

Original Article

Antibacterial effects of medicinal plant extracts against *Lactococcus garvieae*, the etiological agent of rainbow trout lactococcosis

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Abstract: Eight medicinal plants were assessed for antimicrobial activity against *Lactococcus garvieae* isolate obtained from diseased *Oncorhynchus mykiss* collected from rainbow trout fish farms in Iran. *Lactococcus garvieae* is among the major pathogens of a large number of fish species cultured in fresh and marine recirculating and net pen production systems. The antibacterial activity of the medicinal plants against *L. garvieae* was evaluated using disc diffusion, well diffusion and minimum inhibitory concentration. Results showed that the extracts and essential oils had a relatively high antibacterial activity against *L. garvieae*. Of the plants studied, the most active extracts were those from the methanol extract of *Peganum harmala*, the essential oil of *Satureja bachtiarica*, the ethanol extract of *Juglans regia* and *Trachyspermum copticum* with minimum inhibitory concentration (MIC) of 105, 126, 510 and 453 µg/ml, respectively. Conversely, some of the extracts such as *Quercus branti* Lindley and *Glycyrrhiza glabra* L. had lower activity against *L. garvieae* with MIC values of 978 and 920 µg/ml, respectively. Plant extracts as natural and environment-friendly compounds can be an important source of antibacterial agents against *L. garvieae*. They may be used for disinfection of instruments and rainbow trout raceways or treatment of the fish.

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Introduction

Lactococcus garvieae is a fish pathogen causing lactococcosis that has become a major problem in fish culture. Lactococcosis is a serious septicemic disease in freshwater culture of salmonid fish and marine culture species, especially when water temperature is over 15°C (Austin and Austin, 2007). Lactococcosis is a kind of streptococcosis caused by *L. garvieae*. Lactococcosis has spread to many countries causing significant economic loss to the rainbow trout industry as in Turkey (Diler et al., 2002), Portugal (Pereira et al., 2004), France and the Balkans (Eyngor et al., 2004). The Iranian *L. garvieae* isolate was diagnosed using a specific PCR assay based on 16S rDNA gene by producing a single band of 1107 bp. Partial analysis of 16S rDNA

of the bacterium was in close genetic relationship with those previously reported for mullet in Taiwan (AF352166) and yellowtail in Japan (AB267897) based on GenBank data. AntibioGram tests on *L. garvieae* isolates showed a high susceptibility to erythromycin, enrofloxacin and chloramphenicol (Sharifiyazdi et al., 2010).

Many bacterial diseases in aquaculture, are controlled by antibiotics. However, continuous use of antibiotics leads to drug resistance and thereby to a reduced efficacy of the drugs. Antibiotics accumulate in the environment and fish, pose a potential risk to consumers and to the environment. Antibiotics (such as oxytetracycline, erythromycin, tetracycline and etc.) and other chemical disinfectants are widely used to prevent bacterial

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Table 1. Characteristics of eight medicinal plants used in this study.

	Parts used	Habit	Local name	Family name	Scientific name
1	Seed	Herb	Esfand	Nitrariaceae	<i>Peganum harmala</i>
2	Root	Herb	Shirin bayan	Fabaceae	<i>Glycyrrhiza glabra</i> L.
3	Seed	Herb	Zenian	Umbelliferae	<i>Trachyspermum copticum</i>
4	Leaves	Tree	Mort	Myrtaceae	<i>Myrtus communis</i> L.
5	Leaves	Tree	Barge gerdo	Juglandaceae	<i>Guglans regia</i>
6	Seed	Tree	Balout	Fagaceae	<i>Quercus branti</i> Lindley
7	Leaves	Herb	Baboneh gavi	Asteraceae	<i>Tanacetum parthenium</i> L.
8	Leaves	Herb	Marzeh koohi	Lamiaceae	<i>Satureja bachtiarica</i> Bung

disease in fish. The rapidly expanding aquaculture industry in Iran has suffered from heavy economic losses due to bacterial pathogens, particularly *Streptococcus iniae* and *L. garvieae*, which are the major agents of streptococcosis in rainbow trout (Akhlaghi and Keshavarzi, 2002; Akhlaghi 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). For organic fish production it is essential to develop antibacterial treatments that are made from materials with natural sources. Medicinal herbs contain physiologically active gradients that over the years have been exploited in traditional medicine for the treatment of various ailments because of having anti-microbial properties (Kelmanson et al., 2000; Srinivasan et al., 2001; Ghasemi Pirbalouti et al., 2011; Negi et al., 2011).

