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Research Paper

Use *Carum copticum* essential oil for controlling the *Listeria monocytogenes* growth in fish model system

Soghra Rabiey[1], Hedayat Hosseini[2], Masoud Rezaei[3]

[1]Department of Fisheries, Faculty of Marin Sciences, Tarbiat Modares University, Noor, Iran.
[2]Department of Food Sciences and Technology, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[3]Department of Seafood Science and Technology, Faculty of Marine Sciences, Tarbiat Modares University, Noor, Iran.

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Abstract

This study was conducted to evaluate the antibacterial effect of *Carum copticum* essential oil (Ajowan EO) against *Listeria monocytogenes* in fish model system. Ajowan EO chemical composition was determined by gas chromatography/mass spectral analysis and the highest concentration of *Carum copticum* essential oil without any significant changes on sensory properties of kutum fish (*Rutilus frisii kutum*) was assigned. Then the inhibitory effect of Ajowan EO at different concentrations in presence of salt and smoke component was tested on *L. monocytogenes* growth in fish peptone broth (FPB), kutum broth and cold smoked kutum broth at 4 °C for 12 days. Ajowan EO completely decreased the number of *L. monocytogenes* in FPB after 12 days of storage, however, antimicrobial effect of EO significantly reduced in kutum and cold smoked kutum broth. Addition of 4% NaCl and smoke component improved the anti-listerial activity of Ajowan EO in all fish model broths.

Key words: *Carum copticum*, *Listeria monocytogenes*, fish model systems, Hurdle technology, *Rutilus frisiikatum*.

Introduction

Listeria monocytogenes is the agent of listeriosis, a disease with low incidence rate (0.1 to 11.3 cases per million of population), but high mortality rate (28%) (Souza *et al.*, 2008). This pathogen can grow at a wide range of temperature (1 to 45 °C), pH (4.4 to 9.6), high salt content (100 g.L^{-1}), water activity (*aw*) below 0.93 and under aerobic, microaerophilic, and anaerobic (Feldhusen, 2000; Basti *et al.*, 2006). Because of being psychotropic, these bacteria can be considered as a dangerous pathogenic agent in foods stored at refrigerator temperature (Campos *et al.*, 2011). *L. monocytogenes* is widespread in nature and can be found in soil, foliage and the faeces of animals and humans and can be introduced into coastal regions and aquaculture ponds by animal manure and human waste (Feldhusen, 2000). Recently many researchers reported the occurrence of *L. monocytogenes* in raw and processed fish; Pao *et al.* (2008) found *L. monocytogenes* 23.5% in catfish, 5.7% in trout, 10.3% in tilapia and 10.6% in salmon purchased from internet and local retail markets (Pao *et al.*, 2008). Basti *et al.* found populations of *L. monocytogenes* greater than 10^2 cfu.g^{-1} in 2.6% of silver carp and 5.1% of smoked silver carp purchased in fish farms, 10% of salted Caspian anadromous shad and 20% of smoked silver carp purchased in a fish market were also contaminated (Basti *et al.*, 2006). A study which was conducted on the prevalence of *L. monocytogenes* in gravlax salmon processing line showed occurrence of *L. monocytogenes* in salmon samples (41%), food contact surfaces (32%); non-food contact surfaces (43%) and of food handlers' samples (34%) (Cruz *et*

Send correspondence to Hedayat Hosseini. Department of Food Sciences and Technology, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: hedayat@sbmu.ac.ir.

