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Effect of orally administered azadirachtin on non-specific immune parameters of goldfish *Carassius auratus* (Linn. 1758) and resistance against *Aeromonas hydrophila*

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ABSTRACT

Modulation of the immune responses using active bio-ingredients as a possible prophylaxis measure has been novel prospect for aquaculture. The present study evaluated the effects of azadirachtin EC 25% on non-specific immune responses in goldfish *Carassius auratus* and resistance against pathogenic bacteria *Aeromonas hydrophila*. The experimental trial for effects of azadirachtin on immuno-haematoloical parameters in goldfish was conducted by feeding the various levels of azadirachtin as control T_0 (without azadirachtin), T_1 (0.1%), T_2 (0.2%), T_3 (0.4%), T_4 (0.8%) and T_5 (1.6%) for a period of 28 days. Fishes were challenged with *A. hydrophila* 28 days post feeding and relative percentage survival (%) was recorded over 14 days post infection. Immuno-haematoloical (total erythrocyte count, total leukocyte count, haemoglobin, packed cell volume, NBT activity, phagocytic activity, serum lysozyme activity, myeloperoxidase activity, total immunoglobulin) and serum biochemical parameters (serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and blood glucose) of fishes were examined at 14 and 28 days of feedings. Fish fed with azadirachtin, showed significantly ($p < 0.05$) enhanced TEC, TLC, Total Ig, total protein, NBT activity, serum lysozyme activity and myeloperoxidase level in different treatment groups in comparison with control group. Similarly, SGOT, SGPT and blood glucose level were found to be significantly ($p < 0.05$) high but PCV and Hb did not differ significantly ($p > 0.05$) in the treatment groups compared to control groups. Azadirachtin at the concentration of 4 g kg^{-1} showed significantly ($p < 0.05$) higher relative percentage survival (42.60%) when compared with the control against *A. hydrophila* infection. This study indicated that azadirachtin EC 25% (4 g kg^{-1}) showed higher NBT activity, serum lysozyme, protein profiles, leukocyte counts and resistance against *A. hydrophila* infection and thus, can be used as a potential immunostimulant in aquaculture.

1. Introduction

Trade of ornamental fish and aquarium keeping is most popular in developed countries in the world and is gaining popularity in many developing countries too. The export potential of ornamental fishes from India is of the order of US $ 30 million [1]. The intense culture practices and transport facility with negligence about sanitary aspects facilitate disease susceptibility in fish. The main drawbacks for intensive commercial production of ornamental fish are associated with diseases including bacterial, viral and parasitic infection. In addition, commercial aquaculture has been negatively hampered by infectious diseases resultant economic loss [2]. Conventional approaches such as use of disinfectants and antimicrobial drugs have shown limited success in prevention or control of aquatic animal diseases. Furthermore, there is growing concern about the use and abuse of antibiotic and antimicrobials in aquaculture [3]. The radial approach in disease management should be based on application of preventive measures, rather than disease control or treatment that is likely to be more cost-effective. One of the most revolutionised technology that has been evolved in response to these problems is "immunostimulants" which overcome the lacuna of vaccines and probiotics [4]. Recently, many synthetic and herbal immunostimulants have been reported to enhance the immune status of fish by enhancing the phagocytic, lysozyme and complement activities and also the immunoglobulin level against a number of causative extremities [5]. A large number of plants have been used in traditional medicine for the treatment and control of many diseases [6]. The use of natural plant products has been reported as antistress, for growth promotion, appetite stimulation,

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tonic and immunostimulation, and to have aphrodisiac and antimicrobial properties in finfish and shrimp larviculture due to the presence of active principle components such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids, and essential oils [7–9]. Herbs, rich sources of immune-enhancing substances, are used in many countries to promote health, increase the body's natural resistance to infection and in prevention and treatment of various diseases [10]. Herbal products are cheaper source for therapeutics and have greater accuracy compared with chemotherapeutic agents, and offer a viable solution for all the problems which aquaculture faces today.

One Indian plant that is most studied worldwide is Neem, *Azadirachta indica* A. Juss (syn. *Melia azadirachta*). A cornerstone of the Ayurvedic tradition, neem is known as "*sarva roga nivarak*" or "healer of all ailments" in India. Preliminary studies have revealed that water soluble part of alcoholic extract of *A. indica* leaves possessed hypoglycemic, hypolipidemic, hepatoprotective, antifertility, hypotensive and anti-serotonin activity [11,12]. The oil from the leaves, seeds and bark of neem *A. indica* possesses a wide spectrum of antibacterial action against both Gram-negative and Gram-positive microorganisms, including *Mycobacterium tuberculosis* and streptomycin resistant strains [13]. Some of the bioactive compounds responsible for neem's antibacterial property are azadirachtin, nimbidin, nimbin, nimbinin, nimbidinin, nimbidic acid [14]; nimbolide; margolone, margolonone and isomargolonone [15], limnoids and tetranotriterpenoids [16]. Neem (*A. indica*) is the classic example of biopesticide. It has been identified by WHO/UNEP as environmentally 'powerful' natural pesticides. The use of natural products including plant extracts in the treatment of some parasitic diseases like myxobolasis, trichodinosis, gyrodactylosis, argulosis, scuticocliates, etc., in farmed tropical freshwater fish has been reported [17,18]. Azadirachtin has been found to be effective against *Argulus* spp. [19] and has also been evaluated for its impact on physiological and serum biochemical parameters in *Carassius auratus* [20].

