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Melaleuca alternifolia essential oil prevents alterations to purinergic enzymes and ameliorates the innate immune response in silver catfish infected with *Aeromonas hydrophila*

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ABSTRACT

Aeromonas hydrophila infection represents a major impediment to the development of aquaculture, leading to important economic losses. Over the last few years, different methods have been used to counteract and minimize the negative effects of this infection, such as the use of *Melaleuca alternifolia* essential oil, popularly known as tea tree oil (TTO), that possess a bactericide action against *A. hydrophila*. The purinergic system develops an important role in the inflammatory response, principally due to involvement of adenosine triphosphate (ATP) in the inflammatory process, as well as by the anti-inflammatory properties of adenosine (Ado), a molecule that is controlled by NTPDase, 5′-nucleotidase and adenosine deaminase (ADA) enzymes. Thus, the aim of this study was to investigate the involvement of purinergic enzymes in the pathogenesis of *A. hydrophila* infection, and whether the purinergic pathway and innate immune response are involved in the protective effects of TTO in silver catfish (*Rhamdia quelen*) experimentally infected with *A. hydrophila*. Our results revealed that *A. hydrophila* infection increased seric NTPDase and 5'-nucleotidase activity, while ADA activity decreased. Also, the seric levels of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) increased in the infected fish, while the seric level of anti-inflammatory interleukin-10 (IL-10) decreased. Treatment with TTO was able to prevent the impairment of purinergic enzymes and improve the innate immune response through the modulation of cytokine response during *A. hydrophila* infection. In summary, prophylactic therapy with TTO can be considered an important approach to improve the immune response and consequently avoid the inflammatory process in fish infected with *A. hydrophila*.

1. Introduction

Aquaculture plays an increasingly important role in food production worldwide; however, intensive and stressful rearing conditions make farmed fish highly susceptible to different infectious diseases, which contribute to mortality and economic losses [1,2]. Among fish diseases, bacterial infections present significantly increasing problems in intensive aquaculture, due to few effective solutions available at preventive and therapeutic levels. In this sense, *Aeromonas hydrophila*, an important Gram-negative pathogen commonly found in the aquatic environment, is an important pathogen particularly of freshwater aquaculture, and the causative agent of epizootic ulcerative syndrome [3]. The main clinical signs

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observed in fish naturally or experimentally infected are ulcerations, injury to the caudal fin with hemorrhagic foci, congestion and hyperemia in hepatic tissue, integumentary depigmentation and erosion of the fins [2]. Recently, the use of feed additives, prebiotics, probiotics and therapeutic baths using essential oils has increased considerably in an attempt to cure bacterial disease and/or improve the immune system and health of fish [4–6].

Melaleuca alternifolia essential oil, popularly known as tea tree oil (TTO), is an Australian native plant used in traditional medicine worldwide principally due to its antimicrobial and anti-inflammatory activity [7,8]. A study conducted by Souza et al. [9] demonstrated that TTO was able to prolong the life of silver catfish, *Rhamdia quelen*, experimentally infected with *A. hydrophila*, showing 88% therapeutic efficacy, possibly to due anti-inflammatory properties. In this sense, a recent study published by Baldissera et al. [10] demonstrated that the purinergic system, an important pathway involved in the inflammatory response, is involved in the immune response of silver catfish during *A. hydrophila* infection. Thus, our hypothesis is that TTO may exert anti-inflammatory activity in silver catfish infected with *A. hydrophila* via the purinergic system, and consequently improve the immune response against this infection.

The purinergic system develops important functions in the body, such as regulation of the immune response [11], that occur due to interaction of purine nucleotides with purinoreceptors present in the plasma membrane of cells. The extracellular purine nucleotide adenosine triphosphate (ATP) can interact with type P2 purinoreceptors, triggering secretion of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-2, IL-6 and IL-7 [12]. Moreover, the nucleoside adenosine (Ado) interacts with type P1 purinoreceptors, inducing the production of anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 [13]. Nucleotide enzymatic regulation initiates with E-NTPDase that hydrolyzes ATP into adenosine diphosphate (ADP) and adenosine monophosphate (AMP). Degradation continues with 5'-nucleotidase activity that hydrolyzes AMP into Ado. Finally, Ado is desaminated by adenosine deaminase (ADA) into inosine [14]. Recently, Boiago et al. [15] demonstrated that alterations to ectoenzymes of the purinergic system contribute directly to the inflammation process and disease pathophysiology of other important Gram-negative bacteria, such as *Salmonella gallinarum*.