In spite of tremendous efforts to provide an alternative to medicinal plants with minimum side effects, easy accessibility and excellent compatibility, future clinical trials and standardization of medicinal plants are still required as an important steps in drug discovery (Sarwar et al., 2011). This paper describes the in vitro use of eight Iranian medicinal plants as antibacterial against lactococcosis caused by *L. garvieae* in rainbow trout.

Materials and methods

Plant materials: Eight medicinal plants were collected from herbal medicine shop and their

identity was confirmed using monographs by Mozaffarian (1996; Table 1).

Extract preparation: Dried plant material was pulverised (200 g) and subjected to hydro-distillation (2000 ml distilled water) for 4 h using a Clevenger-type apparatus. The leaves and seeds of some of the plants were shade dried and ground into a powder (100 g), macerated in 200 ml of methanol or ethanol, filtered and dried at 35 °C using a rotary vacuum. The extract of samples were stored in universal bottles and refrigerated at 4 °C prior to further analyses.

Bacterial strain: *Lactococcus garvieae* was isolated from the infected rainbow trout (*Oncorhynchus mykiss*) from a commercial aquaculture farm in Fars Province, Iran. The isolate was identified as *L. garvieae* using conventional morphological as well as biochemical tests. It was then confirmed by molecular methods (Sharifiyazdi et al., 2010). The bacteria were kept frozen in 15% glycerol, 85% saline solution or Brain Heart Infusion (BHI) 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. An initial bacterial suspension containing 10⁷ 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 methods of Lennette (1985) were used with some modification

Table 2. Inhibition zones and MIC values produced by antibacterial activity of the medicinal plants against *L. garvieae*.

	Plant species	Extraction	Disc diffusion (mm)*	Well diffusion (mm)*	MICs values (µg/ml)	Disc content (µg)
1	<i>Peganum harmala</i>	Methanol extract	28±2.30	39±3.43	105	-
2	<i>Glycyrrhiza glabra</i> L.	Ethanol extract	14±2.15	13±1.20	920	-
3	<i>Trachyspermum copticum</i>	Ethanol extract	18±3.13	21±3.14	453	-
4	<i>Myrtus communis</i> L.	Essential oil	16±2.53	20±1.23	672	-
5	<i>Juglans regia</i>	Ethanol extract	19±4.12	22±3.15	510	-
6	<i>Quercus branti</i> Lindley	Ethanol extract	12±3.80	15±1.93	978	-
7	<i>Tanacetum parthenium</i> L.	Essential oil	15±1.96	17±3.15	824	-
8	<i>Satureja bachtiarica</i> Bung	Essential oil	25±4.70	29±5.12	126	-
9	Erythromycin	-	32±1.54	35±2.61	-	15
10	Ampicillin	-	22±3.87	24±3.49	-	25
11	Flofenicol	-	29±2.82	33±2.67	-	30
12	Enrofloxacin	-	27±1.42	30±2.11	-	5
13	Trimethoprim	-	28±2.30	28±2.30	-	20

to determine the growth inhibition of plant extracts and essential oils on the bacterium. BHI agar 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, Amkcaicin, Penicillin, Cephalexin, Cefazolin and Cefixime (10 µg) were used as positive controls. Bacterial growth inhibition was determined using the diameter of the inhibition zones around the discs (mm). The growth inhibition diameter was calculated as an average of three measurements, from three different directions. All tests had three replicates.

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).

