Combined effects of *Zataria multiflora* boiss essential oil and nisin on the shelf-life of refrigerated rainbow trout (*Onchorynchus mykiss*) fillets

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Abstract

The effects of *Z. multiflora* essential oil (EO) and nisin (N) on fresh rainbow trout (*Onchorynchus mykiss*) fillets were studied during 18 days storage at 4 ºC. Bacteriological and organoleptical evaluation were performed on fresh rainbow trout as the functions of treatment and storage time. Treatments included the following: A (control), E_1 (treated samples with 0.2 % EO), E_2 (treated samples with 0.4 % EO), N (treated samples with 150000 IU nisin/ kg), E_1N (treated samples with Nisin and 0.2 % EO) and E_2N (treated samples with nisin and 0.4 % EO). Total viable count (TVC) reached the value of 7 log cfu/g on days 9-12 for control sample and on days 15-18 for E_1N and E_2N samples. Meanwhile, in the sensory evaluation test, shelf life of rainbow trout fillets was 9 days for control samples and 12 days for other treated samples.

Key words: Rainbow trout fillet, Shelf life, *Zataria multiflora* ,Boiss Essential oil, Nisin.

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Introduction

Many efforts have been done to supply fresh seafood rather than frozen or other processed products with high quality according to increasing consumers' demands (Hassan, 2002; Fernández et al., 2009). It is well known that fish are in group of very perishable food, due to large amounts of free amino acids, volatile nitrogen bases, highly unsaturated fatty acids and higher final pH (Liston, 1980; Razavi Shirazi, 2001). Chemical, enzymatic and microbial activities during storage cause to loss of quality and limit the freshness and shelf life of fish (Özogul et al., 2006; Özyurt et al., 2009). In order to prevent the spoilage of fresh fish, various compounds such as chemical antimicrobials and antioxidants are used (Lu et al., 2010). Since the awareness of chemical preservative problems has arisen in recent years, consumers increasingly demand for minimally processed food with natural products as alternative preservatives (Tassou et al., 2000; Delaquis et al., 2002; Valero and Salmeron, 2003; Govaris et al., 2010). In this situation, natural additives such as essential oils and nisin is used to extend the shelf life of different food, to develop natural preservative with high antioxidant and antibacterial effects that could extent the shelf life of fish (Burt, 2004; Fernández et al., 2009; Mexis et al., 2009; Frangos et al., 2010).

Essential oils possess antibacterial, antioxidant, antiviral and anti-mycotic properties (Burt, 2004). The antimicrobial and anti-oxidative properties of essential oils are mainly due to their phenolic compounds, i.e. carvacrol and thymol (Dormana et al., 2003; Burt, 2004; Lee et al., 2005). *Z. multiflora* Boiss belongs to *Lamiaceae* family (Gandomi et al., 2009) and possess carvacrol and thymol as main phenolic compounds and p-cymene as main non-phenolic compounds, respectively (Sharififar et al., 2007). According to previous studies, *Z. multiflora* essential oil (EO) is active against *Bacillus cereus*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Aspergillus flavus*, *Listeria monocytogenes*, *Escherchia coli* and others in brain and heart infusion broth (Akhondzadeh et al, 2007; Gandomi et al., 2009).

Nisin is a polypeptide bacteriocin produced by certain strains of *Lactococcus lactis*. It acts against Gram positive and also Gram negative bacteria in food with combination of other antimicrobial factors or essential oils extracts of plants (Nykänen et al., 1998; Lu et al., 2010; Govaris et al., 2010). Antibacterial effects of nisin together with cinnamon and oregano essential oil have been studied on the quality of fish and meat products (Govaris et al., 2010; Lu et al., 2010).

With regard to fish, there are no studies in the literature on the antimicrobial effects of nisin in combination of Z. *multiflora* Boiss EO, on the shelf life of fish model system. Thus the aim of this study was to determine the bacteriological and sensory changes of refrigerated rainbow trout fillets using of either nisin or *Z. multiflora* Boiss essential oil and their combination during storage at 4 ºC.