al., 2008). Several sporadic cases and outbreaks of listeriosis associated with seafood products have been reported recently: an outbreak (29 cases, nine deaths) in New Zealand associated with fish or molluscan shellfish (Lennon *et al.*, 1984); six to nine cases (two deaths) in Sweden caused by 'gravad' rainbow trout (Ericsson *et al.*, 1997); five cases in Finland associated with vacuum-packed, cold-smoked rainbow trout (Miettinen *et al.*, 1999); a case associated with fish consumption (Facinelli *et al.*, 1989). Since, concern about the side effects of chemical antimicrobial agents has been arisen in recent years, attention is shifting towards natural preservatives particularly plant essential oils as alternatives in foods. Both plant essential oils as well as similar compounds in wood smoke have shown promise as natural antimicrobials (Holley and Patel, 2005). Essential oils (EOs) are aromatic and volatile oily liquid extracted from different part of aromatic plants (Burt, 2004; Cruz *et al.*, 2008; Campos *et al.*, 2011). These oils are "generally regarded as safe" (GRAS), have broad spectrum of antimicrobial activity and pleasant odors and taste and can be used in food industry for their perfume, flavour and preservative properties (Burt, 2004; Oussalah *et al.*, 2006; Goudarzi *et al.*, 2011). Ajowan (*Carum copticum*) is grassy, annual, essential oil bearing plant which grown in Iran, India, Pakistan and Egypt. Ajowan essential oil is rich in monoterpenes such as thymol, ρ-cymene and γ-terpinene and it may be used as a natural anti-bacterial agent (Zargari, 1988). Many researchers have demonstrated the antibacterial activity of essential oils such as Ajowan EO against some foodborne pathogens (Sabanadesan *et al.*, 2000; Rani and Khullar, 2004; Oliveira *et al.*, 2010; Goudarzi *et al.*, 2011). However there aren't more studies on the effects of Ajowan EO against *L. monocytogenes* in fish model systems and its synergistic activity with NaCl. Thus, the aim of this work was to study the antimicrobial effect of Ajowan EO, salt, smoke component and their combination against *L. monocytogenes* in fish model systems in order to optimize in real fish products design.

Materials and Methods

Plant essential oil

Carum copticum (Ajowan EO) was supplied from Golgatre Essential Oil Co., Mashhad, Iran, and stored in brown bottles at 4 °C prior to use.

GC/MS analysis

The components of the EO were identified by Varian 3400 GC-MS system equipped with a DB-5 fused silica column (30 m x 0.25 mm i.d.); oven temperature was 40 °C to 240 °C at a rate of 4 °C. Transfer line temperature was 260 °C. Carrier gas was helium with a linear velocity of 31.5 $cm.s^{-1}$, split ratio 1/60. In addition, ionization energy was 70 eV, scan time 1 s, and mass range 40-300 amu. The components of the oil were identified by comparison of their mass spectra with those of a computer library or with authentic compounds or with the data published in the literature (Adams, 2011).

Sensory analysis

For sensory analysis kutum fish were filleted and divided into 40 g portions, one portion was dipped in 80 mL sterile 0.2% agar solution as a control, another portions were dipped in 80 mL of 0.2% agar solutions containing 0.1 to 0.6% concentrations of Ajowan EO for 15 min in room temperature. After draining off the excess liquid, the samples were placed in bags and stored at 4 °C for 24 h. Then samples were cooked in a steam-cooker for 10-15 min at 90 ± 2 °C and served warm in dishes coded with 3-digit random numbers and presented in individual booths to each panelist for evaluation. An eight-member trained panel was used, the panelists were asked to evaluate odor and flavour of fillet for on a scale from 10 to 0. According to score, acceptability was determined as having a score of over 6 (Puwastien *et al.*, 1999; Mahmoud *et al.*, 2004).

Bacterial strain and preparation of inoculums

L. monocytogenes PTCC 1298 from Iranian Research Organization for Science and Technology, Tehran, Iran, was used in this study. It was cultivated in Brain Heart Infusion broth (BHI) at 37 °C for 18-24 h. One hundred microlitres (100 uL) of culture were transferred to modified fish peptone broth [FPB containing 1% of sodium chloride; 0.5% of yeast extract; and 3.4% of fish peptone] and were incubated for 24 h at 37 °C. FPB bacterial cultures were diluted in saline peptone solution [0.1% bacteriological peptone; 0.85% sodium chloride solution] and used to obtain final populations of 10^6 $cfu.mL^{-1}$ for inoculation in FPB, kutum and cold smoked kutum broth (Reis *et al.*, 2011).

