Aquaculture 495 (2018) 452–457

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Hematology and biochemistry of *Colossoma macropomum* co-infected with *Aeromonas hydrophila* and monogenean *Anacanthorus spathulatus* after treatment with seed extract of *Bixa orellana*

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ARTICLE INFO

Keywords:
Fish farming
Phytotherapic
Urucum
Chemical composition
Monogenean
Aeromoniosis

ABSTRACT

This study evaluated the use *in vitro* and *in vivo* of *Bixa orellana* seed extracts on the hematological and biochemical parameters of *Colossoma macropomum* co-infected with bacterium and monogenean, *Anacanthorus spathulatus*. The extract presented antimicrobial and antiparasitic activity *in vitro* against the pathogens. Fish supported the toxicity test and *in vivo* assay used 180 fish distributed in six treatments in triplicate: non-parasitized fish non-injected with *A. hydrophila*; non-parasitized fish non-injected exposed to acetone 0.2%; parasitized fish injected non-treated with the extract; parasitized fish injected treated with 125 μg mL^{-1} of extract in 2 h bath for two consecutive days; parasitized fish injected treated with 250 μg mL^{-1} of extract in 2 h bath for two consecutive days; parasitized fish injected treated with 125 μg mL^{-1} of extract for 12 h. After last bath, the fish were examined. Acetonic extract showed minimal inhibitory concentration of 125 μg mL^{-1} against *A. hydrophila* in the *in vitro* test and 100% efficacy against monogenean with therapeutic baths in relation to control non-treated in the *in vivo* test. *Aeromonas hydrophila* infection did not cause mortality. The gross pathology analysis showed ascites and hemorrhagic liver, kidney and spleen. Increased hemoglobin concentration, mean corpuscular hemoglobin concentration and total number of erythrocytes was followed by a decrease in hematocrit percentage in non-treated fish compared to basal group. Lymphocytes from fish in the control group were higher than in the other groups. Glucose was higher in treated fish than that found in those of basal and control. The results demonstrated that *B. orellana* bath was an effective alternative to treat fish diseases.

1. Introduction

Monogenean helminths are common in fish grown in intensive systems, where high storage densities, low water renewal and high organic matter content contribute to the parasite reproduction and development (Pavanelli et al., 2008). Monogeneans have a direct life cycle and reproduce rapidly under suitable conditions. Typically, primary agents are not causal in fish mortality, except in the case of high infestations (Jerônimo et al., 2014). They present a sclerotic structure, called haptor used for attachment on the host, increasing the pathogenicity, causing tissue injury mainly in the gill epithelium, with consequent increase of the mucus production, skin hemorrhage, anorexia and fish death (Pavanelli et al., 2008). Furthermore, the lesions produced serve as a portal of entry to systemic bacterial infections (Xu et al., 2007). Among the bacterioses frequent in fish farm, the Gram-negative bacterium *Aeromonas hydrophila* stands out, which can generate severe sepsis and cause great losses in intensive breeding systems (Silva et al., 2012), therefore its control has become a challenge in fish farm.

For treatment of infectious diseases in fish, several chemotherapeutic agents such as formalin, potassium permanganate, hydrogen peroxide, praziquantel, mebendazole, and other antibiotics (Pavanelli et al., 2008). However, inappropriate use has resulted in negative effects such as drug resistance, immunosuppression, environmental

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https://doi.org/10.1016/j.aquaculture.2018.06.026
Received 25 March 2018; Received in revised form 7 June 2018; Accepted 10 June 2018
Available online 12 June 2018
0044-8486/

contamination and human health risks (Vivekanandhan et al., 2002; Miranda and Rojas, 2007). This fact has stimulated research on new sources of drugs, and those of vegetable origin have presented satisfactory results in the prevention and treatment of infectious diseases in fish, including those caused by parasites and bacteria (Reverter et al., 2014; Brum et al., 2017; Andrade et al., 2016).

Bixa orellana, commonly known as "urucum" in Brazil, belongs to Bixaceae family, is original from the tropical forests of Central and South America (Lorenzi and Matos, 2002), has been used in food industry by its capacity in to preserve the quality of food (Braga et al., 2007) and in pharmacy industry. Their seeds contain flavonoids and alcaloids (Fleischer et al., 2003) rich in carotenoids and the most common is the bixin comprising approximately 80% of total carotenoid contents (Preston and Rickard, 1980). Furthermore, extracts of urucum seeds present biological activities, such as antibacterial, antiparasitic and antioxidant (Di Mascio et al., 1990; Andrade et al., 2016; Medina-Flores et al., 2016). Therefore, this study evaluated the *in vitro* antibacterial activity of the acetone extract of urucum seeds against *A. hydrophila* and *in vivo* on hematological and biochemical parameters of *Colossoma macropomum* naturally parasitized by monogenean *Anacanthorus spathulatus* and experimentally infected by *A. hydrophila*.

2. Materials and methods

2.1. Seed extracts preparation

The seeds of *B. orellana* were donated by the company *Chr. Hansen,* Valinhos, Brazil. Three different extracts were prepared using acetone 100%, [(water/ethanol 1:1)/acetone 1:1] and ethanol 70%, in the proportion of 25 g of seeds by 100 mL solvent. For extraction, the seeds were exposed to ultrasound bath (Unique, model USC 3300) for 20 min to remove the solvent with the aid of rotary evaporator (Fisatom, model 801) and preserved in dark flasks in −20 °C until the use. The extracts were named as: acetonic, hydro alcoholic/acetonic and hydro alcoholic 70%.