Materials and methods

Zataria multiflora

Z. multiflora was collected from Shiraz (Fars province, Iran) and was identified by Medical Plants' Herbarium of Jihad Daneshgahi Tehran, Iran. The air-dried aerial parts were subjected to steam distillation for 4 h using the Clevenger-type apparatus (Rahnama et al., 2009). The extracted essential oil was analyzed by GC-MS (Agilent, model 6890 GC and model 5973 mass detector, America). Separation of active compounds of *Z. multiflora* EO was achieved on a HP-5MS (30 m x 0.25 mm ID x 0.25 μm film thickness). In addition helium was used as the carrier gas with a flow rate of 0.8 ml/min. The oven was programmed at an initial temperature of 50 ºC for 5 min. Temperature was increased to 240 ºC with a rate of 3 ºC/ min and then increased to 300 ºC with a rate of 15 ºC/min and held for 3 min. The injector temperature was set in 290 ºC. The MS was run in the electron ionization mode set at 70 eV. The ion source temperature was maintained at 220 ºC (Rahnama et al., 2009).

Fish Samples and Preparation

72 fresh farmed rainbow trout (*O. mykiss*) with the average weight and length of 400 g and 270 mm, respectively, were obtained from a farm located on Sari (Mazandaran, Iran) in May, 2010. The fish were transported to the laboratory within 20 min of harvesting in insulated boxes containing ice. The fish were then eviscerated, headed and filleted by hand (approximately each fillet was uniformed according to 100 g in weight). The fillet samples were then randomly divided into six treatments, namely: A (control samples without nisin and EO), E_1 (treated samples with 0.2 % EO), E_2 (treated samples with 0.4 % EO), N (treated samples with 150000 IU nisin/ kg), E_1N (treated samples with 150000 IU nisin/ kg and 0.2 % EO) and E_2N (treated samples with 150000 IU nisin/ kg and 0.4 % EO); each with 3 replicates. Essential oil was added onto the surface (two sides) of each fillet and gently massaged by hand to obtain homogenous distribution of EO. Nisin (Serva, Heidelberg, US) was also sprayed homogenously by syringe in two sides of fillets. Then samples were vacuum packaged (Henkelman, 200 A, Netherland) and stored at 4 ºC. Sampling was carried out on day: 0, 3, 6, 9, 12, 15 and 18 of storage.

Microbiological analysis

Microbiological counts were determined by placing a 10 g sample in 90 ml of 0.85% NaCl solution, and homogenizing with a stomacher (Moulinex, France) for 60s. From this dilution, other decimal dilutions were prepared and plated in the appropriate media. Meanwhile, the total viable counts (TVC) were determined using tryptic soy agar (TSA, Merck) after incubation for 48h at 25 ºC (AOAC, 2005). The inoculated plates were incubated at 4 ºC for 10 days for Psychrotrophilic (PTC) count (Mcfaddin, 2000). In Enterobacteriaceae count (EBC), violet red bile glucose agar (VRBGA, Merck) was used (Sallam, 2007). Incubation was carried out at 25 ºC for 48h. The large colonies with pink halos were counted. Lactic acid bacteria (LAB) were enumerated on de Man Rogosa

Sharpe agar (MRS, Merck) incubated at 30 ºC for 2-3 days (Sallam, 2007). The microbiological data were transformed into logarithms of the number of colony-forming units (CFU/g).

Organoleptic evaluation

The attributes of cooked fish (i.e. odor, taste and color) were evaluated by a panel of 10 experienced judges on each day of sampling. Fish samples were baked in oven (at 105 ºC) for 20 min and immediately presented to the panelists. They were asked to score odour using a 1-15 hedonic scale (with 15 corresponding to the most liked sample and 1 corresponding to least liked samples), the taste using a 1-10 hedonic scale (where 1 is undesirable and 10 is desirable) and the color using a 1-6 hedonic scale (Ranken et al., 1993).

Statistical analysis

All measurements were replicated three times for each treatment and mean values ± standard deviations were reported for each case. Analysis of variance (ANOVA), least significant difference ($P<0.05$), Kruskal-Willis test and Mann Whitney U test were performed to evaluate the significance of differences among mean values, using the SPSS 15.0 for windows, SPSS Inc.