Preparation of fish peptone broth (FPB)

FPB was prepared with 3.4% of fish peptone, 0.5% yeast extract at two levels of salt 0 and 4%. The medium was divided into 9.9-mL aliquots in tube, sterilized for 15 min at 121 °C, and maintained at 4 °C overnight before inoculation. In order to dissolve Ajowan essential oil in fish model systems bacteriological agar at concentration level of 0.15% was used (Oliveira *et al.*, 2010; Reis *et al.*, 2011).

Preparation of kutum and cold smoked kutum broth

Kutum and cold smoked kutum broth were prepared according to Nilsson *et al*. Kutum fish (*Rutilus frisii kutum)* samples were boiled with distilled water for 10 min in a ra-

tio of 2:1 (w/v). The suspension was filtered in coffee filter. The juice was buffered with 5.98 g.L^{-1} of KH_2PO_4 and 9.75 g.L^{-1} of K_2HPO_4, and pH was adjusted to 6.2 with 1 mol.L^{-1} HCL. The broth was made at two levels of salt, 0 and 4%. The juices were divided into 9.9-mL aliquots in sterile tube, sterilized for 15 min at 121 °C and kept at 4 °C overnight before inoculation (Nilsson *et al.*, 1999).

Treatments

FPB, kutum broth and cold smoked kutum broth were inoculated with 0.1 mL of saline peptone solution containing 10^6 cfu.mL^{-1} of *L. monocytogenes*, so that the final cell numbers on broth were 10^4 cfu.mL^{-1}. The treatments were as follows: Ajowan EO (0%, 0.05%, 0.15%, and 0.3% v/v) added to NaCl 4% (w/v) or not.

Enumeration of microorganisms in fish model systems

In all fish model experiments, bacterial populations were enumerated at days of 0, 4, 8, 12 by pure plating 1 mL of appropriate dilutions in PALCAM Listeria Selective Agar, with incubation at 37 °C for 48 h.

Statistical analysis

All fish model experiments were done at least three times. Logarithm of bacterial counts were subjected to analysis of variance using ANOVA SPSS 16 (SPSS Inc. Chicago, IL, USA). Differences between means were tested through Duncan and values of $p < 0.05$ were considered significantly different. Sensory data were analyzed using Duncan and One-sample t-test(Mahmoud *et a.l*, 2004; Solomakos *et al.*, 2008a, Solomakos *et al.*, 2008b).

Results

Determination of EO constituents

The chemical compositions of Ajowan EO are shown in Table 1. Seventeen (17) compounds representing 98.8% of Ajowan EO were identified. The main components were thymol (57.18%), ρ-cymene (22.55%), γ-terpinene (13.07%) and *trans*-Anethole (1.7%). A number of studies on Ajowan EO content and constituents have been performed in Iran. Khajeh *et al.* showed that essential oil of Ajowan contained eight main compounds, including thymol (49%), γ-terpinene (30.8%), ρ-cymene (15.7%) and β-pinene (2.1%)(Khajeh *et al.*, 2004). Oroojalian *et al.* detected 12 component, include thymol (48.9%), ρ-cymene (21.8%), γ-terpinene (21.3%) and β-pinene (2.6%) (Oroojalian *et al.*, 2010). Goudarzi *et al.* showed that the main components were thymol (36.7%), ρ-cymene (21.1%), γ-terpinene (36.5%). Compared to other studies we found higher amounts of thymol and less ρ-cymene (Goudarzi *et al.*, 2011).

Table 1 - Phytochemical composition of Ajowan EO (*Carum copticum* essential oil).

