Effect of *Zataria multiflora* Boiss essential oil on the growth of *Staphylococcus aureus* in the light salted fillets of silver carp (*Hypophthalmichthys molitrix*)

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Abstract

The effects of different concentrations of *Zataria multiflora* essential oils at concentrations of 0, 0.005, 0.015, 0.045, 0.135, 0.405 and 0.810% on the growth of *Staphylococcus aureus* were studied in a food model system, light salted fillets of silver carp (*Hypophthalmichthys molitrix*) at storage temperature of 10ºC for 21 days. The results showed that there was no significant difference in bacterial growth between samples treated with different concentrations of *Z. multiflora* essential oil and control group immediately post-inoculation. However, there was significant difference in growth of *S. aureus* between samples treated with concentration of 0.135 of *Z. multiflora* essential oil and control samples ($P<0.05$) on 2 and 6 days post-storage. No significant difference was observed on the growth of *S. aureus* in samples treated with lower concentrations of *Z. multiflora* (below 0.045%) compared with control group ($P>0.05$) except of 1-day post-storage. The most inhibitorial effects were observed in the samples treated with 0.405% and 0.810% of *Z. multiflora* essential oil up to 9 and 12 days post- storage, respectively.

Keywords: *Staphylococcus aureus*, Light salting, Silver carp fillet, *Zataria multiflora* essential oil

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Introduction

There are numerous reports of infections and food poisoning transmitted pathogens through raw and processed products (Prasad and Seenayya, 2000). Two percents of total sea food products in the world is consumed in the form of salted and smoked fish (Basti et al., 2006). *Staphylococcus aureus* is one of the common bacterial food pathogens causing food poisoning worldwide (Basti et al., 2006). In a study by Arkoudelos et al. (2003), *S. aureus* and *Salmonella typhimurium* survived up to 90 days in salted sardine. Human infections can be transmitted by fish or the water environment. Factors such as season, nutrition and immune system of the person can also impact the extent of the infection. Food microbial contaminations can be constrained by some preservatives such as organic acids and also by some physical and chemical methods (Youzbashi, 2001). Today, due to harmful effects of chemical food preservatives, there is a demand for use of natural preservatives derived from plant, animal and microbial resources which improve the human immune system (Burt, 2004). Furthermore, the world health organization has recently called for a worldwide reduction in the consumption of salt in order to reduce the incidence of cardio-vascular disease. To reduce the level of salt in the processed foods and maintain the safety of human foods, using natural preservatives such as essential oils of plants are the best choice (Ultee et al., 2002; Radmehr, 2006; Vandendriessche, 2008). *Z. multiflora* (namely Avishan-e Shirazi) is one of the plants with known anti-bacterial and antioxidant effects (Burt, 2004). Although the anti-bacterial properties *Z. multiflora* essential oils against some pathogenic bacteria including *S. aureus* is well known (Radmehr, 2006; Akhondzadeh et al., 2007; Fazeli et al., 2007 ;Moosavy, 2007) , such antibacterial properties have been demonstrated just in laboratory scale using artificial media. Fish meat is one of the important food products that can be rapidly contaminated by human pathogenic microorganisms (Wang and Shelef, 1992; Prasad and Seenayya, 2000; Barakat et al., 2004). In a study by Goulas and Kontominas (2007), the combined treatment of modified atmosphere packaging (MAP: 40% CO2/ 30% O2/ 30% N2) and oregano essential oil were studied on the shelf-life of lightly salted cultured sea bream (*Sparus aurata*) fillets stored under refrigeration. Salting had a noticeable preservative effect while oregano oil had a strong antioxidant activity. Also they showed that the fillets treated with 0.8% oregano oil were still sensorly acceptable. The objective of this study was to evaluate the antibacterial effects of *Z. multiflora* essential oils (0.005, 0.015, 0.045, 0.135, 0.405 and 0.810%) on the growth of *S. aureus* in a food model system, (light salted fillet of silver carp with NaCl less than 5 %), under an unfavorable refrigeration storage provided at 10ºC for 21 days to poisoning dose limit $>10^6$ (Varnam and Evans, 1991).