Results

According to GC-MS analysis, the main phenolic and non-phenolic active compounds in *Z. multiflora* essential oil consist of thymol (59.50 %), p- cymene (13.60 %), carvacrol (5.6 %) and γ-terpinene (4.3 %).

Microbiological changes

The changes in microbial flora of rainbow trout fillets as a function of treatment and storage time are shown in Fig. 1 a-d. Initial total viable counts (Fig. 1a) of fresh rainbow trout were 3.65 log cfu/g. Treated samples (E_1, E_2, N, E_1N and E_2N) significantly ($P<0.05$) showed decreases in growth of bacteria in comparison with control group immediately post-inoculation. The increase in TVC was found in all treatments during storage at 4 ºC. TVC reached the value of 7 log cfu/g (microbiological upper limit for fresh fish used by Salam, 2007), during days 9 to 12 for control sample (A), day 12-15 for treated samples with 0.2 % EO (E_1) and treated samples with 0.4 % EO (E_2), day 15 for treated samples with nisin (N), and 15-18 day for treated samples by nisin and 0.2 % EO (E_1N) and treated by nisin and 0.4 % EO (E_2N). The use of *Z. multiflora* essential oil (0.2 and 0.4 %) and nisin resulted in microbiological shelf life extension of 3 days, while the use of combination of nisin with *Z. multiflora* essential oil (E_1N and E_2N) resulted in microbiological shelf life extension of 6 days, comparing with control samples. The initial Psychrotrophilic bacteria (PTC) count of rainbow trout fillets in control and treated samples, ranged from 1.62 in E_2 samples to 3.25 log cfu/g in control samples (Fig. 1b).There were significant differences ($P<0.05$) between control and treated samples by nisin and *Z. multiflora* essential oil (0.2 and 0.4 %) in day zero immediately post-inoculation. The PTC bacteria showed a growth pattern same as that of TVC during storage. They exceed 7 log cfu/g on day 12-15 of storage for control samples, E1, E2 and N treatments

and day 18 of storage for treatment of nisin and 0.2 % EO (E_1N) and 0.4 % EO (E_2N), respectively. There were significant differences ($P<0.05$) between control and E_1, E_2 and N treated samples at day 15. The initial and final counts of these bacteria for control samples were less than abovementioned bacteria (Fig. 1c). There were no significant ($P<0.05$) differences on EBC count immediately after treating the fresh rainbow trout fillets. Control, E_1, E_2 and N samples exceeded the 7 log cfu/g at day 12-15 of storage, while treated samples with nisin and essential oil exceeded the 7 log cfu/g at day 15- 18 (Fig. 1c). LAB were found to be members of the natural microbial flora of fresh rainbow trout fillets (Fig. 1d) with 2.12 log cfu/g. Using nisin and *Z. multiflora* essential oil either separately and in combination, significantly ($P<0.05$) reduced the LAB counts. In this study, all treatments showed significant effect ($P<0.05$) on reduction of LAB count. E_2N had the maximum effect with a 1.86 log cfu/g reduction in LAB, followed by E1N and E2 samples. All samples (control and treated) did not reach the upper acceptability limit for fresh water species throughout the 18-day storage period. Two way ANOVA analysis showed that the effects of treatments and storage duration and them interaction were significant ($P<0.05$) on TVC, PTC, EBC and LAB counts.

