

# *In Vitro* Inhibitory Activity of the Leaf Methanol Extract of Green Tea (*Camellia sinensis*) against *Lactococcus garvieae* and *Aeromonas hydrophila* Isolated of Rainbow Trout (*Oncorhynchus mykiss*)

P. Akbary

Department of Marine Sciences, Fisheries Group, Chabahar Maritime University, Chabahar, Iran

Email: [paria.akbary@gmail.com](mailto:paria.akbary@gmail.com)

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## Abstract

*Camellia sinensis* Linn is a well-known medical herb that grows in various parts of the world. In the current study, the antimicrobial activity of methanol extract from *C. sinensis* leaves against *Lactococcus garvieae* and *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*) was investigated. The growth inhibitory effects of green tea extract was determined by disc diffusion method (3 times on different days), minimum inhibitory concentration (MIC) using serial dilution and minimum bactericidal concentration (MBC). For the *L. garvieae* and *A. hydrophila*, the zone diameter inhibition (ZDI) of extract ranged 2.30 - 16.5 mm. The highest ZDI (16.50 ± 1.12 mm) for *L. garvieae* was observed at 100 mg·ml<sup>-1</sup> and for *A. hydrophila* (16.20 ± 0.95 mm) at 250 mg·ml<sup>-1</sup> concentration of green tea extract (P < 0.05). At 20, 30, 40 and 100 mg·ml<sup>-1</sup> concentrations of extract, ZDI values of *A. hydrophila* and *L. garvieae* showed significant difference (P < 0.05). The lowest MIC value for the extract was 0.8 mg·ml<sup>-1</sup> against both *L. garvieae* and *A. hydrophila*. From the results of the present study, it can be concluded that methanol extract of *C. sinensis* leaves could be effective for the inhibition of *A. hydrophila* and *L. garvieae* in rainbow trout.

## Keywords

Antibacterial Activity, *Camellia sinensis*, Disc Diffusion, Plant Extract

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## 1. Introduction

During the last decades, there has been a continuous growth of aquaculture industries all over the world and

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such intensive production would experience disease problems. Infectious diseases that occur as sporadic events in wild fish populations may cause high mortalities when appearing in intensive fish farming [1].

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, and pose a potential risk to consumers and to the environment [2].

Antibiotics (such as oxytetracycline, erythromycin, tetracycline and etc.) and other chemical disinfectants are widely used to prevent bacterial disease in fish. The rapidly expanding aquaculture industry has suffered from heavy economic losses due to bacterial pathogens, particularly *Lactococcus garvieae* [3] [4] and *Aeromonas hydrophila* [5] in rainbow trout.

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 [6] [7]. 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 antimicrobial properties [8]-[11].

*Camellia sinensis* Linn (Family Theaceae) known as green tea, is the second most common beverage consumed worldwide next to water [12]. It is an evergreen shrub or tree which is mainly cultured in Iran, China and India. Green tea is made from unfermented leaves and contains the highest concentration of powerful antioxidant; green tea has a number of pharmacological activities such as anticancer, lipid lowering, neuromuscular, blocking action, immunomodulatory effect antiviral, antibacterial [13], antioxidant [14]. A large number of phytoconstituents like alkaloids (caffeine, theobromine), proteins, enzymes, carbohydrates, lipids, polyphenols, carbohydrates, tannins, vitamins and minerals have been reported to be present in this plant [15] [16].

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 step in drug discovery [5]. On the basis of current knowledge, there is not much information on antibacterial effects of *C. sinensis* against *L. garvieae* and *A. hydrophila*. Therefore, the aim of this the objective of the present study was to assess the antibacterial property of the leaf methanol extract of *C. sinensis* against *L. garvieae* and *A. hydrophila* isolated from rainbow trout (*O. mykiss*) and to provide useful information on the efficacy of antimicrobial treatments in rainbow trout (*O. mykiss*).

