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ANTIBACTERIAL ACTIVITY OF IRANIAN MEDICINAL PLANTS AGAINST *STREPTOCOCCUS INIAE* ISOLATED FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract - *Streptococcus iniae* is among the major pathogens of a large number of fish species cultured in fresh and marine recirculating and net pen production systems. Ten Iranian medicinal plants were assessed for their antimicrobial activity against *Streptococcus iniae* isolates obtained from diseased *Oncorhynchus mykiss* (Salmonidae; Walbaum, 1972) collected from fish farms in Iran. The antibacterial activity of ethanol extracts of *Punica granatum, Quercus branti, Glycyrrhiza glabra* and essential oils of *Heracleum lasiopetalum, Satureja bachtiarica, Thymus daenensis, Myrtus communis, Echinophora platyloba, Kelussia odoratissima* and *Stachys lavandulifolia* against *Steptococcus iniae* was evaluated by disc diffusion and serial dilution assays. Most of the extracts and essential oils showed a relatively high antibacterial activity against *Streptococcus iniae*. Of the plants studied, the most active extracts were those obtained from the essential oils of *Satureja bachtiarica, Echinophora platyloba, Thymus daenensis* and the ethanol extract of *Quercus branti.* Some of the extracts were active against *Streptococcus iniae*. Two essential oils showed lower MIC values; *Heracleum lasiopetalum* (78 μg/ml) and *Satureja bachtiarica* (39 μg/ml). The essential oil of *Satureja bachtiarica* could be an important source of antibacterial compounds against the *Streptococcus iniae* isolated from rainbow trout.

Key words: Iranian medicinal plants, extract, antibacterial activity, *Streptococcus iniae*

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INTRODUCTION

Streptococcus iniae is a major fish pathogen causing streptococcosis. Streptococcosis of cultured fish has become a major problem in many countries, for example Iran. This disease caused by *Streptococcus iniae* accounts for significant economical losses in the aquaculture industry worldwide (Shoemaker and Klesius, 1997). It leads to mortality, reduced growth and unmarketable appearance. Streptococci can cause acute infections in fish, resulting in a greater than 50% mortality rate over a period of 3-7 days. Rainbow trout (*Oncorhynchus mykiss*) is one of the most cultured fish species in Iran and is gaining popularity in other parts of the world. We have found that *Streptococcus iniae* is the most commonly isolated bacterium from diseased rainbow trout in aquaculture farms in Iran.

Bacterial diseases in aquaculture are mainly controlled by antibiotics. However, continuous intensive use of antibiotics is undesirable as this leads to the development of drug resistance and thereby to a reduced efficacy of the drugs. In the public health context, antibiotic resistance can be transferred to environmental and human pathogenic bacteria (Al-

derman and Hastings, 1998; MacMillan, 2001). In addition, antibiotics accumulate in the environment and fish, posing a potential risk to consumers and to the environment in general. Antibiotics (such as oxytetracycline, erythromycin, tetracycline and etc.) are widely used to prevent bacterial disease in fish. However, the indiscriminate use of antibiotics by fish farmers can lead to the emergence of drug-resistant strains and can create serious public health problems because some streptococci are zoonotic agents (Ghittino et al., 2003). The rapidly expanding aquaculture industry in Iran has suffered heavy economic losses due to bacterial pathogens, particularly *Streptococcus iniae* and *Lactococcus garvieae*, which are the major agents of streptococcosis in rainbow trout (Akhlaghi and Keshavarzi, 2002; Akhlagh and Mahjoor, 2004).

Increased public awareness of the negative effects caused by overexposure to synthetic chemicals has led to the search for "green solutions", such as organic and synthetic chemical-free food products (Abutbul et al., 2004). To enable organic fish production it is essential to develop antibacterial treatments that are based on materials from natural sources. Medicinal herbs contain physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments as they have anti-microbial properties (Kelmanson et al., 2000; Srinivasan et al., 2001).