No.	Phytochemicals	RT[a]	RI[b]	%
1	α-Pinene	11.35	931	0.29
2	β-Pinene	13.45	974	0.43
3	β-Myrcene	14.28	990	0.34
4	α-Phellandrene	14.89	1002	0.065
5	α-Terpinene	15.54	1015	0.311
6	ρ-Cymene	16.21	1028	22.55
7	β-Phellandrene	16.29	1030	0.541
8	γ-Terpinene	17.93	1062	13.07
9	α-Terpinolene	19.18	1087	0.095
10	α-Terpineol	24.92	1203	0.155
11	L-Carvone	27.97	1269	0.908
12	trans-Anethole	28.68	1284	1.7
13	Thymol	29.73	1307	57.18
14	Carvacrol	29.84	1310	0.524
15	3-Dodecen-1-Al	36.51	1465	0.161
16	Apiol	42.73	1623	0.566
	Total identified			98.886

[b]Retention time.
[a]Retention index relative to n-alkane series on the DB-5 column.

Sensory evaluation

Scores of samples treated with different concentrations of Ajowan EO are shown in Figure 1. Results showed that the organoleptic properties of kutum fillet treated with Ajowan EO were acceptable by the panelists at the levels of 0.1%, 0.2% and 0.3% but unacceptable at the higher level of EO. The panel detected no difference ($p > 0.05$) in aroma between untreated and 0.1-0.6% EO treated fillet. Sensory studies showed that the fillet treated with EO at concentration above than 0.3% had a score lower than 6 due to undesirable flavour and may not be acceptable to some of the consumers. 0.1 and 0.2% EO treated fillet had no signifi-

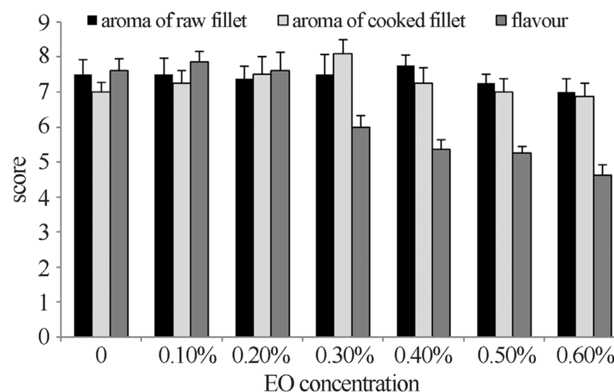

Figure 1 - Scors of organoleptic properties of kutum fillets treated with Ajowan EO.

cant ($p > 0.05$) different with untreated sample but significant different with 0.3% EO treatment ($p < 0.05$).

Effect of Ajowan EO on *L. monocytogenes* populations in FPB

The antimicrobial effects of Ajowan EO at 0.05%, 0.15%, and 0.3% on *L. monocytogenes* in FPB are shown in Figure 2. In the control sample *L. monocytogenes* initial count of 3.8 log cfu.mL^{-1} increased up to 8.5 log cfu.mL^{-1} at the end of storage at 4 °C Addition of Ajowan EO at 0.05%, 0.15% and 0.3% in FPB showed a high inhibitory effect against *L. monocytogenes* at the end of storage time. In 0.05% treatment, numbers of *L. monocytogenes* were declined to 0.8 log cfu.mL^{-1} after 12 days of storage. In broth with 0.15% and 0.3% Ajowan EO, *L. monocytogenes* populations immediately after inoculation reduced by 0.5-1.5 log cfu.mL^{-1}. No growth of viable cells was observed from 4 day and up to the end of the incubation trial. Addition of 4% NaCl significantly improved Ajowan EO performance ($p < 0.05$). In FPB with 4% NaCl and 0.05% Ajowan EO *L. monocytogenes* count completely inhibited from the 8th day, while in broth without salt the initial populations of *L. monocytogenes* decreased to 0.8 log cfu.mL^{-1} at the end of storage period. Moreover *L. monocytogenes* population in FPB containing 4% salt was 0.4 log cfu.mL^{-1} less than broth without salt ($p < 0.05$).

