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Determination of components and *in vitro* antistreptococcal properties of *Mentha piperita* L., *Satureja khuzistanica* Jamzad, *Matricaria recutica* L., *Zataria multiflora* Boiss. and *Rosmarinus officinalis* L. hydroethanolic extracts

Tafi A.A.[1*]; Meshkini S.[2]; Tukmechi A.[3]; Alishahi M.[4]; Noori F.[5]

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Abstract

Streptococcosis is an infectious disease with significant economic and sanitary repercussions for trout farms in the world. The objective of this study was characterize the chemical constituents and *in vitro* antibacterial properties of *Mentha piperita* L., *Satureja khuzistanica* Jamzad, *Matricaria recutica* L., *Zataria multiflora* Boiss and *Rosmarinus officinalis* L. extracts against *streptococcus iniae.* The chemical components of the selected hydroalcoholic extracts were also analyzed by GC/MS. The most constituents were menthol (24%) in the *M. piperita* L., carvacrol (85.88%) in the *S. khuzistanica* Jamzad, guaiazulene (25.6%) in the *M. recutica* L. carvacrol (51.12%) in the *Z. multiflora* Boiss and α-pinene (12.5%) in the *R. officinalis* L. Among plant extracts under study *Z. multiflora* had the lowest MIC (4.896 mg ml^{-1}) and MBC (9.792 mg ml^{-1}) and the maximum MIC and MBC were belonged to *M. piperita* (18.55 mg ml^{-1}) and *R. officinalis* (33.645 mg ml^{-1}) respectively. Also, the inhibition zone diameter of bacteria had determined by disc diffusion method and compared to erythromycin. The highest and the lowest Inhibition zone diameter were belonged to *Z. multiflora* (14.43±0.55 mm) and *M. recutita* (13.23±0.35 mm) respectively. Results showed that *Z. multiflora* extract exhibited highest antibacterial effect against *S. iniae* among all plant extracts in this study.

Keywords: Herbal extracts, Antistreptococcal property, Minimum inhibitory concentration, Minimum bactericidal concentration

1-Department of Fisheries, Faculty of Natural Resources, Urmia University, Urmia, Iran.
2-Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
3-Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
4-Department of Clinical Science, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
5-Department of Aquaculture, Artemia and Aquaculture Institute, Urmia University, Urmia, Iran
*Corresponding author's Email: aliakbartafi@gmail.com

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Introduction

Research directed towards the development of new antimicrobial agents is necessary for several reasons like the emergence and rapid spread of drug resistant against bacterial pathogens and raising the spectra of terrestrial and aquatic animals' untreatable disease (Payne and Tomasz, 2004). Moreover, experts rightly warn that if a new antimicrobial agent is not continued we will not able to improve veterinary and sensitive improving aquaculture industry (Charles and Grayson, 2004). It is therefore imperative to find/develop safer and more potent compounds for the future that are not toxic to the host (Amin *et al.*, 2010).

There is an increasing interest in medical plants as new sources of natural antibacterial agents in aquaculture (Jeevan *et al.*, 2004). There are many reports about antibacterial components of some medicinal plants against aquatic animals disease agents (Issabeagloo *et al.*, 2012; Roomiani *et al.*, 2013). According to researchers reports wide spectra of infectious bacteria are sensitive to medicinal plant extracts (Zlotkin *et al.*, 1998; Agnew and Barnes, 2007; Bairwa *et al.*, 2012; Mesbah *et al.*, 2015).

The well-known and widely used peppermint (*Mentha piperita* L.) (Lamiaceae) is a cultivated natural hybrid of *M. aquatica* L. (water mint) and *M. spicata* L. (Spearmint). Although a native genus of the Mediterranean region, it is cultivated all over the world for flavor, fragrance, medicinal, and pharmaceutical applications. Mint essential oils are generally used externally for antipruritic, astringent, rubefacient, antiseptic, and antimicrobial purposes (Zargari, 1990).

Satureja species are native to warm temperate regions and may be annual or perennial (Tepe, 2015). Significant proportions of *Satureja* species are plants that have an important role in the pharmaceutical industry. *Satureja khuzistanica* Jamzad (Marzeh-e-Khuzestani in Persian) is one of the endemic *Satureja* species that grows in foothills, limestone gaps, and the south-western parts of Iran. The aerial parts of this species have antiparasitic, carminative, digestive and diuretic properties (Ghodrati *et al.*, 2015). The essential oil and extract of *S. khuzistanica* have biological compounds including antibacterial, antifungal, antioxidant and anti-inflammatory properties (Sahin *et al*., 2003; Tampieri *et al*., 2005).

