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GROWTH RESPONSE OF *VIBRIO PARAHAEMOLYTICUS* AND *LISTERIA MONOCYTOGENES* IN SALTED FISH FILLETS AS AFFECTED BY *ZATARIA MULTIFLORA* BOISS. ESSENTIAL OIL, NISIN, AND THEIR COMBINATION

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ABSTRACT

The growth of *Vibrio parahaemolyticus* (at inoculation levels, 10^3 and 10^5 cfu/g) and *Listeria monocytogenes* (at inoculation level, 10^3 cfu/g) were investigated in salted fish fillets (brine salted at 4% NaCl) as affected by different concentrations of *Zataria multiflora* Boiss. essential oil (EO), nisin (N) and their combinations at 8C (and 4C only for *L. monocytogenes*) during 21 days. The growth of *V. parahaemolyticus* was inhibited at 8C (in control group) and reached <2 $\log_{10}$ cfu/g at the first day of study for inoculation level of 10^3 cfu/g. At inoculation level of 10^5 cfu/g, this inhibitory effect was significantly ($P < 0.05$) affected by adding EO. The significant inhibitory effect of N alone and synergistic inhibitory effect of EO with N were also observed during the study. For *L. monocytogenes*, the obvious significant inhibitory effect of EO and its combination with N was observed at 4C. Considering the organoleptic effect of the EO used in this study, the best inhibitory effects of EO in combination with N for *V. parahaemolyticus* and *L. monocytogenes* were obtained at combinations of EO = 0.405% and $n = 0.75$ µg/mL, and EO = 0.405% and $n = 0.25$ µg/mL, respectively.

PRACTICAL APPLICATION

The synergistic inhibitory effect of *Zataria multiflora* Boiss. essential oil and nisin on *V. parahaemolyticus* and *L. monocytogenes*, which was obtained in this study can improve the scope of essential oil function in food industry.

INTRODUCTION

Salt-cured products based on fish are traditional and highly regarded products around the world (Feldhusen 2000). Salt-curing of food is an effective hurdle for microbial growth. However, in the case of salt-cured fish, it has been shown that some foodborne bacterial pathogens such as *Vibrio parahaemolyticus* and *Listeria monocytogenes* are not eliminated during salt-curing (Vilhelmsson *et al.* 1997; Barat *et al.* 2006; Basti *et al.* 2006; Lorentzen *et al.* 2010).

The salt-cured fish is mostly used in dishes that are heat treated before consumption, but the food safety risk of *V. parahaemolyticus* still has to be considered because of the risk of cross contamination and undercooking (Lorentzen *et al.* 2010). Furthermore, raw rehydrated salt-cured fish is also used in some recipes (Lorentzen *et al.* 2010).

Humans are frequently exposed to *L. monocytogenes*, and high numbers may be ingested during consumption of certain types of food. Fish products, cheese and salads were the products that were most heavily contaminated with this organism. A previous study showed that the average yearly exposure to 3, 5 and 6 log *L. monocytogenes* occurred 19.3, 3.8 and 0.8 times, respectively through the consumption of these products (Notermans and Hoornstra 2000).

There is a constant striving to produce safer food and to develop new antimicrobial agents. *Zataria multiflora* Boiss. is

a plant belonging to the Laminaceae family that geographically grows only in Iran, Pakistan and Afghanistan (Ali *et al*. 2000; Hosseinzadeh *et al*. 2000). This plant has the local name of avishan shirazi (in Iran) and traditional uses such as antiseptic, anesthetic and antispasmodic (Hosseinzadeh *et al*. 2000).This plant is extensively used as flavor ingredients in a wide variety of food in Iran. The main constituents of the essential oil (EO) of this plant are phenolic compounds such as carvacrol and thymol (Shaffiee and Javidnia 1997; Ali *et al*. 2000). Moosavy *et al*. (2008) reported the inhibitory effect of *Z. multiflora* Boiss. EO alone and in combination with nisin (N) on *Salmonella typhimurium* and *Staphylococcus aureus* in a liquid food model system (commercial barely soup).

In the present study, we investigated the efficacy of *Z. multiflora Boiss*. EO, N and their combination on *V. parahaemolyticus* and *L. monocytogenes* in salted fish fillets.