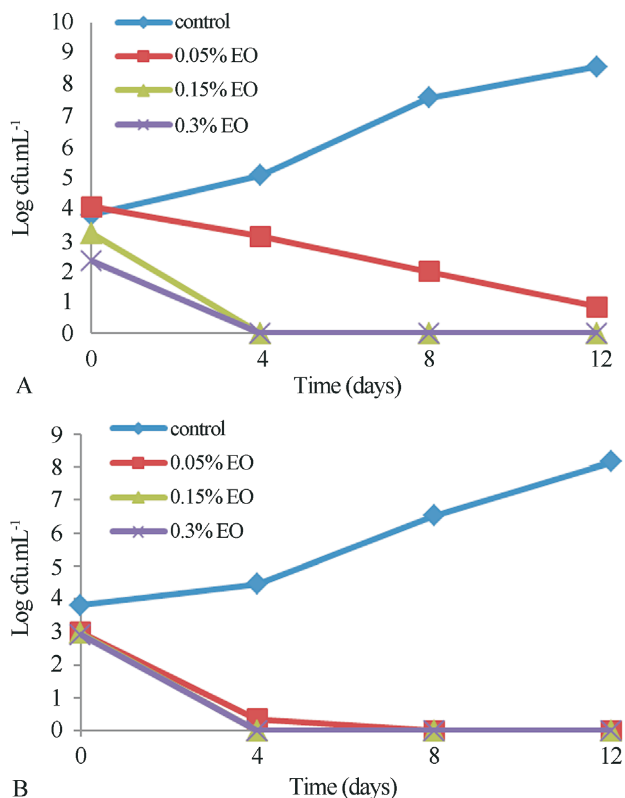

Figure 2 - Survivors curves for *Listeria monocytogenes* in; A-fish peptone broth; B- fish peptone broth plus 4% NaCl for 12 days at 4 °C.

Effect of Ajowan EO on *L. monocytogenes* populations in kutum broth

The antimicrobial effects of EO at 0.05%, 0.15%, and 0.3% on *L. monocytogenes* in kutum broth are shown in Figure 3. After 12 days of storage at 4 °C, the *L. monocytogenes* populations increased in kutum broth by 3.9 log cfu.mL^{-1}, the final counts were 8.1 log cfu.mL^{-1}. In 0.05% treatment initial populations of the *L. monocytogenes* were significantly increased during incubation time, and final populations of the pathogen in this treatment were 2.4 log cfu.mL^{-1} lower than those of the control. In 0.15% EO treatment number of bacteria did not significantly change during incubation period ($p > 0.05$). Addition of EO at 0.3% showed a higher inhibitory effect as compared to the addition at 0.15%. In 0.3% treatment, populations of *L. monocytogenes* showed a reduction of 1.7 log cfu.mL^{-1} after 12 days of storage. In 0.15% and 0.3% treatment final population of listeria were at least 4.2-5.8 log cfu.mL^{-1} less than control sample at the end of storage (day 12). Addition of 4% NaCl in kutum broth significantly improved Ajowan EO efficacy ($p < 0.05$).In broth containing 0.15% EO and 4% NaCl, *L. monocytogenes* population re-

