100 g dried plant was ground, homogenized in distilled water with a ratio of 1:5 and exposed to hydro-distillation using a Clevenger-type apparatus at room temperature ( $25 \pm 2$  °C) for approximately 3 h (17). The EO on top of the distillate was collected and dried over anhydrous sodium sulfate (0.5 g) to remove water. The isolated MSO was stored under refrigerated condition until further use. All media and other chemicals were obtained from Merck, Germany. The commercial MCE was also purchased from Gol Adonis Daru (Tehran, Iran). According to the instruction of the company, 1 g of powdered carrot was dissolved in 10 ml methanol and extracted with a shaker at room temperature for 24 h. The extract was filtered through Whatman filter paper no.3, concentrated in a rotary evaporator at  $40 \pm 1$  °C and stored at refrigerated temperature until further use. Total phenolic compounds and antioxidant activity of MCE were determined as  $310.89 \pm 15.56$  mg catechin/kg fresh weight and  $85.24 \pm 12.03$   $\mu$ mol Trolox/100 g, respectively.

### Gas chromatography-mass spectrometry (GC-MS) analysis of *Mentha spicata* essential oil

Gas chromatography-mass spectrometry (GC-MS) analysis of MSO was performed on an Agilent 7890/5975C GC-MS system (USA) fitted with HP-5MS 5% phenyl methylsiloxane capillary column (30 m length  $\times$  0.25 mm i.d. and 0.25  $\mu$ m 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. MSO analysis

was also run on Agilent 7890/5975C GC-MS system 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.

### Bacterial strains

*S. aureus* (ATCC 6538) and *L. monocytogenes* (ATCC 19118) were purchased from the culture collection of the Iranian Research Organization for Science and Technology, Tehran, Iran. The bacterial strains were cultured in Brain Heart Infusion broth (BHI) at 37 °C for 24 h and immediately used to make appropriate inoculum dose (5 log CFU/ml) in BHI broth for further experiment (18).

### Experimental design

A homemade fish soup as the experimental food model was prepared according to the method described by traditional manual. Fresh rainbow trout was purchased from a local fish market (Kermanshah, Iran) and immediately transferred to the laboratory under refrigerated condition. The obtained fillets were used for preparation of soup. The fish soup contained trout fillet, olive oil, parsley, chopped onion, salt, red pepper and spices, prepared in 500 mL screw capped flask and autoclaved at 121 °C for 15 min. After cooling, the samples were inoculated with 5 log CFU/ml of *S. aureus* and *L. monocytogenes*. Then, concentrations of MSO (0, 0.1 and 0.2%) separately and in combination with MCE (0, 0.25 and 0.5%) were added into the fish soups. All designated soup groups were immediately stored at three temperatures of  $4 \pm 1$  °C (refrigerated temperature),  $9 \pm 1$  °C (abused temperature) and  $25 \pm 1$  °C (room temperature), and sampling was carried out at days 0, 3, 6, 9, 12 and 15 for further analysis. For each day, 10 ml of each designated group was homogenized with 90 ml of 0.1% peptone water for 1 min in a stomacher at room temperature, diluted using ten-fold serial dilution for plating onto Bard Parker agar (incubated at 37 °C for 48 h) and Palcam *listeria* selective agar (incubated at 30 °C for 48 h) (1).

### Sensory evaluation

To determine the sensory effects of addition of MSO and MCE to the un-inoculated homemade fish soup, nine panelist evaluated the products using a nine point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely) for overall liking of the soup and also liking of the appearance and aroma. Samples labelled with three-digit random numbers were placed in small white plastic glasses and served immediately after being heated at approximately  $20 \pm 1$  °C.