The sensory characteristics of salted fish supplemented with *Z. multiflora* Boiss. EO were also evaluated.

MATERIALS AND METHODS

Plant Material

Z. multiflora Boiss. was collected in Shiraz province of Iran and identified by the Institute of Medicinal Plants, Medical University of Tehran, Iran. The method of extraction and percentages of components of the EO (as determined by gas chromatography–mass spectrometry [GC-MS]) were described by Moosavy *et al*. (2008).

N

N containing 2.5% active N was purchased from Sigma-Aldrich Inc. (Dorset, UK, EC 215-807-5). N stock solution was prepared with 0.02 M HCl (pH 1.6), and was filter sterilized through a 0.45-μm sterile, disposal and nonpyrogenic syringe filter (Macherey-Nagel, Düren, Germany).

Experimental Design

To assess the effect of different concentrations of *Zataria multiflora* Boiss. EO, N and their combinations (used in brine during the salting of fish fillets) on the *V. parahaemolyticus* and *L. monocytogenes*, the experiment was arranged in a factorial design. This design included five levels of EO (0, 0.045, 0.135, 0.405 and 0.81%) and three levels of N (0, 0.25 and 0.75 μg/mL) according to our previous studies (Basti *et al*. 2007; Moosavy *et al*. 2008), two storage temperatures (T: 8C as an improper refrigeration temperature for both organism and 4C for *L. monocytogenes*), and repeated examinations for growth (viable count) in salted fish at 10 intervals (0, 1, 2, 3, 6, 9, 12, 15, 18 and 21 days). All experiments were conducted in independent triplicate.

Preparation of Inoculums

The inoculums of *V. parahaemolyticus* American Type Culture Collection (ATCC) 17802 and *L. monocytogenes* ATCC 19118 obtained from Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran) were prepared according to the method described by Basti *et al*. (2007). The *V. parahaemolyticus* and *L. monocytogenes* broth cultures were adjusted to optical density (OD) (absorbance) of 0.1 at 600 nm, using a Spectronic 20 spectrophotometer (Milton Roy Company, Ivyland, PA). These adjustments gave cell concentrations c. 3×10^7 and 7×10^7 cfu/mL for *V. parahaemolyticus* and *L. monocytogenes*, respectively as determined in preliminary trials. Dilutions of *V. parahaemolyticus* and *L. monocytogenes* cultures ($OD_{600\,nm} = 0.1$) were prepared in 0.1% (w/v) peptone water (Merck, KGaA, Darmstadt, Germany). For *V. parahaemolyticus*, 1.5% NaCl was added to the diluents. The tubes (containing c. 3×10^7 and 3×10^5 *V. parahaemolyticus* and c. 3×10^5 *L. monocytogenes* per mL) were used for inoculation.

Preparation of Fish Fillets

Hypophthalmichthys molitrix (silver carp) were caught from a fish farm in Mazandaran (Northern Province of Iran). The fish were further skinned, filleted and cut into square pieces (8×3 cm about 30 g). The fillets were packed in plastic bags and immediately transferred to the Atomic Energy Organization of Iran, Nuclear Research Center for Agriculture and Medicine, Amirabad, Tehran, Iran. The fillets were exposed to gamma irradiation (5 KGy, using cobalt-60 irradiator) to eliminate any likely background of *V. parahaemolyticus* and *L. monocytogenes*. At the same day, the irradiated fillets were transported in refrigeration condition to the laboratory. Five randomly selected samples of the irradiated fillets were examined for *V. parahaemolyticus* and *L. monocytogenes* according to the method of American Public Health Association (2001) and none of them were detected.

Fat, protein and moisture content (%) of five randomly selected fillets were determined according to Association of Analytical Communities (1998), which (mean ± standard deviation [SD]) were 2.14 ± 0.21, 23.16 ± 0.21 and 73.9 ± 0.22, respectively.

Preparation of NaCl Brine Substrate and Salted Fish

Four percent NaCl (Merck, KGaA) brine (w/v) was prepared. Then, EO and N were added in different amounts to satisfy the experimental design.