## 2. Materials and Methods

### 2.1. Extract Preparation

*C. sinensis* medicinal plant was collected from herbal medicine shop in Fars province, Iran, and identified by botanists in the Department of Biology, Shiraz University, Shiraz, Iran. A concentrated extract of this plant was prepared from the leaf according to the method of Nayak *et al.* [17] the seeds of the plant were shade dried and ground into a powder (1 g), macerated in 5 ml of ethanol, filtered and dried at 35°C using a rotary vacuum. The extract of sample was stored in the bottle and refrigerated at 4°C prior to further analyses.

### 2.2. Bacterial Strain

*L. garvieae* and *A. hydrophila* strains were isolated from the infected rainbow trout (*Oncorhynchus mykiss*) from a commercial aquaculture farm in Fars Province, Iran. To isolate above bacteria, specimens from internal organs of diseased fish were cultured using blood agar by streaking method and incubation at 30°C overnight aerobically. On the next day colonies showing characteristics of test bacteria were selected for further studies such as Gram staining, biochemical testing. It was then confirmed by molecular methods [18]-[21].

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 isolates. The cultures were shaken (100 rpm) at 27°C for 48 h. The culture was adjusted to obtain turbidity comparable to that of the turbidity of MC, Farland 0.5 standard. An initial bacterial suspension containing 10<sup>7</sup> CFU/ml was made from the flask broth culture. Subsequent dilutions were made from the above suspension, which were then used in tests.

### 2.3. Minimal Inhibitory Concentration Assay by Serial Dilution Method

Minimum inhibitory concentrations (MICs) were determined by broth dilution method in culture tubes [22] with some modification. In the tube dilution assay, the extract was initially prepared at 50 mg/ml. Then standard bacterial suspension and different concentrations of extract (0.34, 0.68, 1.3, 2.56, 5 and 9.75) were added to tubes containing 1.9 ml Muller-Hinton Broth (MHB, Merck). Each tube was inoculated with 0.1 ml of suspension containing  $10^7$  CFU/ml of each bacterium and incubated at 27°C for 24 h. The negative control tube received no antimicrobial agent, and the positive control tube received no concentration of extract. The tubes were examined for visible growth or lack of growth for each dilution of test bacteria. Turbidity indicated growth of the microorganism and the MIC was the lowest concentration where no growth was visually observed [22].

### 2.4. Minimum Bactericidal Concentration Assay

The MBC values of the extract were determined by the drop plate method from the tubes, where apparently no visible growth found according to Kowser and Fatema [23]. Some modifications were made to the method. The Minimal Bactericidal Concentration (MBC) assay was performed as an adjunct to the MIC and was used to determine the concentration of extract was lethal to the target bacteria *in vitro*. From each MIC broth tube without visible growth, 25 µl volume of the broth was aliquot onto Nutrient agar and spread across the entire surface of the plate. Then the dilution of the sub cultured MIC tube was recorded on each plate and incubated at 25°C for 24 h. The MBC plates were examined for colony growth or lack of growth for each dilution sub cultured. No growth indicated that the extract was bactericidal at that dilution. Growth indicated that the extract was bacteriostatic but not bactericidal at that dilution.

### 2.5. Antibacterial Assay by Disc Diffusion Method

The disc diffusion assays of Lennette [24] were used with some modification to determine the antibacterial effect extract on all test bacteria. Muller Hinton (MH) 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 1.9 ml MH agar inoculated with 0.1 ml of bacterial suspension suspension (0.1 ml of 0.5 McFarland Standard). The extracts were dissolved in dimethyl sulfoxide (DMSO, 15 µL). Sterile paper discs (5 mm in diameter) were impregnated with 0.02 ml of different concentrations of extract (10, 20, 30, 40, 100, 200 and 250 mg/ml) placed onto MH agar. The plates were incubated at 25°C overnight. Negative controls were prepared using the same solvent employed to dissolve the plant extract. Tetracycline (30 µg) antibiotic disc were prepared from Difco. were tested in the same conditions as positive controls used as positive controls [6]. Inhibition zones in mm (without disc paper diameter) around discs were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extract against test microorganisms. The experiment was repeated in triplicate and the mean of diameter of the inhibition zones was calculated.