Chamomile (*Matricaria recutica* L.), a member of the daisy family, is one of the most widely used and well-documented medicinal plants in the world. It is widely distributed in Asian tropics and subtropics areas and grows freely everywhere. The chamomile volatile oils are shown to have antimicrobial activity against certain species of bacteria, fungi and viruses *in vitro*; however, Gram-positive bacteria were found more susceptible than Gram-negative ones (Gholipour Kanani *et al*., 2012). The beneficial effects are related to different classes of therapeutically interesting ingredients such as essential oil components, flavonoids, and coumarin derivatives (Moricz *et al*., 2012).

Zataria multiflora Boiss is a plant belongs to the Labiatae family that is distributed only in Iran, Pakistan and Afghanistan. It is greatly used for medicinal and condimental purposes in these countries. This plant with the

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vernacular name of Avishan Shirazi in Iran has several traditional uses such as antiseptic (Zargari, 1990), anesthetic (Sharif Rohani *et al.*, 2007) and antispasmodic (Zargari, 1990; Nakhai *et al*., 2007). Most aspects of their medicinal use are related to the essential oil which contains various levels of thymol and/or carvacrol, phenolic derivatives with strong and wide-spectrum antimicrobial activity (Dorman and Deans, 2000; Mohagheghzadeh *et al*., 2000). *Z. multiflora* Boiss is a perennial herbaceous plant, distributed in central and southern of Iran (Amin *et al.*, 2010).

Rosemary (*Rosmarinus officinalis* L.) originally grows in southern Europe. Its herb and oil are commonly used as spice and flavoring agents in food processing for its desirable flavor, high antioxidant activity and lately as antimicrobial agent. Rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram positive and Gram-negative bacteria (Rozman and Jersek, 2009).

Streptococcus is a genus of coccus (spherical) Gram-positive bacteria belonging to the phylum Firmicutes and the order Lactobacillales (lactic acid bacteria), which normally live on the body of humans or animals and may cause disease (sometimes severe) in both humans and animals especially in fish (Weinstein *et al*., 1997; Agnew and Barnes, 2007). *Streptococcus iniae* has become one of the most serious aquatic pathogens in the last decade causing high losses in farmed marine and freshwater finfish in warmer regions. This species is capable of causing invasive disease and outbreaks in aquaculture farms (Agnew and Barnes, 2007).

This study aims to determine the composition and antimicrobial activities of *M. piperita* L., *S. khuzistanica* Jamzad, *M. recutica* L., *Z. multiflora* Boiss , and *R. officinalis* L. hydro alcoholic extracts against *S. iniae.*

Materials and methods

Plant material

Herbal samples (aerial organs of *M. piperita* L., *S. khuzistanica* Jamzad, *M. recutica* L. *Z. multiflora* Boiss. and *R. officinalis* L.) were collected from local areas in Khuzestan Province of Iran and identified in the Department of Botany, Faculty of Agriculture, Urmia University, Iran. After identification the plants were transferred to central laboratory of Urmia University and thoroughly washed in running water to remove debris and dust particles and then rinsed in distilled water. Plant samples were air-dried and ground. Twenty grams of grinded powders from each plant was soaked in 100 ml solvent (ethanol (95 %) + distilled water) (50:50) for 15 min with occasional shaking at 60˚C. After they were dissolved, the materials were filtered through Buchner funnel and Whatman No.1 filter paper. Then, the filtrates were evaporated using rotary evaporator and concentrated (Khakzadie *et al.*, 2015).

Identification of extracts' components by GC/MS

GC/MS analyses were performed on a Thermo Finnegan capillary gas chromatograph directly coupled to the mass spectrometer system (model GC TRACE; TRACE MS plus). HP-5MS non-

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polar fused silica capillary column (30m×0.250 mm, 0.25µm film thickness) was used. Temperature profile was as follows: at first, the temperature of the oven was fixed on 40°C for 2 min. and then increased to 160°C with the temperature rate of 3°C min^{-1}, and finally increased to 280°C at 5°C min^{-1} for 2 min. The carrier gas was helium at a flow rate of 1 ml min^{-1}, and ionization energy was 70 eV (Van den and Kratz, 1963).