The fish fillets were dipped into 1,000 mL of the sterilized NaCl brine (containing designated amounts of EO and N) in screw-capped flasks and kept at 5C under agitation condition for 24 h. Then, the samples were transferred to sterile Petri

dishes and kept in a laminar flow biological safety cabinet for 15 min. This allowed extra moisture to evaporate before being inoculated with test organisms. Each treated fillet was weighed again into a 25-g unit.

Salt concentration in the five randomly selected finished products was evaluated according to Association of Analytical Communities (1975) (the salt content was expressed as % NaCl in water phase of muscle), which (mean ± SD) was 3.1 ± 0.83.

Inoculation and Storage of Fillets

A spot inoculation method was used to inoculate the organisms (at levels c. 1×10^5 and 1×10^3 cfu/g for *V. parahaemolyticus* and 1×10^3 cfu/g for *L. monocytogenes*) on treated fillets. Then the fillets were air-dried in laminar flow biological safety cabinet for 20 min for further attachment of the cells.

To evaluate the growth response of *V. parahaemolyticus* and *L. monocytogenes*, samples were tested initially (0 day) by viable count on thiosulfate citrate bile salt sucrose (TCBS) agar (Merck, KGaA) and polymyxin acriflavin lithium-chloride ceftazidime esculin mannitol (PALCAM) Listeria selective agar (Merck, KGaA) supplemented with PALCAM Listeria selective supplement (Merck, KGaA), respectively. Then, the remaining fillets were transferred aseptically to stomacher bags (BagFilter, Interscience, Saint Nom la Breteche, France) followed by incubation at defined temperatures with viable count on TCBS and PALCAM Listeria selective agars at nine intervals (1, 2, 3, 6, 9, 12, 15, 18 and 21 days). The growth state of the organisms in combinations at appropriate time intervals with no detectable count on the selective agars (<2 $\log_{10}$ cfu/g; minimum detection limit for plate count) was also investigated by most probable number (MPN) method.

All experiments were conducted in independent triplicate. The mean of viable counts and corresponding SD of the means are shown in the results section. The main and interactive effects of EO and N concentrations on *V. parahaemolyticus* and *L. monocytogenes* were evaluated by analysis of variance (ANOVA) and Tukey procedure, using the Statistical Package for the Social Sciences (SPSS) 16.0 statistical software (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL). Results were considered statistically significant when $P \leq 0.05$.

Sensory Evaluation

Sensory-derived effects of adding *Z. multiflora* Boiss. EO to salted fish fillets were evaluated by an acceptance test on fried samples. The seven salted fish fillets from each treatment of EO (0, 0.045, 0.135, 0.405 and 0.81%) were evaluated by a panel of seven judges, consisting of scientific staff of the Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, chosen for their experience in the sensory analysis of food. Each panelist evaluated the samples by rating, using a 9-point scale where 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = dislike extremely, for various characteristics such as (appearance) color, odor and flavor. The variability of acceptance or liking of samples was analyzed by ANOVA and Fisher's least significant difference procedure, using SPSS 16.0 statistical software (SPSS 16.0 for Windows, SPSS Inc.).

RESULTS

Chemical Composition of *Z. multiflora* Boiss. EO

GC-MS analysis resulted in identification of 12 components representing 91.9% of the oil. The major compound was phenolic monoterpene carvacrol (71.1%); other important compounds were gammaterpinene (7.34%), alpha-pinene (4.26%) and eucalyptol (3.37%).

Factor Effects

At 8C, *V. parahaemolyticus* and *L. monocytogenes* enumerations were terminated at the time of sensory rejection, which was the day of nine for all the combinations.

The growth of *V. parahaemolyticus* (at both inoculation levels) was inhibited at 8C and the $\log_{10}$ cfu/g of the organism reached <2 (<–0.69 $\log_{10}$ MPN/g; complete inhibitory effect) (data not shown) and 4.39 (Table 1) at the first day of study for inoculation levels of 10^3 and 10^5 cfu/g, respectively. As the results show (Table 1), this inhibitory (at inoculation level of 10^5 cfu/g) was significantly ($P < 0.05$) affected by adding EO, e.g., at EO = 0.045% (minimum concentration level of EO used in this study), the $\log_{10}$ cfu/g of the organism reached 3.77 and <2 (0.71 $\log_{10}$ MPN/g) at the days 1 and 6 of study, respectively.

