



Antibacterial Activity of *Zataria multiflora* Boiss Essential Oil against Some Fish Spoilage Bacteria

Saber Barkhori-Mehni ^a, Saeed Khanzadi ^a, Mohammad Hashemi ^{b,*}, Mohammad Azizzadeh ^c

^a Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

^b Department of Nutrition, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

^c Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

*Corresponding author. E-mail address: mo_hashemi@hotmail.com

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ABSTRACT

Background: The aim of this study was to investigate antimicrobial effect of *Zataria multiflora* Boiss essential oil (EO) against six fish spoilage bacteria for evaluation of its potential utilization in the preservation of minimally processed fish products.

Methods: Firstly, GC-MS analysis of the EO was performed to determine its chemical composition. Then, antibacterial effect of the EO in a range of 0.031 to 4 mg/ml was tested against different fish spoilage bacteria such as *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*, *Escherichia coli* and *Bacillus subtilis* by broth microdilution method to determine minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations.

Results: GC-MS results showed that phenolic components such as carvacrol (51.55%) and thymol (25.49%) were predominant constituents of the EO. *Zataria multiflora* Boiss EO exhibited strong antimicrobial activity against all tested bacteria. *Shewanella Putrefaciens* was the most sensitive bacteria with MBC value of 0.5 mg/ml.

Conclusion: According to the results, this EO could be used as an important natural alternative to prevent bacterial growth in food specially seafood products to preserve them against bacterial spoilage.

1. Introduction

One of the main problems in food industry is increase of resistant bacteria to the current antimicrobial agents and side effects of synthetic antimicrobials consumption [1].

Therefore food producers concern about consumers safety and try to achieve new natural antimicrobial compounds due to their tendency for

Consumption of food without chemical preservatives [2, 3].

Recently, attention to the use of preservatives with plant sources are increasing and many researchers have focused on antimicrobial and antioxidant effects of extracts and EOs from medicinal plants [4- 10].

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EOs have shown antibacterial, antioxidant, antiviral and anti-mycotic properties in different studies [110]. Generally, the major components of EOs determine their biological properties. Variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components and aliphatic components can be found in EOs obtained from different parts of the aromatic plants [4, 11]. These components are dependent on the plant genotype and are influenced by several factors as geographical origin as well as environmental and agronomic conditions [4].

Therefore application of these EOs requires detailed knowledge about their chemical composition and their antimicrobial effect against food spoilage or pathogenic bacteria [12].

Zataria multiflora Boiss, with the Iranian name of Avishan-e-Shirazi, is a plant belonging to the Lamiaceae family [8]. It is extensively used as a flavor ingredient in a wide variety of food like dairy products in Iran. There are also commercial pharmaceuticals with formulae based on *Z. multiflora* Boiss EO that are used commonly for the treatment of respiratory tract infections as an antiseptic, antitussive and irritable bowel syndrome treatment [8, 13]. A large number of studies have determined compounds of different plants in Lamiaceae family and have demonstrated antimicrobial properties of their EOs against some food spoilage or pathogenic bacteria [2, 14-16].

Fish are recognized as very perishable food due to large amounts of free amino acids, volatile nitrogen bases, highly unsaturated fatty acids and higher final pH [13]. The main cause of chemical deterioration in fresh or minimally processed fish is activity of typical seafood spoilage microorganisms [17]. *Shewanella putrefaciens* and *Pseudomonas spp.* were identified as the specific spoilage organisms in any seafood [18]. Moreover, some foodborne pathogenic bacteria such as *Aeromonas hydrophila* are able to grow at low temperatures during storage or distribution [16, 18].

Therefore it is required to apply new preservatives in fish and fish products, not only for improving the product's shelf life extension but

also for ensuring their microbiological safety. Accordingly, the aim of the present study was 1) to determine the chemical composition of *Z. multiflora* Boiss EO by GC/MS analysis and 2) to evaluate *in vitro* antibacterial activity of the EO against fish spoilage bacteria such as *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*, *Escherichia coli*, *Bacillus subtilis* for evaluation of its potential utilization in the preservation and safety of minimally processed fish products.