Materials and methods

Z. multiflora was collected from Shiraz Province, Iran and was identified by the Institute of Medical Plants, Tehran, Iran. Air-dried aerial parts of the plant were subjected to steam distillation for 2 h using Clevenger-type apparatus. The essential oil yield of the air-dried material was analysed

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by gas chromatography mass spectrometry (GC-MS) (Thermoquest2000, UK). The chromatograph was equipped with DB5 capillary column (30_0.25mm ID_0.25 mm film thickness) and the data were acquired under the following temperature conditions: initial temperature at 50ºC; program rate at 2.5ºC; final temperature at 265ºC and finally injector temperature at 250ºC were adjusted. The carrier gas was Helium and the split ratio was 120. The MS was run in the electron ionization mode, using ionization energy of 70 eV (Akhondzadeh et al., 2007). A lyophilized culture of *S. aureus* (ATCC 6538) obtained from the culture collection in the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Iran, was used in this study. The lyophilized culture was grown in tube containing 10 ml of Brain Heart Infusion (BHI) broth (Merck) at 35ºC for 18 h before use (Akhondzadeh et al., 2007). A 13×100 mm sterile cuvette containing 5 ml of sterile BHI broth was provided. The bacterial cells of *S. aureus* grown in the broth were adjusted to an optical density (OD) of 0.02 at 600 nm using the Spectronic 20 spectrophotometer (Milton Roy Company, USA). This adjustment gave a cell concentration of 1×10^7 cfu ml^{-1}. The number of cells in the suspension was estimated by duplicate plating from ten-fold serial dilutions of sterile 0.1% peptone water (Merck) on BHI agar and the bacterial colonies were counted after 24 h incubation at 35ºC. Finally, fish fillets were inoculated with *S. aureus* at 1×10^3 cfu /cm^3. (Akhondzadeh et al., 2007). A brine water of 4% NaCl (w/v) (Merck) was prepared using distilled water in the sterile screw capped flasks. Lecithin as stable oil-water emulsifier at 1% (v/v) and agar-agar stabilizer at 0.05% (w/v) (Merck) were added to the flasks. The flasks were then autoclaved at 121ºC for 15 min. *Z. multiflora* essential oil at concentrations of 0.005, 0.015, 0.045, 0.135, 0.405 and 0.810 % as treatments along with a control group (without *Z. multiflora* essential oil) were prepared. Fresh fillet of silver carp obtained from a fish farm were transported at 4ºC to the laboratory of Iranian Atomic Energy Organization and several pieces of 25 gram fillets with 3×8 cm^2 in size were immediately prepared and were steriled by Gamma Ray at a dose of 3 KGray (Nyka¨nen et al., 2000). A number of 12 fillets, (with chemical analysis of 76.8% moisture, 2% fat, 23% protein), were placed in each flask containing high concentrations of *Z. multiflora* essential oils. The flasks were then placed in a refrigerator at 5ºC for 24 h. Each treatment was included in tri-replicates. After 24 h, the fillets were transferred into the new empty sterile bags and each treatment was inoculated with 10^3 cfu/cm^3 in 10 points of surface of each fillet (Barakat et al., 2006). The bag cabs were firmly closed, labeled and placed in an incubator at unfavorable refrigeration storage temperature of 10ºC. To obtain the limit of toxicity dose of *S. aureus,* i.e $>10^6$ cfu/g, the bacteria were cultured from each treatment immediately at post-inoculation and on days 1, 2, 3, 6, 9, 12, 15, 18, 19 and 21. Each fillet of each treatment was removed and 225 ml sterile 0.1% (w/v) peptone water (Merck) added to it. Samples were then blended in a stomacher 400 (Lab Blender 400, Seward Medical Ltd., London, UK) for 3 min and diluted in sterile 0.1% (w/v) peptone water prior to inoculation on duplicate plates of enrichment culture of Baird–Parker agar (Merck) containing egg yolk and potassium telluride (Merck) (American Public Health Association, APHA, 1992)

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and then incubated at 37ºC for 48 h. The number of *S. aureu*s was determined as cfu/g of test samples.

The sensory-derived effects of adding *Z. multiflora* essential oil were evaluated by an acceptance test (Krishnamurthy et al., 2007) in samples treated with 0.135, 0.405 and 0.810% *Z. multiflora* essential oil in 1 and 3 days post-incubation**.** An eight-member trained panel was used to evaluate sensory color, odor, and overall acceptability attributes of fish meat treated with essential oils. Panelists were selected among students of the faculty before evaluation and treated fish meat samples cooked in a steam-cooker for 30 min by them. Each sample was served warm in dishes coded with 3-digit random numbers and presented in individual booths to each panelist for evaluation. A 3-point hedonic scale was used to score sensory attributes, where:

1: not acceptable, 2: relatively acceptable, 3: highly acceptable.

The effects of different concentrations of *Z. multiflora* essential oil on growth of *S. aureus* were evaluated using SPSS 15.0 statistical software (SPSS 15.0 for windows, SPSS Inc.) and analyzed the logarithm of total count of the bacteria by Tukey Test. Results were considered statistically significant when $P \leq 0.05$.

