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O USO DO EUGENOL CONTRA *Aeromonas hydrophila* E SEU EFEITO SOBRE PARÂMETROS HEMATOLÓGICOS E IMUNOLÓGICOS EM JUNDIÁS (*Rhamdia quelen*)

DISSERTAÇÃO DE MESTRADO

Fernando Jonas Sutili

Santa Maria, RS, Brasil
2014

O USO DO EUGENOL CONTRA *Aeromonas hydrophila* E SEU EFEITO SOBRE PARÂMETROS HEMATOLÓGICOS E IMUNOLÓGICOS EM JUNDIÁS (*Rhamdia quelen*)

Fernando Jonas Sutili

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Mestre em Farmacologia

Orientador: Prof. Dr. Bernardo Baldisserotto

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elaborada por
Fernando Jonas Sutili

Como requisito parcial para obtenção do grau de
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RESUMO

Dissertação de Mestrado
Programa de Pós–graduação em Farmacologia
Universidade Federal de Santa Maria

O USO DO EUGENOL CONTRA *Aeromonas hydrophila* E SEU EFEITO SOBRE PARÂMETROS HEMATOLÓGICOS E IMUNOLÓGICOS EM JUNDIÁS (*Rhamdia quelen*)

AUTOR: FERNANDO JONAS SUTILI
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Na aquicultura o eugenol tem sido utilizado e recomendado como anestésico para várias espécies de peixes. Além disso, este produto tem atraído a atenção de pesquisadores devido a suas propriedades quimiopreventivas, antiinflamatórias e antioxidantes, bem como, o seu potencial antimicrobiano. O objetivo deste estudo foi avaliar a atividade do eugenol contra o patógeno de peixes *Aeromonas hydrophila* e seu efeito sobre parâmetros hematológicos e de imunidade natural em jundiás (*Rhamdia quelen*). O eugenol mostrou fraca atividade contra *A. hydrophila in vitro*, mas *in vivo*, a uma concentração subinibitória (10 mg/L) promoveu a sobrevivência de jundiás infectados. *In vitro* o eugenol (50 µg/mL) reduziu a atividade hemolítica do sobrenadante de *A. hydrophila* em eritrócitos de peixe. A exposição de jundiás ao eugenol (5 e 10 mg/L) através de banhos durante cinco dias não alterou os parâmetros hematológicos e imunológicos estudados neste trabalho. Com base nestes resultados, o eugenol pode ser usado para tratar ou prevenir infecções bacterianas em peixes.

Palavras-chave: *Aeromonas*. Sistema imune inato. Peixe, Jundiá. Bactérias. Hematologia.

ABSTRACT

Master Dissertation
Post-Graduate Course in Pharmacology
Universidade Federal de Santa Maria

THE USE OF EUGENOL AGAINST *Aeromonas hydrophila* AND ITS EFFECT ON HEMATOLOGICAL AND IMMUNOLOGICAL PARAMETERS IN SILVER CATFISH (*Rhamdia quelen*)

AUTHOR: FERNANDO JONAS SUTILI
ADVISOR: BERNARDO BALDISSEROTTO
Date and place of the defense: Santa Maria, February 25th, 2014.

In aquaculture, eugenol have been used and recommended as anesthetics for several fish species. Moreover, this product has attracted the attention of researchers because of its chemopreventive, anti-inflammatory and antioxidant properties, as well as, its antimicrobial potential. The aim of this study was to evaluate the activity of eugenol against the fish pathogen *Aeromonas hydrophila* and eugenol's effect on hematological and natural immune parameters in silver catfish (*Rhamdia quelen*). *In vitro*, eugenol showed weak activity against *A. hydrophila*, but *in vivo*, at a subinhibitory concentration (10 mg L^{-1}), it promoted survival in infected silver catfish. Eugenol (50 µg mL^{-1}) reduced the hemolytic activity of *A. hydrophila* supernatant *in vitro* in fish erythrocytes. Subjecting catfish to eugenol baths (5 and 10 mg L^{-1}) for five days did not alter the hematological and immunological parameters studied in this work. Based on these results, eugenol can be used to treat or prevent bacterial diseases in fish.

Keywords: *Aeromonas*. Innate immune system. Fish. Silver catfish. Bacteria. Hematology.

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INTRODUÇÃO

Plantas medicinais e os seus compostos ativos têm sido amplamente usados na medicina veterinária e humana desempenhando um papel significativo na aquicultura (DIREKBUSARAKOM, 2004). De maneira contrária, o uso de fármacos sintéticos para controlar doenças em animais vem sendo amplamente criticado por seus impactos negativos como, por exemplo, acumulação residual nos tecidos, desenvolvimento de resistência à droga pelos microrganismos e imunossupressão. Assim, na aquicultura tem se dado maior atenção ao uso de fitoquímicos como agentes profiláticos e terapêuticos (MAQSOOD *et al*., 2011).

Fitoquímicos, tais como polifenóis, alcalóides, quinonas, terpenóides, flavonóides e lectinas têm-se mostrado como alternativas muito eficazes aos antibióticos e outros compostos sintéticos. Estes produtos apresentam potencial antimicrobiano, podendo ser utilizados como agentes profiláticos e terapêuticos contra agentes patogênicos de peixes (CITARASU, 2010) ou como moduladores da resposta imune, conferindo ativação precoce sobre os mecanismos de defesa não específicos de peixes (HARIKRISHNAN *et al*., 2011).

Fitoquímicos podem ser usados não só como agentes profiláticos e terapêuticos contra doenças infecciosas, mas também como promotores de crescimento, agentes anti-estresse e estimuladores do apetite (CITARASU, 2010). Adicionalmente, estes compostos se apresentam como alternativas mais baratas no combate a doenças em peixes, além de apresentarem toxicidade ambiental reduzida em comparação aos pesticidas sintéticos ou fármacos convencionais (PARK *et al*., 2011).

Como alternativa aos fármacos sintéticos na gestão da saúde dos peixes e com papel significativo na aqüicultura, o objetivo deste estudo foi determinar a eficácia do fitoquímico eugenol (composto majoritário do óleo de cravo) contra o patógeno de peixes *Aeromonas hydrophila* e seu efeito sobre parâmetros hematológicos e imunológicos naturais em jundiás (*Rhamdia quelen*).

1. OBJETIVOS

1.1. Objetivo geral

Avaliar a atividade antibacteriana *in vitro* e *in vivo* do eugenol contra a bactéria *Aeromonas hydrophila*, bem como avaliar o potencial do eugenol sobre o reforço da imunidade não especifica de jundiás *Rhamdia quelen.*

1.2. Objetivos específicos

Determinar a Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM) do eugenol frente à bactéria *A. hydrophila;*

Verificar o potencial do eugenol em inibir a atividade hemolítica de *A. hydrophila* em eritrócitos de peixes;

Verificar a utilização do eugenol na água como agente terapêutico em jundiás experimentalmente infectados com *A. hydrophila;*

Verificar os efeitos da adição do eugenol na água sobre parâmetros hematológicos de jundiás;

Verificar os efeitos da adição do eugenol na água sobre parâmetros de imunidade não especifica de jundiás.

2. REVISÃO BIBLIOGRÁFICA

2.1. Eugenol

O eugenol (2-metoxi-4-prop-2-enilfenol) ($C_{10}H_{12}O_2$) é um fenilpropanóide, componente principal (70-90%) do óleo de cravo (KEENE *et al*., 1998), encontrado também em vários outros óleos essenciais de plantas. É uma molécula extremamente versátil incluída como ingrediente em cosméticos, perfumes, bebidas, alimentos e preparações dentárias (KAMATOU *et al*., 2012).

Nos últimos anos o eugenol tem atraído a atenção de muitos pesquisadores devido à suas propriedades antimicrobianas, antiparasitárias, analgésicas, antiinflamatórias e antioxidantes (KAMATOU *et al*., 2012). Na aquicultura o eugenol juntamente com o óleo de cravo, têm sido utilizados e recomendados como anestésicos alternativos para várias espécies de peixes (JAVAHERY *et al*., 2012).

2.2. Eugenol como antibacteriano

Durante séculos produtos naturais têm sido utilizados no o tratamento de infecções microbianas. Numerosas moléculas provenientes de óleos essenciais têm demonstrado a capacidade de inibir o crescimento de vários agentes patogênicos. Muitos trabalhos têm confirmado a atividade antibacteriana do eugenol contra vários patógenos como *Listeria monocytogenes, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Helicobacter pylori, Staphylococcus epidermidis, Streptococcus pneumoniae* e *Streptococcus pyogenes,* (FILGUEIRAS, VANETTI, 2006; KAMATOU *et al*., 2012; DEVI *et al*., 2012).

Muitos estudos também foram realizados avaliando a atividade do eugenol frente a diversos fatores de virulência bacterianos. O eugenol foi responsável por inibir em mais de 60% a adesão, e em 90% a formação de biofilmes de *P. aeruginosa* (EL-ABED *et al*., 2011). O eugenol inibiu a produção de listeriolisina por *Listeria monocytogenes* (FILGUEIRAS,

VANETTI, 2006) e em concentrações subinibitórias apresentou atividade inibitória do quorum sensing em *P. aeruginosa, Chromobacterium violaceum* e *E. coli* suprimindo também a produção de fatores de virulência, incluindo violaceína, elastase, piocianina, e formação de biofilmes nestas bactérias (ZHOU *et al*., 2013). Eugenol também suprimiu a produção de exotoxinas produzidas por *S. aureus*. As concentrações de 16, 32 e 64 µg mL^{-1} de eugenol inibiram a hemólise de eritrócitos de coelho causada por *S. aureus*, com uma redução de aproximadamente 50, 80 e 100%, respectivamente, quando comparados ao controle (QIU *et al*., 2010).