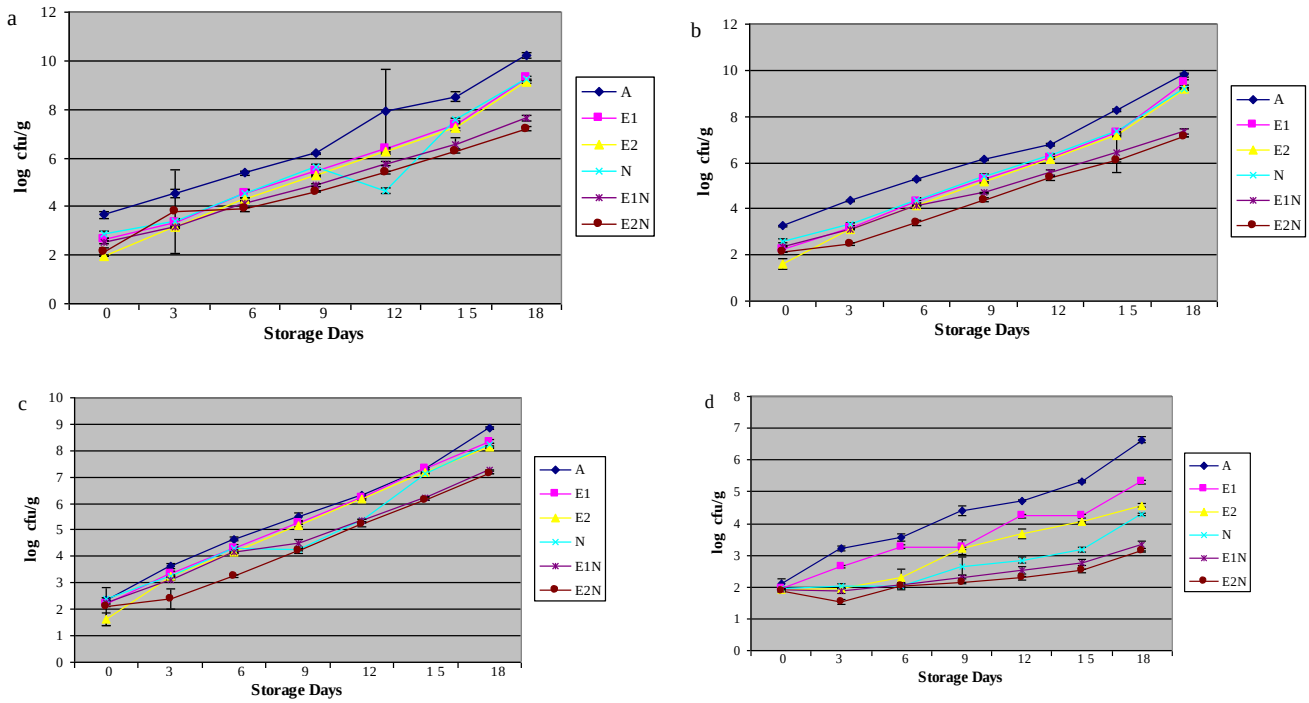
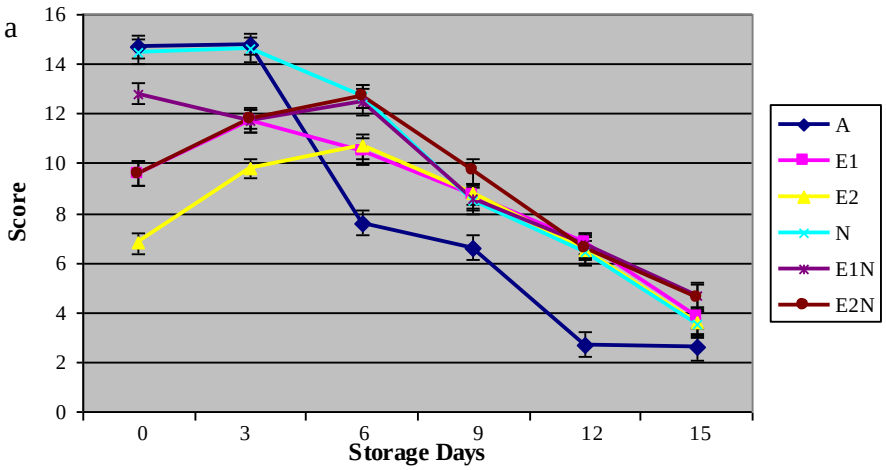