The Iranian medicinal plants, including *Heracleum lasiopetalum, Satureja bachtiarica, Thymus daenensis, Myrtus communis, Punica granatum, Quercus branti, Glycyrrhiza glabra, Echinophora platyloba, Kelussia odoratissima* and *Stachys lavandulifolia* have been utilized as traditional medicines by the indigenous people of Chaharmahal va Bakhtiari in Iran (Ghasemi Pirbalouti, 2009a). This paper describes the use of some Iranian medicinal plants as treatment against streptococcal disease caused by *Streptococcus iniae* in rainbow trout.

MATERIALS AND METHODS

Plant material

The ten medicinal plants were collected from mountain areas of Zagross, Chaharmahal va Bakhtiari district (altitude: 2000-2500 m asl, latitude: 30°-31°, longitude: 50°-51°), during May–Sep, 2009 (Table 1). Their identity was confirmed using monographs by Ghahraman (1987–1989), Mozaffarian (2007), Rechinger (1963-1998), and voucher specimens were deposited at the Research Centre of Medicinal Plants, Islamic Azad University, Shahrekord Branch, Iran.

Table 1. Iranian medicinal plants used in this study

Scientific name	Family name	Local name	Habit*	Parts used
Punica granatum L.	Punicaceae	Golnar	S	Flowers
Quercus branti Lindley	Fagaceae	Balout	T	Seed
Echinophora platyloba DC.	Apiaceae	Khosharizeh	S	Aerial plant
Heracleum lasiopetalum Boiss.	Apiaceae	Kereson	H	Fruit
Kelussia odoratissima Mozaff.	Apiaceae	Kelus	H	Leaves
Glycyrrhiza glabra L.	Fabaceae	Shirin bayan	H	Roots
Satureja bachtiarica Bung.	Lamiaceae	Marzeh Koohi	H	Aerial plant
Stachys lavandulifolia Vahl.	Lamiaceae	Lolopashmak	H	Flowers
Thymus daenensis Celak	Lamiaceae	Oushon	H	Aerial plant
Myrtus communis L.	Myrtaceae	Mort	T	Leaves

*Habit: T: Tree, S: Shrub, H: Herb

Extract preparation

Dried plant material was powered (200 g) and subjected to hydro-distillation (2000 ml distillated water) for 4 h using a Clevenger-type apparatus according to the method recommended in British pharmacopoeia (British Pharmacopoeia, 1988). The leaves, seeds and flowers of some of the plants were shade dried and ground into a powder (100 g), macerated in 200 ml of ethanol 70%, filtered and dried at 35°C under rotary vacuum (Model Zirbus 302®, Italy). The extract samples were stored in universal bottles and refrigerated at 4°C prior to use.

Bacterial strain

Streptococcus iniae was isolated from the infected rainbow trout (*Oncorhynchus mykiss*) from a commercial aquaculture farm in Iran and kindly donated by Dr. Mostafa Akhlaghi, Department of Fish Health, Faculty of Veterinary, Shiraz University, Shiraz, Iran. The isolate was identified as *Streptococcus iniae* using conventional morphological as well as biochemical tests. The bacteria were kept frozen in 15% glycerol, 85% saline solution or Brain Heart Infusion (BHI, Merk, Germany) broth, in aliquots, at -70 °C until used. For infection trials, 100 ml of BHI broth was inoculated with 50 µL of the frozen isolate. The cultures were shaken (100 rpm) at 27 °C for 24 h. Absorbance at 600 nm of known bacterial densities was determined to obtain a standard calibration curve (data not shown). An initial bacterial suspension containing 10^7 CFU/ml was made from the flask broth culture. Subsequent dilutions were made from the above suspension, which were then used in tests.