2. Materials and Methods

2.1. Materials

Aerial parts of *Zataria multiflora* Boiss plant was purchased at the full flowering stage in spring of 2015 from Shiraz, Fars province, Iran. The taxonomic identification of plant materials was confirmed by Herbarium Department of Academic Center For Education, Culture and Research, Karaj, Iran. The Lyophilized cultures of bacterial strains (*Aeromonas hydrophila* ATCC 7965, *Pseudomonas aeruginosa* ATCC 10662, *Pseudomonas fluorescens* ATCC 17386, *Shewanella putrefaciens* PTCC 861214 *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6051) were obtained from microbial collection of Department of Food Hygiene, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. All culture media were purchased from Merck Company (Merck, Germany) as well.

2.2. Extraction and Analysis of EO

Zataria multiflora Boiss EO was extracted by steam distillation method [19]. Briefly, 100 g of dried parts of *Zataria multiflora* plant was separately grounded and placed with water (900 cc) in distillation flask. The flask was coupled to a Clevenger type apparatus and heated at 100°C for 3 h and finally the upper liquid (EO) was isolated from the Clevenger apparatus. This procedure repeated several times to obtain enough EO for further experiments. The obtained EO was dehydrated over anhydrous sodium sulfate, filtered by 0.22 µm filters and were stored at 4°C. Yield of

EO isolation was calculated by weighting the obtained EO each time and reported as percentage of EOs per 100 g of the plant.

GC/MS analysis of EO was performed using a Gas Chromatograph (Agilent 7890A/5975C) equipped with a Chrome-pack CP-Sil 8 CB capillary column (50 m x 250 μ m x 0.12 μ m).

Flow rate of Helium was 5 ml/min. The column temperature was initially 50 °C, held for 2 min, and then gradually increased to 120 °C at a 4 °C/min rate, then to 200 °C at a 2 °C/min rate, and finally increased to 280 °C, held for 8 min. The MS procedure was performed with ionization energy of 70 eV using a Mass Spectrophotometer detector and CTC CombiPAL for liquid and headspace. The compounds were identified by comparing their retention indices with those of authentic samples and mass spectral data available in the library (Wiley-VCH 2001 data software, Weinheim, Germany) [9].

2.3. Antibacterial activity assay

The antibacterial activity of the *Zataria multiflora* Boiss EO was determined using Micro-dilution method [2, 20]. Firstly, bacterial suspensions (*Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*, *Escherichia coli* and *Bacillus subtilis*) were prepared by culturing the bacteria in 9 ml of BHI broth and incubation at 37 °C for 24, except for *P. fluorescens* which incubated at 30°C for 24h. The suspensions were confirmed to the 0.5 McFarland standard turbidity while being in log phase and serially diluted (1:10) to achieve the desired concentration (1.5×10^6 cfu/ml). The EO was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma–Aldrich) and the obtained solution firstly diluted to the highest concentration (40 mg/ml) as a stock solution and then serial two-fold dilutions were made in a concentration range 0.31 - 40 mg/ml in distilled water. The 96-well micro-plates were prepared by distributing 160 μ l of BHI broth and 20 μ l of the inoculums (1.5×10^6 cfu/ml) into each well. Aliquots of 20 μ l of the EO, at the concentration of 40 mg/ml, were added into the first wells. Then, 20 μ l of its serial two-fold

dilutions were transferred into consecutive wells. Wells without any bacteria (180 μ l of BHI broth and 20 μ l of the EO) and wells without any EO (180 μ l of BHI broth and 20 μ l of the inoculums) were considered as negative and positive control respectively. The final volume in each well was 200 μ l, the final concentration of the EO was in a range between 0.031 to 4 mg/ml and the final concentration of bacterial suspensions was approximately 1.5×10^5 cfu/ml. All experiments were carried out in triplicate. The lowest concentrations with no visible bacterial growth were regarded as the minimum inhibitory concentration (MIC) values of the EO. The minimum bactericidal concentration (MBC) values were determined by inoculating 10 μ L of none turbid wells on BHI agar and the lowest concentrations with no visible bacterial growth on the agar were regarded as the MBC values of the EO.

3. Results and Discussion

Chemical constituents of *Zataria multiflora* Boiss EO are presented in Table 1. The results indicated that main components of the EO were carvacrol (51.55%), Thymol (25.49%), p -cymene (5.23%) and γ -terpinene (4.44%), respectively. The MIC and MBC values of *Zataria multiflora* Boiss EO against six known fish spoilage bacteria are summarized in Table 2. As it can be seen, all bacterial strains displayed a notable susceptibility to the EO. *Shewanella putrefaciens* was the most sensitive bacteria (MIC: 0.1 mg/ml and MBC: 0.5 mg/ml) and *Aeromonas hydrophila* and *Pseudomonas aeruginosa* were the most resistant bacteria to the antibacterial effect of *Zataria multiflora* Boiss EO with the same MIC (0.5 mg/ml) and MBC (4 mg/ml) values.