Dano à membrana é considerado o principal mecanismo pelo qual fenilpropanoides como eugenol exercem os seus efeitos antibacterianos. O mecanismo de ação do eugenol foi avaliado sobre membranas bacterianas de *L. monocytogenes*, *S. pyogenes*, *Proteus vulgaris* e *E. coli* observando-se alterações na composição da membrana. O estudo revelou que o eugenol provoca lise celular induzida por vazamento do conteúdo de proteínas e lipídios (OYEDEMI *et al*., 2009).

A terapia combinada é muitas vezes necessária no tratamento de infecções graves e no intuito de reduzir o risco de desenvolvimento de estirpes resistentes. A interação sinérgica de eugenol com ampicilina e gentamicina foi investigada através de estudos de tempo de morte celular. Após 60 min de tratamento, a taxa de morte em unidades de bactérias avaliadas por UFC/mL (unidades formadoras de colônias) foi maior quando o eugenol foi combinado com antibióticos, sugerindo uma interação sinérgica (MOON *et al*., 2011). Este efeito sinérgico pode ser explicado pelo fato do eugenol ser capaz de danificar a membrana das bactérias (OYEDEMI *et al*., 2009).

2.3. Eugenol como modulador da resposta imune

Eugenol às vezes é descrito como um estimulante com base em seu efeito sobre alguns parâmetros imunológicos em peixes, e em outras vezes, ele é descrito como um inibidor de certos eventos imunológicos. Anestesia causada por óleo de cravo em truta arco-íris (*Oncorhynchus mykiss*) não alterou a atividade hemolítica do complemento, mas 24 h após a anestesia reduziu a explosão respiratória produzida por leucócitos sanguíneos (KANANI *et al*., 2013). Linfopenia, neutrofilia e aumento da atividade da lisozima foram observados em truta arco-íris 24 h após anestesia com óleo de cravo (KANANI *et al*., 2011) .

HALDER *et al*. (2011) mostraram que o óleo de cravo diminui a resposta imune mediada por células, provavelmente por meio da redução da secreção de linfocinas e aumenta a imunidade humoral em ratos. Eugenol foi responsável pela inibição de IL-1 e IL-6 por macrófagos (RODRIGUES *et al*., 2009) exercendo efeitos imunomoduladores e antiinflamatórios através da inibição da ação de lipopolissacarídeos (LPS), envolvendo um possível mecanismo de supressão de NF-kB (BACHIEGA *et al*., 2012). Além disso, o eugenol previne o aumento de cortisol em *R. quelen* submetidos a estresse agudo (CUNHA et al., 2010), diminuindo os efeitos imunodepressivos provocados por este hormônio (BARCELLOS *et al*., 2004).

2.4. *Aeromonas hydrophila*

O gênero *Aeromonas* é essencialmente onipresente na biosfera microbiana, podendo ser isoladas a partir de praticamente todos os nichos ambientais em ecossistemas bacterianos existentes. Estes incluem habitats aquáticos, peixes, alimentos, animais domésticos, espécies de invertebrados, aves, insetos, carrapatos, e os solos naturais. Hoje, o gênero é considerado não só como um importante patógeno causador de doenças de peixes e outras espécies ectotérmicas, mas também como o agente etiológico responsável por uma variedade de complicações infecciosas, como em pessoas imunocompetentes e imunocomprometidas (JANDA, ABBOTT, 2010).

As *Aeromonas* estão classificadas dentro da família Aeromonadaceae. São bastonetes Gram-negativos, anaeróbios facultativos, não formadores de esporos, capazes de utilizar diferentes carboidratos no seu metabolismo (JOSEPH, CARNAHAN, 2000). O crescimento bacteriano ocorre preferencialmente entre 20 e 25 ºC, mas pode ocorrer também a 37ºC, em meios artificiais de cultura, sendo a espécie *Aeromonas hydrophila* um dos principais agentes patogênicos na aquicultura (AOKI, 1999).

A patogênese da *A. hydrophila* é multifatorial e depende da secreção de inúmeros fatores extracelulares que influenciam na virulência bacteriana (YU *et al*., 2005). Hemolisinas, amilases, quitinases, elastases, aerolisinas, nucleases, gelatinases, lecitinases, lipases e proteases são produtos extracelulares biologicamente ativos de espécies de *Aeromonas* que promovem a virulência (PEMBERTON *et al*., 1997),

Para *A. hydrophila* os sinais clínicos em peixes podem variar de lesões de pele, superficiais ou profundas, a quadros típicos de septicemia. As lesões de pele podem se apresentar como áreas de hemorragia e necrose de extensão variada, que podem progredir para úlceras que acometem geralmente o tecido muscular. Nos quadros de infecção sistêmica são observados exoftalmia, abdômen distendido contendo líquido serosanguinolento e presença de petéquias hemorrágicas nas vísceras (PAVANELLI *et al*., 2002).

3. MANUSCRITO

The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*)

Fernando Jonas Sutili, Luiz Carlos Kreutz, Mirela Noro, Leticia Trevisan Gressler, Berta Maria Heinzmann, Agueda Castagna de Vargas and Bernardo Baldisserotto

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The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*)

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ABSTRACT

The aim of this study was to evaluate the activity of eugenol against the fish pathogen *Aeromonas hydrophila* and eugenol's effect on hematological and natural immune parameters in silver catfish (*Rhamdia quelen*). *In vitro*, eugenol showed weak activity against *A. hydrophila*, but *in vivo*, at a subinhibitory concentration (10 mg L^{-1}), it promoted survival in infected silver catfish. Eugenol (50 µg mL^{-1}) reduced the hemolytic activity of *A. hydrophila* supernatant *in vitro* in fish erythrocytes. Subjecting catfish to eugenol baths (5 and 10 mg L^{-1}) for five days did not alter the hematological and immunological parameters studied in this work. Based on these results, eugenol can be used to treat or prevent bacterial diseases in fish.

Keywords: Aeromonas, Innate immune system, Fish, Silver catfish, Bacteria, Hematology

1. Introduction

Medicinal plants and their active compounds have been widely used in veterinary and human medicine and currently play a significant role in aquaculture (Direkbusarakom, 2004). These products present antimicrobial potential and they can be used as prophylactic and therapeutic agents against fish pathogens (Citarasu, 2010) or as immunostimulants that confer early activation on the non-specific defense mechanisms of fish (Harikrishnan et al., 2011). The therapeutic use of essential oils, extracts and isolated compounds from plants in the treatment of diseases or as immune response modulators in fish can be accomplished by baths (Harikrishnan et al., 2003) or by incorporation into feed (Pachanawan et al., 2008).

Eugenol is the major component (70-90%) of clove oil (Keene et al., 1998) and is found in several other plant essential oils. In aquaculture, eugenol and clove oil have been used and recommended as an alternative anesthetic for several fish species (Javahery et al., 2012). In addition, the low cost of obtaining eugenol, as well as its antimicrobial potential (Filgueiras and Vanetti, 2006; Qiu et al., 2010), further promote its use in aquaculture. Eugenol has attracted the attention of researchers because of its chemopreventive effects as well as its anti-inflammatory and anti-oxidant properties (Yogalakshmi et al., 2010; Leem et al., 2011).

Eugenol is an effective anesthetic for silver catfish (*Rhamdia quelen*) (Cunha et al., 2010). This is a native species largely grown in southern Brazil that responds easily to induced spawning and is well adapted for cultivation (Barcellos et al., 2004a). Given the above, the aim of this study was to determine the effectiveness of eugenol against the fish pathogen *Aeromonas hydrophila* and its effect on hematological and natural immune parameters in silver catfish.

2. Materials and methods

2.1. *Antibacterial assay*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of eugenol for *A. hydrophila* cells were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI, 2008), following the methodology described by Dal Pozzo et al. (2011). Three strains of *A. hydrophila*, which were isolated from infected fish obtained from local fish farms, and *A. hydrophila* ATCC 7966 were used. Eugenol diluted in ethanol and incorporated in a Mueller-Hinton broth (MHB) (Himedia Laboratories) at concentrations of 6400, 3200, 1600, 800, 400, 200, 100 μg mL^{-1} (in triplicate) was tested. The inoculum was prepared in saline solution from cultures grown in Mueller-Hinton agar (Himedia Laboratories) ($1x10^8$ colony forming units (CFU) mL^{-1}; 0.15 OD600 nm) (30 °C/24 h). Ten microliters ($1x10^5$ CFU) of inoculum was added to each well containing eugenol. The microplates were incubated at 30 °C for 24 h under aerobic conditions. The same procedure was performed on an ethanol control.