2.2. Extract composition and bixin quantification

The samples were weighed and 10 mg of each extract was diluted in 600 μL de DMSO-d_6 to be analyzed by Nuclear Magnetic Resonance (NMR) of 300 MHz (Bruker - model Fourier 300). Each spectrum was examined and compared with NMR of the isolated substances to determine the presence of bixin in the samples.

The seed extracts (10 mg) obtained from acetone 100%, [(water/ethanol 1:1)/acetone 1:1] and ethanol 70% were diluted in chloroform and the solutions obtained had their volume adjusted to 100 mL with the same solvent. An aliquot of 10 mL of each solution was withdrawn and diluted to 50 mL of $CHCl_3$, to obtain three solutions of 20 mg L^{-1}. These solutions were analyzed in a spectrophotometer UV–Vis. After that, the concentration of bixin was determined using the expression $A = acb$, where $\boldsymbol{A}$ represents the absorbance of chloroform solution extraction read in the spectrophotometer, $\boldsymbol{c}$ represents the bixin concentration in the solution (g L^{-1}), $\boldsymbol{b}$ represents the optic way (1 cm) and $\boldsymbol{a}$ represents the absorption coefficient of bixin in $CHCl_3$ (2826) at λ_{max} 470 nm in accordance with Tocchini and Mercadante (2001). The bixin amount was determined considering the extract mass used in each dilution. The ratio of bixin/geranylgeraniol was determined as shown in the equation, ratio of bixin/geranylgeraniol = Σ Hβ/H^{-1}.

2.3. Antibacterial activity

The strain *A. hydrophila* (125 FG) was isolated from a diseased fish at the Aquaculture Center of the Paulista State University (CAUNESP), Jaboticabal, SP and was preserved in glycerol 20% at −80 °C in the Laboratory of Bioprospection and Biotechnology (INPA-National Institute of Amazon Research).

Antibacterial activity test was performed using three extracts: acetonic, hydroalcoholic/acetonic and hydroalcoholic 70% to determine the most active to posterior *in vivo* test. The determination of Minimum Inhibitory Concentration (MIC) was realized in Mueller-Hinton (HIMEDIA) broth by the micro dilution method in 96-wells microplates (Eloff, 1998). Firstly, the extracts were solubilized in culture medium added with 5% DMSO to posterior successive dilutions of the extracts were made (1000; 500; 250; 125; 62,5; 31,25; 15,65; 7,81 e 0 μg.mL^{-1}), in triplicates. After added 95 μL of each extract, 5 μL of bacterial inoculum was added and calculated by McFarland 0.5 (1.5×10^8). The microplate was incubated at 30 °C for 24 h, and after this period, it was revealed using 40 μL chloride of 2, 3, 5-triphenyltetrazole (20 mg.mL^{-1}). Bacterial growth was revealed in red and the wells that did not show bacterial growth remained colorless and the MIC was considered the lowest extract concentration with no bacterial growth. For negative control, a solution of Mueller-Hinton broth + 5% DMSO + bacterial inoculum was used to certify the antibacterial activity of the extract. For positive control the antibiotic oxytetracycline was used in serial dilutions (125, 62.5, 31.25, 15.6 and 7.8 μg·mL^{-1}).

The microplate used for MIC was also analyzed for Minimum Bactericidal Concentration (MBC). An aliquot of 100 μL of each concentration was inoculated in plates containing Mueller-Hinton agar and incubated at 30 °C for 24 h. The MBC was considered the lowest extract concentration where did not show cellular growth on the agar (considered 99.9% of bacterial death).

2.4. Fish and parasites

Juveniles of tambaqui were obtained from a commercial fish farm located in Manaus, AM, Brazil. At the sampling site, 10 fish samples were anesthetized with clove oil (20 mg L^{-1}) (Inoue et al., 2011) and euthanized to confirm the presence of monogenean parasites using a dissection microscope (Zeiss Stemi 2000-C, Carl Zeiss, Oberkochen, Germany). The fish were kept for 15 days in 500 L tanks, in static systems before the assays, with constant aeration to improve the infestation level. The fish were fed to apparent satiation twice a day (9 a.m. and 4 p.m.) with commercial diet (NUTRIPISCIS 36% crude protein). Fish management was according to the ethics of animal use by the Brazilian Society of Laboratory Animal Science (COBEA).

2.5. In vivo assay

2.5.1. Experimental infection

Aeromonas hydrophila was cultured in agar Mueller-Hinton 24 h before experimental infection. The inoculum was prepared with saline solution 0.85% at 3×10^7 CFU mL^{-1} measured by the McFarland scale for posterior intraperitoneal injection with 100 μL. Clinical signs were observed and the mortality verified for 10 days.

2.5.2. Therapeutic bath

Acetonic extract was chosen for therapeutic baths as presenting the highest antibacterial activity (Table 1) and has been shown previously efficacious as antiparasitic against monogeneans and tambaqui was tolerant to tested concentrations (Andrade et al., 2016).

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the seeds of *Bixa orellana* extracts against *Aeromonas hydrophila*.

Extracts	MIC (μg·mL^{-1})	MBC (μg·mL^{-1})
Hydroalcoholic 70%	250	> 1000
Hydroalcoholic/acetonic	250	> 1000
Acetonic	125	> 1000
Oxytetracycline[a]	31.25	> 1000

[a] Positive control.