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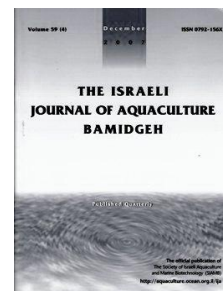
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Effect of Dietary Supplementation of *Achyranthes aspera* Seed on the Immune System of *Labeo rohita* Fry

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Key words: *Achyranthes aspera*, immunostimulation, myeloperoxidase, rohu

Abstract

Labeo rohita (rohu) fry (0.547 ± 0.01 g) were fed one of four diets containing 0 (control), 0.1, 0.5, or 1.0% *Achyranthes aspera* seed. After 30 days, the fry were immunized with chicken red blood cells (c-RBC). Blood samples were collected 7, 14, and 21 days after immunization. Significantly ($p < 0.05$) higher average weight (2.565 ± 0.02 g) and SGR were obtained in fry fed the 1.0% diet compared to others. The increase in average weight was directly related to the increasing dose of seed. FCR was significantly ($p < 0.05$) lower in fry fed the 1.0% diet while total serum protein, albumin, and globulin were higher in treated groups than in the unsupplemented control. The antigen-specific antibody titer level was significantly ($p < 0.05$) lower in rohu fed the control diet than in rohu fed the supplemented diets. Titer levels were 32-128, 128-256, 256-1024, and 256-1024 in the 0, 0.1, 0.5, and 1.0% diets, respectively. Serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP) were significantly ($p < 0.05$) higher in rohu fed the control diet than in rohu fed the supplemented diets while myeloperoxidase was lower in the former. Among the treated groups, myeloperoxidase was significantly ($p < 0.05$) higher in rohu fed the 1.0% diet ($1.561\text{--}2.558$, λ 450 nm) than in those fed other diets. The present study documents the immunostimulatory properties of *A. aspera* seed and finds that a dose of 1.0% might be suitable for rohu fry.

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Introduction

The aquaculture industry faces the challenge of disease outbreaks. Farmed species are increasingly exposed to pathogens, while prophylactic treatment with antibiotics is no longer acceptable and, for many pathogens, ineffective (Watts et al., 2001). Alternative strategies are essential to prevent disease and sustain production. Immunostimulants enhance immunocompetence and disease resistance (Ganguly et al., 2010). Used as a dietary supplement, some immunostimulants increase disease resistance in fish by improving the non-specific arm of the immune defense system (Selvaraj et al., 2005; Kamilya et al., 2008; Ye et al., 2011). The non-specific immune system of fish is highly advanced and in some respects superior to the mammalian immune system (Watts et al., 2001). Therefore, the use of immunostimulants is gaining importance as a prophylactic measure in aquaculture. So far, these substances have not shown any of the negative side effects that antibiotics and live vaccines have on consumers and the environment, and they are generally classified as biological response modifiers (Anderson, 1992; Secombes, 1994). Immunostimulants can activate fish immune functions, even in immuno-suppressive states caused by toxic or stress situations, and thereby reverse the deleterious effects mediated by stress (Sahoo and Mukherjee, 2002, 2003).

Carps account for about 82% of the total fish production in India. *Labeo rohita* (rohu) is an economically important carp species. Outbreaks of disease hinder aquaculture production in the country. Fish in early developmental stages are more susceptible to disease than mature fish due to the poor immune competency of young fish. It is essential to boost the immune system exogenously for successful larviculture. *Achyranthes aspera* stimulates the immunity of mammals. When administered orally, it enhances c-RBC agglutinating antibody responses and other non-specific immune parameters in fish (Rao and Chakrabarti, 2005). The present investigation evaluates the optimum dose of *A. aspera* seed for effective stimulation of immunity in *Labeo rohita* fry.

Materials and Methods

Culture conditions. Fry of the Indian major carp, *Labeo rohita* (rohu), were obtained from Chatterjee Brothers' Fish Farm, Mogra, West Bengal. The rohu were acclimatized for 48 h in outdoor cement 165-l tanks, then divided into four groups and stocked separately in recirculating culture systems. At the start of the experiment, the rohu weighed 0.547 ± 0.01 g. The stocking density was 15 fry/15-l aquarium. Each recirculating system consisted of four glass aquaria that held fish plus one 55-l glass aquarium with a filter (Sharma and Chakrabarti, 2003). Water temperature ranged 30–31°C and pH 7.5–8.15. Dissolved oxygen was maintained above 5 mg/l by an aerator.

Four diets (40% protein) were prepared containing 0 (control), 0.1%, 0.5%, and 1.0% *A. aspera* seed along with dry fish powder, wheat flour, cod liver oil, and vitamin-mineral premix (Table 1). Rohu were fed at 5% of their body weight daily in two portions, at 09:00 and 17:00. There were four replicates of each feeding regime.