The aqueous extract of neem bark possesses anticomplement activity, acting on both the alternative and the classical pathway of complement activation in human serum. The aqueous extract of leaf also possesses potent immunostimulant activity as evidenced by both humoral and cell-mediated responses [21,22]. Neem oil has been shown to possess immunostimulant activity by selectively activating the cell-mediated immune mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge [23]. Enhanced primary and secondary antibody responses have been observed in *Oreochromis mossambicus* administered with azadirachtin, a triterpenoid derived from the seed kernel of *A. indica* [24]. However, application of multifunctional herbal bioactive compounds in ornamental and aquaculture system is recent activity, and few studies have been carried out in fish [25]. Keeping these aspects in view, the present study was conducted to investigate the effects of azadirachtin enriched diet on immuno-haematological profiles in goldfish and disease resistance against *Aeromonas hydrophila* infection.

2. Materials and methods

2.1. Experimental fish and their maintenance

Disease free mature goldfish (*C. auratus*) having average weight (20.33 ± 0.50 g) were procured from Kurla ornamental fish market, Mumbai, India. The fish were acclimatized for 15 days in laboratory condition in 500 L FRP tanks at 25–27 °C under continuous aeration. Fishes were fed with commercial goldfish pelleted diet @ 3% of body weight twice a day.

2.2. Experimental diets

The experimental diet was prepared with slight modification following the method of Rao et al. [26]. The commercial Azadirachtin EC 25% was procured from SOM Phytopharma Industries Pvt. Ltd., Hyderabad, India. Commercial ingredients such as fish meal, wheat flour, cod liver oil, vitamin and mineral mixture premix were procured from local market. Experimental diets were prepared by mixing all the ingredients in required quantity (Table 1) along with water to form dough and calculated amount of oil was added to the dough before cooking. Further, completely cooled dough was mixed with vitamins, mineral mixture and five of the experimental diets contained azadirachtin in different concentrations as 0.1%, 0.2%, 0.4%, 0.8% and 1.6% (Table 1) as per the treatments. The dough was pressed through a hand pelletizer to get uniform sized pellets. These pellets were dried at 40 °C for 12 h. After drying, pellets were packed in polythene bags, sealed airtight and labelled according to the different concentrations of treatments for further use.

2.3. Experimental design

The experiment was performed in rectangular plastic tubs (80 × 57 × 42 cm, 150 L capacity) covered with perforated lids and the water used for rearing was drawn from bore well. The acclimatized fishes were randomly distributed into six distinct experimental groups including control. Each group had three replicates and completely randomized design (CRD) was followed to set up the experiment. Fifteen fish were stocked in each tub. The experimental trial for effects of azadirachtin on haemato-immunological parameters in goldfish was conducted by feeding the various levels of azadirachtin as control T_0 (without azadirachtin), T_1 (0.1%), T_2 (0.2%), T_3 (0.4%), T_4 (0.8%) and T_5 (1.6%) for a period of 28 days. The fish were fed with the experimental diet at the rate of 3% of body weight twice a day at 09:00 and 17:00 h to approximate satiation for 28 days. The all physico-chemical parameters of water such as temperature (25 ± 2 °C), pH (7.2 ± 0.4), dissolved oxygen (5.2 ± 0.5 mg L^{-1}) and ammonia (0.01 ± 0.005 mg L^{-1}) were in optimum range during the experimental period. Total fifteen fishes from each treatment were sampled and blood was drawn on 14th and 28th days for different haemato-immunological assays. Remaining fishes were challenged with *A. hydrophila* 28 days post feeding and relative percentage survival (%) was recorded over 14 days post infection.

2.4. Collection of blood and separation of serum

Each fish was anesthetized with clove oil (Merck, Germany) at 50 μl per litre of water before collecting blood samples from fish.

Table 1
Composition of experimental diets (g%).

Ingredients	T_0	T_1	T_2	T_3	T_4	T_5
Fish meal	25	25	25	25	25	25
Soya flour	20	20	20	20	20	20
Corn flour	12	12	12	12	12	12
Wheat flour	15	15	15	15	15	15
Rice bran	10	10	10	10	10	10
GOC	12	11.9	11.8	11.6	11.2	10.4
CMC	1	1	1	1	1	1
Veg. oil	4	4	4	4	4	4
Vit. & mineral[a]	1	1	1	1	1	1
Azadirachtin	–	0.1	0.2	0.4	0.8	1.6

[a] Composition of vitamin–mineral mix (Agrimin) (quantity kg^{-1}), Vitamin A – 6,25,000 IU; Vitamin D3 – 62,500 IU; Vitamin E – 250 mg; Nicotinamide – 1 g; Cu – 312 mg; Co – 45 mg; Mg – 6 g; Fe – 1.5 g; Zn – 2.13 g; I – 156 mg; Se – 10 mg; Mn – 1.2 g; Ca – 247.34 g; P – 114.68 g; S – 12.2 g; Na – 5.8 mg; K – 48.05 mg.