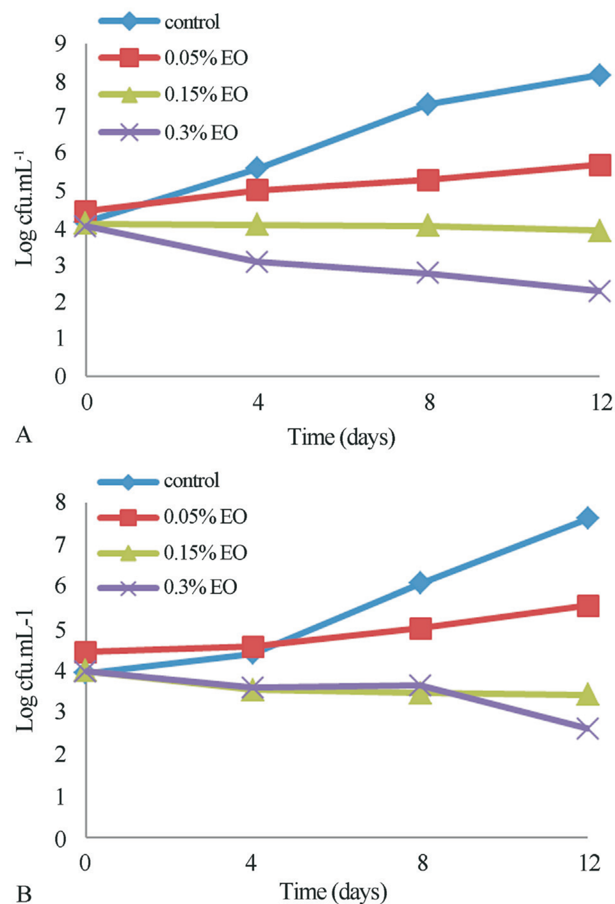

Figure 3 - Survivors curves for *Listeria monocytogenes* in; A- kutum broth; B- kutum broth plus 4% NaCl for 12 days at 4 °C.

duced by 0.5 log cfu.mL^{-1} after 12 days of storage. The number of *L. monocytogenes* in kutum broth containing 0.05% EO and 4% salt was significantly lower than kutum broth containing 0.05% EO during test period although this different wasent significant at the end of storage (12 day). Moreover *L. monocytogenes* count in kutum broth with 4% NaCl were 0.5 log cfu.mL^{-1} less than on kutum broth at 12 day ($p < 0.05$).

Effect of Ajowan oil EO *L. monocytogenes* populations in cold smoked kutum broth

According to Figure 4 after 12 days storage at 4 °C, the number of *L. monocytogenes* had increased 2.6 log cfu.mL^{-1} in cold smoked kutum broth without any salt or EO. In 0.05% EO treatment initial populations of *L. monocytogenes* were increased by 1.8 log cfu.mL^{-1}. In 0.15% EO treatment number of bacteria did not significantly change in incubation period ($p > 0.05$). In 0.3% treatment *L. monocytogenes* populations decreased from 3.8 log cfu.mL^{-1} to 3.1 log cfu.mL^{-1} ($p < 0.05$). Addition of 4% NaCl in cold smoked kutum broth (Figure 4) significantly improved Ajowan EO efficacy ($p < 0.05$) from 8th day on.

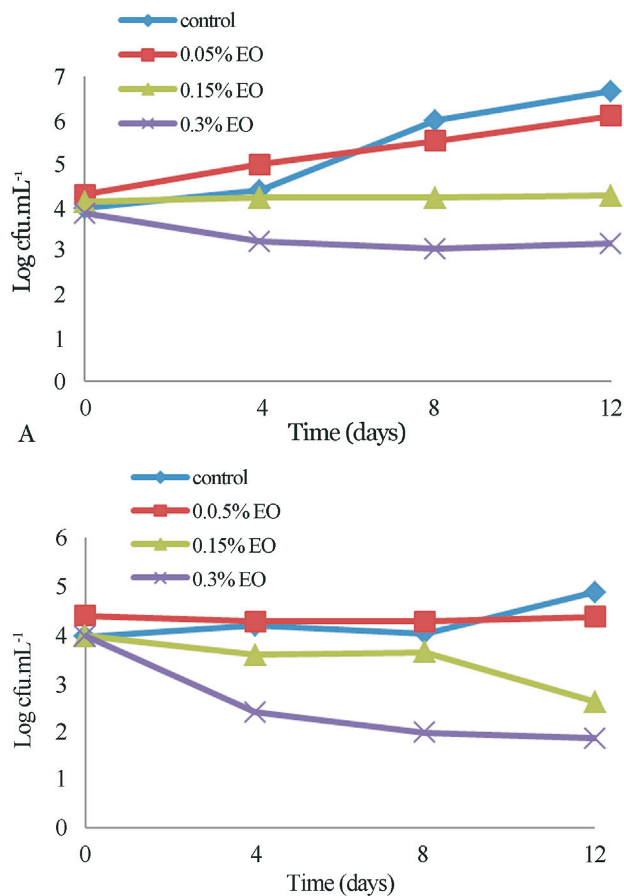

Figure 4 - Survivors curves for *Listeria monocytogenes* in; A- cold smoked kutum broth; B- cold smoked kutum broth plus 4% NaCl for 12 days at 4 °C.