Injection and sampling. Chicken red blood cells (c-RBC) were collected in Alsever's solution one day prior to injection of the fish and washed thrice with phosphate buffered saline (PBS; pH 7.4). A 20% (v/v) suspension of PBS and c-RBC (80:20) was made and fish were anesthetized with MS-222 (Sigma, USA) and injected intraperitoneally with 10 μ l of the PBS:c-RBC suspension with a 30G x 5/16 (0.30 x 8 mm) needle (Hindustan Syringes and Medical Devices Ltd.). During this period, the rohu were not weighed to prevent mortality. After injection, the fish were released into culture tanks. Blood samples were drawn from the caudal veins of five fish from each aquarium with a 30G x 5/16 (0.30 x 8 mm) needle (Hindustan Syringes and Medical Devices Ltd.) on days 7, 14, and 21 after immunization. The samples from each aquarium were pooled and served as replicates. Each treatment had four replicates. Samples were allowed to clot, stored overnight at 4°C, and centrifuged at 400 x g for 10 min. Serum was stored at -

Table 1. Composition of control and experimental diets.

Ingredient	Diet (g <i>A. aspera</i> /kg diet)			
	0	0.1	0.5	1.0
Dry fish powder	583.3	583.3	583.3	583.3
Wheat flour	402.7	401.7	397.7	392.7
Cod liver oil	10.0	10.0	10.0	10.0
Vitamin-mineral premix	4.0	4.0	4.0	4.0
<i>Achyranthes aspera</i>	0	1.0	5.0	10.1

20°C until use. Fish were weighed individually after collection of the blood samples and weights taken on day 21 were considered final body weights. Specific growth rate (SGR) was calculated as $100(I_{n\ wt} - I_{n\ wi})/t$, where W_i and W_t are the initial and final body weights and t is the time in days. The food conversion ratio was calculated as dry weight of consumed food by individual fish/wet wt gain of individual fish.

Biochemical assays. Total serum protein, albumin, and globulin fractions were determined by the method of Lowry et al. (1951) using a Microplate Reader (BioTek, Synergy HT, NY, USA). Total protein, albumin, and globulin help to understand the nutritional status and health condition of fish. A low level of total protein may be due to a liver or kidney disorder, or to improper digestion and absorption of protein.

The antigen-specific antibody response was determined by hemagglutination assay. Chicken blood was collected in Alsever's solution (1:3) and the cells were washed in PBS and resuspended to 2% (v/v) in PBS. Serum (50 μ l) was serially diluted in PBS in a 96-well round-bottomed microplate. An equal volume of c-RBC (2%) was added to each well and the plate was kept at room temperature for 1 h, then overnight at 4°C. The reciprocal of the highest dilution that agglutinated was considered the hemagglutination antibody titer.

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP) were determined using diagnostic kits (Siemens Healthcare Diagnostics Ltd., Baroda, India). SGOT, also known as aspartate aminotransferase, is usually found in the liver, muscle, kidney, etc. SGPT, known as alanine aminotransferase, is largely found in the liver. These are released into the serum in case of damage to any of these tissues. An elevated amount of these amino transferases are indicators of tissue damage, although SGPT is more specific for liver. ALP are enzymes found primarily in the liver, bone, intestine, kidney, etc. A high level of ALP indicates liver and bone disorder, although rapid bone growth during the developmental stage may cause elevated ALP in the blood. Optical density was measured at 340 nm.

Total myeloperoxidase content of the serum was measured according to Quade and Roth (1997). Optical density was measured at 450 nm. Myeloperoxidase is the most abundantly expressed lysosomal protein stored in azurophilic granules of neutrophils. It produces hypochlorous acid from hydrogen peroxide and chloride ions during the neutrophil's respiratory burst. Further, it oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as an oxidizing agent. Hypochlorous acid and tyrosyl radical are cytotoxic, so they are used by the neutrophil to kill bacteria and other pathogens. Release of myeloperoxidase by neutrophils and monocytes during inflammation plays an important role in the innate immune response

Statistical analysis. Data were compiled as means \pm SE and analyzed by using one-way analysis of variance (ANOVA) and Duncan's multiple range test (Montgomery, 1984). Statistical differences were considered significant when $p < 0.05$.

Results

There was no fish mortality during the study period. The average final weight was significantly highest in rohu fed the 1.0% diet (Table 2). The average weight was directly related to the increase of seed in the diet. Like average weight, SGR was significantly highest in fish fed the 1.0% diet. FCR was significantly lowest in fish fed the 1.0% diet and inversely related to seed dose.