### 2.6. Statistical Analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). A comparison of antibacterial activity of the extract against *L. garvieae* with *A. hydrophila* was evaluated by applying a two tailed-unpaired t-test. The comparison of antibacterial activities in the different concentrations of extract for each test bacteria were evaluated by using one-way analysis of variance (ANOVA) and Duncan multiple comparisons test respectively. Bacterial strains were considered to be significantly different if  $P < 0.05$ . All statistics were performed using SPSS for windows version 16, Chicago, IL, USA.

## 3. Results

**Table 1** presents diameters of inhibition zones exerted by the different concentration of the ethanol extract of *C. sinensis* leaves and the standard (tetracycline) towards tested microorganisms. *C. sinensis* leaf extract was more effective against the Gram positive strain (*L. garvieae*) and the Gram negative strain (*A. hydrophila*) than tetracycline. At 20, 30, 40 and 100 mg·ml<sup>-1</sup> concentrations, the extract showed lower activity against *A. hydrophilic* than that reported for *L. garvieae* ( $P < 0.05$ ). For *L. garvieae*, the extract showed maximum inhibitory zone of 16.5 mm at 100 mg·ml<sup>-1</sup>, but for *A. hydrophila* strain, the extract exhibited maximum inhibitory zone of 16.20

**Table 1.** The inhibition zones around the discs (mm) produced by antibacterial activity of different concentrations of *Camellia sinensis* (mg·ml<sup>-1</sup>) and standard antibiotics (tetracycline) against test bacteria.

Concentration (mg·ml <sup>-1</sup> ) bacterial strains	10	20	30	40	100	200	250	Tetracycline
<i>A. hydrophila</i>	2.56 ± 4.44 <sup>a/a</sup>	2.36 ± 4.09 <sup>a/ab</sup>	2.73 ± 4.73 <sup>a/ab</sup>	2.53 ± 4.38 <sup>a/abc</sup>	13.83 ± 0.7 <sup>a/abcd</sup>	16.13 ± 1.47 <sup>a/cd</sup>	16.20 ± 0.95 <sup>a/c</sup>	13.03 ± 1.20 <sup>a/abcd</sup>
<i>L. garvieae</i>	2.5 ± 4.33 <sup>a/a</sup>	4.76 ± 4.12 <sup>b/ab</sup>	7.63 ± 0.50 <sup>b/abc</sup>	8.03 ± 0.86 <sup>b/abc</sup>	16.5 ± 1.12 <sup>b/c</sup>	15.93 ± 0.6 <sup>a/c</sup>	16.36 ± 0.90 <sup>a/c</sup>	12.43 ± 0.87 <sup>a/bc</sup>

Each value represents the mean ± standard error (SE) of triplicates. Data are identified by Tukey's test. The growth inhibition values where are similar in columns are identified by the same letter before slash. The growth inhibition values where are similar ( $P > 0.05$ ) in rows are identified by the same letter after slash.

mm at 250 mg·ml<sup>-1</sup>.

Results of MIC and MBC determination (**Table 2**) showed that MIC and MBC values for the leaves extract of *C. sinensis* against the two strains (*L. garvieae* and *A. hydrophila*) were the same (0.8 and 1.1 mg·ml<sup>-1</sup> respectively).

#### 4. Discussion

In recent years, an explosive spread of multidrug-resistant (MDR) bacterial pathogens has become a serious concern worldwide in terms of public health and economic effects. 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 [6] [7]. For organic fish production it is essential to develop antibacterial treatments that are made from materials with natural sources.