The significant inhibitory ($P < 0.05$) effect of N was observed during the storage of the treated filets and the number of organism reached 2.66 $\log_{10}$ cfu/g (after 3 days) and 2.10 $\log_{10}$ cfu/g (after 2 days) for N concenterations of 0.25 and 0.75 μg/mL, respectively.

The inhibitory effect of EO was also significantly ($P < 0.05$) increased by adding the N even at its minimum concentration level (0.25 μg/mL) used in this study, e.g., at the combination of EO = 0.045% and n = 0.25 μg/mL, the $\log_{10}$ cfu/g of the organism reached 3.46 and <2 (0.66 $\log_{10}$ MPN/g) at the days 1 and 3 of the study, respectively.

For *L. monocytogenes* (at 8C; Table 2), although the significant ($P < 0.05$) inhibitory effect of EO, N and their combination was observed, but even at their highest concentration used in this study, the $\log_{10}$ cfu/g of the organism remained at level ≥2 during the study. The obvious significant ($P < 0.05$) inhibitory effect of EO, and its combination with N was observed at storage temperature of 4C (Table 3), e.g., at

TABLE 1. GROWTH RESPONSE (VIABLE COUNT) OF *VIBRIO PARAHAEMOLYTICUS* (AT INOCULATION LEVEL OF 10^5 CFU/G) IN FISH FILLETS (BRINE SALTED AT 4% NaCl) AS AFFECTED BY DIFFERENT CONCENTRATIONS OF EO, N AND THEIR COMBINATIONS DURING 21 DAYS OF STORAGE AT 8C

		$\log_{10}$ cfu/g ± SD on sampling days									
EO (%)	N (μg/mL)	0	1	2	3	6	9†	12	15†	18	21
0	0	5.39 ± 0.01	4.39 ± 0.01	3.46 ± 0.01	3.45 ± 0.01	3.44 ± 0.01	3.42 ± 0.01				
0	0.25	5.30 ± 0.00	4.34 ± 0.02	3.33 ± 0.02	2.66 ± 0.1	2.1 ± 0.17	2.1 ± 0.17				
0	0.75	5.30 ± 0.00	4.37 ± 0.01	2.1 ± 0.17	2 ± 0.00	2 ± 0.00	1.43 ± 0.09				
0.045	0	5.01 ± 0.01	3.77 ± 0.01	3.26 ± 0.01	2.56 ± 0.07	0.71 ± 0.05*	<–0.69*				
0.045	0.25	4.99 ± 0.00	3.46 ± 0.01	2.99 ± 0.04	0.66 ± 0.13*	<–0.69*					
0.045	0.75	4.98 ± 0.02	3.33 ± 0.01	0.56 ± 0.06*	<–0.69*						
0.135	0	4.99 ± 0.01	3.41 ± 0.01	2.97 ± 0.02	0.45 ± 0.15*	<–0.69*					
0.135	0.25	4.99 ± 0.00	2.20 ± 0.17	2.10 ± 0.17	0.54 ± 0.08*	<–0.69*					
0.135	0.75	4.99 ± 0.01	0.45 ± 0.07*								
0.405	0	4.99 ± 0.00	2.95 ± 0.05	2.10 ± 0.17	<–0.69*	<–0.69*					
0.405	0.25	4.99 ± 0.01	2 ± 0.00	<–0.69*							
0.405	0.75	4.99 ± 0.01	<–0.69*								
0.81	0	4.99 ± 0.01	<–0.69*								
0.81	0.25	4.99 ± 0.00	<–0.69*								
0.81	0.75	4.99 ± 0.01	<–0.69*								

* $\log_{10}$ MPN/g (The growth state of the organisms in combinations at appropriate time intervals with no detectable count on thiosulfate citrate bile salt sucrose agar was investigated by MPN method).

† *V. parahaemolyticus* was enumerated until the time of sensory rejection which was the day of nine for all the combinations.

EO, essential oil; MPN, most probable number; N, nisin; SD, standard deviation.

combinations of EO = 0.405% and $n = 0$ μg/mL, and EO = 0.045% and $n = 0.25$ μg/mL, the $\log_{10}$ cfu/g of the organism decreased to < 2 during the first day of the study; complete inhibitory effect (<–0.69 $\log_{10}$ MPN/g) was observed at combinations of EO = 0.81% and $n = 0$ μg/mL, and EO ≥ 0.405% and $n \geq 0.25$ μg/mL.