Disc diffusion assay

The disc diffusion method of Iennette (1985) was used with some modification to determine the growth inhibition of plant extracts and essential oils on the bacterium. BHI agar (Merck, Germany) was used to prepare the culture medium and autoclaved at 121°C for 15 min. Briefly, plates (8-cm diameter) were prepared with 10 ml agar inoculated with 1 ml of bacterial suspension. Sterile paper discs (6 mm in diameter) were impregnated with 60 µL of dilutions of known extract concentrations (100 µg/disc) and incubated at 35°C for 18 h. The extracts were dissolved in dimethyl sulfoxide (DMSO, 15 µL) before being tested for antimicrobial activity. Discs (6 mm diameter) of Ampicillin, Amkcacin, Penicillin, Cephalexin, Cefazolin and Cefixime (10 µg) were used as positive controls. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs (mm). The growth inhibition diameter was an average of three measurements, taken at three different directions. All tests were performed in triplicate.

Well diffusion assays

Four equidistant holes were made in the agar using sterile cork borers (Q=6 mm). 20 µL of each extract and essential oil was added to the holes using a micropipette (Sagdic et al., 2007).

Serial dilution

The minimal inhibitory concentration (MIC) value was determined by serial dilution assay. The MIC was defined as the lowest concentration of the compound to inhibit the growth of the microorganism to 50%. All extracts were initially tested at 10000 µg/ml and serially diluted to 19 µg/ml. Each tube was inoculated with 5 ml of bacterial suspension at a density of 10^7 CFU/ml and incubated at 37° C for 48 h. The growth of microorganisms was observed as turbidity determined by measuring the optical density at 600 nm with a spectrophotometer (Eppendorf AG, Germany). Erythromycin was included as a positive control in each assay. Extract-free solution was used as a negative control. Control tubes were incubated under the same condition. All assays were carried out in triplicate. The inhibition demonstrated by the extracts is expressed by the following equation (Zampini et al., 2005):

$$\text{Inhibition \%} = [(OD_c - OD_t) / OD_c] \times 100$$

where OD_c is the OD_{600} for the negative control (con-

Table 2. Antibacterial tests of the investigated plants in different assay against *Streptococcus iniae* (100 µg.disc^{-1})

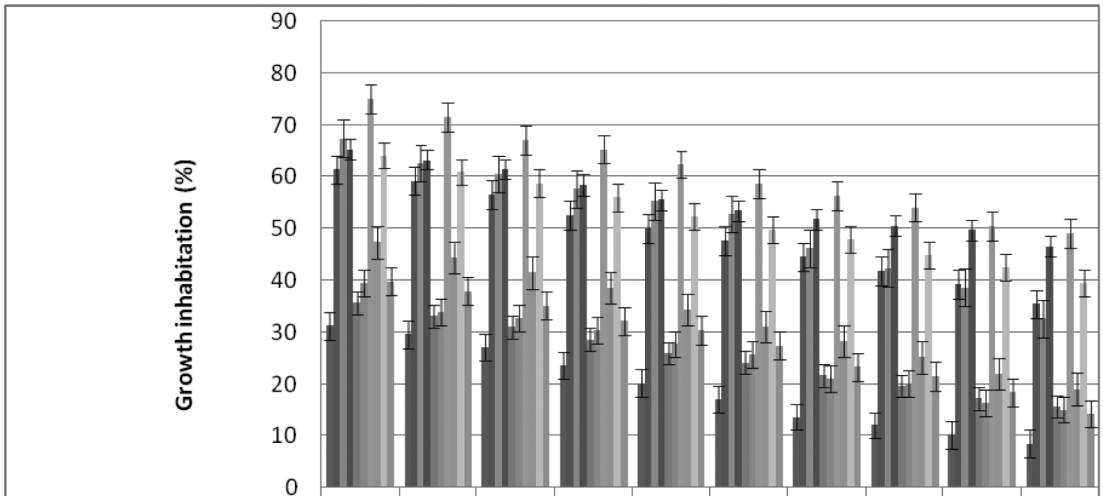
Plant species	Extraction	Disc diffusion [a]	Well diffusion [a]	Part used [b]
Punica granatum L.	Ethanol extract	10.33±1.53	15.83±2.02	FL
Quercus branti Lindley.	Ethanol extract	13.67±1.15	19.50±0.50	SE
Echinophora platyloba DC.	Essential oil	17.00±2.00	23.17±2.25	AP
Heracleum lasiopetalum Boiss.	Essential oil	-	-	FR
Kelussia odoratissima Mozaff.	Essential oil	11.33±0.58	12.67±2.08	L
Glycyrrhiza glabra L.	Ethanol extract	13.67±1.15	12..83±1.04	R
Satureja bachtiarica Bung.	Essential oil	20.67±4.16	27.00±2.65	AP
Stachys lavandulifolia Vahl.	Ethanol extract	11.67±1.53	-	AP
Thymus daenensis Celak.	Essential oil	19.00±1.73	25.00±3.00	AP
Myrtus communis L.	Essential oil	15.67±0.58	13.50±1.50	LE
Control (DMSO)	-	-	-	15 µL
Ampicillin	-	-	-	10 µg
Penicillin	-	-	-	10 µg
Amkcacin	-	10.67±1.15	-	10 µg
Cephalexin	-	11.83±1.61	-	10 µg
Cefazolin	-	12.33±1.89	-	10 µg
Cefixime	-	14.33±1.76	-	10 µg
Probability	-	*P<00.1*	*P<00.1*	