In the present study, the activity of green tea extract was higher than of that of tetracycline for all tested microorganisms. Also higher inhibition was detected against *L. garvieae* in comparison with *A. hydrophila* at 20, 30, 40 and 100 mg·ml<sup>-1</sup> concentrations (**Table 1**). These observations are likely to be the result of the differences in cell wall structure between Gram-positive and Gram-negative bacteria [25]. The Gram-negative outer membrane acts as a barrier to many environmental substances including antibiotics [26] Tariq and Reyaz [27]. The methanolic extract of *Camellia sinensis* showed the antimicrobial activity against *Bacillus subtilis*, and *Enterococcus* sp. It reveals the highest zone of inhibition around the bacterial colonies when compared with standard antibiotics erythromycin, tetracycline and ampicillin. Also they reported that one gram of *Camellia sinensis* leaves extract contained 0.7 gram of phenolic compounds, while flavonoid content was 14 mg·gram<sup>-1</sup> of *Camellia sinensis* leave extract. One gram of leaf extracts contained 0.11 gram of reducing power. The highest antimicrobial activity of tea is due to presence of catechins and polyphenols which damages bacterial cell membrane [27].

The antibacterial activity of *Camellia sinensis* tea extracts was selective and depends upon the concentration, type of the extracts and bacterial species [28]. In this work different concentrations of leaf extracts were used against two strains of bacteria and the highest zone of inhibition was observed against *L. garvieae* (16.5 ± 1.12 mm) at 100 mg·ml<sup>-1</sup> and for *A. hydrophila* (16.20 ± 0.95 mm) at 250 mg·ml<sup>-1</sup> concentration of leaf extracts.

MIC and MBC values for the leaves extract of *C. sinensis* against the two strains (*L. garvieae* and *A. hydrophila*) were 0.8 and 1.1 mg·ml<sup>-1</sup> respectively (as shown **Table 2**). Aligiannis *et al.* [29] have proposed a classification of plant extracts on the basis of their MIC values: strong inhibition: MIC < 500 µg·ml<sup>-1</sup>; moderate inhibition: 600 µg·ml<sup>-1</sup> < MIC < 1500 µg·ml<sup>-1</sup> and low inhibition: MIC > 1600 µg·ml<sup>-1</sup>. On the basis of this classification, the leaf extract exerts a strong inhibitory activity on all tested bacteria. The comparison of MICs and MBCs values allows a better evaluation of antibacterial effect of bioactive compounds. According to Biyiti *et al.* [30], a substance is bactericidal when the ratio MBC/MIC < 2, and is bacteriostatic if the ratio MBC/MIC > 2. The MIC and MBC are often near or equal values; so, it can be concluded that leaf extract of *C. sinensis* has a bactericidal effect on the mentioned bacteria. The present results are comparable with other study [31]. They showed that all the *Salmonella typhi* isolates showed *C. sinensis* MICs 400 - 600 µg·ml<sup>-1</sup> and *Vibrio cholerae* Ogawa isolates had *C. sinensis* MICs of 400 - 600 µg·ml<sup>-1</sup>.

Finally, in this study, we found that the methanol extract of *C. sinensis* leaves is a potential source of natural antibacterial against *L. garvieae* and *A. hydrophila* isolated from rainbow trout. It might be used for disinfection

**Table 2.** Determination of MIC (by serial dilution assay) and MBC in different concentrations of *Camellia sinensis* (mg·ml<sup>-1</sup>) against all test bacteria.

Concentration (mg·ml <sup>-1</sup> ) bacterial strains	2	1.5	1.1	0.8	0.6	0.47	0.35	0.26	0.20	0.15	0.10	0.08	0.06	0.04
	MIC/ MBC													
<i>A. hydrophila</i>	-/-	-/-	-/-	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
<i>L. garvieae</i>	-/-	-/-	-/-	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

(+) Visible growth of each microorganism; (-) No growth of each microorganism.

of instruments and rainbow trout raceways. Further work should be performed to describe its *in vivo* antibacterial activities in more detail in fish.

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