In broth with NaCl, 0.05% EO showed bacteriostatic effect and 0.15% EO decreased *L. monocytogenes* population by 1.3 log cfu.mL^{-1}. The greatest decrease in populations (2.1 log cfu.mL^{-1}) was observed for 0.3% EO in broth with 4% NaCl.

Discussion

Recently many tests have been carried out in synthetic growth media in order to evaluate the EO efficacy against spoilage and food-borne pathogens. However, results obtained in this food model media (*e.g.* meat broth, vegetables broth, milk broth) may be more useful prior to further application in real food, rather than those observed using laboratorial media, since these food models media may assist in the optimized final application of EOs and would also reflect the nutrient availability and composition of food produce (Gutierrez *et al.*, 2009). Some authors used from such synthetic growth media to evaluate antimicrobial activity of plant essential oil and other antimicrobial component. Reis *et al.* (2011) evaluated the inhibitory effect of *Lippia sidoides* extract and lactic acid bacteria (LAB) against *L. monocytogenes* in model fish systems include fish peptone broth, fish broth and fish homogenate (Reis *et al.*, 2011). Oliveira *et al.* evaluated effect of combined application of thymol and carvacrol with lactic and acetic acid against *Staphylococcus aureus* in meat broth and in a food model(Oliveira *et al.*, 2010). Munoz *et al.* investigate the antimicrobial properties of plant extracts on the growth and viability of *L. monocytogenes* in laboratory medium and broccoli juice (Muñoz *et al.*, 2009). Souza *et al*. evaluated effect of *Origanum vulgare* essential oil against *Staphylococcus aureus* in nutrient broth, meat broth and in a meat model (Souza *et al.*, 2009). Prior to this study, sensory analyses were carried out to determine the highest concentration of EO without any organoleptic undesirable changes. Concentration of 0.3% was selected and EO at 0.05%, 0.15% and 0.3% were added in FPB, kutum broth and cold smoked kutum broth to evaluate antimicrobial activity. The results from this study showed that Ajowan EO showed strong antibacterial activity in FPB. In this model Ajowan EO at concentrations of 0.05%, 0.15% and 0.3% caused a sharp drop in *L. monocytogenes* count after 4 days and completely inhibited pathogen at the end of storage (day 12). In Oroojalian *et al.* study, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Ajowan EO against *L. monocytogenes* was 0.025% (Oroojalian *et al.*, 2010).The major constituents of Ajowan EO were thymol, γ-terpinene, and ρ-cymene. It has been shown that EOs containing phenolic compounds, *e.g.* thymol, carvacrol, γ-terpinene, and ρ-cymene, have high levels of antibacterial activity (Burt, 2004; Holley and Patel, 2005; Goudarzi *et al.*, 2011). In kutum and cold smoked kutum broth this effect significantly decreased, generally the plant

extracts efficacy decreased in the food model media, by comparison with the in vitro control media because the rich nutrients in food model media compared to laboratory media may enable bacteria to repair damaged cells faster (Burt, 2004; Gill *et al.*, 2002).

In kutum and cold smoked kutum broth, 0.15% EO showed bacteriostatic effect and number of bacteria did not significantly change during the incubation period, therefore this concentration can be considered as MIC of EO against *L. monocytogenes* in these mediums.

The results demonstrated a synergic effect of Ajowan EO and NaCl, addition of 4% salt significantly improved antimicrobial activity of Ajowan EO in FPB, kutum broth and cold smoked kutum broth. As the final concentration of NaCl in flesh of light salted fish is about 4% (Wilson and Droby, 2000), this concentration of NaCl has been applied in this model study. Synergistic effect between NaCl and plant essential oil has been observed in other studies. The combined use of NaCl and clove powder in mackerel muscle extract has totally prevent growth and histamine production by *Enterobacter aerogenes* (Wendakoon and Sakaguchi, 1993).Synergism between NaCl and mint oil against *S. enteritidis* and *L. monocytogenes* has been recorded in taramosalata (Tassou *et al.*, 1995). Antilisterial activity of garlic essential oil also improved by NaCl in BHI broth (Razavi Rohani *et al.*, 2011).