2.2. *Fish and water quality*

Silver catfish fingerlings (10.3 ± 0.40 g) were used to study survival, and silver catfish juveniles (70.0 ± 5.5 g) were used to determine immunological and hematological parameters. All fish were transferred from a local fish culture to the laboratory, where they were maintained in continuously aerated tanks with controlled water parameters (21.0 - 23.0 °C, pH: 7.2 - 7.8, dissolved oxygen levels: 5.0 - 7.0 mg L^{-1}). Fish were acclimated for seven days,

and dissolved oxygen and temperature were measured with a YSI oxygen meter (Model Y5512). The pH was measured using a DMPH-2 pH meter (Digimed). Total ammonia levels were determined according to Verdouw et al. (1978), and un-ionized ammonia (NH_3) levels were calculated according to Colt (2002).

2.3. *Experimental design*

First, an experiment was conducted to evaluate the survival of fish infected with *A. hydrophila* and exposed to eugenol. Silver catfish fingerlings were anesthetized (50 mg L^{-1} eugenol) (Cunha et al., 2010), and 100 μL of an *A. hydrophila* solution (ATCC 7966; $1.5x10^9$ CFU; 1.2 OD600 nm) was inoculated intramuscularly on the right latero-dorsal side of each fish. The *A. hydrophila* concentration was determined in a previous experiment (data not shown). The treatments (in triplicate, n = 8) were as follows: control (silver catfish inoculated with 100 μL sterile saline); negative control (infected fish left untreated); gentamycin 10 mg L^{-1} (Belem-Costa and Cyrino, 2006); and eugenol 5 and 10 mg L^{-1}. These eugenol concentrations were chosen because concentrations from 20 to 50 mg L^{-1} are an anesthetic for silver catfish (Cunha et al., 2010). The exposure was performed through baths (1 h daily for five days) (the same management was performed for control group), and *A. hydrophila*-related mortality was monitored in each group for five additional days for a total of 10 days. Eugenol was previously diluted 1:10 in 95% ethanol and added to the bath water. Fish were fed once a day to satiation with commercial feed, and uneaten food, other residues and feces were removed 30 min after feeding. Dead fish were also removed daily. The bacterial re-isolation was made from lesions and kidneys of dead fish. Morphological and biochemical characteristics were analyzed according to Quinn (1994) for bacterial identification and characterization.

In the second experiment, healthy silver catfish juveniles were exposed to the same concentrations of eugenol as in the first experiment (baths for 1 h daily for five days). The treatments (in triplicate, n = 5) were as follows: control (water only); ethanol (at the same concentration used for the dilution of the highest eugenol concentration); eugenol 5 and 10 mg L^{-1}. After this period, silver catfish from all treatments were anesthetized (as described in the first experiment) for collection of blood and phagocytic cells to evaluate hematological and immunological parameters.

The methodology of these experiments was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process nº 046/2010).

2.4. *Hemolysis assay*

The hemolysis assay was modified from methodology described by Qiu et al. (2010). A β-hemolytic strain was selected from the strains investigated during the MIC-test. This strain was cultured in MHB in subinhibitory eugenol concentrations (0-control, 25 and 50 µg mL^{-1}), at 30 ºC for 24 h, and prepared at a concentration of $2.1x10^{9}$ CFU mL^{-1} (1.75 OD600 nm). Bacterial cultures were centrifuged (5500 x g, 4 ºC, 1 min), the supernatant was collected, and the residual cells were removed using a 0.2 µm filter. Prior to the addition of 100 µL of defibrinated fish blood, a 200 µL volume of culture supernatant was diluted to a volume of 1000 µL through the addition of phosphate-buffered saline (PBS) in triplicate. After incubation for 90 min at 37 °C, the unlysed blood cells were pelleted by centrifugation (5500 x g, room temperature, 1 min). The hemolytic activity of the supernatant was detected by measuring its optical density at 540 nm. The percent hemolysis was calculated by comparison with the control.

2.5. *Blood collection*

Blood samples for the hematological studies were collected from the caudal vein using sterile heparinized syringes. To determine immunological parameters, blood was allowed to clot in ice-chilled water for 2 h and then centrifuged (600 x g, 4 ºC, 10 min), and serum aliquots were stored (-18 ºC) until use. Blood smears were prepared immediately after sampling, air-dried and submitted to Wright-Giemsa staining. Hematocrit, hemoglobin, and erythrocyte and leukocyte counts were determined for whole blood (Barcellos et al., 2004b; Noro and Wittwer, 2012).

2.6. *Phagocytosis assay*

To collect phagocytic cells, anesthetized fish were placed in ice-chilled water, and the coelomic cavity was injected with 3 mL of ice-cold sterile PBS (pH 7.4). After 1 min, the PBS-containing phagocytic cells were collected from the coelomic cavity and pelleted by centrifugation (600 x g), counted and suspended in RPMI media containing 1% fetal bovine serum (FBS; Cultilab, Brazil) to a final concentration of 10^6 mL^{-1}. The phagocytic activity of coelomic cells was determined for each fish using *Candida albicans* coupled to Fluorescein Isothiocyanate (FITC), according to the manufacturer's instructions (Sigma, St Louis, USA). For the phagocytic assay, 10 µL of FITC-labeled *C. albicans* (10^9 mL^{-1}) was thoroughly mixed with 200 µL of coelomic cells (10^6 mL^{-1}) in RPMI medium (1% FBS), and two aliquots of 100 µL each were layered over a circular (13-mm diameter) glass coverslip lamina placed inside the wells of a 24-well tissue culture plate and incubated at 22 ºC for 15 min. Following that, the wells were washed three times with PBS to remove both non-adherent cells and non-phagocytosed FITC-labeled *C. albicans*. The coverslip was removed, washed

once more in PBS, quenched with Evans blue, fixed with standard histological solution and mounted over a microscopic lamina. To determine the phagocytosis index (PI), 100 cells were counted using an epifluorescent microscope, and the number of cells containing engulfed FITC-labeled *C. albicans* was registered for each fish (Kreutz et al., 2011).

2.7. *Respiratory burst assay*

The production of superoxide anion (O^{2-}) was determined by the reduction of Nitroblue Tetrazoliun (NBT, Sigma-Aldrich) (Behera et al., 2010). Heparinized blood from each fish was mixed with NBT solution and incubated for 30 min at 25 ºC. Next, 50 µL of this mixture was added to 1 mL of N,N dietilmethyilformamide (Sigma-Aldrich) and centrifuged at 6000 x g for 5 min. The supernatant was collected, and its optical density was determined (OD540 nm).

2.8. *Peroxidase activity*

The peroxidase content of fish serum was performed following the protocol of Quade and Roth (1997) with some modifications. Exactly 10 µL of fish serum was diluted with 90 µL of Ca^{2+}, Mg^{2+} and phenol red-free Hank's solution in flat bottomed 96-well plates. Then, 35 µL of OPD (o-phenylenediamine dihidrochloride) in citrate (0.2 M) and phosphate buffer (0.1 M, pH 5.3) was added. The peroxidase reaction was stopped after 5 min by adding 35 µL of hydrochloric acid (HCl 3 M). Plates were read with a spectrophotometer at 450 nm.

2.9. *Serum agglutination activity*

The natural agglutination activity of fish serum was investigated using "U"-shaped 96-well plates. Serum was diluted two-fold in PBS (pH 7.4) that contained Ca^{2+} and Mg^{2+}, and an equal volume of formalin-killed *A. hydrophila* in suspension (0.1 OD550 nm) was added to each well. The plates were incubated for 2 h at 25 ºC and then overnight at 4 ºC; the serum titer was defined as the logarithm of the highest dilution of the serum that caused complete agglutination of the bacterial cell (Kreutz et al., 2011).

2.10. *Serum hemolytic activity*

The titer of the hemolytic activity of serum was determined using fresh serum (without freezing) from each fish. The serum was diluted in Hank's solution (containing 1 mM Mg^{2+}, 10 mM EGTA and 6.7 mM HEPES, pH 7.2) in "U"-shaped 96-well plates. Serum was diluted two-fold, and rabbit erythrocytes (6% in Hank's medium with 10 mM EGTA) were added to each well. The serum titer was determined to be the logarithm of the highest dilution of the serum that caused complete hemolysis of the erythrocytes.

2.11. *Statistical analysis*

The homogeneity of variances between groups was tested with the Levene test. Comparisons between different groups were made using one-way ANOVA and Tukey's test or Kruskal-Wallis test when appropriate (Statistica 7.0 Software). Fish survival was compared using Kaplan-Meier survival analysis with the Logrank test (SPSS 18 Software). The minimum significance level was set at $P \leq 0.05$.

3. Results and discussion

The mean water quality parameters were a temperature of 21.7 ± 0.3 °C, dissolved oxygen level of 6.6 ± 0.2 mg L^{-1}, pH of 7.5 ± 0.05, total ammonia of 1.35 ± 0.05 mg L^{-1} and non-ionized ammonia of 0.016 ± 0.0008 mg L^{-1}.

3.1. *Eugenol versus Aeromonas hydrophila*

The MIC (Minimum inhibitory concentration) values for eugenol against *A. hydrophila* ranged from 800 to 3200 μg mL^{-1}, and the MBC (Minimum bactericidal concentration) values ranged from 1600 to 3200 μg mL^{-1} (Fig. 1a). Aligiannis et al. (2001) proposed a classification for plant materials based on MIC results: a MIC up to 500 μg mL^{-1} is considered strong inhibition, moderate inhibition is a MIC between 600 and 1500 μg mL^{-1}, and a MIC above 1600 μg mL^{-1} is considered weak inhibition. According to this classification, eugenol showed moderate inhibition against only one strain of *A. hydrophila* and weak inhibition against the other strains. Plant constituents, such as terpenoids and phenylpropanoids, have a stronger antibacterial effect on Gram-positive compared to Gram-negative organisms (Dorman and Deans, 2000). Membrane damage is considered the major mechanism by which phenylpropanoids, such as eugenol, exert their antimicrobial effects (Devi et al., 2010). The resistance of Gram-negative bacteria may be due to the complexity of their double layer cell membrane in comparison with the single membranes of Gram-positive bacteria (Savage, 2001).