Results

The $\log_{10}$ (cfu ml^{-1} or g^{-1}) of *S. aureus* in silver carp fillets treated with different concentrations of *Z. multiflora* essential oil at 0, 0.005, 0.015, 0.045, 0.135, 0.405 and 0.810 % at storage time are showed in Table 1. The obtained results showed that the bacterial growth in **s**amples treated with different concentrations of *Z. multiflora* essential oil had not significant difference compared with control group immediately post-inoculation but samples treated with concentration of 0.135 of *Z. multiflora* essential oil had a significant decrease on the growth of *S. aureus* compared with control samples ($P<0.05$) on 2 and 6 days post-storage. No significant difference was seen on the growth of *S. aureus* in samples treated with lower concentrations of *Z. multiflora* (below 0.045%) compared with control group ($P>0.05$) except on 1 days post-storage. The inhibitoriest effects were seen in the samples treated with 0.405% and 0.810% of *Z. multiflora* essential oil up to 9 and 12 days post- storage, respectively.

The results of organoleptic evaluation of treated fillets showed that samples treated with 0.135 and 0.405% essential oil were highly acceptable (scale 3) on 3 days post- incubation while samples treated with 0.810% essential oil were relatively acceptable (scale 2) 1 day post-incubation. Other treated samples were not evaluated sensory acceptable.

Discussion

Various studies were carried out on using of different preservatives for increasing shelf life of fish fillets and delaying microbial growth (Goulas and Kontominas, 2007; Motalebi et al., 2010; Rostamzad et al., 2010) as the antibacterial activity of plant essential oils and their components could be considered suitable for application on food products. The current knowledge on potential antagonistic and synergistic features of such plants were presented by Burt (2004). *Zataria multiflora* Boiss is a native Iranian herb and its essential oil is vastly used as a food preservative and medical drug (Burt, 2004)**.** In the present study fillets treated with essential oil at concentration 0.405%

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showed an improved organoleptic property compared with other treatments. Of course sensory acceptance depends on type of fish feeding, season of catching, fish size as well as other environmental factors. Lastly it is directly related to microbial activity in

Table 1: Log_{10}(cfuml^{-1} or g $^{-1}$) of *S. aureus* in silver carp fillets samples treated with different concentrations of *Z. multiflora* essential oil (Log ±SD)

Time (day)	day							
Z. multiflora e. o.conc (%)	0	1	2	3	6	9	12	15
0.00	2.95^{a}±0.048	3.14^{a}±0.009	5.01^{a}±0.577	6.41^{a}±0.015	8.11^{a}±0.131	8.80^{a}±0.086	-	-
0.0005	3.04^{a}±0.039	3.74^{b}±0.007	5.32^{b}±0.020	6.39^{a}±0.001	7.69^{a}±0.004	7.68^{a}±0.001		
0.015	3.04^{a}±0.039	3.89^{b}±0.005	5.32^{b}±0.002	6.38^{a}±0.001	7.69^{a}±0.043	7.65^{a}±0.001	-	-
0.045	3.07^{a}±0.036	3.84^{b}±0.031	6.32^{a}±0.001	7.66^{a}±0.004	8.56^{a}±0.092	-	-	-
0.135	3.04^{a}±0.039	3.63^{b}±0.010	4.70^{c}±0.008	6.08^{a}±0.003	6.61^{b}±0.010	7.34^{a}±0.039	7.30^{b}±0.003	-
0.405	2.99^{a}±0.043	3.00^{a}±0.033	3.41^{d}±0.016	4.30^{b}±0.021	5.88^{c}±0.005	5.66^{b}±0.571	7.47^{b}±0.014	7.86^{a}±0.00
0.810	2.95^{a}±0.048	1.95^{c}±0.241	2.46^{e}±0.151	3.04^{c}±0.039	4.11^{d}±0.033	5.90^{b}±0.005	6.43^{c}±0.016	8.00^{a}±0.012^{a}

Different letters showing significant difference (P<0.05).