On day 7, total serum protein content was significantly higher in fish fed the 0.5% and 1.0% diets (Fig. 1). On days 14 and 21, it was significantly highest in fish fed the 1.0% diet. In the 0.5% and 1.0% diets, total serum protein showed an increasing trend with day after immunization. The albumin level was significantly higher in fish fed the 0.5% and 1.0% diets throughout the study but there was no significant difference in albumin between these two groups 7 and 14 days after immunization. Like albumin, the globulin level was significantly higher in fish fed the 0.5% and 1.0% diets 7 days after immunization and in fish fed the 1.0% diet on days 14 and 21. The highest globulin level was recorded on day 21 in fish fed the 1.0% diet.

Table 2. Effect of dietary *Achyranthes aspera* seed on mean final average weight, specific growth rate (SGR), and food conversion ratio (FCR) of rohu (n = 4).

	Diet (g <i>Achyranthes aspera</i> seed/kg diet)			
	0	0.1	0.5	1.0
Avg wt	1.502±0.04 ^d	1.661±0.05 ^c	1.935±0.04 ^b	2.565±0.02 ^a
SGR	1.740±0.05 ^d	1.919±0.05 ^c	2.178±0.03 ^b	2.664±0.01 ^a
FCR	1.718±0.08 ^a	1.430±0.07 ^b	1.122±0.05 ^c	0.785±0.01 ^d

Values in a row followed by different superscripts significantly ($p < 0.05$) differ.

Hemagglutination antibody titer was significantly higher in fish fed the 0.5% and 1.0% diets on days 14 and 21 after immunization, respectively. The control group had significantly lower values throughout the study period. Values ranged 32-128, 128-256, 256-1024, and 256-1024 in the 0, 0.1%, 0.5%, and 1.0% diets, respectively.

SGOT was significantly higher in the control group. Among the treated fish, the highest SGOT was obtained in fish fed the 0.1% diet. Similarly, SGPT was significantly higher in the control than in the treated fish. Among the treated groups, SGPT was significantly lowest in fish fed the 1.0% diet. There was an inverse relationship between SGPT and seed dose. Both SGOT and SGPT tended to decrease with time, regardless of the feeding regime. ALP decreased as the seed dose increased on days 14 and 21 and was significantly higher in the control fish, except on day 21 when there was no significant difference between fish fed the 0.1% and control diets. Myeloperoxidase was highest in fish fed the 1.0% diet throughout the study and increased with duration.

