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**ORIGINAL ARTICLE**

# Evaluation of antimicrobial activities of some Iranian herbal medicines essential oils against standard and isolated strain of *Lactoccocus garvieae*

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**ABSTRACT**
*Members of Iranian medical plants such as Mytrus communis, Satureja bachtiarica, Satureja khuzestanica and Mentha longifolia have antimicrobial effects on the pathogenic bacteria and they have been known and used from past by native people. The increasing prevalence of antibiotic resistant bacteria has led to a demand for new agents that could be used to decrease the prevalence of bacterial disease. The aim of this study was to determine the antimicrobial activity of the essential oils of these medicine herbs on Lactococcus garvieae. The essential oils of Satureja bachtiarica (MIC: 50 µl/ml, MBC: 200µl/ml) and Satureja khuzestanica (MIC: 100µl/ml, MBC: 400µl/ml) showed the best antibacterial effect on Lactococcus garvieae.The results indicate that these essential oils have appropriate antibacterial properties agaisnt Lactococcus garvieae. Therefore, they can be suggested to purify and evaluate the active substance of the essential oils for future application as antibacterial agent.*
***Key Words:*** *Lactococcus garvieae, essential oils, MIC and MBC.*

## INTRODUCTION

The seven species of *Lactoccocus garvieae* are included of *L. garvieae, L. fujiensis, L. piscium, L. chungagensis, L. plantarum, L. raffinolactis and L. lactis* (1). But the only pathogenic kind of these bacteria is *L. garvieae* (2). This gram positive bacteria cause the *Lactoccocosis* of different kind of fish species worldwide (3). Also this emergent pathogen has been detected from buffalos, cattle, freshwater shrimp and profitable important fish (4). This bacteria also, reported as a pathogenic agent of mastitis in cows and the existence of it in raw-milk and raw-milk cheese cause it become more importance (5). In addition, the isolation of *Lactoccocus garvieae* infection in human that describes the zoonotic possibility of this pathogen (6). *L. garvieae* has been detected from different kinds of fish in several parts of the world such as South Africa, Europe, Australia, Japan and Turkey (7) and also has been isolated from rainbow trout farms of Iran especially during the spring and summer in Iran (8). Thus, *L. garvieae* causes important economic losses by the high elevation rate of mortality to rainbow trout industry (9). The widely use of antibiotics such as erythromycin, tetracyclin and etc to prevent bacterial disease in fish (10, 11). Although antibiotics have controlled a large number of bacterial disease in aquaculture, the drug resistance and the accumulation of drugs in fish, environment and recurrent infection pretend risk to environment and consumers (9). Recent efforts have been done to discover the new antimicrobial compounds of different kinds of sources such as animals, microorganisms and plants. Herbal medicine recently, have been used to treat several infectious diseases (12). *Satureja khuzestanica* J. is a property of native plants of *Laminacea* family that is distributed in mountain areas and in fractures of limestone of southern parts of Iran (13). The highest of this subshrub and branched stem is about 30 cm and also covered with white hairs (14). The antibacterial, antifungal and antiviral components of some medical plants essential oils have been studied but the main antibacterial compound of these elements is carvacrol (13). The utilization of some Iranian medicine plants such as *Satureja bachtiarica* and *Myrtus communis* by the people of west of Iran as traditional medicines have been done (15). In folk medicine *Myrtus communis* L. has been used as a traditional remedy for treatment infectious disease, diarrhea, and dysentery and also used as anti-inflammation and anti-septical agents (16). *Mentha longifolia* is one of the species of *Mentha* genus. This

plant family has been used for treatment ulcerative colitis, bronchitis, anti-inflammation and etc. (17). The main compound of essential oil of *Satureja bachtiarica* is included Thymol and Gama-Tripinen (18). This study describes the *in vitro* use of some Iranian medicinal plants as treatment against *Lactococcosis* that caused by *Lactoccocus garvieae* in rainbow trout.

## MATERIAL AND METHOD

**Plant material:** two Iranian medical herbs consist of *Satureja bachtiarica* and *Mentha longifolia* had been collected from mountain areas of Zagross, Chaharmahal va Bakhtiari District (altitude: 2000-2500 m asl, latitude: 30-31˚, longitude: 50˚-51˚), during May-Sep, 2013, two Iranian medical plants which comprise *Mytrus comminus* and *Satureja khuzestanica* were collected from a well-known market of herbal medicine in Isfahan and Shahrekord, Iran. Then, the identification of these herbal medicine established by using the monographs by Rechinger (19), Mozaffarian (20) and Ghahraman (21) at the Researches (enter of medicinal plants, Islamic Azad University, Shahrekord Branch, Iran.