In the *in vivo* test to evaluate the effect of eugenol on fish infected with *A. hydrophila*, there was 100% survival in the non-infected group. In the infected fish groups treated with the antibiotic gentamycin or 5 and 10 mg L^{-1} eugenol, survival was 75, 37 and 66%, respectively.

The infected fish group that did not receive any treatment had 33% survival. Survival in the group treated with 10 mg L^{-1} eugenol did not differ statistically from that of the group treated with the antibiotic and was significantly higher than for the negative control group (untreated infected fish) (Fig. 1b). *Aeromonas hydrophila* was re-isolated from the lesions and kidneys of dead fish.

The pathogenesis of *A. hydrophila* is multifactorial and depends upon the secretion of numerous extracellular factors that influence virulence (Yu et al., 2005). Hemolysins, amylases, chitinases, elastases, aerolysins, nucleases, gelatinases, lecithinases, lipases and proteases are all biologically active extracellular products of *Aeromonas* species that promote virulence (Pemberton et al., 1997). An alternative strategy for the treatment of bacterial infections is to target bacterial virulence. This strategy provides promising opportunities to reduce pathogenicity and its consequences without placing immediate life-or-death pressure on the bacterium (Cegelski et al., 2008; Qiu et al., 2010).

According to Filgueiras and Vanetti (2006), eugenol inhibits the production of listeriolysin by *Listeria monocytogenes*. Eugenol exhibited quorum sensing inhibitory activity on *Pseudomonas aeruginosa*, *Chromobacterium violaceum* and *Escherichia coli* biosensors. At sub-inhibitory concentrations, the production of virulence factors was inhibited, including violacein, elastase, pyocyanin, and biofilm formation (Zhou et al., 2013). Eugenol also suppresses the production of exotoxins produced by *Staphylococcus aureus*. Concentrations of 16, 32 and 64 µg mL^{-1} of eugenol inhibited hemolysis of rabbit erythrocytes caused by *S. aureus* with a reduction of approximately 50, 80 and 100%, respectively, when compared to controls (Qiu et al., 2010).

Therefore, this study investigated the potential of eugenol to be an inhibitor of hemolysis caused by *A. hydrophila* in fish erythrocytes. The maximum anesthetic concentration of eugenol for silver catfish (50 µg mL^{-1}) (Cunha et al., 2010) and an

intermediate concentration were tested. *Aeromonas* cells cultured with 25 µg mL^{-1} of eugenol did not significantly reduce (10%) the hemolytic activity of *A. hydrophila* supernatant in fish erythrocytes when compared to controls. However, there was a significant reduction (35%) of hemolysis in fish erythrocytes at 50 µg mL^{-1} (Fig. 1c). The effect of eugenol in the *in vivo* test was most likely not due to its bactericidal potential, and it is possible that eugenol at subinhibitory concentrations can decrease *A. hydrophila* virulence.

3.2. *Eugenol effect on hematological and immunological parameters*

Medicinal plants contain many active compounds known to have immunomodulatory properties in fish through inhibitory or stimulatory effects on the immune system (Harikrishnan et al., 2011). This study evaluated the effects of eugenol on hematological and immunological parameters of silver catfish for a period longer than normally used for anesthesia and at lower concentrations. To the best of our knowledge, no data are available about the effects of eugenol on these parameters in fish for such a long exposure time. After five days of baths, no significant changes were observed in the hematological analysis. Only fish exposed to ethanol showed significantly higher hematocrit compared to controls (Table 1). In this study, the blood parameter values for all treatments were similar to basal values reported for the species (Tavares-Dias et al., 2002; Borges et al., 2004; Barcellos et al., 2004b)

Coelomic cells from silver catfish exposed to eugenol did not show a significant difference in phagocytic activity relative to controls (Fig. 2a). Similarly, eugenol baths did not change the production of superoxide anion (O^{2-}), a direct indicator of the respiratory burst from phagocytic cells (Fig. 2b) and serum myeloperoxidase activity (Fig. 2c). The natural bacterial agglutination titer, measured against formalin-inactivated pathogenic *A. hydrophila*,

and serum hemolytic activity were not significantly different compared to the control group (Fig. 2d and 3e).

Eugenol is sometimes described as a stimulant based on its effect on some immunological parameters in fish, and at other times, it is described as an inhibitor of certain immunological events. Anesthesia caused by clove oil in rainbow trout (*Oncorhynchus mykiss*) did not alter the hemolytic activity of the alternative complement, but it depressed respiratory burst activity for 24 h after anesthesia (Kanani et al., 2013). Lymphopenia, neutrophilia and increased lysozyme activity were observed in rainbow trout 24 h post-anesthesia with clove oil (Kanani et al., 2011).

Halder et al. (2011) showed that clove oil decreases the cell-mediated immune response, likely by reducing the secretion of lymphokines and augmenting humoral immunity in rats. Eugenol was responsible for the inhibition of IL-1 and IL-6 production by macrophages (Rodrigues et al., 2009) and exerted immunomodulatory/anti-inflammatory effects by inhibiting lipopolysaccharide (LPS) action through the possible mechanism of NF-kB suppression (Bachiega et al., 2012). The observed effect of eugenol on fish experimentally infected with *Aeromonas* was most likely due to anti-inflammatory and antioxidant effects (Rodrigues et al. 2009; Yogalakshmi et al., 2010; Leem et al., 2011; Halder et al., 2011; Bachiega et al., 2012) associated with its direct effects on bacteria. Additionally, in *R. quelen* submitted to acute stress, eugenol also prevents the rise of cortisol (Cunha et al., 2010). High plasma cortisol levels cause secondary immunosuppression (Barcellos et al., 2004a,b).

Eugenol in subinhibitory concentrations promoted the survival of silver catfish infected with *A. hydrophila* and significantly reduced *in vitro* hemolysis of fish erythrocytes caused by the same bacteria. These results, along with the non-alteration of immunological and hematological parameters evaluated by this study, strengthen the case to use eugenol not only as a sedative or anesthetic for fish but also as a therapy or in combination with other

drugs for treating or preventing disease in fish. With its broad range of pharmacological and biological properties, eugenol is a priority for aquaculture research.

Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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Figure legends

Fig. 1. A – Eugenol activity against *Aeromonas hydrophila*. (MIC) Minimum inhibitory concentration. (MBC) Minimum bactericidal concentration. B – Survival of silver catfish infected with *A. hydrophila*. (*) indicates a significant difference relative to control as determined by Kaplan-Meier survival analysis with Logrank test ($P \leq 0.05$). SS –fish inoculated with sterile saline; ATB –fish inoculated with *A. hydrophila* and treated with antibiotic (10 mg L^{-1} gentamicin); EU10 and EU5 – fish inoculated with *A. hydrophila* and treated with eugenol (5 or 10 mg L^{-1}); CA – control, fish inoculated with *A. hydrophila* and not treated. C – Hemolytic activity of *A. hydrophila* culture supernatant treated with subinhibitory concentrations of eugenol. (*) indicates a significant difference relative to control (0 µg mL^{-1}) as determined by one-way ANOVA and Tukey's test ($P \leq 0.05$).

Fig. 2. Natural immunological parameters of silver catfish exposed to eugenol through baths (1 h daily for five days). A – Phagocytic index for cells collected from the coelomic fish cavity; B – Respiratory burst activity; C – Serum myeloperoxidase activity; D – Serum agglutination activity; E – Serum hemolytic activity; WC – water control; EC – ethanol control; EU5 – eugenol 5 mg L^{-1}; EU10 – eugenol 10 mg L^{-1}. There was no significant difference between groups ($P>0.05$).

Table 1. Blood responses of silver catfish following five-day eugenol baths.

Blood parameters	Treatment group			
	Control	Ethanol	Eugenol (5 mg L^{-1})	Eugenol (10 mg L^{-1})
Hematocrit (%)	33.5 ± 1.0	38.6 ± 1.2*****	36.8 ± 1.7	34.7 ± 0.8
Hemoglobin (g dL^{-1})	8.7 ± 0.9	9.3 ± 1.5	8.3 ± 1.1	10.8 ± 1.5
Total protein (g dL^{-1})	4.6 ± 0.2	5.0 ± 0.2	4.8 ± 0.1	4.5 ± 0.1
Erythrocytes (10^6 μL^{-1})	1.78 ± 0.08	2.04 ± 0.13	1.99 ± 0.04	1.87 ± 0.05
Total leukocytes (10^3 μL^{-1})	30.1 ± 3.4	25.3 ± 2.5	30.5 ± 4.1	27.0 ± 3.6
Lymphocytes (10^3 μL^{-1})	13.7 ± 2.1	12.1 ± 1.9	13.8 ± 1.7	12.1 ± 1.8
Heterophils (10^3 μL^{-1})	16.0 ± 2.9	13.4 ± 3.7	16.4 ± 3.0	15.9 ± 3.0
Monocytes (10^3 μL^{-1})	0.5 ± 0.16	0.32 ± 0.13	0.39 ± 0.25	0.30 ± 0.15
Thrombocytes (10^3 μL^{-1})	21.3 ± 3.0	24.7 ± 2.4	23.0 ± 4.0	29.4 ± 6.3
MCV (fl)	195.6 ± 8.9	189.8 ± 12.2	181.5 ± 17.3	186.9 ± 6.9
MCHC (g dL^{-1})	24.6 ± 2.6	23.9 ± 3.8	23.0 ± 3.0	27.0 ± 4.5

(*) indicates a significant difference relative to control as determined by one-way ANOVA and Tukey's test ($P \leq 0.05$).