### Statistical analysis

All experiments were done in triplicate. The analysis was performed using SPSS 16.0 (version 16; SPSS Inc,

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 *Mentha spicata* essential oil

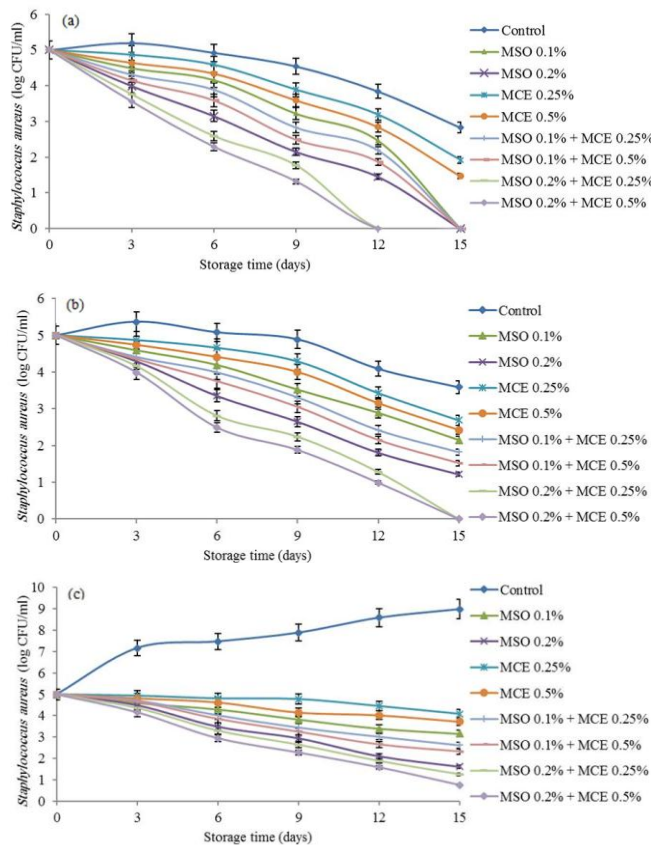
The chemical compositions of MSO together with retention indices are presented in Table 1. The MSO was mainly comprised from carvone (78.76%) and limonene (11.50%).

**Table 1** Composition of essential oil of *Mentha spicata* identified by GC-MS

No.	Compound	Composition %	Retention time (min.)
1	$\beta$ -Myrcene	0.25	450
2	Limonene	11.50	509
3	$\gamma$ -Terpinene	0.16	553
4	Menthone	1.01	703
5	Menthol	1	713
6	Terpinen-4-ol	0.99	720
7	$\alpha$ -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	$\beta$ -Bourbonene	1.23	981
16	trans-Caryophyllene	1.04	1021
17	$\gamma$ -Amorphene	0.21	1048
18	$\alpha$ -Amorphene	0.16	1058
	Others	0.11	-
	Total	100	

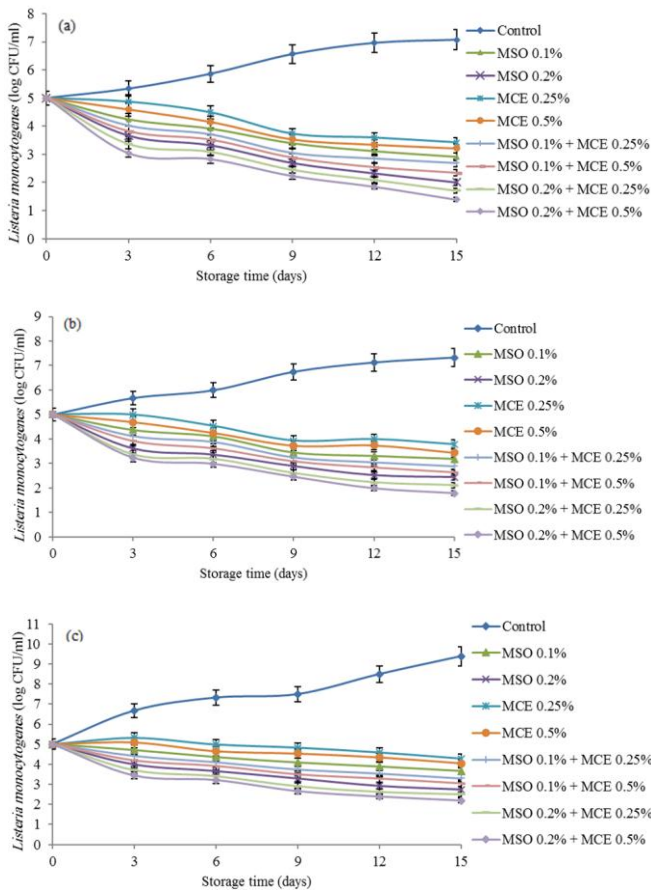
### Effects of natural compounds against bacterial pathogens in fish soup

Based on our findings, in control groups, the initial *S. aureus* count of 5 log CFU/ml was reached to 2.83, 3.59 and 8.99 log CFU/ml after 15 days of storage at 4, 9 and 25 °C, respectively (Fig. 1a-c).



