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Achyranthes aspera extract against *Pseudomonas fluorescens* enhance antibody production in *Labeo rohita*

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Abstract
Indian major carp, rohu, *(Labeo rohita)* (30±6g), were fed with two types of diets; an experimental diet, containing root extract (0.5%) of *Achyranthes aspera* as an ingredient and control diet without the root extract. Experimental rohu were injected intraperitoneally with *Pseudomonas fluorescens* (3.2×10^{-6} CFU ml^{-1}) and bloods were sampled 14 and 28 days post immunization. Though hemagglutination antibody (HA) titers were always higher in the test group than the control group, antigen-specific antibody and total serum globulin levels peaked on day 14 after immunization and steadily reduced towards day 28. Beside this, the results showed that dietary supplementation of *A. aspera* in test group showed significantly increased serum antibody titer (3280) and growth factors (41.1%) of *L. rohita* ($P<0.05$) compared with the control group throughout the experimental period. The RNA/DNA ratio was significantly ($P<0.05$) higher in test group of fish than the control group on days 7 and 14. These results showed the immunostimulatory activity of the prepared diet containing root extract of *A. aspera*.

Keywords: *Labeo rohita*, *Achyranthes aspera*, Immunopotentiation, Hemagglutination, Antigen-specific, Growth promotor

1. Introduction
The Indian major carp, the rohu, *Labeo rohita*, is an important commercial fish in our aquaculture and also a vital source of the protein food supply for the people of Bangladesh [1]. *Aeromonas* and *Pseudomonas* are the major bacteria fish pathogens which widely distributed in aquatic organisms in nature [2]. The use of herbal compounds as immunostimulants has been increasing rapidly in aquaculture to avoid the indiscriminate use of hazardous antibiotics. The practices of antibiotics and pesticides are avoiding because of their damaging effect. Consequently, there is an urgent need for alternative protein and oil sources suitable for aquafeed to ensure sustainability of both the aquaculture and fisheries industries [3]. It is however essential that these feed resources must not compete with human food grade interest [4, 5]. Reported that the ethanol and chloroform extracts of seeds of *Achyranthes aspera* shows mild to moderate antibiotic activity against *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa.* [6] studied the various extracts of the leaves and callus of the plant also shows antimicrobial activity. Such diets are also being promoted in aquaculture as a means of overcoming the immunosuppressive effects of stress that commonly occur in intensive fish production, even though few published reports support the claims. Much of the recent work on the effect of dietary supplementation of immunostimulants on fish health has focused on β-glucans, although other immunostimulants, such as bovine lactoferrin, have also shown promise [7]. Salar-bec, a vitamin premix and Ergosan, comprising an unspecified plant extract and alginate stimulated the antibody production against *Aphanomyces invadans* in snakeheads, *Channa striata* [8]. *Quillaja saponin* mixture was reported as acting as growth stimulant forcommon carp, *Cyprinus carpio* through diet [9]. The Indian major carp, the rohu, *L. rohita*, is an important commercial fish in India. Disease problems have resulted in losses to Indian farmers. Several plant sources had been screened and *Achyranthes aspera*, a herb belonging to the family Amaranthaceae was found to potentiate the immunity in mice [10]. Plants can be a considerable source of replacement for fish meal in aquaculture diets given their steadily increasing production, high availability, relatively adequate nutritional quality and better economic value [11]. *L. rohita* species were feeding which diets was containing graded levels of *Achyranthes aspera.* The aim of the present investigation was to test the

ability of *A. aspera* added to the diet to potentiate the immune response, antibody production and growth performance of this fish against *Pseudomonas fluorescens*.

2. Materials and Methods

2.1 Sample collection

Indian major carp, rohu, *(Labeo rohita)* (30±6g), were obtained from the local fish market Ma Fatema Fish hatchery Jessore, Bangladesh during February 2017. Fish were cultured in experimental pond in JUST campus. Fishes were divided into four groups (Twenty five fishes/group) under two feeding regimes, control: four groups test: four groups. Temperature, pH and TDS (total dissolved solid) ranged from 30±0.7 ºC, 6.84±0.08 and 422±0.34 respectively during the experiment. Dissolved oxygen level will maintain above 5 mg/l throughout the experiment.

2.2 Experimental diet and feeding

A. aspera (Common name: Apang) was found in the field near the department. Roots of the plant were collected, washed with tap water and made into small pieces. Leaves (dried) were pulverized using an electrical grinder and root extract were collected manual pressure by iron grinder and extracted with distilled water at 100°C for 4 h, centrifuged at 5,000 rpm for 15 min, and filtered using what man no.1 filter paper. The residues obtained after evaporation of ethanol was kept in sterilized screw cap glass container and stored at -20ºc until use. In experimental diet (30% protein), this root extract was added along with other ingredients such as fishmeal, wheat flour, cod liver oil, vitamin and mineral premix. Normal diet was prepared using the same ingredients, except root extract (Table 1). Test groups was fed with experimental diet, and control groups was fed with normal diet. Feeding was started 4 weeks prior to the immunization and continued till the end of the experiment. Both groups of fish was be fed at 1% of body weight [12].