Organoleptic Effects of *Z. multiflora* Boiss. EO

Table 4 shows the mean acceptability scores of the salted fish fillet samples containing EO. The most preferred sample was the fillet treated by 0.405% EO during the salting process. In

TABLE 2. GROWTH RESPONSE (VIABLE COUNT) OF *L. MONOCYTOGENES* IN FISH FILLETS (BRINE SALTED AT 4% NaCl) AS AFFECTED BY DIFFERENT CONCENTRATIONS OF EO, N AND THEIR COMBINATIONS DURING 21 DAYS OF STORAGE AT 8C*

		$\log_{10}$ cfu/g ± SD on sampling days									
EO (%)	N (μg/mL)	0	1	2	3	6	9†	12	15†	18	21
0	0	3 ± 0.04	3.43 ± 0.02	4.20 ± 0.02	5.74 ± 0.03	8.86 ± 0.01					
0	0.25	2.97 ± 0.03	3.32 ± 0.01	4.34 ± 0.02	5.09 ± 0.08	8.12 ± 0.01					
0	0.75	3.04 ± 0.08	2.98 ± 0.05	4.27 ± 0.03	5.10 ± 0.02	7.90 ± 0.01					
0.045	0	2.97 ± 0.03	3.45 ± 0.01	4.40 ± 0.01	5.54 ± 0.01	7.95 ± 0.00					
0.045	0.25	3.08 ± 0.04	3.40 ± 0.02	4.32 ± 0.02	5.48 ± 0.01	7.86 ± 0.07					
0.045	0.75	3 ± 0.04	2.95 ± 0.05	4.25 ± 0.02	5 ± 0.04	7.89 ± 0.01					
0.135	0	2.97 ± 0.03	3.25 ± 0.01	4.17 ± 0.03	5.23 ± 0.02	6.9 ± 0.05					
0.135	0.25	3 ± 0.04	3.14 ± 0.03	4.20 ± 0.03	5.23 ± 0.02	6.90 ± 0.01					
0.135	0.75	3 ± 0.04	2.93 ± 0.08	4.17 ± 0.03	4.97 ± 0.03	6.85 ± 0.01					
0.405	0	2.95 ± 0.05	2.56 ± 0.07	3 ± 0.04	3.70 ± 0.01	5.87 ± 0.00	6.96 ± 0.00				
0.405	0.25	3 ± 0.04	2.67 ± 0.06	2.93 ± 0.08	3.59 ± 0.11	5.70 ± 0.04	7.29 ± 0.01				
0.405	0.75	3 ± 0.04	2.92 ± 0.06	2.98 ± 0.03	4.30 ± 0.02	5.32 ± 0.02	8.54 ± 0.01				
0.81	0	2.84 ± 0.06	2.59 ± 0.11	2.29 ± 0.11	3 ± 0.04	5.57 ± 0.01	6.97 ± 0.00				
0.81	0.25	2.95 ± 0.09	2.26 ± 0.24	2.59 ± 0.11	3.23 ± 0.02	4.36 ± 0.19	6.63 ± 0.07				
0.81	0.75	2.90 ± 0.05	2.26 ± 0.24	2.62 ± 0.15	3.14 ± 0.03	4.33 ± 0.06	6.56 ± 0.03				

* All combinations showed significant ($P < 0.05$) difference.

† *L. monocytogenes* was enumerated until the time of sensory rejection which was the day of nine for all of the combinations.

EO, essential oil; N, nisin; SD, standard deviation.