This synergic mechanism would be due to increasing effect of thymol on permeability of microorganism plasma membrane by perturbation of the lipid fraction and also inhibitory effect of NaCl on intracellular enzyme (Wendakoon and Sakaguchi, 1993; Gutierrez *et al.*, 2009). It has been shown that with a higher saline concentration, a greater bacterial surface hydrophobicity may facilitate EO penetration or contact with microorganism (Angienda and Hill, 2011). This could explain why it was possible to inhibit bacterial growth by combining EOs and saline. In this study the bacterial counts found for the FPB, kutum and cold smoked kutum broth added 4% salt without any EO were significantly lower than the counts obtained for the broth controls ($p < 0.05$).

According to Figures 3 and 4 *L. monocytogenes* growth in cold smoked kutum broth was significantly lower than kutum broth, *L. monocytogenes* count in the cold smoked kutum broth was 2 log cycle less than kutum broth after 12 days of storage at 4 °C. Results showed inhibitory effect of smoke and salt against this bacterium. Sabanadesan *et al*. (2000) evaluated liquid smoke for its antilisterial activity in salmon fillet and found smoking for 4 h resulted in a 1.5 log cfu.mL^{-1} reduction of *Listeria innocua* when smoking was done for 12 h, it gave a 3 log cfu.mL^{-1} reduction(Sabanadesan *et al.*, 2000). Poysky *et al*. reported that the use of smoke reduced the minimum heat required to kill *L. monocytogenes* in salmon steaks from 82 °C to 67 °C (Poysky *et al.*, 1997). Niedziela *et al.* asses the antimicrobial effect of salting and smoking on *L. monocytogenes* in salmon fillet and found there was no significant growth in the smoked samples, whereas in the salted-only samples the number of bacterium increased by between 2-5 log cycles (Niedziela *et al.*, 1998). In the same study commercially available phenols and formaldehyde from wood smoke in concentrations found in smoked products tested for their antimicrobial properties against *L. monocytogenes* in TSB with added salt at a concentration similar to that in smoked salmon, these experiments have shown that phenols and salt have a bacteriostatic, not bactericidal, effect but salt and formaldehyde have bactericidal effect. Sunen *et al*. (2003) reported smoke had a synergistic inhibitory effect with salt and vacuum packaging on both *L. monocytogenes* and *A. hydrophidae* in rainbow trout (Sunen *et al.*, 2003). The main purposes of smoking are development of aroma, color, flavor and preservation of food via antioxidant and antibacterial activity. The antimicrobial effect of smoking is due to the activity of some of the smoke component such as phenols, alcohol, organic acids, carbonyls, hydrocarbons that is result to wood burning (Jay, 2000).

Conclusion

The results of this study demonstrated the advantages of hurdle technique in fish safety via application of antilisterial factors such assmoke components, low temperature (4 °C), salt at 4%, and use of Ajowan EO without any undesirable changes in organoleptic properties of fish. The lowest growth of *L. monocytogenes* was observed in cold smoked kutum broth with 4% salt and 0.3% Ajowan EO. Final population of *L. monocytogenes* in cold smoked kutum broth containing 0.3% Ajowan EO plus 4% NaCl (1.8 log cfu.mL^{-1}) was 6.2 log cfu.mL^{-1} less than kutum broth (without any salt and EO) with final population of 1.8 log cfu.mL. Among the tested factors 0.3% Ajowan EO with 2 log cfu.mL^{-1} reduction on initial number of bacteria was more effective on inhibition of *L. monocytogenes* growth. Proteins, fats and other compounds which existing in fish matrix reduced the inhibitory effects of Ajowan EO on *L. monocytogenes*, while NaCl and smoke components stimulated this antilisterial effect.

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