**Figure 1** Changes in *Staphylococcus aureus* of commercial fish soup during storage at  $4 \pm 1$  °C (refrigerated temperature) (a),  $9 \pm 1$  °C (abused temperature) (b) and  $25 \pm 1$  °C (room temperature) (c) for 15 days. Each number is the mean  $\pm$  standard error taken from different experiments. The result was expressed as mean  $\pm$  standard error.

The initial *L. monocytogenes* count of 5 log CFU/ml was reached to 7.07, 7.32 and 9.38 log CFU/ml after 15 days of storage at 4, 9 and 25 °C, respectively (Fig. 2a-c). The growth rates of *S. aureus* and *L. monocytogenes* were significantly affected by different concentrations of MSO (0.1 and 0.2%) at different storage temperatures ( $P < 0.05$ ). In comparison with control group, all samples treated with MSO significantly delayed the growth of bacterial pathogens during the storage time ( $P < 0.05$ ). The group treated with MSO 0.2% exhibited the best antibacterial effectiveness against the microorganisms ( $P < 0.05$ ). At the end of study period, the viable counts of *S. aureus* in samples treated with MSO 0.2% were 2.83, 2.37 and 7.37 log CFU/ml lower than control group during storage at 4, 9 and 25 °C, respectively. Regarding *L. monocytogenes*, the corresponding values were 5.07, 4.88 and 6.62 log CFU/ml lower than control group



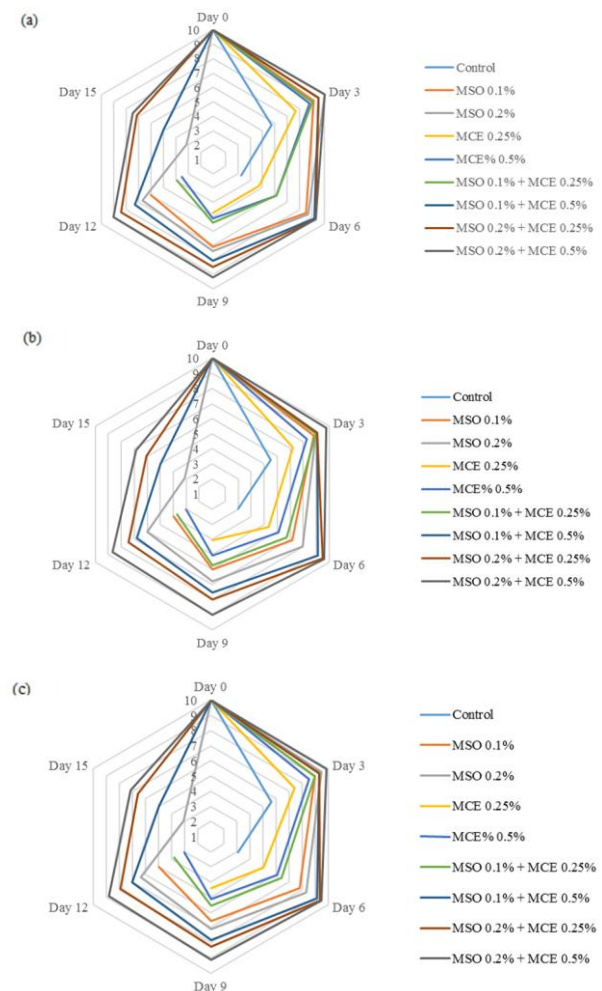
**Figure 2** Changes in *Listeria monocytogenes* of commercial fish soup during storage at  $4 \pm 1$  °C (refrigerated temperature) (a),  $9 \pm 1$  °C (abused temperature) (b) and  $25 \pm 1$  °C (room temperature) (c) for 15 days. Each number is the mean  $\pm$  standard error taken from different experiments. The result was expressed as mean  $\pm$  standard error.

