In vivo and In vitro Antibacterial Activities of Some Essential Oils of Lamiaceae Species on Aeromonas salmonicida Isolates from Cultured Rainbow Trout, Oncorhynchus mykiss

1Gulten Okmen, 1Aysel Uğur, 3Nurdan Sarac and 2Tulin Arslan
1Department of Biology, Faculty of Science and Arts, 
2Department of Aquaculture, Faculty of Fisheries, 
Mugla University, 48121 Koteşli-Mugla, Turkey
3Medical Laboratory Program, Vocational School of Health Services, 
Mugla University, 48700 Marmaris-Mugla, Turkey

Abstract: In this study, usability of plant essential oils from Lamiaceae species for Aeromonas salmonicida infections was investigated under in vitro and in vivo conditions. A total of 18 A. salmonicida strains were obtained from the organs and tissues of locally cultured rainbow trout. All strains were 100% resistant to penicillin, trimethoprim and bacitracin. All strains showed widest resistance profile to antibiotics. Antibacterial effects of essential oils obtained through water distillation of aerial parts of 4 Lamiaceae species (Origaniun comites, Oreganum vulgare sp. hirtum, Thymbra spicata var. intricata and Satureja thymbra) were high on 17 out of 18 strains and inhibition zones ranged between 14-25, 12-26, 10-30 and 10-33 mm, respectively but none of them was effective on A. salmonicida FC84 strain. The most sensitive bacteria to Origanum essential oils was A. salmonicida FC29 strain and the most effective essential oil was S. thymbra’s. Therefore, MIC value of this essential oil on FC29 strain was determined. Then, the MIC value (800 μg) of this essential oil were injected to experimental fishes for in vivo studies. Injection of the most effective dose of S. thymbra essential oil and its several dilutions (400, 200, 100 μg mL⁻¹) caused toxic effect and total mortality of experimental fishes. The essential oil at the doses with low or no toxic effect did not increase the bactericidal activity of fish blood indicating that it does not protect rainbow trout against A. salmonicida infections. On the other hand, S. thymbra essential oil at non-toxic low dosages can be used as immune stimulant.

Key words: Antimicrobial activity, fish pathogenic Aeromonas species, plant essential oil, immune stimulant, trout, toxic effect

INTRODUCTION

Aquaculture industry has shown fast growth over several decades and became one of the significant source of high protein food for the ever growing world human population. This fast growth and increased production intensity, however, brought along some important problems such as infectious diseases causing high economical losses or their treatments with chemical agents which raise concerns on human and environmental health.

As in other organisms, fungus, parasites, viruses and bacteria can cause infections in fish but since, the expansion of aquaculture bacterial pathogens have been one of the leading factor of high economical losses (Ispir et al., 2004). Antimicrobial agents have been widely used to treat infections caused by bacteria including Aeromonas hydrophila, A. salmonicida, Edwardsiella vibrio anguillarum and Yersinia ruckeri. Frequent use of antimicrobial agents in aquaculture as in other uses, however causes selective pressure for antimicrobial resistance in the exposed bacterial flora consisting of both target and non-target pathogens (Midvdet and Lingaas, 1992; World Health Organization, 1999). Development of antibiotic resistance and reduced efficacy of the contemporary drugs due to intense usage of antimicrobial agents has been identified in fish pathogens (Midvdet and Lingaas, 1992; World Health Organization, 1999; Castro-Escarpulli et al., 2003). Additionally, antibiotic resistance can be transmitted horizontally from one bacterium to another and this way it can be passed through to human pathogens. Furthermore, since antimicrobial agents in aquaculture are administered via feed which is dispersed in water usage of antimicrobial

Corresponding Author: Gulten Okmen, Department of Biology, Faculty of Science and Arts, Mugla University, 48121 Koteşli-Mugla, Turkey
agents in aquaculture generates direct negative impact on the receiving aquatic environment (Aoki, 1992; Smith et al., 1994; Kerry et al., 1996; Herwig et al., 1997; Saavedra et al., 2004).

Finally, antibiotics can accumulate in fish and the environment which creates a potential risk both for the consumers and environmental health (FAO/WHO, 2006). All these and similar outcomes reported from diverse agricultural industries increased public awareness towards the negative impacts of indirect exposures to antimicrobial agents. This increasing public awareness led to search for green solutions such as organic food products that are free of organic or synthetic chemicals. Development of alternative antibacterial treatments is necessary for organic fish production (Abutbul et al., 2004). Medicinal plants have been used for treatment of common infections since ancient times (Rios and Recio, 2005). In recent years, many studies were also conducted in order to evaluate the antibacterial activity of various plant extracts on different fish pathogens (Muniruzzaman and Chowdhury, 2004; Biradar et al., 2007; Asha et al., 2008; Castro et al., 2008; Turker et al., 2009). *A. salmonicida* is one of the oldest known fish pathogens. It was first reported in the literature as the causative agent of furunculosis in 1894 (Williamson, 1928; McCarthy and Roberts, 1980). *A. salmonicida* infections range from superficial to deep skin lesions without systemic association to ulcer disease to a typical, gram negative bacterial septicemia (Noga, 1996).

Furunculosis mainly affects salmonids cultured in freshwater and can cause high mortality rates. Antibiotics and vaccines can be effectively used to control furunculosis break outs in farmed salmonids. However, chemotherapeutic administration can have drawbacks as mentioned before, it can cause the development of antibiotic resistance in bacteria. Additionally, it can decrease growth rates (Midtyng and Lillevang, 1998) increase production cost, produce possibility of lower market value (Manning, 1998) and cause injection risks related to vaccination (Pirhonen et al., 2003).

Plant genus such as *Origaniun*, *Thymbra* and *Satureja* belonging to Lamiaceae family have rich (>2%) essential oil contents (Baser et al., 1993). Many studies conducted so far showed that their essential oils have high antimicrobial activity on microorganisms (Karaman et al., 2001; Yu et al., 2004; Sarac, 2005; Vaginas et al., 2007; Bendahou et al., 2008; Kotan et al., 2008; Sarac and Ugrur, 2008; Sarac et al., 2009; Ugrur et al., 2009) suggesting that they might be used as an alternative antibacterial agents in aquaculture industry too. Taking this proven high antimicrobial activity into consideration, the goal in this study was to evaluate antibacterial activity of Lamiaceae species on antibiotic resistant *A. salmonicida* strains isolated from locally farmed rainbow trout (*Oncorhynchus mykiss*).

**MATERIALS AND METHODS**

**Plant material:** The plant specimens *O. onites* L., *O. vulgare* sp. *hirtum* (Link) Litzwaert, *S. thymbra* L., *T. spicata* L. var. *intricata* P.H. Davis were collected at the flowering stage between May and July, 2007 from different localities of Mugla province, Turkey. All plant specimens used in the study were deposited at the Herbarium of the Biology Department of Mugla University, Mugla, Turkey.

**Fish materials:** Fourteen rainbow trout from the fish farms in Fethiye and Koycegiz (Mugla, Turkey) showing the symptoms of furunculosis were brought to laboratory on ice for isolation of bacteria.

**Isolation of essential oils:** The essential oils of air-dried aerial parts of plant samples were obtained by hydrodistillation using a Clevenger type apparatus.

**Isolation and identification of A. salmonicida:** The samples of gills, liver, kidney, spleen, skin and internal lesions of the fishes were homogenized for 2 min in stomacher (Kema Keur Pro 200) containing alkaline peptone water (1/10 w/v). They cultured on Tryptic Soy Broth (TSB) and incubated at 22°C for 48 h.

The pure culture inoculated in Tryptic Soy Agar (TSA) and Coomassie Brilliant Blue (CBB) Agar medium and incubated at 22°C for 48 h. The typical *A. salmonicida* colonies on TSA (Hirvela-Koski et al., 1988) and CBB Agar medium (Markwardt et al., 1989) were selected. Stock cultures of each strain were maintained for short periods at room temperature on TSA slants at +4°C and for longer storage they were frozen at -20°C in 20% (w/v) glycerol.

The isolates were identified with conventional methods. The isolates were first tested for gram reaction. Bacteria identification was restricted to gram-negative isolates. Bacterial groups were determined on the criteria of shape, motility, catalase and oxidase reactions and the Hugh and Leifson glucose oxidation-fermentation test (OF basal medium, Merck). API 20E systems (bioMerieux, France) were furthermore used in order to identify the bacteria at species level.

**In vitro antimicrobial assays:** Antimicrobial activities of the essential oils were assayed by the Disc Diffusion Method (Bauer et al., 1966; Collins et al., 1995;
Murray et al., 1995). The inoculum sizes of the bacteria were prepared by using 100 mL McFarland tubes to give a concentration of 1×10^6 bacteria mL^-1. Mueller-Hinton Agar (MHA) sterilized in a flask and cooled to 45-50°C were distributed (15 mL) to 9 cm sterilized petri dishes after homogeneous injection of 0.5 mL bacteria cultures. The plates were held for 15-20 min at room temperature. Each essential oil (20 mL) was individually applied to 6 mm sterile discs (Schleicher and Schuell, Keene, NH). Discs were placed on the solid agar medium with a slight fingertip pressure. Then, plates were incubated at 22°C for 48 h. At the end of incubation period, diameters of the inhibition zones were evaluated in millimeters. Discs of penicillin (10 U), amoxicillin + clavulanic acid (20 + 10 μg), azlocillin (75 μg), chloramphenicol (5 μg), piperacillin (50 μg), norfloxacin (10 μg), rifampin (5 μg), sulfisoxazole (300 μg), trimetoprim (5 μg), tetracycline (30 μg), bacitracin (0.04 μU), trimetoprin/sulfamethoxazole (1.25/23.75 μg), ofloxacin (5 μg), oxolinic acid (2 μg) and nalidixic acid (30 μg) were used as positive controls.

Studies were performed in triplicates and the developing inhibition zones were compared with those of reference discs.

**Determination of Minimum Inhibitory Concentration (MIC):** Minimum Inhibitory Concentrations (MICs) of the most effective essential oil was determined by Broth Dilution Method (Collins et al., 1995) against the strain which was the most resistant to tested antibiotics but sensitive to the essential oil.

The essential oil were serially-diluted from 800-10 μg mL^-1 in test tubes using 10% Tween 80 in distilled sterile water. Bacterial suspension was incubated in Brain Heart Infusion Broth (BHIB) overnight at 22°C and then standardized to 1.5×10^6 cfu mL^-1 in No.: 0.5 McFarland tubes. About 100 μL freshly prepared bacterial suspensions were pipetted into tubes containing Mueller Hinton Broth (MHB). About 100 μL of the essential oil dilutions were then added to the culture tubes containing a final volume of 2 mL. A positive control containing the bacterial culture without the essential oil and a negative control containing only MHB were also included to the test panel. All tests were carried out in triplicates. The test tubes were incubated for 24 h at 22°C. The minimum concentrations at which no visible growth was observed were defined as the MICs.

**In vivo antimicrobial assays:** In vivo studies were conducted in the research and development unit of a commercial rainbow trout farm located in Fethiye, Mugla, Turkey. The 1 week prior to injection of essential oils, fish collected from the outdoor production units were brought inside for acclimation in a concrete tank (1 m³). Their mean weight and mean total length were 117.6±23.4 g and 21.3±1.4 cm (N = 70), respectively. After injections, fish were stocked to the previously assigned 100 L cylindrical fiberglass tanks. All tanks received spring water with a constant water temperature of 8.5±0.2°C and pH of 7.6. Water exchange rate in cylindrical tanks was kept around 5 L min^-1. At this exchange rate, dissolved oxygen concentration stayed above 10 mg L^-1.

Before the collection of blood or injection of essential oils feeding of experimental fish was ceased for 48 h and except one occasion all fish were lightly anesthetized using 2-phenoxy ethanol (1:20,000). Then, they were randomly assigned to the treatment groups and each group contained 4-5 fish. Before placing fish to their designated tanks each treatment group injected with the predetermined doses of essential oils intraperitonally. The same amount (0.2 mL) of sterile Phosphate Buffer Solution (PBS, pH = 7.0) were given to the 1st control group and the 2nd control group received no injection. The experiment was carried out in duplicates.

Injection of *S. thymbra* essential oil was done in two occasions. In the first occasion, fish were injected with the effective dose of 800 μg as predetermined in the MIC test. Nevertheless, fish injected with this dose never gain their balance back and died shortly after the injections. Then, two sets of 5 fish were injected again without anesthetizing in order to eliminate the possibility of over anesthesia. All these fish also, never gain their balance back their respiration stopped and they died within 15-30 min pursuing the injections. Therefore in the 2nd occasion lower doses of 400, 200, 100, 50 and 10 μg *S. thymbra* essential oil were injected. Again all fish injected with 400, 200 μg essential oil died shortly after the injections. Only one fish survived from 100 μg group. Total 80% of fish survived in 50 μg group and all fish survived in 10 μg group while no mortality occurred in the control groups. Blood samples were collected from the randomly selected half of the surviving fish at 24 and 48 h following the injections in order to determine the effects of *S. thymbra* essential oil on bactericidal activity of fish blood.

**Bactericidal activity of serum:** Bactericidal activity of the serum was determined by following the procedure of Kajita et al. (1990). An equal volume (100 mL) of serum and bacterial suspension were mixed and incubated for 1 h at 22°C. Blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile PBS at a ratio 1:10. The serum-bacterial mixture (100 mL) was plated onto nutrient agar and plates were incubated for 48 h at 22°C. The number of viable bacteria was determined by counting the growing colonies.
RESULTS AND DISCUSSION

Total 50 different presumptive *A. salmonicida* isolates were obtained on TSA medium inoculated with samples taken from the gills, liver, kidney, spleen, skin and internal lesions of the cultured rainbow trout showing the symptoms of furunculosis. Based on the colony morphologies on CBB medium, microscopic examinations and biochemical tests, 18 isolates were identified as *A. salmonicida*. Table 1 shows the antibiotic sensitivity profiles of these 18 strains against 15 antibiotics commonly used for *A. salmonicida*. All strains were found to be 100% resistant to penicillin, trimetoprim and bacitracin. Their resistances to sulfisoxazole, amoxicillin-clavulanicacid, trimetoprim/sulfamethoxazole, phosphomycin, azlocillin, nalidixic acid, oxytetracycline, rifampin, tetracycline, norfloxacin and ofloxacin were 89, 83, 78, 72, 67, 61, 55, 50, 44, 17, 11 and 6%, respectively.

Essential oils of all 4 plant species inhibited the growth of 18 out of 17 different *A. salmonicida* strains (Table 2). Inhibition zones of *O. onites*, *O. vulgare* sp. *hirtum*, *T. spicata* var. *intricata* and *S. thymbra* essential oils on these 17 strains ranged between 14-25, 12-26, 10-30 and 10-33 mm. Nonetheless, none of the 4 essential oils was effective on *A. salmonicida FC84* strain.

Essential oil of *O. onites* was highly effective on *A. salmonicida FC9*, *FC43*, *FC94-2* and *FC95* and prevented their growth within an inhibition zone of 25 mm. It was the least effective against *A. salmonicida FC98* (Table 2). *O. vulgare* sp. *hirtum* essential oil produced the largest inhibition zone of 26 mm for FC43 and FC94-1 strains but it was least effective against *A. salmonicida FC98*. *T. spicata* var. *intricata* essential oil had the highest antibacterial activity against *A. salmonicida FC29* and its activity was much lower on FC93 and FC98 strains. *S. thymbra* essential oil were found highly effective on FC43 and FC92 strains producing inhibition zones of 33 and 32 mm, respectively.

Overall, *A. salmonicida FC9* was determined to be the most sensitive strain to all 4 essential oils evaluated and this strain showed the highest sensitivity against the essential oil obtained from *S. thymbra*. The MIC test demonstrated that the most effective *S. thymbra* essential oil dose on *A. salmonicida FC9* was 800 µg. For this reason *in vivo* studies were conducted by using the *S. thymbra* essential oil starting with the most effective dose. However, this dose caused total mortality of all fish due to toxic effect. Lower doses of *S. thymbra* essential oil (400, 200 and 100 µg) were also toxic to rainbow trout with a mean total weight of 117.6±23.4 g. Toxic effect of *S. thymbra* essential oil dropped at 50 µg dose and disappeared at a lower dose of 10 µg. Nevertheless, blood serum of the fish exposed to these lower doses had no bactericidal activity against *A. salmonicida FC92*. All serum bactericidal activity evaluations with blood serum collected before (at 0 h) at 24 and 48 h after injection of *S. thymbra* essential oil yielded over 300 ecfu mL⁻¹