[a:] Diameter of inhibition zone in mm.
[b:] part used (organ tested): FL: flower, FR: fruit, LE: leaves, R: Roots, SE: seed, AP: Arial parts.
- : no inhibition.
Values are means of three replications ± SD.

taining no extract) and Od_t is the OD_{600} for the sample treated with the antimicrobial compounds.

RESULTS

The growth inhibition value of the extracts and essential oils on the bacterium strain is shown in Table 2. The extracts from the different plant species studied showed antibacterial activities, with the diameters of the inhibition zone ranging from 10 to 26 mm (Fig 1a,b). There were significant differences ($P \leq 0.01$) in the antibacterial activities of plant extracts. Among the plants tested, the essential oils of *Satureja bachtiarica, Thymus daenensis* and extracts of *Echinophora platyloba* aerial parts (leaves and stem) and the ethanol seed extract of *Quercus branti* showed the best antibacterial activity and could effectively inhibit the growth of *Streptococcus iniae* (Table 2).



	10000	5000	2500	1250	625	312	156	78	39	19
Punica granatum L.	31.25	29.65	27.16	23.61	20.22	17.16	13.66	12.03	10.07	8.41
Qurcus brantii Lindley.	61.29	59.09	56.49	52.48	49.99	47.62	44.51	41.81	39.2	35.42
Echinophora platyloba DC.	67.45	62.59	60.44	57.5	55.19	52.76	46.15	42.31	38.58	32.63
Heracleum lasiopetalum Boiss.	65.3	63.27	61.35	58.3	55.42	53.38	51.74	50.44	49.6	46.51
Kelussia odoratissima Mozaff.	35.64	33.1	31.01	28.64	26.04	24.23	21.69	19.71	17.28	15.7
Glycyrhiza glabra L.	39.53	33.89	32.76	30.33	27.73	25.69	21.18	20.1	16.38	14.96
Satureja bachtiarica Bung.	74.96	71.57	67.05	65.36	62.37	58.52	56.26	54	50.33	49.03
Stachys lavandulifolia Vahl.	47.28	44.4	41.46	38.58	34.28	31.06	28.24	25.19	21.97	19.09
Thymus daenensis Celak.	64.05	60.84	58.64	55.87	52.31	49.77	47.79	44.85	42.53	39.48
Myrtus communis L.	39.77	37.9	35.08	32.2	30.39	27.45	23.32	21.57	18.47	14.28

Fig 1. Effect of extract and essential oil on growth *Streptococcus iniae* by serial dilution assay (μg/ml)

Table 3. Minimal inhibitory concentration of medicinal plant extracts and essential oils to *Streptococcus iniae* (μg/ml)