during storage at 4, 9 and 25 °C, respectively. The viable counts of the pathogenic bacteria were significantly affected by MSO concentrations (0.1 and 0.2%) more than MCE concentrations (0.25 and 0.5%) used at all temperatures ( $P < 0.05$ ). Indeed, MCE could not inhibit the growth of bacterial strains; as the final bacterial population of *S. aureus* in treated samples with MCE 0.5% at 4, 9 and 25 °C were found to be 1.47, 2.42 and 3.72 log CFU/ml, respectively. Regarding *L. monocytogenes*, the corresponding values at 4, 9 and 25 °C were 3.21, 3.78 and 4.04 log CFU/ml, respectively. However, the results of the present study showed that treated fish soups with MCE 0.25% and 0.5% had lower population of *S. aureus* and *L. monocytogenes* compared to control ones ( $P < 0.05$ ). Samples treated with different concentrations of MSO in combination with MCE, reduced the growth of *S. aureus* and *L. monocytogenes* at 4, 9 and 25 °C. As it can be seen (Fig. 1a-c and 2a-c), the following sequence inhibition effect on *S. aureus* and *L. monocytogenes* was observed in treated soups: MSO 0.2% + MCE 0.5% > MSO 0.2% + MCE 0.25% > MSO 0.2% > MSO 0.1% + MCE 0.5% >

MSO 0.1% + MCE 0.25% > MSO 0.1% > MCE 0.5% > MCE 0.25%. The results of the present study demonstrated that antibacterial effects of different concentrations of MSO separately and in combination with MCE were higher at 4 and 9 °C than 25 °C (Fig. 1a-c and 2a-c).

### Effects of natural compounds on sensory properties of fish soup

The results of sensory evaluation of soup samples stored at refrigerated temperature showed that there were no significant differences between the appearance or aroma of samples treated with MSO at concentrations of 0.1 and 0.2% ( $P > 0.05$ ) (Fig. 3a-c). Nevertheless, there were significant differences for hedonic scores for aroma and appearance of samples containing MCE at concentrations of 0.25 and 0.5% compared to control group ( $P < 0.05$ ). The control samples presented significant lower sensory values for all the attributes studied ( $P < 0.05$ ).



**Figure 3** Sensory attributes (appearance (a), aroma (b) and overall acceptability (c)) of homemade fish soup during storage at  $4 \pm 1$  °C for 15 days. Each number is the mean  $\pm$  standard error taken from different experiments. The result was expressed as mean  $\pm$  standard error.

## Discussion

In the present study, the MSO was mainly comprised from carvone (78.76%) and limonene (11.50%). According to the published results by Chauhan et al., (10) the major components of MSO obtained from North-West Himalaya, India were carvone (76.65%), followed by limonene (9.57%) and 1,8-cineole (1.93%). In another study (19), carvone (40.8%), limonene (20.8%) and 1,8-cineole (17.0%) were reported as the main constituents of MSO, which is good in agreement with our findings. The chemical compositions of the EOs from plants and spices may different depend on geographical and climate conditions, the method used for drying and isolation of the EO and also the plant growth phase (2).

Based on our findings, in control groups, *S. aureus* and *L. monocytogenes* counts were survived/increased with storage time (Fig. 1a-c and 2a-c). Emiroğlu et al., (28) reported that initial *S. aureus* count for minced beef meat (4.2 log CFU/g) was increased up to 6.7–6.9 log CFU/g after 10 days of storage at refrigerated condition. Shahbazi et al., (18) also found that the initial count of 5 log CFU/g of *S. aureus* and *L. monocytogenes* for raw chicken meatball was reduced during refrigerated storage and reached to 3.3 and 4.86 log CFU/g at the end of designated study period (day 12), respectively. Khezrian and Shahbazi, (4) found that the population (5 log CFU/g) of *L. monocytogenes* inoculated to the minced camel meat sample was gradually increased with 7.33 log CFU/g after 14 days' storage at refrigerated condition