According to the results of GC/MS analysis (Table 1), carvacrol and thymol were determined as major components of *Zataria multiflora* Boiss EO that many studies reported their notable antioxidative and antimicrobial effects [2,8,15,21].

Lambert et al (2001) reported carvacrol and thymol as the main phenolic compound and p -cymene as main non-phenolic compound of this EO which was similar to results of the present study [22].

Table 1: Chemical composition of *Zataria multiflora* Boiss essential oil by GC-MS analysis.

Component	%
1-R- α -pinene	1.68
α -Phellandrene	0.10
(+)-4-Carane	1.12
P-Cymene	5.23
γ -Terpinene	4.44
Borneol	0.11
Terpinen-4-ol	0.39
Alpha Terpineol	0.21
Bornyl acetate	0.20
Thymol	25.49
Carvacrol	51.55
Valencen	0.60
Caryophyllene oxide	0.32
Total	91.44%

Table 2: Antimicrobial activity of the *Zataria multiflora* essential oil by microdilution method.

Bacterial Strains	MIC (mg/ml)	MBC (mg/ml)
<i>Aeromonas hydrophila</i> (ATCC 7965)	0.5	4
<i>Pseudomonas aeruginosa</i> (ATCC 10662)	0.5	4
<i>Pseudomonas fluorescens</i> (ATCC 17386)	0.5	1
<i>Shewanella putrefaciens</i> (PTCC 861214)	0.1	0.5
<i>Bacillus subtilis</i> (ATCC 6051)	0.5	1
<i>Escherichia coli</i> (ATCC 25922)	0.25	0.5

As shown in Table 2, the EO showed notable antibacterial activities against studied bacteria.

Aeromonas hydrophila and *Pseudomonas aeruginosa* were the most resistant bacteria and showed similar sensitivity to the EO. Although in a former study on antibacterial effect of thyme EO by disk diffusion method, *Pseudomonas fluorescens* was more resistant than *Aeromonas hydrophila* which was not in line with results of the present study [12]. This difference in obtained results may be related to the different strains of bacteria tested for microdilution method and different bioactive chemical compounds of the EOs due to evaluation of different EOs; although there are a lot of other factors that can affect chemical composition of EOs such as differences in environmental conditions (seasonal, geographic and climate conditions) [4, 23].

Antimicrobial effect of EOs is not directed to a specific mechanism due to the existence of different chemical components. All identified mechanisms of EOs against bacteria include disruption of the cell walls, destroying of cytoplasmic membrane and membrane proteins, loss of cell contents, coagulation in the cytoplasm, and dysfunction of the system activated proton transfer [4, 20]. Antimicrobial mechanism of carvacrol and thymol as two major constituents of the EO is based on their ability to disintegrate the outer membrane of Gram-negative bacteria, release lipopolysaccharides and increase the permeability of the cytoplasmic membrane to ATP [22].

Most previous studies concerning the antimicrobial effect of EOs have indicated that Gram-negative bacteria are generally less susceptible than Gram-positive bacteria [1, 7-9, 16, 23, 24]. In the present study, MIC and MBC values of *Zataria multiflora* Boiss EO on different tested bacteria indicated notable sensitivity of Gram-negative bacteria. Almajano et al (2008) also reported excellent antibacterial activity of Thyme EO against Gram-negative bacteria such as *Escherichia coli* and *Salmonella* spp [25] which was completely consistent with results of the present study. Akhondzadeh et al (2014) also reported higher antibacterial activity of *Zataria multiflora* Boiss EO on Gram negative bacteria [2].

4. Conclusion

The current results revealed that *Zataria multiflora* Boiss EO is active against Gram-negative bacteria involved in fish spoilage and can be used as an effective natural food preservative.

Therefore this EO or their main active components could be potential candidates to be used as natural alternatives for further application in food preservation to delay or inhibit the spoilage bacterial growth and to extend shelf life or safety of these products. On the other hand, the confirmation of antimicrobial efficiency and organoleptic impact of this EO in foodstuffs need to be evaluated.

Conflict of Interest

Authors declare no conflict of interest.

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