TABLE 3. GROWTH RESPONSE (VIABLE COUNT) OF *L. MONOCYTOGENES* IN FISH FILLETS (BRINE SALTED AT 4% NaCl) AS AFFECTED BY DIFFERENT CONCENTRATIONS OF EO, N AND THEIR COMBINATIONS DURING 21 DAYS OF STORAGE AT 4C

EO	N	$\log_{10}$ cfu/g ± SD on sampling days									
(%)	(μg/mL)	0	1	2	3	6	9	12	15	18	21
0	0	3.40 ± 0.02	3.45 ± 0.01	3.55 ± 0.02	3.90 ± 0.00	4.54 ± 0.01	5.80 ± 0.01	6.00 ± 0.00	6.41 ± 0.02		
0	0.25	3.03 ± 0.02	3.40 ± 0.03	3.53 ± 0.01	3.90 ± 0.01	4.52 ± 0.01	5.80 ± 0.01	5.91 ± 0.00	6.38 ± 0.02		
0	0.75	3.05 ± 0.06	3.38 ± 0.06	3.5 ± 0.01	3.85 ± 0.01	4.40 ± 0.02	5.75 ± 0.01	5.92 ± 0.00	6.28 ± 0.02		
0.045	0	3.03 ± 0.02	2.98 ± 0.03	2 ± 0.00	3.46 ± 0.01	4.23 ± 0.02	5.74 ± 0.01	5.91 ± 0.00	6.2 ± 0.03		
0.045	0.25	3.05 ± 0.06	<2	3.46 ± 0.01	3.83 ± 0.01	4.32 ± 0.02	5.66 ± 0.01	5.83 ± 0.01	5.95 ± 0.00	6.04 ± 0.00	
0.045	0.75	2.98 ± 0.03	<2	3.38 ± 0.02	3.81 ± 0.01	4.23 ± 0.02	5.59 ± 0.01	5.74 ± 0.01	5.9 ± 0.00	6.3 ± 0.02	
0.135	0	3 ± 0.04	2.98 ± 0.03	3.45 ± 0.01	3.80 ± 0.01	4.28 ± 0.02	5.63 ± 0.01	5.81 ± 0.01	5.94 ± 0.00	6.38 ± 0.02	
0.135	0.25	2.97 ± 0.03	<2	3.36 ± 0.02	3.78 ± 0.01	4.11 ± 0.03	5.53 ± 0.01	5.71 ± 0.01	5.88 ± 0.00	6.25 ± 0.02	
0.135	0.75	3.05 ± 0.06	<2	3.21 ± 0.01	3.75 ± 0.01	4.08 ± 0.04	5.40 ± 0.02	5.62 ± 0.01	5.83 ± 0.01	6.20 ± 0.03	
0.405	0	2.95 ± 0.05	<2	2.10 ± 0.17	3.72 ± 0.01	3.91 ± 0.00	5.10 ± 0.03	5.67 ± 0.01	5.85 ± 0.01	6.11 ± 0.13	
0.405	0.25	2.98 ± 0.03	<–0.69*								
0.405	0.75	2.93 ± 0.06	<–0.69*								
0.81	0	2.95 ± 0.05	<–0.69*								
0.81	0.25	3.1 ± 0.02	<–0.69*								
0.81	0.75	3.06 ± 0.14	<–0.69*								

* $\log_{10}$ MPN/g (The growth state of the organisms in combinations at appropriate time intervals with no detectable count on PALCAM agar was investigated by MPN method).

EO, essential oil; MPN, most probable number; N, nisin; SD, standard deviation.

general, the samples treated by 0.045, 0.135 and 0.405% EO were acceptable to the panelist.

DISCUSSION

Despite the strong antimicrobial activity against food-borne pathogens and spoilage microorganisms shown by EOs (Tassou and Nychas 1995; Tassou *et al*. 2000; Rhayour *et al*. 2003; Bagamboula *et al*. 2004; Oussalah *et al*. 2006), their practical application is currently limited because of their undesirable flavor and the changes they cause in food products (Palmer *et al*. 2001; Yamazaki *et al*. 2004). Leistner and Gorris (1995) suggested that food preservation by multiple preservatives in small amounts was superior to preservation by a large amount of a single preservative in order to both secure microbial stability and safety and maintain the sensory, nutritive and economic properties of foods. The synergistic effect between EOs (and their constituents) and other antimicrobial substances such as N has been conclusively demonstrated (Pol and Smid 1999; Ettayebi *et al*. 2000; Periago and Moezelaar 2001; Pol *et al*. 2002; Yamazaki *et al*. 2004; Rajkovic *et al*. 2005). Basti *et al*. (2007) found significant inhibitory effect of *Z. multiflora* Boiss. EO on gram negative and gram positive bacteria (*S. typhimurium and S. aureus*) in brain heart infusion broth. Moosavy *et al*. (2008) also obtained significant inhibitory effect of *Z. multiflora* Boiss. EO on both *S. typhimurium* and *S. aureus* in a liquid food (barley soup) model system, which was increased by adding N at 25 and 8C for *S. aureus*, and only at 8C for *S. typhimurium*. In this study, we obtained similar results on *V. parahaemolyticus* and *L. monocytogenes* in salted fish fillets. Our results (Tables 1–3) showed significant inhibitory effect of EO on the studied microorganisms, which was significantly increased by adding N.