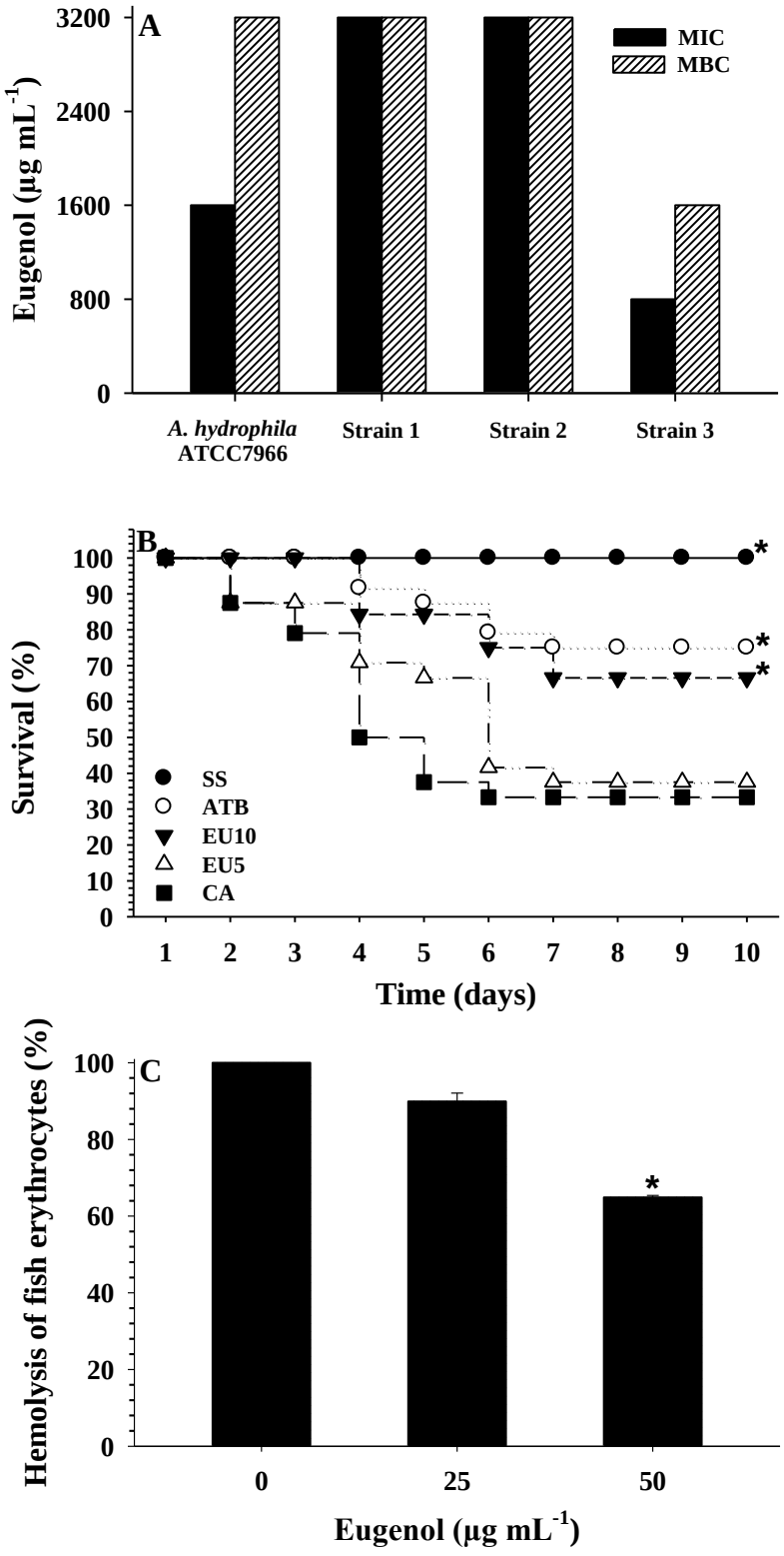
A
3200
2400
1600
800
0
Eugenol (µg mL-1)
MIC
MBC
A. hydrophila ATCC7966
Strain 1
Strain 2
Strain 3
B
100
90
80
70
60
50
40
30
20
10
0
Survival (%)
SS
ATB
EU10
EU5
CA
1
2
3
4
5
6
7
8
9
10
Time (days)
C
100
80
60
40
20
0
Hemolysis of fish erythrocytes (%)
0
25
50
Eugenol (µg mL-1)

Figura 1

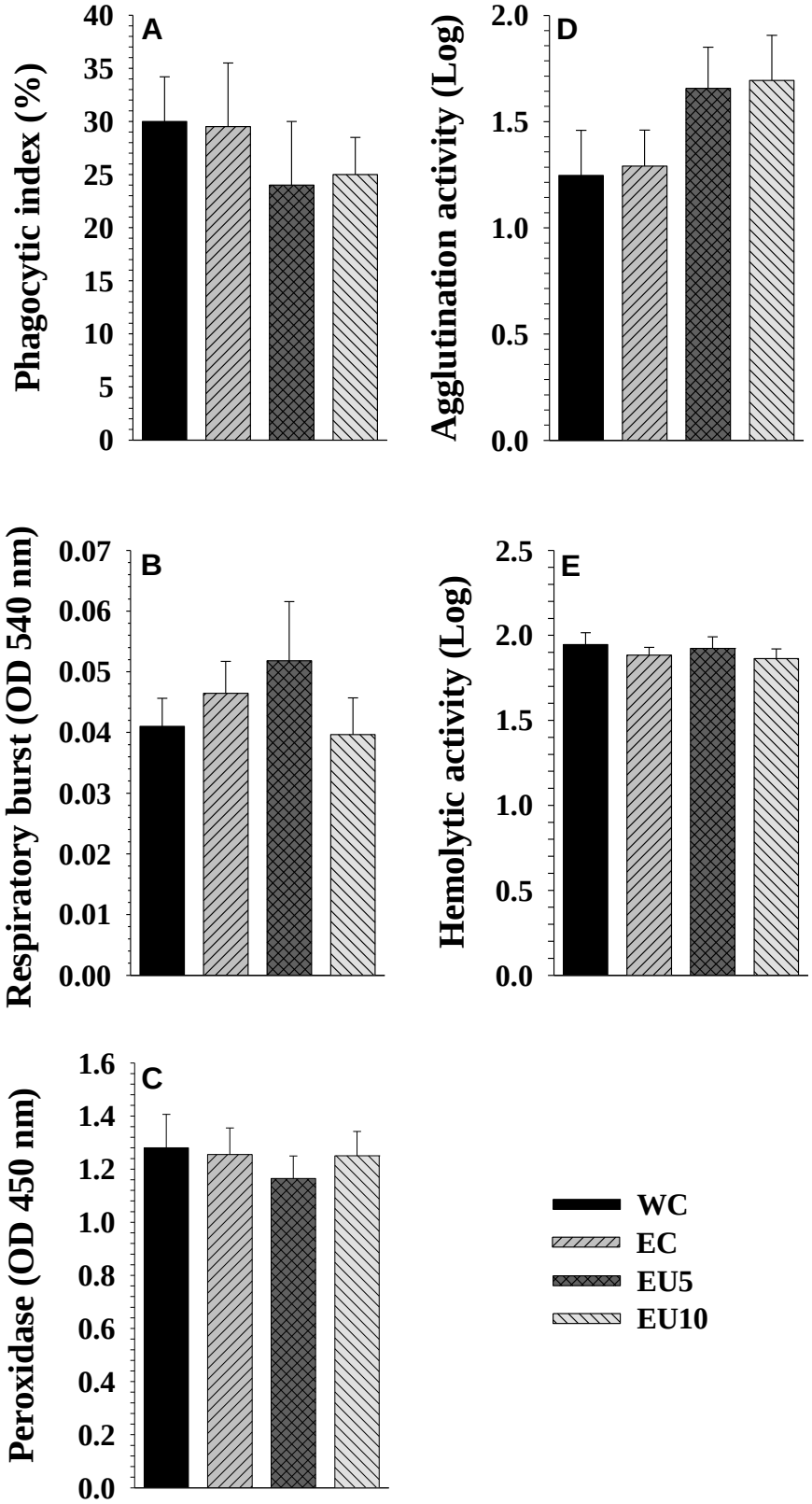
A
Phagocytic index (%)
40
35
30
25
20
15
10
5
0
D
Agglutination activity (Log)
2.0
1.5
1.0
0.5
0.0
B
Respiratory burst (OD 540 nm)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
E
Hemolytic activity (Log)
2.5
2.0
1.5
1.0
0.5
0.0
C
Peroxidase (OD 450 nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WC
EC
EU5
EU10

Figura 2

4. CONCLUSÃO

- *In vitro*, o eugenol, apresentou atividade antibacteriana fraca à moderada contra *A. hydrophila*, com valores de CIM (concentração inibitória mínima) variando entre 800-3200 µg/mL e valores de CBM (concentração bactericida mínima) entre 1600-3200 µg/mL.