Bacteria preparation and morphological, physiological and biochemical determination tests

The bacterial strain *streptococcus iniae* was provided by bacteria collection of Laboratory for Microbiology, Faculty of Sciences, Ghent University (LMG 3740). After preparation, bacteria strain was subjected to the some standard morphological, physiological and biochemical tests (Chang *et al.,* 2002). Gram stain and cellular morphologies were examined at scale 1000x. Catalase activity was determined by flowing drops of 3% hydrogen peroxide down the colonies of bacteria on a clean glass slide. Growth features were determined by culturing bacteria in nutrient broth provided at temperatures (10 and 37 ˚C), salinities (1.5 and 5 % NaCl) and pH (7.5 and 9). Motility was determined from a hanging drop preparation of bacteria using phase contrast microscopy and inoculation of bacteria into brain heart infusion agar. Acid from carbohydrates was performed using inoculation of the bacteria into nutrient broth containing the respected D-glucose and Lactose. Hydrolysis of arginine and lysine was examined (Soltani *et al.,* 2005).

Antibacterial susceptibility measured by disc diffusion

The antibacterial activity of the plant extracts was determined by disc diffusion according to NCCLS (National Committee for Clinical Laboratory Standard) guidelines 1999. Briefly, 100µL of suspension of *Streptococcus iniae* (half McFarland 1.5×10^8 cfu mL^{-1}, calculated by McFarland tubes) was spread out on Mueller-Hinton Agar (CONDALAB, Spain) plates and sterile 6mm discs, each containing 10µL of plant extract placed on the microbial lawns. Antibiotic discs including Erythromycin (15µg) (PADTANTEB, Tehran, Iran) were also included. The tests were carried out in triplicate and plates were incubated at 37^{o}C for 24h. Inhibition zone diameters of bacteria were measured after the incubation period and reported in mm (NCCLS, 1999).

Determination of minimum inhibitory concentrations and minimum bactericidal concentrations

Minimum inhibitory concentration (MIC) values were determined by broth microdilution as recommended by NCCLS (NCCLS, 1999). Serial two-fold were obtained from the hydroalcoholic extracts of *M. piperita*, *S. khuzistanica*, *M. recutica*, *Z. multiflora* and *R. officinalis* in brain heart infusion broth (BHI) [QUELAB, Canada] in 96-well micro titer plates. Fresh bacterial suspensions (*S. iniae*), prepared from overnight grown cultures in BHI, were added to give a final concentration of 1.5×10^8 cfu ml^{-1}. For each plant serial dilution, a control group of bacteria, a medium control group, and an extract control group were also included.

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The microplates were incubated at 37°C for 24h and the first dilution with no turbidity (no growth) was considered as the MIC. Minimal bactericidal concentrations (MBC) were determined by spreading 10µl of the contents of the MIC wells (all wells without turbidity) that showed no bacterial growth on Mueller Hinton Agar (MHA) [CONDA LAB, Spain] plates followed by incubation at 37°C for 24h. The first well with colony counts of <5 was considered to be negative for growth and reported as the MBC.

Statistical analysis

Inhibition zone diameters data of the plant extracts and erythromycin antibiotic against *Streptococcus iniae* were analyzed in SPSS statistical software (v. 20) using one-way ANOVA followed by least significant differences Dunnett's test (p<0.05).

Results

According to gas chromatography–mass spectrometry (GC-MS) analysis, plant extracts compositions of *M. piperita*, *S. khuzistanica*, *M. recutica*, *Z. multiflora* and *R. officinalis* are presented in Table 1. The main components were Menthol (24.6%) followed by Menthone (18.4%) for *M. piperita*, Carvacrol (85.88%) for *S. khuzestanica*, Guaiazulene (25.6%) followed by (E)-β-Faranesens (20.1%) and Chamazulene (12.4%) for *M. recutita*, Carvacrol (51.12%) followed by Gamma-terpinene (10.35%) for *Z. multiflora* and α-pinene (17.5%) followed by 1,8-cineole (10.2%) for *R. officinalis*.

Results of some standard morphological, physiological and biochemical tests to identify bacterial strain are shown in Table 2.

Table 1: Chemical composition of hydroalcoholic extracts of plants.