Based on this evidence, the aim of this study was to investigate the involvement of purinergic enzymes and serum levels of cytokines in the pathogenesis of *A. hydrophila* infection, and whether the purinergic pathway and innate immune response are involved in the protective effects of TTO in *R. quelen* experimentally infected with *A. hydrophila*.

2. Materials and methods

2.1. Plant material and TTO characterization

M. alternifolia essential oil was purchased from Vimontti S/A (Santa Maria, Brazil). The essential oil was extracted by the water vapor drag method using a Clevenger extractor.

Oil composition and yield were analyzed using gas chromatography (GC) carried out using an Agilent Technologies 6890N GC-FID system, equipped with a DB-5 capillary column (30 m × 0.25 mm × 2.5 μm film thickness) connected to a flame ionization detector (FID). The injector and detector temperatures were set to 250 °C. The carrier gas was helium, at a flow rate of 1.3 mL/min. The thermal program was 100–280 °C at a rate of 10 °C/min. Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors. The injection volume of the TTO was 1 μL [8,16]. GC-mass spectroscopy (GC-MS) analyses were performed on an Agilent Technologies AutoSystem XL GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (250 °C). The transfer line temperature was 280 °C. Helium was used as carrier gas (1.5 mL/min) and the capillary columns used were an HP 5MS (30 m × 0.25 mm × 2.5 μm film thickness) and an HP INNOWax (30 m × 0.32 mm × 0.50 mm film thickness). The temperature program was the same as that used for the GC analyses. Essential oil injected volume was 1 μL.

Identification of TTO components was performed on the basis of retention index (RI), determined with reference to a homologous series of *n*-alkanes, C_7–C_{30}, under identical experimental conditions, compared with a mass spectra library search (NIST and Wiley), and with the mass spectra literature data of Adams [17]. The relative amounts of individual components were calculated based on the GC peak area (FID response).

2.2. Aeromonas hydrophila isolate

The isolate of *A. hydrophila* used in this study was obtained from a naturally infected silver catfish, identified by phenotypic testing and 16S rDNA sequencing, prepared in saline solution from cultures grown in Mueller–Hinton agar (HiMedia Laboratories).

2.3. Animal model and water quality

Forty-two silver catfish juveniles (50.5 ± 2 g; 19.5 ± 1.3 cm) were used as the experimental model for determination of enzymes of the cholinergic and adenosinergic systems in liver tissue. All fish were transferred from a local fish culture to the laboratory, where they were maintained in continuously aerated tanks with controlled water parameters (22.0–24 °C, pH 7.2–7.6, dissolved oxygen levels: 5.5–7.5 mg/L). Fish were acclimated for 14 days, and dissolved oxygen and temperature were measured with a YSI oxygen meter (model Y5512, Ohio, USA). The pH was measured using a DMPH-2 pH meter (Digimed, SP, Brazil). Total ammonia levels were determined according to Verdouw et al. [18], and un-ionized ammonia (NH_3) levels were calculated according to Colt [19].

2.4. Study design

The silver catfish were assigned into six groups (A–F), with seven animals each. Groups A, B and C were composed of uninfected animals, while animals in groups D, E and F were infected. Groups A and D were used as negative and positive controls (without treatment), respectively. Animals from groups B and E were exposed to prophylactic treatment with 50 μL/L TTO for 7 days, 1 h per day, while groups C and F received 450 μL/L ethanol (used to dilute the TTO) for 7 days, 1 h per day. TTO was previously diluted 1:10 in 95% ethanol and added to the bath water. The therapeutic protocol was based on a recent study published by Souza et al. [9]. Fish were fed once a day to satiation with commercial feed, and uneaten food, other residues and feces were removed 30 min after feeding.

After 7 days of prophylactic treatment, groups D, E and F were inoculated intramuscularly with 100 μL of *A. hydrophila* solution (2.1×10^9 colony forming units: OD600 = 1.7–1.9) on the right laterodorsal side of each fish. Uninfected groups received the same dose of sterile saline by the same route.

The methodology used in this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria under protocol number 074/2014.