TABLE 4. MEAN RATING FOR ACCEPTABILITY OF FISH FILLETS (BRINE SALTED AT 4% NaCl) AS AFFECTED BY DIFFERENT CONCENTRATIONS OF EO (WHICH USED IN BRINE)

EO (%)	Mean rating ± SD
0	7.86 ± 0.69[a]
0.045	7.57 ± 0.78[a]
0.135	7.71 ± 0.75[a]
0.405	8.1 ± 0.69[a]
0.81	4.14 ± 0.37[b]

Means followed by the same letters are not significantly ($P < 0.05$) different.

EO, essential oil; SD, standard deviation.

Because EO containing mainly phenolic compounds and N act on the bacterial cytoplasmic membrane, their antibacterial activity could be enhanced by treatments involving combinations of them (Yamazaki *et al*. 2004). For *V. parahaemolyticus*, it could be further explained that phenolic constituents of EO act synergistically with N because of interaction with the outer membrane of the gram-negative cells, alteration in outer membrane structure, causing the loss of functional permeability barrier and resulting in an increased permeability for N (Gänzle *et al*. 1999; Ettayebi

et al. 2000). In contrast to the results obtained by Moosavy *et al*. (2008) and the findings of Kannappan *et al*. (2004), which indicated the disability of N to inhibit the growth of gram-negative bacteria (*S. typhimurium* in barely soup at 25 and 8C and *V. parahaemolyticus* in fish fillet at 37C, respectively), our findings in this study (Table 1) showed the significant effect of N on *V. parahaemolyticus* at 8C. This inhibitory effect may be described by the sensitivity of the organism to temperatures below the lower limit for its growth (Varnam and Evans 1991) that influenced the effect of N on *V. parahaemolyticus*.

According to the results (Table 3), for *L. monocytogenes* the obvious significant ($P < 0.05$) inhibitory effect (complete inhibitory effect; <–0.69 $\log_{10}$ MPN/g) of EO and its combination with N was observed at lower storage temperature of 4C. This was similar to the findings of Ultee *et al*. (1998) and Pol and Smid (1999) on *Baciluus cereus* and *L. monocytogenes* in laboratory media. This increased sensitivity at lower temperatures could be explained by the altered fluidity of the membrane, which is the primary target for N and EOs. At lower temperatures, the proportion of unsaturated fatty acid chains of the lipids is increased resulting in a higher membrane fluidity (Pol and Smid 1999), which could facilitate the incorporation of lipophilic compounds as N or carvacrol (the main constituent of some EOs such as *Z. multiflora* Boiss. EO).

According to the sensory analysis (Table 4), the undesirable sensory effect of EO was observed at concentrations 0.81% used in this study. Therefore, this strong synergistic inhibitory effect of EO by N reduces the total quantity of the preservatives incorporated in food products for adequate inhibitory effect on the bacteria and prevents any likely undesirable sensory effect of the EO.

CONCLUSION

According to the results, the growth (viable count) of *V. parahaemolyticus* and *L. monocytogenes* were significantly ($P < 0.05$) inhibited by *Z. multiflora* Boiss. EO, N and their combination (synergistic effect) in salted fish fillet in this study. Considering the organoleptic effect of the EO used in this study, the best inhibitory effects of EO in combination with N for *V. parahaemolyticus* and *L. monocytogenes* were obtained at combinations of EO = 0.405% and $n = 0.75$ μg/mL, and EO = 0.405% and $n = 0.25$ μg/mL, respectively. This synergistic effect, which was obtained in this study, can improve the scope of EO function in food industry.

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