Mentha piperita		*Satureja khuzestanica*		*Matricaria recutita*		*Zataria multiflora*		*Rosmarinus officinalis*	
Compound	**(%)**	**Compound**	**(%)**	**Compound**	**(%)**	**Compound**	**(%)**	**Compound**	**(%)**
Menthofuran	5.2	α- pinene	0.26	Limonene	0.5	Thujene	0.19	α- pinene	17.5
Isomenthone	5.9	β-Myrcene	0.29	-Terpineneγ	0.5	Alpha-pinene	4.26	Camphene	6.3
Menthone	18.4	α-Terpinene	1.49	(E)-β-Faranesens	20.1	Beta-pinene	0.43	β- pinene	3.5
Menthol	24.6	p-Cymene	3.11	Germacrene-D	3.1	Beta-myrcene	0.85	p-cymene	3.0
1,8-cineole	6.0	β-Phellandrene	3.19	α-Muurolene	0.8	Eucaliptol	3.37	1,8-cineole	10.2
1-limonene	4.1	□-Terpinene	1.14	Germacrene-A	0.5	Gamma-terpinene	10.35	1-limonene	2.4
Linalool	4.8	Linalool	0.91	Z-γ-Bisabolene	2.6	Linalool	5.68	Linalool	2.6
β-caryophyllene	2.5	Borneol	0.36	Caryophyllene oxide	1.2	Thymyl methyl ether	0.47	Camphor	4.2
Borneol	0.7	Terpinene-4-o1	0.65	Spathulenol	1.7	Carvacrol methyl ether	0.46	Borneol	9.6
α-terpineol	0.7	Thymol	0.19	α -Bisabolol oxide B	7.3	Carvacrol	51.12	4-terpineol	2.6
Pulegone	14.4	Carvacrol	85.88	α -Bisabolol	7.3	Trans-caryophyllene	0.41	Verbenone	8.6
Terpinen-4-ol	4.1	(Z)-Caryophyllene	0.15	Chamazulene	12.4	Globulol	2.32	Borneol acetate	3.1
		β-Bisabolene	0.21	α- Bisabolol oxide A	1.9			Tricyclene	0.5
		Caryophyllene oxide	0.18	Guaiazulene	25.6			3-octanone	0.8
				Hexadecanole	5.6			β-myrcene	1.6
				n-nonadecane	1.4			Crysanthenone	1.0
				Sclarene	0.4			Geraniol	1.8
				n-pentacosane	0.5			Caryphyllene oxide	1.7

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Table 2: Morphological, physiological and biochemical characteristics of *Streptococcus iniae* (LMG 3740) + = positive reaction/growth, – = negative reaction/growth.

Gram	Morphology	Colony Size	Catalase activity	Motility	Growth						Acid from		Hydrolysis of	
					Temperature °C		NaCl % (after 72 h)		pH (after 72 h)					
					10	37	1.5	5	7.5	9	D-glucose	Lactose	Arginine	Lysine
+	cocci	<0.5mm	-	-	-	+	+	+	+	+	+	-	+	-

Antibacterial effects of the plant extracts are presented in Table 2. The MIC and MBC values of *M. piperita, S. khuzistanica, M. recutica, Z. multiflora, R. officinalis* against *S. iniae* and inhibition zone diameters are presented in Table 3. Table 3 indicates MIC and MBC of erythromycin against *S. iniae*.

Table 3: MIC and MBC of plant extracts against *Streptococcus iniae* and comparison of inhibition zone diameters.

Plant extracts	MIC (mg ml^{-1})	MBC (mg ml^{-1})	Inhibition zone diameter (mm) (Mean±SD)*	
			Plant extracts	Erythromycin
Mentha piperita	18.550	18.550	13.97±0.50^{a}	16.07±0.58^{b}
Satureja khuzestanica	10.870	10.870	14.20±0.30^{a}	16.07±0.58^{b}
Matricaria recutita	8.285	16.570	13.23±0.35^{a}	16.07±0.58^{b}
Zataria multiflora	4.896	9.792	14.43±0.55^{b}	16.07±0.58^{b}
Rosmarinus officinalis	16.822	33.645	14.20±0.61^{a}	16.07±0.58^{b}

* Inhibition zones diameters were subjected to analysis of variance (SPSS, One-Way ANOVA) followed by least significant differences Dunnett's test. The different superscript alphabets in the same row are significantly different at $p<0.05$.

Table 4: MIC and MBC of Erythromycin against *Streptococcus iniae.*

Concentration (µg ml^{-1})							
500	250	125	62.5	31.25	15.62	7.81	3.90
					MBC	MIC	

Discussion

In recent years, plant species have been used in the focus of scientific scrutiny due to their unique phytochemical contents. As it is well-known, these compounds have excellent pharmacological properties (Tepe, 2015).

Many authors have suggested that the differentiated effects of *M. piperita* L. (Talpur, 2014; Trevisan *et al*., 2017), *S. khuzistanica* Jamzad (Tepe, 2015), *M. recutica* L. (Owlia *et al*., 2007) *Z. multiflora* Boiss. (Khakzadie *et al*., 2015) and *R. officinalis* L. (Issabeagloo *et al*., 2012) preparations on selected microorganisms depend on their composition, the characteristics of the plant material, and concentration used. Ndakalimwe *et al*., (2015) reported that 4% *Aloe vera* powder supplemented diet