**Preparation of extracts:** Dried plant materials were pulverized (200g) and subjected to hydro-distillation (2000 ml distillated water) for 4h using a Clevenger-type apparatus according to method recommended in British pharmacopoeia (22). The extract samples were stored in universal bottles and refrigerated at 4˚C prior to use.

**Bacterial strain:** *L. garvieae* was isolated from the infected rainbow trout from a well-known commercial aquaculture farm in Iran. It was kindly given by Dr. Namatollahi, Department of fish health, Faculty of veterinary medicine, Shahrekord University, Shahrekord, Iran. The isolated was identified as *L. garvieae* using conventional morphological as well as biochemical tests. Bacteria were kept frozen in 15% glycerol and 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 frozen isolate. The cultures were shaken (100 rpm) at 27˚C for 24h 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 dilution were made from the above suspension, and then used in tests.

**Antimicrobial test (Disc diffusion assay):** The disc diffusion method of Lennette [23] was used with some modification to determined rate of inhibition grown of bacteria by plant essential oils. BHI agar (Merk, Germany) was used to prepare the culture medium and was autoclaved at 121˚C for 15min. briefly, plates (8 cm in diameter) were prepared with 10 ml agar inoculated with 1 ml of each bacterial suspension. Sterile paper discs (6 mm in diameter) were impregnated with dilution of known extract concentration and incubated at 35˚C for 24h. The essential oils were dissolved in dimethyl sulfoxid (DMSO) before the test for antimicrobial activity. Erythromycin concentration used as positive control. Bacterial growth 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.

**Serial dilution:** The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) values were determined by serial dilution assay. The MIC was the lowest concentration at which bacteria failed to grow in liquid media, but viable when 100 µl samples were plated on agar media [24] each tube was inoculated with 5 ml of bacterial suspension at a density of $10^7$ CFU/ml and incubated at 37˚C for 48h. The growth of microorganisms was observed as turbidity, determined by the measure optical density at 600 nm, by spectrophotometer (Eppendorf, AG, Germany). Erythromycin was included as positive control. Control tubes were incubated under the same condition. All assays were carried out in triplicate. The inhibition demonstrated by the extract is expressed by the following equation [25].

$$Inhibition100\% = \frac{(OD_c - OD_t)}{OD_c} \times 100$$

$OD_c$: is the $OD_{600}$ for the negative control (containing no extract) and $OD_t$ is the $OD_{600}$ for the sample treated with the antimicrobial compounds.

## RESULTS

Table 1 and Figure 1 shows the growth inhibition value of the essential oils on *L. garvieae* by agar disc diffusion assay. The diameters of inhibition zone range of antimicrobial activity of these essential oils were from 18.53mm to 39.2mm. Thus, the significant differences in antimicrobial activities of these herbal essential oils were noticeable ($p<0.05$). Among all plants essential oils that tested, the best antibacterial activity occurred by the essential oils of *Saturja bachtiarica, S. khuzestanica* and *Mytrus comminus.* Subsequent scientific procedures were conducted to control the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of all the selected herbal essential

oils presented in the Table 2 and Figure 2. Therefore, the best antimicrobial activity was demonstrated by the essential oils of *S. bachtiarica* (MIC=50µl/ml, MBC=200µl/ml) and *S. khuzestanica* (MIC=100µl/ml, MBC=400µl/ml). The essential oils of *Mytrus comminus* and *Mentha longifolia* that used in this study showed the less effect than *S.bachtiarica* and *S.khuzestanica.*

**Table 1: shows the growth inhibition value of the essential oils on L. garvieae by agar disc diffusion assay**

| The essential oils | The diameters of inhibition zone |
|---|---|
| *Mentha longifolia* | 18.53±0.56 |
| *Mytrus comminus* | 28.13±0.30 |
| *Satureja bachtiarica* | 34±2.25 |
| *Satureja khuzestanica* | 39.20±0.45 |
| Control - | 0 |
| Control + | 31.4±0.81 |

**Table 2: MIC and MBC of all the selected herbal essential oils**

| Concentration µl/ml | *Mentha longifolia* | *Mytrus comminus* | *Satureja bachtiarica* | *Satureja khuzestanica* |
|---|---|---|---|---|
| 800 | 51.20±1.57 | 98.94±0.11 | 99.75±0.07 | 99.36±0.11 |
| 400 | 26.34±6.60 | 77.55±6.52 | 99.36±0.05 | 99.06±51 |
| 200 | 21.66±12.14 | 60.63±10.06 | 99.06±0.04 | 86.10±3.51 |
| 100 | 27.52±10.10 | 40.37±8.23 | 94.08±2.7 | 64.15±5.34 |
| 50 | 28.09±7.68 | 16.57±9.11 | 80.62±3.23 | 36.84±13.26 |
| 25 | 22.78±8.88 | 18.02±5.25 | 45.33±5.63 | 15.99±9.7 |
| 12.5 | 19.25±13.12 | 17.63±5.11 | 21.07±2.10 | 15.58±4.6 |