Plant species	Extraction	MICs values (μg/ml)
Punica granatum L.	Ethanol extract	>1000
Quercus branti Lindley.	Ethanol extract	625
Echinophora platyloba DC.	Essential oil	312
Heracleum lasiopetalum Boiss.	Essential oil	78
Kelussia odoratissima Mozaff.	Essential oil	>1000
Glycyrrhiza glabra L.	Ethanol extract	>1000
Satureja bachtiarica Bung.	Essential oil	39
Stachys lavandulifolia Vahl.	Ethanol extract	>1000
Thymus daenensis Celak.	Essential oil	312
Myrtus communis L.	Essential oil	>1000

Subsequent experiments were conducted to determine the minimal inhibitory concentration (MIC) of all the selected plant extracts and essential oils. The results are presented in Table 3. The essential oils of *Heracleum lasiopetalum*, *Thymus daenensis, Echinophora platyloba* and *Satureja bachtiarica* and the ethanol extract of *Quercus branti* showed the best antibacterial activities against *Streptococcus iniae* in rainbow trout (Table 3). The MIC values for the active extracts and essential oils ranged between 39-10000 µg/ml (Fig 2).

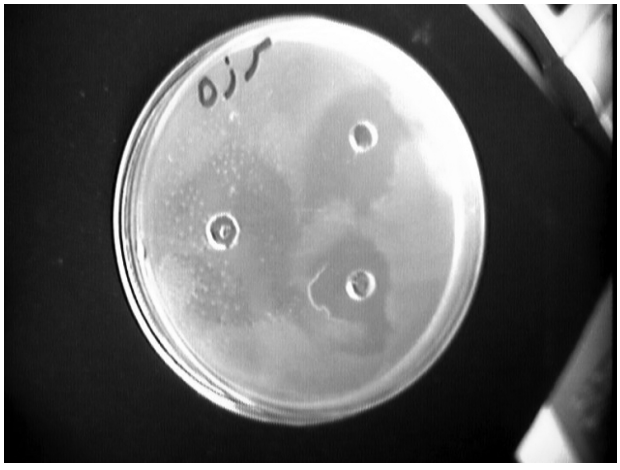


Fig 2a. Antibacterial activities of the essential oil of *Satureja bachtiarica* at 100 µg/well (inhibition zones, mm) against *Streptococcus iniae*.
Growth inhibition was measured and is expressed in millimeters.

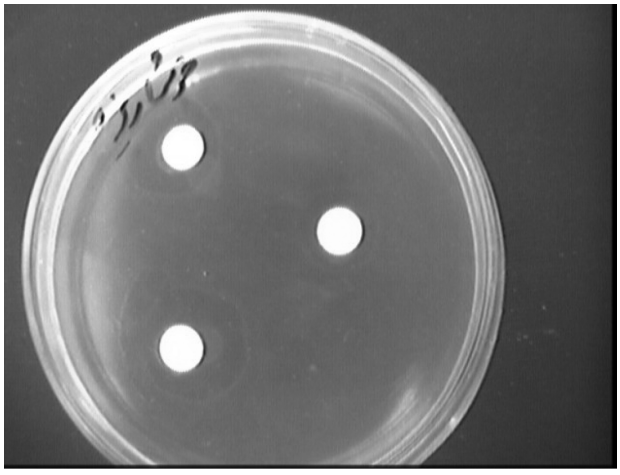


Fig 2b. Antibacterial activities of the essential oil of *Echinophora platyloba* at 100 µg/disc (inhibition zones, mm) against *Streptococcus iniae*.
Growth inhibition was measured and is expressed in millimeters.

The highest level of antibacterial activity against *Steptococcus iniae* was demonstrated by the essential oils prepared from the leaves and stem of *Satureja bachtiarica, Echinophora platyloba* and *Thymus daenensis,* and by the ethanol extract of *Quercus branti* seeds. These preparations exhibited MIC values from 39 to 625 µg/ml. Few essential oils were active against *Streptococcus iniae* with a MIC under 100 µg/ml. The essential oils *Satureja bachtiarica* and *Heracleum lasiopetalum* were found to be effective against *Streptococcus iniae* with low MIC of 39 and 78 µg/ml, respectively. Other extracts showed only a slight inhibition of the tested microorganism.