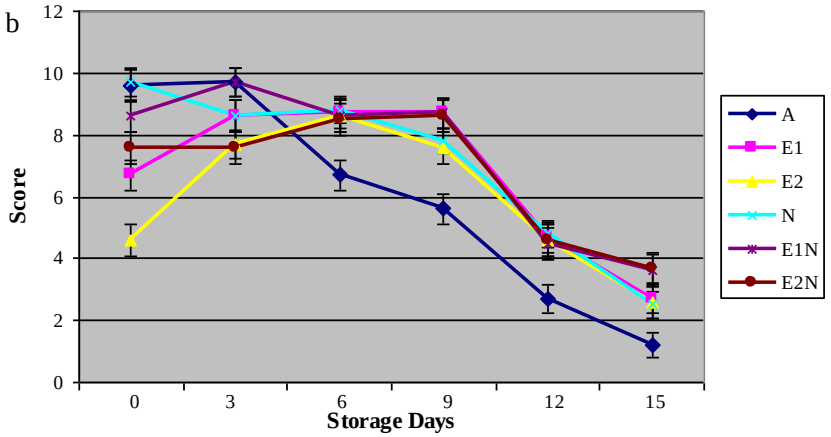
Figure 1: Changes (log cfu/g) in (a): Total Viable count (TVC); (b): Psychrotrophilic (PTC) counts; (c): Enterobacteriaceae counts (EBC) and (d): Lactic acid bacteria (LAB) in untreated and treated rainbow trout fillet stored at 4 ºC. Each point is the mean of three replicate and error bars show SD.

Sensory analysis

The results of the sensory evaluation (odor, taste and color) of cooked fillet samples are presented in Fig. 2a-c. There were significant (P<0.05) differences in odor and taste score between treated and untreated cooked fillets in day 0 (immediately post-inculation). On day 0 of storage control cooked samples had a pleasant odor and taste which are characteristic of fresh fish. Samples treated with nisin also showed a highest score same as control samples. Treated samples with *Z. multiflora* essential oil had the lowest acceptability at day 0 (Fig. 2a-c). Results showed successful application of nisin with essential oil with more pleasant odor and taste at days 0 and 3 (Fig. 2 a-b).

The sensory attributes changes during storage were not the same for treated and untreated samples. While the sensorial score of control and nisin treated samples decreased, the score of other treated samples increased until days 3 and 6 of storage and then started to decrease. From day 6 to end of storage, all treated samples showed higher odor and taste score compared with control samples. There were no significant effects of nisin and *Z. multiflora* essential oil on color score of rainbow trout fillet. Based on sensory evaluation (odor and taste), shelf life of rainbow trout fillets was 9 days for control samples and 12 days for other treated samples.





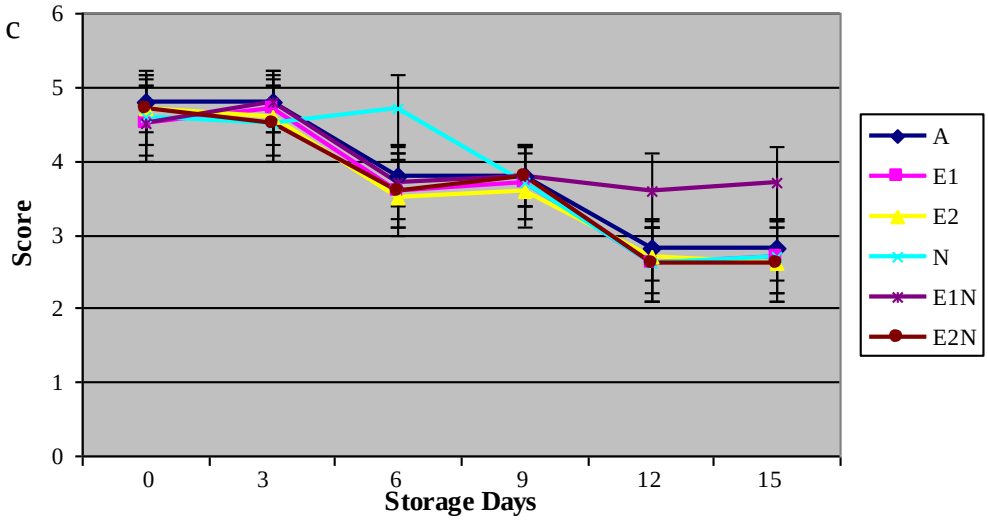


Figure 2. Changes in odor (a), taste (b) and color (c) scores of untreated and treated rainbow trout fillet stored at 4 ºC. Each point is the mean of three replicate and error bars show SD.