- *In vivo*, o eugenol adicionado na água (banhos diários de 1h durante 5 dias) na concentração de 10 mg/L promoveu a sobrevivência de jundiás infectados experimentalmente com *A. hydrophila.*

- *In vitro*, em concentração subinibitória (50 µg/mL) o eugenol reduziu significativamente a hemólise causada pela *A. hydrophila* em eritrócitos de peixes.

- Após banhos diários de 1h durante cinco dias, jundiás expostos ao eugenol não apresentaram mudanças significativas nos parâmetros hematológicos e imunológicos analisados.

- Estes resultados, juntamente com a não alteração dos parâmetros imunológicos e hematológicos avaliados neste estudo fortalecem o uso do eugenol não só como um sedativo ou anestésico para peixes, mas também como terapia ou em combinação com outros medicamentos para o tratamento ou prevenção de doenças em peixes.

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ANEXO

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The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*)

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ABSTRACT

The aim of this study was to evaluate the activity of eugenol against the fish pathogen *Aeromonas hydrophila* and eugenol's effect on hematological and natural immune parameters in silver catfish (*Rhamdia quelen*). *In vitro*, eugenol showed weak activity against *A. hydrophila*, but *in vivo*, at a subinhibitory concentration (10 mg L^{-1}), it promoted survival in infected silver catfish. Eugenol (50 μg mL^{-1}) reduced the hemolytic activity of *A. hydrophila* supernatant *in vitro* in fish erythrocytes. Subjecting catfish to eugenol baths (5 and 10 mg L^{-1}) for five days did not alter the hematological and immunological parameters studied in this work. Based on these results, eugenol can be used to treat or prevent bacterial diseases in fish.

1. Introduction

Medicinal plants and their active compounds have been widely used in veterinary and human medicine and currently play a significant role in aquaculture (Direkbusarakom, 2004). These products present antimicrobial potential and they can be used as prophylactic and therapeutic agents against fish pathogens (Citarasu, 2010) or as immunostimulants that confer early activation on the non-specific defense mechanisms of fish (Harikrishnan et al., 2011). The therapeutic use of essential oils, extracts and isolated compounds from plants in the treatment of diseases or as immune response modulators in fish can be accomplished by baths (Harikrishnan et al., 2003) or by incorporation into feed (Pachanawan et al., 2008).

Eugenol is the major component (70–90%) of clove oil (Keene et al., 1998) and is found in several other plant essential oils. In aquaculture, eugenol and clove oil have been used and recommended as an alternative anesthetic for several fish species (Javahery et al., 2012). In addition, the low cost of obtaining eugenol, as well as its antimicrobial potential (Filgueiras and Vanetti, 2006; Qiu et al., 2010), further promote its use in aquaculture. Eugenol has attracted the attention of researchers because of its chemopreventive effects as well as its anti-inflammatory and anti-oxidant properties (Yogalakshmi et al., 2010; Leem et al., 2011).

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Eugenol is an effective anesthetic for silver catfish (*Rhamdia quelen*) (Cunha et al., 2010). This is a native species largely grown in southern Brazil that responds easily to induced spawning and is well adapted for cultivation (Barcellos et al., 2004a). Given the above, the aim of this study was to determine the effectiveness of eugenol against the fish pathogen *Aeromonas hydrophila* and its effect on hematological and natural immune parameters in silver catfish.

2. Materials and methods

2.1. Antibacterial assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of eugenol for *A. hydrophila* cells were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI, 2008), following the methodology described by Dal Pozzo et al. (2011). Three strains of *A. hydrophila*, which were isolated from infected fish obtained from local fish farms, and *A. hydrophila* ATCC 7966 were used. Eugenol diluted in ethanol and incorporated in a Mueller–Hinton broth (MHB) (Himedia Laboratories) at concentrations of 6400, 3200, 1600, 800, 400, 200, 100 $\mu g\,mL^{-1}$ (in triplicate) was tested. The inoculum was prepared in saline solution from cultures grown in Mueller–Hinton agar (Himedia Laboratories) (1×10^8 colony forming units (CFU) mL^{-1}; 0.15 OD 600 nm) (30 °C/24 h). Ten microliters (1×10^5 CFU) of inoculum was added to each well containing eugenol. The microplates were incubated at 30 °C for 24 h under aerobic conditions. The same procedure was performed on an ethanol control.

2.2. Fish and water quality

Silver catfish fingerlings (10.3 ± 0.40 g) were used to study survival, and silver catfish juveniles (70.0 ± 5.5 g) were used to determine immunological and hematological parameters. All fish were transferred from a local fish culture to the laboratory, where they were maintained in continuously aerated tanks with controlled water parameters (21.0–23.0 °C, pH: 7.2–7.8, dissolved oxygen levels: 5.0–7.0 $mg\,L^{-1}$). Fish were acclimated for seven days, and dissolved oxygen and temperature were measured with a YSI oxygen meter (Model Y5512). The pH was measured using a DMPH-2 pH meter (Digimed). Total ammonia levels were determined according to Verdouw et al. (1978), and un-ionized ammonia (NH_3) levels were calculated according to Colt (2002).

2.3. Experimental design

First, an experiment was conducted to evaluate the survival of fish infected with *A. hydrophila* and exposed to eugenol. Silver catfish fingerlings were anesthetized (50 $mg\,L^{-1}$ eugenol) (Cunha et al., 2010), and 100 μL of an *A. hydrophila* solution (ATCC 7966; 1.5×10^9 CFU; 1.2 OD 600 nm) was inoculated intramuscularly on the right latero-dorsal side of each fish. The *A. hydrophila* concentration was determined in a previous experiment (data not shown). The treatments (in triplicate, $n = 8$) were as follows: control (silver catfish inoculated with 100 μL sterile saline); negative control (infected fish left untreated); gentamycin 10 $mg\,L^{-1}$ (Belem-Costa and Cyrino, 2006); and eugenol 5 and 10 $mg\,L^{-1}$. These eugenol concentrations were chosen because concentrations from 20 to 50 $mg\,L^{-1}$ are an anesthetic for silver catfish (Cunha et al., 2010). The exposure was performed through baths (1 h daily for five days) (the same management was performed for control group), and *A. hydrophila*-related mortality was monitored in each group for 5 additional days for a total of 10 days. Eugenol was previously diluted 1:10 in 95% ethanol and added to the bath water. Fish were fed once a day to satiation with commercial feed, and uneaten food, other residues and feces were removed 30 min after feeding. Dead fish were also removed daily. The bacterial re-isolation was made from lesions and kidneys of dead fish. Morphological and biochemical characteristics were analyzed according to Quinn (1994) for bacterial identification and characterization.

In the second experiment, healthy silver catfish juveniles were exposed to the same concentrations of eugenol as in the first experiment (baths for 1 h daily for five days). The treatments (in triplicate, $n = 5$) were as follows: control (water only); ethanol (at the same concentration used for the dilution of the highest eugenol concentration); eugenol 5 and 10 $mg\,L^{-1}$. After this period, silver catfish from all treatments were anesthetized (as described in the first experiment) for collection of blood and phagocytic cells to evaluate hematological and immunological parameters.

The methodology of these experiments was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process no. 046/2010).

2.4. Hemolysis assay

The hemolysis assay was modified from methodology described by Qiu et al. (2010). A β-hemolytic strain was selected from the strains investigated during the MIC-test. This strain was cultured in MHB in subinhibitory eugenol concentrations (0-control, 25 and 50 $\mu g\,mL^{-1}$), at 30 °C for 24 h, and prepared at a concentration of 2.1×10^9 CFU mL^{-1} (1.75 OD 600 nm). Bacterial cultures were centrifuged ($5500 \times g$, 4 °C, 1 min), the supernatant was collected, and the residual cells were removed using a 0.2 μm filter. Prior to the addition of 100 μL of defibrinated fish blood, a 200 μL volume of culture supernatant was diluted to a volume of 1000 μL through the addition of phosphate-buffered saline (PBS) in triplicate. After incubation for 90 min at 37 °C, the unlysed blood cells were pelleted by centrifugation ($5500 \times g$, room temperature, 1 min). The hemolytic activity of the supernatant was detected by measuring its optical density at 540 nm. The percent hemolysis was calculated by comparison with the control.

2.5. Blood collection

Blood samples for the hematological studies were collected from the caudal vein using sterile heparinized syringes. To determine immunological parameters, blood was allowed to clot in ice-chilled water for 2 h and then centrifuged (600 × *g*, 4 °C, 10 min), and serum aliquots were stored (−18 °C) until use. Blood smears were prepared immediately after sampling, air-dried and submitted to Wright–Giemsa staining. Hematocrit, hemoglobin, and erythrocyte and leukocyte counts were determined for whole blood (Barcellos et al., 2004b; Noro and Wittwer, 2012).