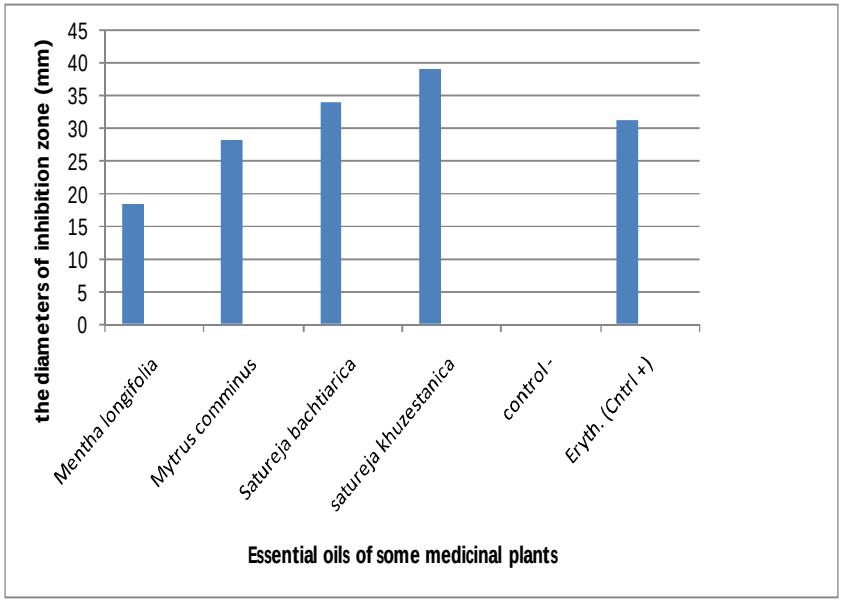


**Figure 1: shows the growth inhibition value of the essential oils on L. garvieae by agar disc diffusion assay**

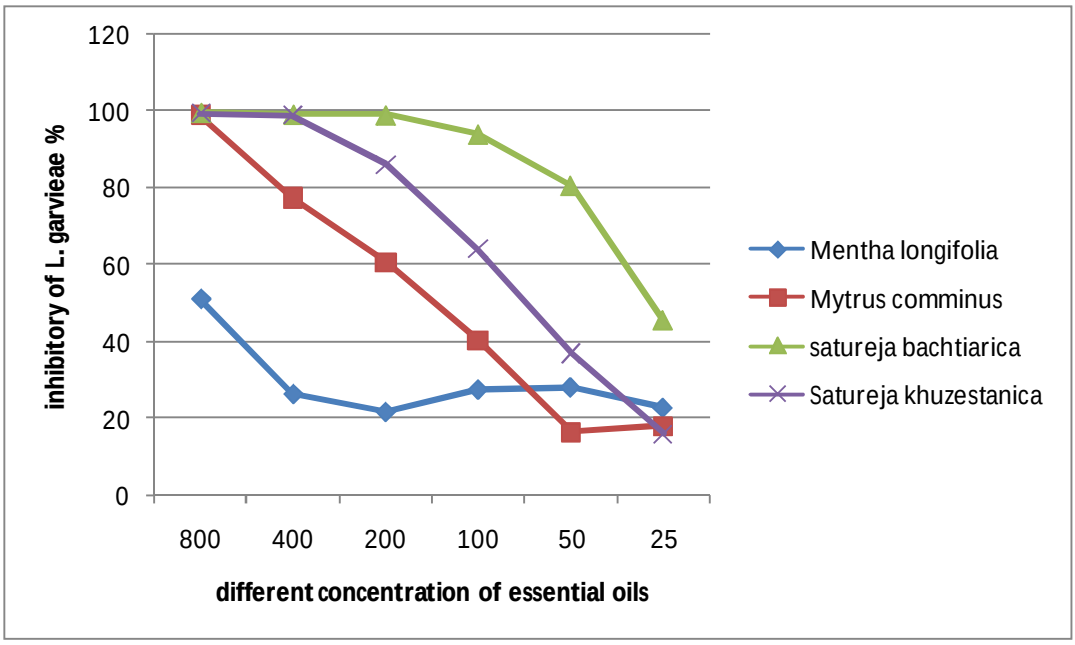


**Figure 2: MIC and MBC of all the selected herbal essential oils. [DIGIT WRITE IN ENGLISH]**

## DISCUSSION

The compound of herbal essential oils could be an important natural source of antimicrobial agents. Thus, not only these natural antibacterial compounds can control *Lactoccocosis* but these natural sources are also not expensive. This study makes known the antibacterial abilities of these essential oils against the *L. garvieae.* The highest efficacy against the bacterial strain caused by the highest percentages of carvacrol and thymol of essential oils (26). The study of Akbari-Shahabi et al. was showed that the highest chemical compound in *S. khuzestanica* is carvacrol (13). In addition, the study that conducted by Majd et al. on the other essential oils of this family plant have the antibacterial activity on gram-positive and gram-negative bacteria (27). The results of these studies are closed to our study. Previous studies showed the essential oil of *S. bachtiarica* has antifungal activities against *Saprolegnia parasitica* that existed in cutaneous lesions caused by *Oncorhynchus mykiss* eggs (15). Besides, the antibacterial activities against *Klebsiella pneumonia*, *E.coli*, *Pseudomonas aeroginosa* and *Staphylococcus aureus* exhibited by essential oils of *Thymus* and *S.bachtiarica* (28). The investigation that had done on the antibacterial effect of some Iranian herbal medicine on *L. garvieae* that isolated from contaminated rainbow trout fish farms in Iran showed that the most active extracts which from the essential oil of *Satureja bachtiarica,* the ethanol extract of *Juglans regia* and *Trachyspermum copticum* and *the methanol extract of Peganum harmala* with minimal inhibitory concentration of 126, 510, 453 and 105 µl/ml (9). The study of Ghasemi Pirbalouti et al. (29) revealed the antibacterial effect of essential oils of *Satureja bachtiarica, Mytrus comminus, Heracleum lasiopetalum, thymus daenesis, Stachys lavandulifolia, Kelussia odoratissima and Echinophora platyloba* were investigated against *Streptococcus iniae* that collected from diseased fish farms was evaluated by serial dilution and disc diffusion, the results were exhibited the most activate essential oils were for *Satureja bachtiarica, Echinophora platyloba, thymus daenesis and the ethanol* extract of *Quercus branti* and the lowest MIC values were for *Heracleum lasiopetalum* (78µl/ml) and *S. bachtiarica* (39 µl/ml). Besides, the results of another study on some Iranian medical herbs on *L. garvieae* showed that the extract of *Mytrus comminus* and *Thymus daenesis* have antibacterial effect against *L. garvieae.