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enhanced immune responses and haemato-biochemical indices of *Oreochromis niloticus* against aquatic pathogenic *Streptococcus iniae*. Antimicrobial activity of *R. officinalis* (Rosemary), *Z. multiflora* (Oregano), *Anethum graveolens* (Dill) and *Eucalyptus globulus* (Eucalyptus) essential oils and extracts against *Lactococcus garvieae* was reported by Mahmoodi *et al.*, (2012). They demonstrated that among four plant extracts they have been studied. *Multiflora* extract with carvacrol (71.1 %) was the most effective plant extract with the highest antibacterial effect against *Lactococcus garvieae.* Thus, in the present study, three hydroalcoholic extracts obtained from these various medicinal plants (*M. piperita, S. khuzistanica, M. recutica, Z. multiflora* and *R. officinalis*) were analyzed by GC/MS (Table 1) to determine their main constituents and evaluated for their antimicrobial effects against gram positive zoonosis pathogenic bacteria *S. iniae*.

All results of bacterial identification tests (Table 2) are compatible with standard characterizations of *S. iniae* reported by Soltani *et al.* (2005). *S. iniae* may colonize in the surface of fish or causes invasive disease associated with 30 to 50 percent mortality in affected fish ponds (Eldar *et al*., 1994). The results of the present study indicated that *S. iniae* was inhibited or eliminated after exposure to the extracts of *M. piperita, S. khuzistanica, M. recutica, Z. multiflora* and *R. officinalis* in culture broth (Table 3). Such delay in inhibition of microbial growth is particularly useful in terms of public health and safety. The difference in microbial susceptibility is attributable to the chemical composition of extracts. The lower effectiveness of some extracts against *S. iniae* in this study (Table 3) might reflect the lower amount of antibacterial compounds in the plants. A possible explanation for could be due to some of the plant extracts may have contained antibacterial constituents but were not present in sufficient concentrations to be effective. Moreover, microorganism's features are among reasons for miscarriage of extracts to eliminate or inhibit microorganisms (Owlia *et al*., 2007).

The results obtained in the present study, referring to the activity of *M. piperita, S. khuzistanica, M. recutica, Z. multiflora* and *R. officinalis* extracts against *S. iniae* are consistent with findings of some authors (Iscan *et al*., 2002; Owlia *et al*., 2007; Issabeagloo *et al*., 2012; Esmaeili *et al*., 2015; Khakzadie *et al*., 2015). This effect is attributable to the considerable concentration of Menthol (24.6%) and Menthone (18.4%) in *M. piperita*, Carvacrol (85.88%) in *Satureja khuzistanica*, Guaiazulene (25.6%), (E)-β-Faranesens (20.1%) and Chamazulene (12%) in *M. recutita*, Carvacrol (51.12%) and Gamma-terpinene (10.35%) in *Z. multiflora* Boiss and α- pinene (17.5%) and 1,8-cineole (10.2%) in *R. officinalis* L. (Table 1). However, in some cases, the results of the present study are a little bit different from findings of some authors (Iscan *et al*., 2002). These differences in the extracts' compositions can be attributed to the several environmental factors such as climatic, seasonal and geographical or ontogenesis variations, plant harvest time, error in the analysis of extracts, time between extraction and analysis of compounds' constituents.

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Antibacterial and antiparasitical effects of *M. piperita*, *S. khuzistanica*, *M. recutica*, *Z. multiflora* and *R. officinalis* extracts in fishes were reported in *in vivo* research of many authors (Abutbul *et al*., 2004; Soltani *et al*., 2010; Gholipour Kanani *et al*., 2012; Khansari *et al*., 2013; Adel *et al*., 2015; Adel *et al*., 2016). These researchers reported promotes growth performance and increases the main humoral immune parameters (both at mucosal and systemic levels) of dietary administration of these herbal extracts to farmed fish.

Mahmoodi *et al.* (2012) reported that *Z. multiflora* extract had the most active antibacterial plant against *L. garvieae* extract among plants in his study , showing both MIC and MBC of 15.6 μg mL^{-1} and inhibition zone diameter 28±0.7 mm. Inhibition zone diameter of herbal extracts under current study have lower antistreptococcal effect than erythromycin, but *Z. multiflora* extract with MIC (4.896 mg mL^{-1}) and MBC (9.792 mg mL^{-1}) has good potentials as antistreptococcal agent in combating *S. iniae* and its inhibition zone diameter (14.43±0.55 mm) has no significant difference beside erythromycin (16.07±0.58 mm) statistically (p<0.05) (Table 3). So *Z. multiflora* may be more acceptable to consumers and the regulatory agencies than chemical compounds. Antibacterial effects of *Z. multiflora* extract on the inactivation or elimination of *S. iniae* will help to develop or modify a new factor used for safety of aquatic animals.

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