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Protection of ornamental gold fish *Carassius auratus* against *Aeromonas hydrophila* by treating *Ixora coccinea* active principles

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ABSTRACT

Herbals such as *Ixora coccinea*, *Daemia extensa* and *Tridax procumbens* were selected to screen *in vitro* antibacterial and immunostimulant activity against the freshwater fish pathogen *Aeromonas hydrophila* using different organic polar and non-polar solvents. Initial screening results revealed that, ethyl acetate extracts and its purified fraction of *I. coccinea* was able to suppress the *A. hydrophila* strains at more than 15 mm of zone of inhibition and positive immunostimulant activity. The purified active fraction, which eluted from H40: EA60 mobile phase was structurally characterized by GC–MS analysis. Two compounds such as Diethyl Phthalate (1,2-Benzene dicarboxylic acid, monobutyl ester) and Dibutyl Phthalate were characterized using NIST database search. In order to study the *in vivo* immunostimulant influence of the compounds, the crude extracts (ICE) and purified fractions (ICF) were incorporated to the artificial diets at the concentration of 400 mg kg^{-1} and fed to the ornamental gold fish *Carassius auratus* for 30 days. After termination of feeding experiment, they were challenged with highly virulent *A. hydrophila* AHV-1 which was isolated from infected gold fish and studied the survival, specific bacterial load reduction, serum biochemistry, haematology, immunology and histological parameters. The control diet fed fishes succumbed to death within five days at 100% mortality whereas ICE and ICF fed groups survived 60 and 80% respectively after 10 days. The diets also helped to decrease the Aeromonas load after challenge and significantly ($P \leq 0.01$) improved the serum albumin, globulin and protein. The diets also helped to increase the RBC and haemoglobin level significantly ($P \leq 0.05$) from the control group. Surprisingly the immunological parameters like phagocytic activity, serum bactericidal activity and lysozyme activity were significantly increased ($P \leq 0.001$) in the experimental diets. Macrophages and erythrocytes were abundantly expressed in the treated groups and the present work concluded that, the Phthalate derivatives from *I. coccinea* helps to stimulate the immune system against *A. hydrophila* challenge in *C. auratus*.

1. Introduction

Ornamental fishes are nowadays rapidly gaining importance because of their aesthetic value and also due to their immense commercial value in the export trade world over [1]. The art of keeping aquarium is ancient and it started around 800 B.C. in China with the gold fish *Carassius auratus*, which is in demand and a popular ornamental fish throughout the world even today. These fishes are often called as “living jewels” due to their varied colour, shape and behaviour [2]. In the ornamental aquaculture sector, ornamental fish breeding, culture and trade provide excellent opportunities as a non-food fishery activity for employment and income generation. It is environment friendly, socially acceptable and involves low investment for adopting as a small scale enterprise with high return. The attractive colouration and quiet disposition of ornamental fish provide a source of joy and peace for people irrespective of age group [3].

Nowadays, ornamental aquaculture industry is facing bacterial disease outbreaks resulting high mortality and loss of economy. Gold fish *C. auratus* has high susceptibility to aeromonads and are commonly valuable for experimental animals [4]. *Aeromonas hydrophila*, gram negative facultative anaerobic short bacillus, causes red fin disease, haemorrhagic septicaemia, motile aeromonad septicaemia and other infections in *C. auratus* [5]. Potential virulence factors of *A. hydrophila*, which contribute to their pathogenicity, include the production of endotoxins, extracellular

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enterotoxins, haemolysin, cytotoxins and protease, the ability to adhere the cells, and the possession of certain surface proteins [6]. Outbreaks of motile aeromonad septicaemia usually occur whether fish are immune compromised due to unpleasant environment or predisposing factors leading to stresses such as temperature, overcrowding, organic pollution and hypoxia.

The current bacterial disease treatment protocols are rather difficult, non effective, un-safety, costly and create so many environmental hazards such as residual effects, biomagnifications and resistant strain development etc [7]. Humoural and cellular immune responses of common carp have been shown to be suppressed during treatment with oxytetracycline, and an increased number of granulocytes were observed in the spleen of treated fish [8]. Increasingly, antibiotic resistance of bacterial pathogens is reported from all areas of aquaculture, ranging from warm water to cold water. The use of antibiotics in the fish/shrimp hatcheries has led to biomagnifications that in turn leads to rejection of the total consignment during export [9]. Marine product development export authority (MPEDA) of India has also banned more than 20 antibiotics due to their bad effects. In order to achieve optimal fish production, better prophylactic, diagnostic and therapeutic measures are warranted during fish farming operations. To develop the alternative practices for disease management in aquaculture, attention should be diverted to find novel drugs, especially from plant sources. Plant-derived compounds act as a better antibacterial, antiviral, immunostimulant and antistress effect in fish and shellfish aquaculture [7,10–12].