Minimal inhibitory concentration: 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 10 µg/ml. Each tube was inoculated with 5 ml of bacterial suspension at a density of 10⁷ 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. 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): Inhibition % = [(OD c –OD t) / OD c] ×100 where ODc is the OD600 for the negative control (containing no extract) and Odt is the OD600 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. There were significant differences in the antibacterial activities of plant extracts ($P \leq 0.01$). Among the plants tested, the essential oil of *Satureja bachtiarica* leaves, the

methanol extract of *Peganum harmala* seeds and the ethanol extract of *Juglans regia* leaves and *Trachyspermum copticum* seeds showed the best antibacterial activity that could effectively inhibit the growth of *L. garvieae* (Table 2).

Subsequent experiments were conducted to determine the minimal inhibitory concentration (MIC) of all the selected plant extracts and essential oils. The essential oil of *Satureja bachtiarica* and the methanol extract of *Peganum harmala* showed the best antibacterial activities against *L. garvieae* in rainbow trout (Table 2). The MIC values for the active extracts and essential oils ranged 105-978 µg/ml. The highest level of antibacterial activity against *L. garvieae* was demonstrated by the methanol extract prepared from the seeds of *Peganum harmala* and ethanol extract of *Trachyspermum copticum* seeds when compared with the results of five antibiotic discs. These preparations showed MIC values from 105 to 453 µg/ml. Other extracts used in this study only showed a slight inhibition of the tested microorganism.

Discussion

Plant extracts as natural and environmentally friendly compounds could be an important source of antibacterial agents against *L. garvieae*. Lactococcosis could be controlled by a health management protocol using disinfectants such as natural antibacterial compound besides employing vaccination of fish against the etiological agent. Such antibacterial with natural sources are not expensive and could be prepared and ordered by registered agencies around the world.

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; Pritam and Purushottam, 2007) showed that a highly positive linear relationship exists between the antioxidant activity, cytotoxic activity and total phenolic content in some spices and herbs. 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).

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 strains. Also, the antimicrobial activity of the essential oils of some *Thymus* spp. that possess large quantities of phenolic monoterpenes, have shown activity against viruses, bacteria, food-derived microbial strains and fungi (Rasooli et al., 2006).

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) and *Streptococcus iniae* (Ghasemi Pirbalouti et al., 2011). 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 suggested 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 the main components (Sefidkon and Jamzad, 2000; Sefidkon et al., 2007). Sefidkon et al. (2007) reported that the anti-bacterial effect (five 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). The essential oil of *Tanacetum parthenium* L. containing camphor showed minimum antibacterial effect on *S. aureus* and *P. aeruginosa* (Saharkhiz et al., 2008). Similarly this essential oil had a low antibacterial effect on *L. garvieae* in this study.

The influence of three medicinal plants, *Ocimum basilicum*, *Adathoda vasica* and *Calendula officinalis* on the biochemical parameters of normal and *Aeromonas hydrophila*-infected fish (*Iabeo rohita*) was assessed using feeds supplemented with 3% concentrations of the three plants extracts. At the culmination of feeding experiment, biochemical analysis of serum for enzymes was undertaken and the highest level of serum glutamate oxaloacetate transferase, serum glutamate phosphor transferase, alkaline phosphatase, were recorded 60.0, 54.1, 26.3 mg dL⁻¹ respectively in *Ocimum basilicum* supplemented group. Serum protein content was also higher in *Calendula officinalis* supplemented group (6.0 mg dL⁻¹). This study revealed that feed supplementation of three medicinal plant extracts alters biochemical parameters to overcome disease induced stress in fishes (John et al., 2011).

In three methods used for extraction of the eight medicinal plants in this study, the highest level of antibacterial activity was demonstrated by the essential oil of the leaves of *Satureja bachtiarica*, the methanol extract of *Peganum harmala*, the ethanol extracts of *Juglans regia* and *Trachyspermum copticum*. Thus they are potential source of natural antibacterial against *L. garvieae* isolated from rainbow trout. They might be used for disinfection of instruments and rainbow trout raceways. Further work should be performed to describe their in vivo antibacterial activities in more detail in fish.

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