- = Not detected because of higher number of *S. aureus* detected (>10^6 cells) in samples.

e. o. Conc= essential oil Concentration

the fish tissue (Goulas and Kontominas, 2007). In a study by Barakat et al. (2004) the effects of garlic oil and combinations of some essential oils including allylisothiocyanate, carvacrol, cinnamaldehyde, citral, cuminnaldehyde, eugenol, isoeugenol, linalool and thymol were studied on the natural microflora in carp meat. Maximum inhibitory effect were observed by carvacrvl, thymol and cinnmaldehyde against *Acinetobacter, Alcaligenes, Bacillus, Flavobacterium, Micrococcus, Moraxella* and *Pseudomonas* and two families of Enterobacteriacea*e* and Vibrionaceae. The carvacrol is one the main components presented in the essential oils of *Z. multiflora* which the inhibitory effect on the growth of *S. aureus* could mainly create due to this component. Also, Bonyadian and Karim (2002) showed inhibitory effects of the volatile oil such as thyme, cumin, menta, oregano and tarragon on the growth of *S. aureus* and the most inhibitory effect recorded by thyme which is a dominant component of *Z. multiflora* essential oil. In a study by Alipour (2007) samples of commercial barley soup (liquid media) treated with concentrations of 0.005, 0.015 and 0.03 % of *Z. multiflora* essential oil showed a significant inhibitory effect (P<0.05) against the growth of *Bacillus cereus* at 15 and 25ºC for 21 days and while the temperature decreased, growth rate of the bacteria decreased. The antimicrobial activity of essential oils is benefited by a decrease in storage temperature (Burt, 2004).Moosavy et al. (2008) studied the effect of different concentrations of *Z. multiflora* essential oils on the growth of *S. aureus* in both temperature of 8 and 25ºC resulted in greater inhibitory effect at 8ºC than 25ºC. Thus viable count of the bacteria could affect by the storage temperature. Also, in this study we used unfavorable refrigeration temperature of

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10ºC at which can enhance the growth of *S. aureus* as well as the spoilage of fish fillet. In addition, the results of other workers (Wang and Shelef, 1992; Tassou et al., 2000; Gutierrez et al., 2008) showed that the effectiveness of essential oils decreased when the experiments were conducted in the *"in vivo* conditions*"*. This could be due to high protein and fat contents of meat which can mask the antimicrobial effect of essential oils. In this study, the effectiveness of *Z. multiflora* essential oil also reduced in the *in vivo* conditions (fish fillet) which contains high protein content therefore it seems that higher concentrations of essential oil is necessary to preserve the food products such as fish fillet in the *in vivo* condition compare with *in vitro* condition, e.g., liquid food products. Essential oil is hydrophobic in nature and so is more soluble in the lipid phase of food while microorganisms sit in the hydrophilic portion. These reactions may reduce opportunity for contact between the antimicrobial substances of *Z. multiflora* essential oil and the target organism. In addition the fat and protein content and pH of food system are important factors affecting the activity of essential oils (Tassou et al., 2000; Burt, 2004).In a study by Singh et al. (2003), different plant essential oils against *Listeria monocytogenes* in hotdogs (rigid media), was reduced the bacterial population but the antimicrobial action was highly dependent on the fat content of hotdogs. Also, Burt (2004) suggested that herbs do have greater effect in broth culture medium than in food as said previously. Barakat et al. (2006) studied the combination effects of NaCl 0.85% and essential oil compounds contains of 0.5% carvacrol and 0.5% thymol on fillets of carp fish and showed that the combination of NaCl saline 0.85% and thymol and carvacrol enhanced storage time at 5 and 10ºC in carp fish fillets which is in agreement with the results obtained in this study. In another study, the antimicrobial effect of *Z. multiflora* essential oil at 0.3%, 0.6%, or 0.9% and nisin at 500 or 1000 IU/g and their combination against *Listeria monocytogenes* was examined in both tryptic soy broth (TSB) and minced beef meat. *Z. multiflora* essential oil at 0.3% possessed a weak antibacterial activity against the pathogen in TSB, whereas at 0.9% showed unacceptable organoleptic properties in minced meat. Thus, only the level of 0.6% of *Z. multiflora* essential oil was further examined against the pathogen in minced meat. Treatment of minced beef meat with *Z. multiflora* essential oil at 0.6% and nisin 1000 IU/g showed stronger inhibitory activity against *L. monocytogenes* (Solomakos et al., 2007). In minced beef meat, the content of protein, fat and moisture were 21.8%, 2.4% and 72.2%, respectively, while in this study these contents were 23%, 2% and 76.8% respectively. Obviously more studies are needed on the antimicrobial properties of essential oils and their compounds before they can be used as food preservatives.Based on studies of Moreira et al. (2005), further studies are needed to investigate the oils incorporation into appropriate food formulations, and evaluate flavor, chemical changes and antimicrobial effect in the whole food system. In conclusion, the obtained results of this study suggest a possible application of *Z. multiflora* essential oil as a food natural preservative in fish fillet. However more studies are required to verify the precise conclusions of essential oil provided at different storage temperatures.

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