2.6. Phagocytosis assay

To collect phagocytic cells, anesthetized fish were placed in ice-chilled water, and the coelomic cavity was injected with 3 mL of ice-cold sterile PBS (pH 7.4). After 1 min, the PBS-containing phagocytic cells were collected from the coelomic cavity and pelleted by centrifugation (600 × *g*), counted and suspended in RPMI media containing 1% fetal bovine serum (FBS; Cultilab, Brazil) to a final concentration of 10^6 mL^{-1}. The phagocytic activity of coelomic cells was determined for each fish using *Candida albicans* coupled to Fluorescein Isothiocyanate (FITC), according to the manufacturer's instructions (Sigma, St Louis, USA). For the phagocytic assay, 10 μL of FITC-labeled *C. albicans* (10^9 mL^{-1}) was thoroughly mixed with 200 μL of coelomic cells (10^6 mL^{-1}) in RPMI medium (1% FBS), and two aliquots of 100 μL each were layered over a circular (13-mm diameter) glass coverslip lamina placed inside the wells of a 24-well tissue culture plate and incubated at 22 °C for 15 min. Following that, the wells were washed three times with PBS to remove both non-adherent cells and non-phagocytosed FITC-labeled *C. albicans*. The coverslip was removed, washed once more in PBS, quenched with Evans blue, fixed with standard histological solution and mounted over a microscopic lamina. To determine the phagocytosis index (PI), 100 cells were counted using an epifluorescent microscope, and the number of cells containing engulfed FITC-labeled *C. albicans* was registered for each fish (Kreutz et al., 2011).

2.7. Respiratory burst assay

The production of superoxide anion (O^{2-}) was determined by the reduction of Nitroblue Tetrazoliun (NBT, Sigma-Aldrich) (Behera et al., 2010). Heparinized blood from each fish was mixed with NBT solution and incubated for 30 min at 25 °C. Next, 50 μL of this mixture was added to 1 mL of *N,N* dietilmethyilformamide (Sigma-Aldrich) and centrifuged at 6000 × *g* for 5 min. The supernatant was collected, and its optical density was determined (OD 540 nm).

2.8. Peroxidase activity

The peroxidase content of fish serum was performed following the protocol of Quade and Roth (1997) with some modifications. Exactly 10 μL of fish serum was diluted with 90 μL of Ca^{2+}, Mg^{2+} and phenol red-free Hank's solution in flat bottomed 96-well plates. Then, 35 μL of OPD (*o*-phenylenediamine dihidrochloride) in citrate (0.2 M) and phosphate buffer (0.1 M, pH 5.3) was added. The peroxidase reaction was stopped after 5 min by adding 35 μL of hydrochloric acid (HCl 3 M). Plates were read with a spectrophotometer at 450 nm.

2.9. Serum agglutination activity

The natural agglutination activity of fish serum was investigated using "U"-shaped 96-well plates. Serum was diluted two-fold in PBS (pH 7.4) that contained Ca^{2+} and Mg^{2+}, and an equal volume of formalin-killed *A. hydrophila* in suspension (0.1 OD 550 nm) was added to each well. The plates were incubated for 2 h at 25 °C and then overnight at 4 °C; the serum titer was defined as the logarithm of the highest dilution of the serum that caused complete agglutination of the bacterial cell (Kreutz et al., 2011).

2.10. Serum hemolytic activity

The titer of the hemolytic activity of serum was determined using fresh serum (without freezing) from each fish. The serum was diluted in Hank's solution (containing 1 mM Mg^{2+}, 10 mM EGTA and 6.7 mM HEPES, pH 7.2) in "U"-shaped 96-well plates. Serum was diluted two-fold, and rabbit erythrocytes (6% in Hank's medium with 10 mM EGTA) were added to each well. The serum titer was determined to be the logarithm of the highest dilution of the serum that caused complete hemolysis of the erythrocytes.

2.11. Statistical analysis

The homogeneity of variances between groups was tested with the Levene test. Comparisons between different groups were made using one-way ANOVA and Tukey's test or Kruskal–Wallis test when appropriate (Statistica 7.0 Software). Fish survival was compared using Kaplan–Meier survival analysis with the Logrank test (SPSS 18 Software). The minimum significance level was set at $P \leq 0.05$.

3. Results and discussion

The mean water quality parameters were a temperature of 21.7 ± 0.3 °C, dissolved oxygen level of 6.6 ± 0.2 mg L^{-1}, pH of 7.5 ± 0.05, total ammonia of 1.35 ± 0.05 mg L^{-1} and non-ionized ammonia of 0.016 ± 0.0008 mg L^{-1}.

3.1. Eugenol versus Aeromonas hydrophila

The MIC (Minimum inhibitory concentration) values for eugenol against *A. hydrophila* ranged from 800 to 3200 μg mL^{-1}, and the MBC (Minimum bactericidal concentration) values ranged from 1600 to 3200 μg mL^{-1} (Fig. 1a). Aligiannis et al. (2001) proposed a classification for plant materials based on MIC results: a MIC up to 500 μg mL^{-1} is considered strong inhibition, moderate inhibition is a MIC between 600 and 1500 μg mL^{-1}, and a MIC above 1600 μg mL^{-1} is considered weak inhibition. According to this classification, eugenol showed moderate inhibition against only one strain of *A. hydrophila*

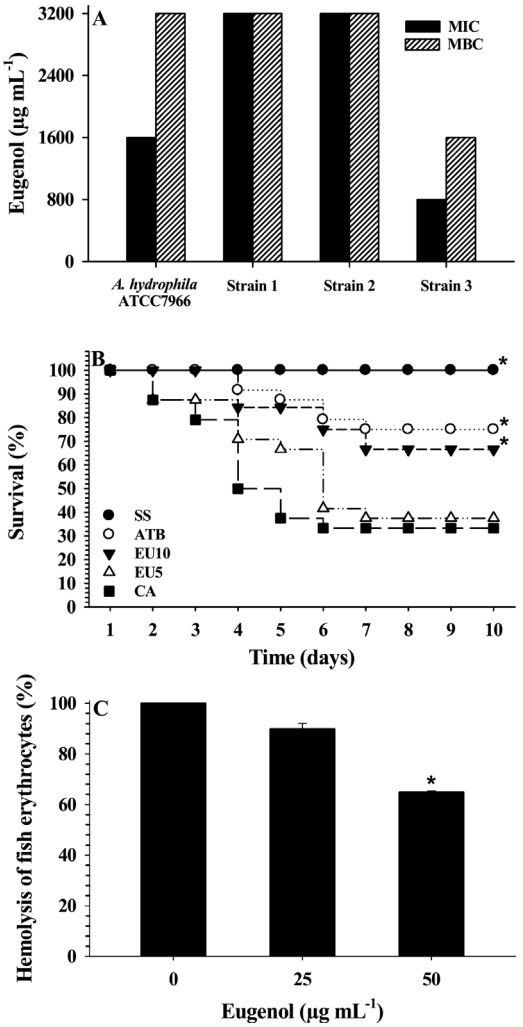

Fig. 1. (A) Eugenol activity against *Aeromonas hydrophila*. (MIC) Minimum inhibitory concentration. (MBC) Minimum bactericidal concentration. (B) Survival of silver catfish infected with *A. hydrophila*. (*) indicates a significant difference relative to control as determined by Kaplan–Meier survival analysis with Logrank test ($P \leq 0.05$). SS—fish inoculated with sterile saline; ATB—fish inoculated with *A. hydrophila* and treated with antibiotic (10 mg L^{-1} gentamicin); EU10 and EU5—fish inoculated with *A. hydrophila* and treated with eugenol (5 or 10 mg L^{-1}); CA—control, fish inoculated with *A. hydrophila* and not treated. C—hemolytic activity of *A. hydrophila* culture supernatant treated with subinhibitory concentrations of eugenol. (*) indicates a significant difference relative to control (0 μg mL^{-1}) as determined by one-way ANOVA and Tukey's test ($P \leq 0.05$).

and weak inhibition against the other strains. Plant constituents, such as terpenoids and phenylpropanoids, have a stronger antibacterial effect on Gram–positive compared to Gram–negative organisms (Dorman and Deans, 2000). Membrane damage is considered the major mechanism by which phenylpropanoids, such as eugenol, exert their antimicrobial effects (Devi et al., 2010). The resistance of Gram–negative bacteria may be due to the complexity of their double layer cell membrane in comparison with the single membranes of Gram–positive bacteria (Savage, 2001).

In the *in vivo* test to evaluate the effect of eugenol on fish infected with *A. hydrophila*, there was 100% survival in the non-infected group. In the infected fish groups treated with the antibiotic gentamycin or 5 and 10 mg L^{-1} eugenol, survival was 75%, 37% and 66%, respectively. The infected fish group that did not receive any treatment had 33% survival. Survival in the group treated with 10 mg L^{-1} eugenol did not differ statistically from that of the group treated with the antibiotic and was significantly higher than for the negative control group (untreated infected fish) (Fig. 1b). *A. hydrophila* was re-isolated from the lesions and kidneys of dead fish.

The pathogenesis of *A. hydrophila* is multifactorial and depends upon the secretion of numerous extracellular factors that influence virulence (Yu et al., 2005). Hemolysins, amylases, chitinases, elastases, aerolysins, nucleases, gelatinases, lecithinases, lipases and proteases are all biologically active extracellular products of *Aeromonas* species that promote virulence (Pemberton et al., 1997). An alternative strategy for the treatment of bacterial infections is to target bacterial virulence. This strategy provides promising opportunities to reduce pathogenicity and its consequences without placing immediate life-or-death pressure on the bacterium (Cegelski et al., 2008; Qiu et al., 2010).