Our findings for the inhibition of *S. aureus* and *L. monocytogenes* in a food model are very good in agreement with previous *in vitro* results (20-22). Aggarwal et al., (20) have demonstrated the strong antimicrobial activity of MSO against *S. aureus* using disk diffusion assay (zone of microbial growth inhibition = 10 mm). Hussain et al., (21) also reported that MSO had good antibacterial activity against some Gram-positive (*S. aureus*, *L. monocytogenes* and *B. cereus*) and Gram-negative (*E. coli*, *Y. enterocolitica*, *Klebsiella pneumoniae*, *S. typhi* and *Pseudomonas aeruginosa*) bacteria with MIC values in the ranges of 0.25-4 µl/ml. The antibacterial mechanism of MSO was suggested to be due to its major compounds especially carvone which is able to interact with cytoplasmic membrane of bacterial cells and subsequently cause the leakage of cellular components (23). 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 (2). According to the literature performed by Bakkali et al., (24), the major compounds of MSO may cause extensive damage of phospholipid bilayer structure, reduced the pH gradient across the bacterial membrane and subsequently inhibited the bacterial barrier function. It has been also reported that a number of compounds in

relatively low concentrations such as *p*-menthone,  $\alpha$ -pinene, cis-isopulegone, terpineol and piperitenone could also be expected to enhance the antimicrobial activity of the EO (2). Tassou et al., (3) demonstrated that a topical application of *M. piperita* EO, which has similar chemical compositions with MSO, could effectively reduce the population of *E. coli* and *S. typhimurium* in minced beef meat. In a recent study (25), MSO at 0.5% concentration had the strong antibacterial effect against microbial spoilage parameters and *L. monocytogenes* of minced camel meat.

It can be concluded that MCE showed a small effect on its own at 25 °C, and needed to be combined with lower temperature, MSO or both of them for maximum bactericidal activity. The *in vitro* antibacterial activity of MCE against Gram-positive (*L. monocytogenes*, *B. cereus*, *B. subtilis* and *S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*, *S. enteritidis*) bacteria has been reported in previous studies (13,26,27) and has being attributed to interaction of its major compounds such as carotol,  $\alpha$ -pinene, and sabinene on the cytoplasmic membrane of bacterial cells. This will cause the formation of transient pores in the plasma membrane, depletion of the ATP pool, amino acids, collapse of crucial ion gradients and ultimately leading to cell death (2). Pajohi et al., (16) studied the effect of *C. cyminum* seed EO and nisin on quality and shelf life of barley soup under refrigerated condition. They reported that application of this antimicrobial agents extended the shelf life of barley soup to 21 days by inhibiting microbial growth, which is good in agreement with our findings. Our results also agreed with Shahbazi et al., (14) who studied the effect of *Z. clinopodioides* EO and nisin as natural preservatives against *B. cereus* and *E. coli* O157:H7 in commercial barley soup. Based on their results, addition of 250 and 500 IU/ml nisin successfully delayed the growth of *B. cereus* and *E. coli* O157:H7 compared with control group after 11 days of storage under refrigerated condition.

Samples treated with different concentrations of MSO in combination with MCE, reduced the growth of *S. aureus* and *L. monocytogenes* at 4, 9 and 25 °C. Previous studies found that the combination of EOs with natural extracts had higher effects than the EOs alone against microbial growth in food models (2,9,28). 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 (2).

The results of the present study demonstrated that antibacterial effects of different concentrations of MSO separately and in combination with MCE were higher at 4 and 9 °C than 25 °C (Fig. 1a-c and 2a-c). The same results were also found in the study of Rajkovic et al.,

(6) who showed the remarkable increase of inhibitory effect of nisin and carvacrol against *B. cereus* and *B. circulans* in vacuum-packed potato puree. Lower growth of most pathogens such as *S. aureus* and *L. monocytogenes* is due to alternation of fatty acid components of lipids in cell membrane of bacteria that cause to interfere with membrane fluidity and lead to death them (7).

Some studies investigated the sensory quality of food products treated with natural EOs or extracts with the aim of predicting the applicability of the food products in terms of consumer acceptance (5,25,29,30). The findings published on this matter among the food stuffs were different possibly due to the sensory properties of the food itself, used concentrations of EOs or extracts as well as storage time and temperature of the products.

### Conclusion

The results of the present study indicated that MSO presented a good source of bioactive compounds such as carvone (78.76%) and limonene (11.50%). Based on our findings, application of MSO in combination with MCE are good alternative compounds to control *S. aureus* and *L. monocytogenes* in homemade fish soup. To our knowledge, this is the first time the antibacterial activity of MSO separately and in combination with MCE has been reported against *S. aureus* and *L. monocytogenes* in homemade fish soup.

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### Conflicts of interest

The authors declare no conflict of interest.

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