Table 1: The amount of nutritional, microbiological and toxicological evaluation of *A. aspera*

Components	Amount
Moisture	4.05%
Proteins	20.54%
Fats	0.903%
Ash	20.25%
Carbohydrates	54.26%
Energy	294 Kcal
Phosphorus	1447.5mg/kg
Microbiological	Nutritive supplement in blood
Toxicological	Good effect on general health (weight)

2.3 Antigen preparation and immunization of fish

Chicken red blood cells (c-RBC) collected in Alsever's solution 1 day before immunization. For immunization, c-RBC will be washed thrice with phosphate buffered saline (pH 7.4) and made 20% suspension in the same solution. Fish anaesthetized with MS-222 (Sigma) 1:10,000 in dechlorinated water. All fishes injected intraperitoneally with 500 Al of 20% suspension of c-RBC in phosphate buffered saline using 1 cm3 syringe with 28G needle [13].

2.4 Sampling

Four fishes from each feeding regime (one fish from each tank) sampled and anaesthetized with MS-222. Blood collected by cutting the tail on days 7, 14, 21 and 28 after immunization in centrifuge tubes and allowed to clot at room temperature. Serum obtained by centrifugation and was stored in plastic vials at -20 ^{0}C for further use. After blood collection, spleen remove for RNA/DNA estimation.

2.5 Serum agglutination titer assay

At day 7, 14, 21 and 28 of the experiment blood sample were collected from each group of fish. Serum samples were collected by following centrifugation. Isolated bacterial cell suspensions were centrifuged in 7000 rpm for 12 min and supernatant was discarded. The resulting plates were washed twice with PBS solution and then plates were re-suspended in PBS. Starting with a dilution of 1:10 (10 µl serum and 90µl PBS) two-fold serial serum dilutions were made in 96-well round bottom micro titer plates by adding 25 µl of diluted serum into the remaining wells plate with 25 µl of bacterial cell suspension was added to each well. The plate was covered with plastic film and incubated at 4 ºC for 2 hrs and 24 hrs incubated at 25 ºC. Result of agglutination titer was determined by using multi-scanner [14].

2.6 Hemagglutination assay

Hemagglutination assay perform according to [15, 16] blood from chicken were collected and mixed with Alsever's solution and blood cells were washed in phosphate buffered saline and 2% c-RBC suspension in phosphate buffered saline prepared. In round bottomed microtiter plate, 50 Al of control/test serum was serially double-diluted in phosphate buffered saline. Equal volumes of 2% c-RBC were added to all the wells and kept for 1 h at room temperature and overnight at 4 ^{0}C. The reciprocal of the highest dilution that gave agglutination was take as the hemagglutinating antibody titer.

2.7 Total serum protein

Five microliters of serum was diluted to 10 ml with distilled water and the protein was determined by using DNA/Protein program pack, Ver. No. 2.00, UV-1600 Series of Shimadzu UV–Visible spectrophotometer.

2.8 Serum globulin level

One hundred microliters of serum was taken in a micro-centrifuge tube and 100 Al of saturated ammonium sulfate added, mixed well and put in a stand for 1 h. After that the tubes were centrifuged at 10,000_g for 10 min and the supernatants removed. The precipitate (globulin fraction) in centrifuge tube was dissolved in 500 Al of distilled water. Then the protein content were determined by the method of [17].

2.9 RNA/DNA ratio

Nucleic acid content of spleen was assayed according to [18]. DNA and RNA were determined using diphenylamine and orcinol, respectively.

2.10 Growth Performance

The growth performance of species weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were determine according to [19].

$$\text{Percentage of weight (g) gain} = \frac{\text{Final weight (g)} - \text{Initial weight(g)}}{\text{Initial ewight (g)}} \times 100$$

$$\text{Percentage of specific growth rate (SGR)} = \frac{\text{Final weight (g)} - \text{Initial weight(g)}}{\text{Initial ewight (g)}} \times 100$$

$$\text{Feed convertion ratio} = \frac{\text{Feed intake per body weight}}{\text{Weight gain}}$$

2.11 Statistical analysis
Values for each parameter measured were expressed at the arithmetic mean ±standard error (SE). Effects of herbal diets on growth performance, hematological and immunological parameters were tested using one-way ANOVA and the mean values were compared by using Duncan's multiple range tests at 0.5% level of significance [20].