Plants are the storehouses and rich sources of safe and cheap chemicals. Many plant-derived compounds have been found to have non-specific immunostimulating properties in animals, of which more than a dozen have been evaluated in fin and shellfishes [13,14]. Sivaram et al. [15] successfully controlled the Vibrio pathogen, and improved the immune system of grouper larviculture using herbal methanolic extracts. These findings suggest that phytochemicals could be an alternative to the chemotherapeutic molecules and safe to use in aquaculture. Also, the herbal immunostimulants *Emblica officinalis*, *Cynodon dactylon* and *Adathoda vasica* improved the immune system and reduced microbial infection in the gold fish *C. auratus* [16], and similar work was carried out by Magdelin [17] on the ornamental fish *Poecilia sphenops* using herbal immunostimulants. The extracts of the flowers and leaves of *Ixora coccinea* have been reported to show cytotoxic, antitumour [18]; antimicrobial, anti-inflammatory [19] and antioxidant activities [20]. The present study focus on the antibacterial and immunostimulant effect of *I. coccinea* extracts on ornamental gold fish *C. auratus* against *A. hydrophila* challenge.

2. Materials and methods

2.1. Source of A. hydrophila

A highly virulent strain of *A. hydrophila* (AHV-1; GenBank: HQ331525.1) and medium virulent strain CMST-1 (GenBank: JX575135.1) used for this study were isolated from infected ornamental gold fish *C. auratus* during massive outbreak of disease in the ornamental fish hatchery at Nagercoil, Kanyakumari District of India [21,22].

2.2. Herbal extracts for in vitro antibacterial activity against virulent A. hydrophila

Three herbal extracts including *I. coccinea*, *Daemia extensa* and *Tridax procumbens* having antibacterial and immunostimulant characteristics were selected following Nadkarni [23] and Citarasu [9]. These were extracted serially with hexane, ethyl acetate and methanol. Aqueous extracts were concentrated using lyophilizer and stored at 4 °C for further use.

2.2.1. In vitro antibacterial activity

Known quantity of different plant organic extracts condensate were impregnated in 5-mm diameter sterile paper discs (Himedia, India) and screened against virulent *A. hydrophila* (AHV-1 & CMST-1) with three replicates of the disc diffusion test as described by Bauer et al. [24]. After drying, the impregnated discs with herbal extracts were carefully dispensed with uniform distances over Muller Hinton agar surface and correct implantation was assured by applying gentle pressure over the disc. Control tests were carried out using sterile discs without impregnation of the herbal extracts. All plates were kept at 35 °C for 24 h incubation. After the incubation, plates were studied for inhibitory zone formation of antibacterial extracts on microbial lawns in agar surface.

2.2.2. In vitro immunostimulant activity

In vitro immunostimulation was carried out following the method of Sritunyalucksana et al. [25] with slight modification. Blood was bled from uninfected *C. auratus* and left to clot at 25 ± 2 °C for 1 h and stored in −80 °C deep freezer for 5 min and thawing to induce lysis. Repeated freeze and thaw cycle resulted in complete haemocyte lysis. The liquid fraction was then collected and designated as the Cell lysate fraction (CLF). One gram of plant extract condensate was dissolved in 100 ml of distilled water (W/v) as stock solutions. Five microlitres of each extracts was incubated with 100 μl of CLF at 25 ± 2 °C for 1 h. The immunostimulant-incubated CLF was used for testing the antibacterial assay with *A. hydrophila* [12]. The 100 μl of immunostimulated CLF was incubated again with 100 μl *A. hydrophila* bacterial culture 1×10^3 cfu ml^{-1} for 30 min at 25 ± 2 °C. Control experiments were performed for *A. hydrophila* incubated with CLF without incubation of immunostimulants herbal extracts. Triplicate samples of 20 μl each were drop-transferred to Tryptic Soy Agar (TSA) (Hi media, India) to obtain bacterial counts (CFU) after incubation at 30 °C for 12 h. Based on the positive immunostimulation results, the extracts were selected for further analysis.

2.3. Purification of the ethyl acetate extract of I. coccinea

Based on the better antibacterial and immunostimulant activities among the herbal extracts, ethyl acetate extract was selected for further works including purification, characterization and *in vivo* delivery. For purification, preparative silica column chromatography (50–80 μm particle size; 30 cm column length; 0.5 ml elution flow rate and three bed volume elution) was used. The column was equilibrated with 100% hexane and one gram of the condensate of the ethyl acetate extract of *I. coccinea* was loaded on the top of the column. Different proportions of the mobile phases such as hexane/ethyl acetate and ethyl acetate/methanol were used for eluting the compounds. The different fractions were collected, concentrated in a rotary evaporator and stored at 4 °C. The fractions were spotted on silica gel plates GF254 (Merck), 20 × 20 cm, 1 mm thick and the chromatogram was developed using, hexane: ethyl acetate (8:2) as mobile phase. The plates were visualized under short UV wavelength. Secondary antibacterial and immunostimulant screening was also performed against the virulent *A. hydrophila* strains using different fractions following the method mentioned the Section 2.2.

2.4. Structural characterization of I. coccinea fraction by GC–MS analysis

The fraction which eluted by H 40: EA 60 mobile phase was selected for GC–MS analysis based on the highest zone of inhibition