DISCUSSION AND CONCLUSION

Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004). There has been no large scale systematic investigation into the relationship between bacterial inhibition and total phenolic content of spices and herbs. Previous studies (Shan et al., 2005) showed that a highly positive linear relationship exists between the antioxidant activity and total phenolic content in some spices and herbs.

The essential oil and extract of some aromatic plants with a higher percentage of carvacrol and thymol (e.g. the mint family, *Lamiaceae*), have a higher efficacy against the bacterial strain (Rasooli et al., 2006). The essential oils of *Thymus daenensis* and *Satureja bachtiarica* contained high levels of phenolics and exhibited antibacterial activity (Ghasemi Pirbalouti, 2009b). Previous studies (Rasooli et al., 2006) on the antimicrobial activity of the essential oils of some *Thymus* spp., most of them possessing large quantities of phenolic monoterpenes, have shown activity against viruses, bacteria, food-derived microbial strains and fungi.

Previous works showed that the essential oil of *Satureja bachtiarica* exhibited antifungal activities against *Saprolegnia parasitica* from cutaneous lesions of *Oncorhynchus mykiss* eggs (Ghasemi Pirbalouti et al., 2009); the essential oils of *Satureja bachtiarica* and *Thymus daenensis* exhibited antibacterial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Ghasemi Pirbalouti et al., 2010b); essential oils of *Myrtus communis*, *Thymus daenensis* and *Satureja bachtiarica* exhibited antimicrobial activities against *Escherichia coli O157:H7, Bacillus cereus*, *Listeria monocytogenes* and *Candida albicans* (Ghasemi Pirbalouti et al., 2010a).

The results of study by Sonboli et al., 2004 indicated the essential oil of *Satureja laxiflora* C. Koch contained a high concentration of oxygenated monoterpenes (76.3%) of which thymol (63.9%) was the major compound followed by carvacrol (4.8%) and geraniol (3.2%), and it exhibited antimicrobial activities against *Candida albicans*, *Aspergillus niger*, *Saccharomyces cerevisiae, Klebsiella pneumonia* and *Enterococcus faecalis.* They reported that a major portion of this antimicrobial activity is due to the thymol present in the oil.

The compounds from the essential oil of *Satureja bachtiarica* included 20% carvacrol and 19% thymol before flowering and 26% carvacrol and 5% thymol at full flowering stage, as main components (Sefidkon and Jamzad, 2000; Sefidkon et al., 2007).

Sefidkon et al., 2007 reported that the anti-bacterial effect (five gram positive bacteria including: *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus*, Staphylococcus sp. and *Staphylococcus aureus*; three gram negative bacteria including: *Klebsiella pneumonia*, *Klebsiella oxytoca and Pseudomonas aeruginosa*) of *Satureja bachtiarica* oil was stronger before the flowering stage, because of a higher percentage of phenolic compounds (thymol and carvacrol).

According to a report (Abutbul et al., 2004) the extract and essential oils of *Rosmarinus officinalis* inhibited the growth of *Streptococcus iniae*.

The findings of this study show that the essential oils of *Satureja bachtiarica, Echinophora platyloba, Thymus daenensis* and the ethanol extract of *Quercus branti* had antibacterial activities against *Streptococcus iniae*. In three methods of antibacterial test, the highest level of antibacterial activity was demonstrated by the essential oil of the aerial parts (leaves and stem) of *Satureja bachtiarica*. The present study suggests that the essential oil of *Satureja bachtiarica* is a potential source of natural antibacterial against *Streptococcus iniae* from cutaneous lesions of rainbow trout (*Oncorhynchus mykiss*). After this screening experiment, further work should be performed to describe the antibacterial activities in more detail as well as *in vivo*.

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