* Moreover, the most effective essential oils were for *S. bachtiarica, Zataria multifora, Thymus daenesis, Thymbra spicata* against the *L. garvieae* (30). This study presented that the essential oils have an effective antibacterial while Turker et al. presented the opposite results that use of alcohol for extracting was more effective compare with water extraction (31). In investigation of Shan et al. on some spices and herbs exhibited the positive linear relationship between total phenolic compound and antioxidant activity. Almost some studies asserted the major role of antimicrobial activity of phenolic compound in some spices and medical herbs (32). The percentages of thymol and carvacrol of some extracts and essential oils of some aromatic plants caused them to have been most effective and that have shown antimicrobial effect (26). Sefidkon et al. reported that the antibacterial effect of *S. bachtiarica* oil was stronger because of high percentages of

thymol and carvacrol (33). Similarly, this essential oil had the highest antibacterial effect on *L. garvieae* in this study. According to the results of this study, essential oils of *S. bachtiarica, S. khuzestanica, Mytrus comminus* and *Mentha longifolia* had antibacterial effect in opposition to *L. garvieae* in serial dilution and disc diffusion method of antibacterial test, the most effective antibacterial activity was demonstrated by essential oil of *S. bachtiarica* in serial dilution method (MIC=50µl/ml MBC=200µl/ml) and in disc diffusion method the highest inhibition zone was demonstrated by *S. khuzestanica* (39.3 mm). In this study, erythromycin use as positive control and it was sensitive to this choice antibiotic. Recently out breaks in Turkey were showed the resistance of *L. garvieae* to penicillin and clindamycin, but it was sensitive to erythromycin, chloramphenicol, oxacin and ampicillin (34). The heavy using of antibiotic in aquaculture caused to appearance resistance in environmental and pathogenic bacteria; therefore, this using needs to be reduced and replaced with unconventional method for treating fish diseases (35, 36). There are some natural substance that used as unconventional bio-pesticides and bio-herbicides in organic agriculture, including: clove oil, thyme oil and pine oil (37, 38).

## CONCLUSION

The results of this study showed that most of the essential oils presented similar antibacterial effect when compared with chemical in a particular bacterial strain, diverging discreetly in relation to the size of the size of the zones of growth inhibition, as well as to the MIC and MBC values. These data suggest the potential application of some herbal preparation alone or used in synthetic drugs for the treatment and prevention of pathogenic associated with multi-resistant bacteria. Furthermore, investigation regarding the *in vitro* and *in vivo* toxicity should be conducted in order to develop such products.

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