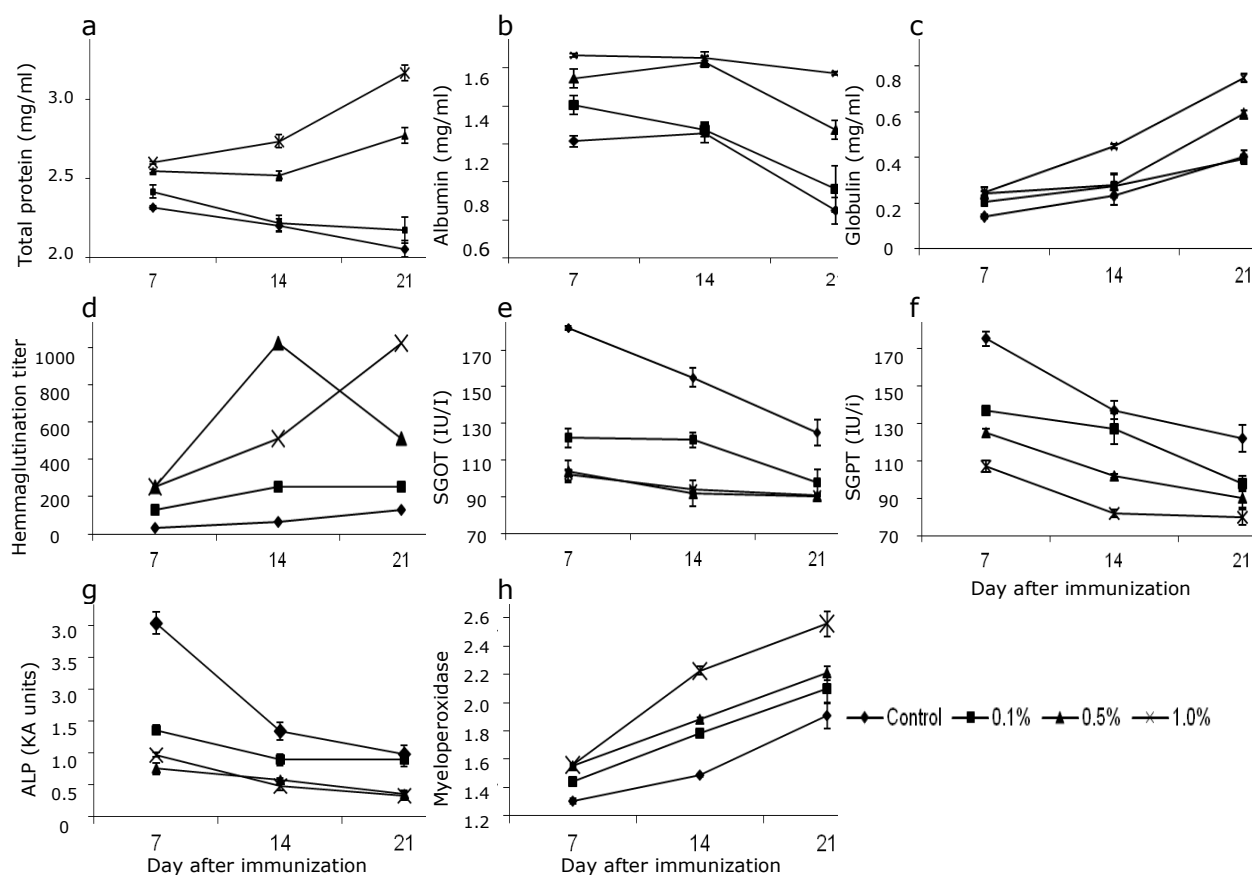


Fig. 1. Levels of (a) total serum protein, (b) serum albumin, (c) serum globulin, (d) hemagglutination titer, (e) serum glutamic oxaloacetic transaminase (SGOT), (f) serum glutamate pyruvate transaminase (SGPT), (g) alkaline phosphatase activity, and (h) myeloperoxidase activity in rohu fed different doses of *Achyranthes aspera* seed.

Discussion

Dietary manipulation for growth and health management is an important research area in aquaculture. In the present investigation, incorporation of *A. aspera* seed enhanced the growth of rohu. There was a direct relationship between seed dose and fish growth. The

lower FCR in fish fed supplemented diets shows better food utilization efficiency while the higher SGR indicates better health status. Similarly, the feed additive, levamisole, had a positive effect on growth promotion and feed efficiency in hybrid striped bass (*Morone chrysops* x *M. saxatilis*; Li et al., 2006). The nutritional value of *A. aspera* seed may influence the health status of rohu. The seed contains fatty acids, a number of oleonic acid and bisdesmosidic triterpenoid based saponins, ecdysterone, and amino acids (Hariharan and Rangaswamy, 1970). Ecdysterone has a pronounced growth promoting effect due to a high rate of protein synthesis (Goerlich-Feldmann et al., 2008).

The present study documents the immunostimulatory properties of *A. aspera* seed. Albumin and globulin are the major serum proteins, and play important roles in the immune response. Fish fed the supplemented diets had higher serum protein and globulin than control fish. Similar results were found with mango kernel incorporated into diets for rohu (Sahu et al., 2007).

The hemagglutination titer level was significantly higher in fish fed the supplemented diets. Hemagglutination titer indicates the response of fish antibodies to the antigen c-RBC. In all treated groups, the response was minimal during the first sampling as it takes time to respond. In fish fed the 0.5% diet, the response was significantly higher on day 14 than on days 7 and 21. Since the effect of antigen c-RBC dropped on day 21, the titer decreased on this day. In fish fed the 1.0% diet, the antibody responded later; the highest response was obtained on day 21. A similar result was found with catla (*Catla catla*) where the hemagglutination titer was significantly higher in those fed 0.5% *A. aspera* seed than in control fish (Rao and Chakrabarti, 2005). Indian major carps are cultured in warm environments that are conducive to pathogen growth. A natural factor such as agglutination may help to overcome diseases earlier than that required to produce specific immunity (Sahoo et al., 2005).

SGOT, SGPT, and ALP were significantly lower in treated fish than in the control. High levels of these enzymes indicate poor physiological status in an organism. Myeloperoxidase is an important enzyme most abundantly present in neutrophil granulocytes. It has antimicrobial activity by utilizing hydrogen peroxide during respiratory burst and producing hypochlorous acid (Dalmo et al., 1997). There is evidence for phagocytic, chemotactic, and bactericidal functions in fish neutrophils and an intense respiratory burst that may be used to assess fish health (Roth, 1993). Reduced activity may indicate the presence of contaminants or stress (Anderson and Siwicki, 1995). Myeloperoxidase was significantly ($p < 0.05$) higher in *Clarias batrachus* that were fed immunostimulants (Kumari and Sahoo, 2006). The higher myeloperoxidase in treated fish in the present study shows that its well-developed immune status can overcome adverse environmental conditions. Study of the functional aspects of neutrophils is useful for evaluating the health status of cultured species.

In conclusion, dietary supplementation of *A. aspera* seed improves immunity in rohu fry and a 1.0% dose is recommended for the early developmental stage of this species. The plant can be easily grown with minimum production costs. Therefore, the seeds can be made available to the aquaculture industry on a commercial scale. The plant's ingredients play a dual role: they directly enhance immunity and indirectly quicken the growth rate. Thus, *A. aspera* helps fish to overcome disease in early vulnerable developmental stages.

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