3. Result and Discussions
3.1 Hemagglutination assay
The hemagglutination antibody (HA) titers were minimum on day 7 and reached a maximum on day 14, gradually decreased thereafter (Fig. 1). Though hemagglutination antibody titers were always higher in test group than control group, the difference was significant ($P_{t\text{-test}}$ =0.008) on day 21. In the present study, the antigen-specific antibody levels were peaked on day 14 in rohu, whereas in tilapia (*Tilapia mosambica*) the peak antibody response was found on day 8 [21]. Incorporation of *A. aspera* in the diet has enhanced serum antibody and serum globulin levels in *L. rohita*. *Achyranthes* has significantly (P<0.05) enhanced the BSA-specific antibody titers than the untreated control group throughout the study period.

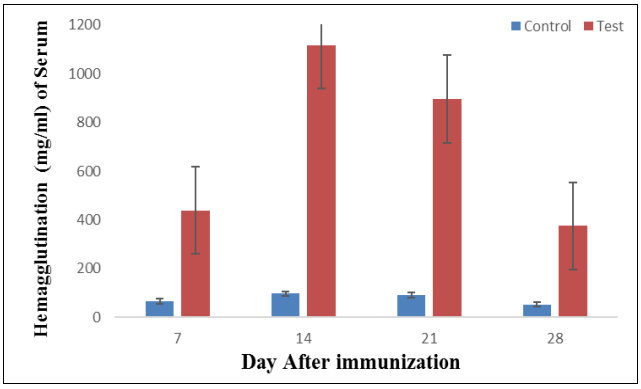

Fig 1: Immunostimulatory effect of experimental diet on antigen-specific antibody response in *L. rohita* determined by hemagglutination. The reciprocal of the highest serum dilution that gave the agglutination was taken as titer. The values represented are the mean ± E.S of four fishes.

3.2 Serum agglutination titer assay
Measurement of serum agglutination titer assay fish diet continues 28 days. After 4 weeks of feeding, fish were immunized, spleen and blood were sampled on weekly intervals for four times after immunization. *Achyranthes* has significantly (P<0.05) enhanced the BSA-specific antibody titers than the untreated control group throughout the study period. The efficiency of antigen clearance was also enhanced in *L. rohita* treated with *Achyranthes*. Serum agglutination titer assay (Table 2) was done on 14th day and 28th day of the experimental period. Test *A. aspera* added diet fed fishes and highest diluted serum (3280) showed positive agglutination (0.29±.02; 0.06±.01) response (Fig. 2). Hemagglutination antibody titers were significantly higher in the test group of fishes compared with the control group [22]. In this present study showed the highest diluted serum (5, 00,143) showed positive agglutination (0.29±.08; 0.06±.07) treated by test group than the control (0.65±.08.02; 0.06±.07).

Table 2: Different immune parameters of *L. rohita* at 14 days and 28 days of the experiment.

Immune parameters	Control		Test	
Serum agglutination	14 days	28 days	14days	28 days
	0.65	0.38	0.29	0.06

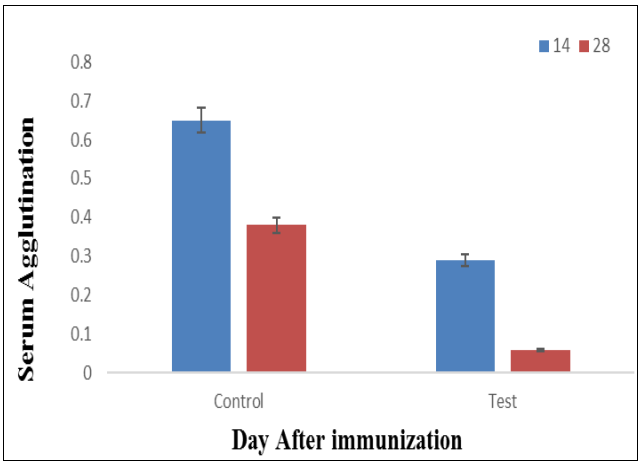

Fig 2: Serum agglutination titer assay of *L. rohita* fed with different doses of *A. aspera* supplemented diets against *P. fluorescent.*

3.3 Serum globulin level
The serum globulin level of test and control group was similar on day 7 of post immunization. Serum globulin levels were significantly (Pt-test=0.047; 0.011) higher in the treated group than the control group on days 14 and 21 and there was no significant ($P_{t\text{-test}}$=0.45) difference between these two groups on day 28 (Fig. 3). Highest level of serum globulin was found on day 14 in both treatments. Similar results were found with other immunostimulants, as total immunoglobulin levels were increased in Atlantic salmon, *Salmo salar*, when fed with lipopolysaccharide coated feed [23]. