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Shirazi thyme (*Zataria multiflora* Boiss) and Rosemary (*Rosmarinus officinalis*) essential oils repress expression of sagA, a streptolysin S-related gene in *Streptococcus iniae*

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ABSTRACT

The pathogenicity of *Streptococcus iniae* in fish is depending on the secreted virulence factors, such as streptolysin S (SLS). It is demonstrated that some plant essential oils can affect the production of bacterial toxins. In this study, broth macrodilution method was first used to determine minimum inhibitory concentrations (MICs) of *Rosmarinus officinalis* and *Zataria multiflora* essential oils against *S. iniae*. Hemolysin assay and real-time PCR analysis were then conducted to investigate the effect of subinhibitory concentrations of the essential oils on SLS major encoding gene, sagA, in two isolates of *S. iniae*. The obtained data showed that both *R. officinalis* and *Z. multiflora* decreased the hemolytic activity of *S. iniae* supernatant, dose-dependently. However, SLS was not inactivated when the essential oils were added to the bacterial culture supernatant. The transcription of sagA was significantly down-regulated during treatment of the bacterial isolates with subinhibitory concentrations of the essential oils. This data suggested that both *R. officinalis* and *Z. multiflora* could be advantageous for the control of streptococcosis caused by *S. iniae*, via inhibition the growth and repression the production of SLS.

1. Introduction

Streptococcus iniae is a gram-positive, hemolytic pathogen that causes high morbidity and mortality in a wide range of commercial fish species in aquaculture industry worldwide (Agnew and Barnes, 2007). *S. iniae* possesses streptolysin S (SLS), a small, pore-forming, oxygen-stable and non-immunogenic cytotoxin that is a proven virulence factor (Nizet, 2002; Wessels, 2005) and responsible for zones of β-hemolysis surrounding colonies on blood agar media, the formation of SLS by group A streptococci was shown to occur during the stationary phase of bacterial growth in the presence of minimal energy resources (Duncan and mason, 1976). It is demonstrated that SLS could affect fish via three categories including promoting the cellular adherence and invasion, increasing resistance to phagocytic clearance, and producing cytolytic injury to animal cells and tissues (Locke et al., 2007). Direct cytotoxicity against fish cells is likely a major factor underlying the virulence role of *S. iniae* SLS (Locke et al., 2007). The cytolytic spectrum of SLS is broad including lymphocytes, neutrophils, platelets and subcellular organelles such as lysosomes, nuclei, and mitochondria (Alouf, 1980; Ginsburg, 1970).

Streptolysin S is encoded by a nine-gene cluster (sagA–sagI) and products of the sag operon are necessary for SLS production (Nizet et al., 2000). The first gene of this operon, sagA, encoded a putative bacteriocin-like prepropeptide. Studies have shown that sagA is the structural gene encoding SLS and the predicted size of the saga propeptide matches the size of mature SLS (Loridan and Alouf, 1986). The remaining gene products of the operon (sagB to sagI) are considered for processing and transporting of the SLS (Fuller et al., 2002). SagA may also possess a regulatory function affecting the expression of SLS and other virulence factors (Biswas et al., 2001).

The rapid development of antibiotic resistance has made it difficult to treat *S. iniae* infections in fish. Therefore, in aquaculture industry, there is a need to develop new therapeutics to aid the prevention and treatment of such bacterial infections. Comprehensive understanding of SLS, can point to new targets for anti-infective therapy. Recently, studies have focused on plant essential oils, due to their potent antimicrobial properties (Solorzano-Santos and Miranda-Novales, 2012). For instance, it has been demonstrated that *R. officinalis* and Z. *multiflora* essential oils were able to suppress the growth of some isolates of *S. iniae* plus a remediation in morbidity and mortality of affected tilapia (Abutbul et al., 2004; Soltani et al., 2013, in press). Also, different studies have been demonstrated not only the safety of oral administration of these medicinal plants but also the their

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positive immunostimulatory effects in some commercial fish species including rainbow trout, common carp (*Cyprinus carpio*) and tilapia (*Oreochromis* sp.) (Abutbul et al., 2004; Sharif Rohani et al., 2011; Soltani et al., 2009, 2010, 2013). Therefore, the use of these medicinal plants as a part of supplementary diet for fish is feasible. However there is no information on the possible effect of these essential oils on virulence factors of *S. iniae*. Therefore, the aim of this study was to assess the anti-*Streptococcus iniae* activity of *R. officinalis* and *Z. multiflora* oils and to investigate the effect of sub-inhibitory concentrations of these essential oils on saga expression, as the major gene encoding SLS.

2. Materials and methods

2.1. Preparation of the plants

Z. multiflora and *R. officinalis* were collected in June 2013 from major growing areas of Fars and Tehran provinces, Iran. Both plants were identified by Herbarium of Institute of Medicinal Plants, Karaj, Iran.

2.2. Extraction of essential oils

The leaves of *Z. multiflora* and *R. officinalis* were dried in an oven equipped with hot air circulation, prior to be grounded. To obtain essential oils, amount of 50 g of powdered leaves were added to 500 ml sterile distilled water and subjected to steam distillation for 3 h using Clevenger-type apparatus. The gas chromatography–mass spectrometry (GC–MS) analysis were performed using Agilent 6890 gas chromatography equipped with a HP-5MS column (30 m length × 0.25 mm i.d., film thickness 0.25 μm) coupled with a Agilent 5973 mass spectrometer. Data were obtained under the following conditions: initial temperature at 50 °C; program rate at 3 °C; final temperature at 240 °C and injector temperature at 290 °C. Helium was used as a carrier gas at a flow rate of 0.8 ml/min. Ionization voltage of mass spectrometer was equal to 70 eV and ionization source temperature was 220 °C.

2.3. Test organisms

Two isolates of *S. iniae*, isolates 201C (GenBank Accession no. KC708484.1) and L2B (GenBank Accession no. KC708485.1), obtained from the Department Of Aquatic Animal Health, Faculty of Veterinary Medicine, University Of Tehran were used. These isolates were previously recovered from a moribund rainbow trout (*Oncorhynchus mykiss*) with typical clinical symptoms of darkening of the body, exophthalmia and sluggish movement in Iran, and were identified using both phenotypical and molecular works by Soltani et al. (2005, 2008). These bacterial isolates were of serotype I and β-hemolytic strains. The lyophilized bacteria were first grown on a defibrinated sheep blood (Darvash, Iran) agar (Merck, Darmstadt, Germany) incubated at 25 °C for 48 h. The second passage was provided on the blood agar as working cultures.

2.4. Antibacterial activity of the essential oils

Minimum inhibitory concentrations (MICs) of *Z. multiflora* and *R. officinalis* essential oils against *S. iniae* were determined using broth macrodilution described by Clinical and Laboratory Standards Institute (CLSI, 2000), with slight modifications. The bacterial isolates were first individually dissolved in a sterilized physiological saline solution (0.9% w/v) to give a final concentration of 10^8 cfu/ml confirmed by viable count. Doubling dilutions of both essential oils were prepared in tubes containing a tryptic soy broth (TSB) (Merck, Darmstadt, Germany) to reach a concentration range of 1 to 0.0017 μl/ml. The dimethyl sulfoxide (DMSO) was used as a solvent at 3%. Bacterial cultures were then added to the oil dilutions with the final concentration of 10^5 cfu/ml in each individual oil dilution. After 24 h incubation at 25 °C, the lowest concentration demonstrating no visible growth was regarded as the MIC. Minimum bactericidal concentrations (MBCs) were confirmed by reinoculation of 10 μl of each bacterial culture on blood agar incubated at 25 °C for 24 h and the lowest concentration of the oils showing no growth was defined as the MBC. Both the MIC and MBC were performed in triplicates.

2.5. Hemolysin assay

The bacterial isolates were first cultured in TSB containing subinhibitory concentrations of each essential oil at 25 °C for 24 h. The bacterial cultures were then centrifuged at 5500 ×*g*, at 4 °C for 1 min. The supernatant was then collected and the residual cells were removed using a 0.2 μm sterile filter. An aliquot of 0.1 ml of each culture supernatant was brought up to 1 ml by the addition of phosphate buffered saline (PBS) followed by the addition of 25 μl of defibrinated rabbit blood. After incubation at 37 °C for 15 min, the unlysed blood cells were pelleted by centrifugation at 5500 ×*g* at room temperature for 1 min. The hemolytic activity of the supernatant was assessed by measuring the optical density (OD) at 543 nm. The control culture supernatant was served as 100% hemolysis, and the hemolytic sample percentage was calculated by comparison to the control culture (Qiu et al., 2011). To investigate whether tested essential oils could directly inhibit hemolytic activity, 5 μl of each undiluted essential oil was added to the 10 ml supernatant of *S. iniae* cultured in TSB. The samples were then incubated at 37 °C for 1 h with constant shaking. The samples were then settled down for 15 min to separate the essential oils (Smith-Palmer et al., 2004). Hemolytic activity was then determined as described above.

2.6. RNA isolation and real-time PCR

Both isolates of *S. iniae* (strains 201C and L2B) were first grown in TSB in the presence of 1/2, 1/4, 1/8 and 1/16 MICs of *R. officinalis* and *Z. multiflora* essential oils at 25 °C until the post-exponential growth phase occurred. The cells were then pelleted by centrifugation at 5000 ×*g* for 1 min at 4 °C. The bacterial RNA was isolated by a simply P total RNA extraction kit (Bio Flux, Japan) according to the manufacturer's instruction. The RNA concentrations were detected from the OD_{260} and the RNA was loaded onto a 2% agarose gel to test degradations. RNA was then reversed transcribed into cDNA using BioRT two-step RT-PCR kit (Bio Flux, Japan) according to the manufacturer's protocol. Nucleic acid purity was assessed by measuring the A260 nm:A280 nm ratio using NanoDrop Spectrophotometer 2000 (Thermo Scientific, USA). The synthesized cDNA was stored at −20 °C until used. The primers used in real-time PCR were designed by a primer express software and are listed in Table 1. The reactions were performed using Rotor-Gene 6000 (Qiagen, Hilden, Germany). The PCRs were carried out in a 20 μl volume and contained QuantiFast SYBR Green premix (Qiagen, Hilden, Germany) as recommended by the manufacturer. Cycling conditions were as follows: 95 °C for 5 min, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. Melt curve analysis was then performed to assess PCR specificity, resulting in a single primer-specific melting temperature. All samples were analyzed in triplicates and a no template control (NTC) was included to check contamination and primer dimer formation. The 16 s rRNA housekeeping gene served as the reference gene. The relative expression levels were analyzed by the comparative

Table 1
Primers used for the real-time PCR assay.

Primer	Sequence (5′ → 3′)	Amplicon length (bp)
sagA–F	ATTTGTATAAGGAGGTAAGC	
sagA–R	AGTGAATTACTTTGGAGGTG	212
16 s-F	TCACTCACGCGGCGTTGCTC	
16 s-R	AACGGCTCACCAAGGCGACG	196