This study evaluated usability of plant essential oils from Lamiaceae species under *in vitro* and *in vivo* conditions for the treatments of *A. salmonicida* infections causing high economical losses in rainbow trout culture. Since, intensive use of antibiotics in fish farming cause development of antibiotic resistance, especially

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AZL: Azlocillin (75 µg); C: Chloramphenicol (5 µg); FF: Phosphomycin (50 µg); NO: Norfloxacin (10 µg); P: Penicillin (10 U); RA: Rifampin (5 µg); ST: Sulphisoxazole (300 µg); TMP: Trimetoprim (5 µg); TE: Tetracycline (30 µg); B: Bacitracin (0.04 U); SXT: Trimetoprim/Sulfamethoxazole (1.25 µg/23.75 µg); OFX: Ofloxacin (5 µg); AMC: Amoxicillin-Clavulanic acid (20 µg + 10 µg); OA: Oxolinic Acid (2 µg); NA: Nalidixic Acid (30 µg); (-): no zone.
Table 2: Antibacterial activity of essential oils of *O. onites*, *O. vulgare* sp. *hirtum*, *T. spicata* var. *intricata* and *S. thymbra* against *A. salmonicida* isolated from cultured rainbow trout

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*A. salmonicida* strains showing multiple antibiotic resistance were preferred for the study. Plant species of which essential oils were obtained are also chosen for their known high antimicrobial activity as demonstrated in previous studies (Karaman et al., 2001; Yu et al., 2004; Sarac, 2005; Vagionas et al., 2007; Bendahhou et al., 2008; Kotan et al., 2008; Sarac and Ugur, 2008; Sarac et al., 2009; Ugur et al., 2009).

All *A. salmonicida* strains obtained from cultured rainbow trout in this study showed multiple antibiotic resistance. *A. salmonicida* strains frequently showed multiple resistance against antibiotics from sulphonamide, tetracycline, amoxicillin, trimetoprim + sulphadimethoxine and quinolone groups which are commonly used in aquaculture (Tsounas et al., 1989; Inglis et al., 1991; Barnes et al., 1994; Dalsgaard et al., 1994). In a recent study, it was also demonstrated that multiple antibiotic resistance is a common phenomenon of *A. salmonicida* isolated from Atlantic salmon with the symptoms of furunculosis (Inglis et al., 2006). Results of this study confirming the outcome of previous investigations illustrated once more the size of problem caused by intensive use of antibiotic in aquaculture. All these accumulated information on adverse effects of oblivious antibiotic use inevitably lead the search on alternative ways for the treatment of bacterial infections in aquaculture.

Essential oils obtained from medicinal plants can provide such an alternative. The results showed that essential oil of *O. onites* had high antibacterial activity against *A. salmonicida* strains FC9, FC43, FC94-2 and FC95, *O. vulgare* sp. *hirtum* essential oil was highly effective on FC43 and FC94-1 strains, *T. spicata* var. *intricata* essential oil had the high antibacterial activity against to FC29 strain and *S. thymbra* essential oil were found highly effective on FC43 and FC29 strains. Nevertheless in vivo studies demonstrated that essential oil of *S. thymbra* or perhaps the other Lamiaceae species can be toxic to rainbow trout at the most effective dose that produces the highest antibacterial activity or at much lower doses. Sadly as indicated by the serum antibacterial activity test at non-toxic low doses essential oils do not provide effective protection against *A. salmonicida* infections.

**CONCLUSION**

These outcomes of the in vivo studies indicated that essential oils of 4 Lamiaceae species could not be used as the replacement of antibiotics. Yet, these results do not exclude the possibility of their other beneficial use at non-toxic low doses. At such doses they can be used as immune stimulant. As immune stimulant, essential oils of Lamiaceae species can help rainbow trout and perhaps many other cultured species to develop resistance against disease factors in turn reducing the need of antibiotics use in aquaculture. Further studies will be conducted in near future in order to evaluate the immune stimulator effects of 4 Lamiaceae species.

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