Discussion

Two main components used for treating of rainbow trout fillets in this study (nisin and *Z. multiflora* essential oil), showed antibacterial effects and prolonged the shelf life of rainbow trout fillet during chilled storage. Based on microbiological study, treatment of fillets by nisin and essential oil prolonged the shelf life of fresh fillets from 3 to 6 days. The effect of essential oil on the extension of shelf life of fresh fish has been also reported by Kykkidou et al. (2009); Mexis et al. (2009) and Frangos et al. (2010). The same effect has also been reported for nisin (Nilsson et al., 1997; Nykänen et al., 2000). The synergistic effect can be clearly seen, since the best result found for samples treated with nisin and 0.4 % EO (E_2N) which followed by E_1N.

Initial total viable counts of fresh rainbow trout were 3.65 log cfu/g in present study. A 2.7 and 3.0 log cfu/g initial bacterial counts were reported for rainbow trout by Mexis et al. (2009) and Frangos et al. (2010), respectively.

Zataria multiflora Boiss is a native Iranian herb, belongs to *Lamiaceae* family, and its essential oil is vastly used as food preservative and medical drug. Phenolic compounds in essential oils have antibacterial effects and categorized as GRAS (generally recognized as safe) (Bagamboula et al., 2004; Burt, 2004). *Z. multiflora* Boiss possess carvacrol, thymol as main phenolic compounds and p-cymene as main non-phenolic compounds, respectively (Sharififar et al., 2007). It has

been reported that the quantity of these compounds can be vary due to harvesting season, plant age, soil, climate, geographical sources, herb drying method and extraction method (Shaffiee and Javidan, 1997; Bagamboula et al., 2004; Shraififar et al., 2007). Carvacrol and thymol are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP (Burt, 2004). It has been reported that Gram-negative of these bacteria are the major group of microorganisms responsible for aerobic spoilage of stored fresh fish at chilled temperature (Ojagh et al., 2010). Studies also showed that carvacrol interacts with the cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains (Ultee et al., 2000). It is well known that the antimicrobial potency of essential oils in food systems is generally reduced when compared to in vitro work, due to presence of fats, carbohydrates, proteins, salts and pH strongly influence the effectiveness of these agents (Burt, 2004). Accordingly, larger amounts of essential oils are required in food systems, thus seriously interfering with the final organoleptical properties. It has been reported that nisin can inhibit a wide range of Gram-positive bacteria by making pores in the cytoplasmic membrane (Driessen et al., 1995) and also inhibit Gram-negative and nisin-resistant Gram-positive bacteria when is used with other methods and materials (Nykänen et al., 2000). Enterobacteriaceae are facultative anaerobic bacteria and psychrotolerant, which have capability of growing at refrigeration temperature (Frangos et al., 2010). It has also reported that they can not compete well with other Gram-negative spoilers (Atrea et al., 2009). Reduction of LAB by 1.1 and 2.6 log cfu/g has reported by Chouliara and Kontominas (2006) and Mexis et al. (2009), respectively in treatments of oregano oil and combination of oregano essential oil with oxygen absorber. Actually, lactic acid bacteria (LAB) are also facultative anaerobic bacteria that can grow under both anaerobic and aerobic conditions (Jay, 1986). They are tolerant to CO_2 that may inhibit growth of other bacteria (Atrea et al., 2009).

By using a combination of nisin and *Z. multiflora* essential oil, both of which have no adverse effects on humans, it may be possible to produce both safer and higher quality product. Meanwhile, present study showed that using of *Z. multiflora* essential oil give an unpleasant sensorial attributes to fresh fillet in initial stage of treatment due to strong flavor of essential oil and also application of nisin with essential oil could reduce this problem and yielded a more pleasant odor and flavor. It has been reported this unpleasant sensorial attributes is due to strong flavor of essential oil (Kostaki et al., 2009; Choobkar et al., 2010). *Z. multiflora* essential oil and nisin either separately or in combination extend the shelf life of rainbow trout fillet for 3 to 6 days in comparison with control samples during storage at 4 °C. The best results were found in E_2N followed by E_1N, regarding to microbiological studies.

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