According to Filgueiras and Vanetti (2006), eugenol inhibits the production of listeriolysin by *Listeria monocytogenes*. Eugenol exhibited quorum sensing inhibitory activity on *Pseudomonas aeruginosa*, *Chromobacterium violaceum* and *Escherichia coli* biosensors. At sub-inhibitory concentrations, the production of virulence factors was inhibited, including violacein, elastase, pyocyanin, and biofilm formation (Zhou et al., 2013). Eugenol also suppresses the production of exotoxins produced by *Staphylococcus aureus*. Concentrations of 16, 32 and 64 μg mL^{-1} of eugenol inhibited hemolysis of rabbit erythrocytes caused by *S. aureus* with a reduction of approximately 50%, 80% and 100%, respectively, when compared to controls (Qiu et al., 2010).

Therefore, this study investigated the potential of eugenol to be an inhibitor of hemolysis caused by *A. hydrophila* in fish erythrocytes. The maximum anesthetic concentration of eugenol for silver catfish (50 μg mL^{-1}) (Cunha et al., 2010) and an intermediate concentration were tested. *Aeromonas* cells cultured with 25 μg mL^{-1} of eugenol did not significantly reduce (10%) the hemolytic activity of *A. hydrophila* supernatant in fish erythrocytes when compared to controls. However, there was a significant reduction (35%) of hemolysis in fish erythrocytes at 50 μg mL^{-1} (Fig. 1c). The effect of eugenol in the *in vivo* test was most likely not due to its bactericidal potential, and it is possible that eugenol at subinhibitory concentrations can decrease *A. hydrophila* virulence.

3.2. Eugenol effect on hematological and immunological parameters

Medicinal plants contain many active compounds known to have immunomodulatory properties in fish through inhibitory or stimulatory effects on the immune system (Harikrishnan et al., 2011). This study evaluated

Table 1
Blood responses of silver catfish following five-day eugenol baths.

Blood parameters	Treatment group			
	Control	Ethanol	Eugenol (5 mg L^{-1})	Eugenol (10 mg L^{-1})
Hematocrit (%)	33.5 ± 1.0	38.6 ± 1.2*	36.8 ± 1.7	34.7 ± 0.8
Hemoglobin (g dL^{-1})	8.7 ± 0.9	9.3 ± 1.5	8.3 ± 1.1	10.8 ± 1.5
Total protein (g dL^{-1})	4.6 ± 0.2	5.0 ± 0.2	4.8 ± 0.1	4.5 ± 0.1
Erythrocytes (10^6 μL^{-1})	1.78 ± 0.08	2.04 ± 0.13	1.99 ± 0.04	1.87 ± 0.05
Total leukocytes (10^3 μL^{-1})	30.1 ± 3.4	25.3 ± 2.5	30.5 ± 4.1	27.0 ± 3.6
Lymphocytes (10^3 μL^{-1})	13.7 ± 2.1	12.1 ± 1.9	13.8 ± 1.7	12.1 ± 1.8
Heterophils (10^3 μL^{-1})	16.0 ± 2.9	13.4 ± 3.7	16.4 ± 3.0	15.9 ± 3.0
Monocytes (10^3 μL^{-1})	0.5 ± 0.16	0.32 ± 0.13	0.39 ± 0.25	0.30 ± 0.15
Thrombocytes (10^3 μL^{-1})	21.3 ± 3.0	24.7 ± 2.4	23.0 ± 4.0	29.4 ± 6.3
MCV (fl)	195.6 ± 8.9	189.8 ± 12.2	181.5 ± 17.3	186.9 ± 6.9
MCHC (g dL^{-1})	24.6 ± 2.6	23.9 ± 3.8	23.0 ± 3.0	27.0 ± 4.5

* Indicates a significant difference relative to control as determined by one-way ANOVA and Tukey's test ($P \leq 0.05$).

the effects of eugenol on hematological and immunological parameters of silver catfish for a period longer than normally used for anesthesia and at lower concentrations. To the best of our knowledge, no data are available about the effects of eugenol on these parameters in fish for such a long exposure time. After five days of baths, no significant changes were observed in the hematological analysis. Only fish exposed to ethanol showed significantly higher hematocrit compared to controls (Table 1). In this study, the blood parameter values for all treatments were similar to basal values reported for the species (Tavares-Dias et al., 2002; Borges et al., 2004; Barcellos et al., 2004b).

Coelomic cells from silver catfish exposed to eugenol did not show a significant difference in phagocytic activity relative to controls (Fig. 2a). Similarly, eugenol baths did not change the production of superoxide anion (O^{2-}), a direct indicator of the respiratory burst from phagocytic cells (Fig. 2b) and serum myeloperoxidase activity (Fig. 2c). The natural bacterial agglutination titer, measured against formalin-inactivated pathogenic *A. hydrophila*, and serum hemolytic activity were not significantly different compared to the control group (Fig. 2d and e).

Eugenol is sometimes described as a stimulant based on its effect on some immunological parameters in fish, and at other times, it is described as an inhibitor of certain immunological events. Anesthesia caused by clove oil in rainbow trout (*Oncorhynchus mykiss*) did not alter the hemolytic activity of the alternative complement, but it depressed respiratory burst activity for 24 h after anesthesia (Kanani et al., 2013). Lymphopenia, neutrophilia and increased lysozyme activity were observed in rainbow trout 24 h post-anesthesia with clove oil (Kanani et al., 2011).

Halder et al. (2011) showed that clove oil decreases the cell-mediated immune response, likely by reducing the secretion of lymphokines and augmenting humoral immunity in rats. Eugenol was responsible for the inhibition of IL-1 and IL-6 production by macrophages (Rodrigues et al., 2009) and exerted immunomodulatory/anti-inflammatory effects by inhibiting lipopolysaccharide (LPS) action through the possible mechanism of NF-kB suppression (Bachiega et al., 2012). The observed effect of eugenol on fish experimentally infected with *Aeromonas* was most likely due to anti-inflammatory and antioxidant effects (Rodrigues et al., 2009; Yogalakshmi et al., 2010; Leem et al.,

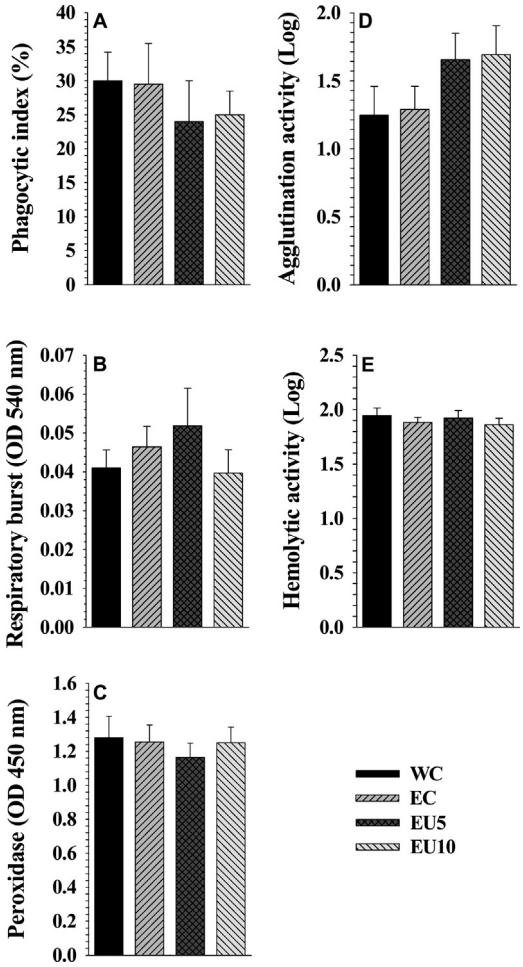

Fig. 2. Natural immunological parameters of silver catfish exposed to eugenol through baths (1 h daily for five days). (A) Phagocytic index for cells collected from the coelomic fish cavity; (B) Respiratory burst activity; (C) Serum myeloperoxidase activity; (D) Serum agglutination activity; (E) Serum hemolytic activity; WC—water control; EC—ethanol control; EU5—eugenol 5 mg L^{-1}; EU10—eugenol 10 mg L^{-1}. There was no significant difference between groups ($P > 0.05$).

2011; Halder et al., 2011; Bachiega et al., 2012) associated with its direct effects on bacteria. Additionally, in *R. quelen* submitted to acute stress, eugenol also prevents the rise of cortisol (Cunha et al., 2010). High plasma cortisol levels cause secondary immunosuppression (Barcellos et al., 2004a,b).

Eugenol in subinhibitory concentrations promoted the survival of silver catfish infected with *A. hydrophila* and significantly reduced *in vitro* hemolysis of fish erythrocytes caused by the same bacteria. These results, along with the non-alteration of immunological and hematological parameters evaluated by this study, strengthen the case to use eugenol not only as a sedative or anesthetic for fish but also as a therapy or in combination with other drugs for treating or preventing disease in fish. With its broad range of pharmacological and biological properties, eugenol is a priority for aquaculture research.

Conflict of interest statement

None declared.

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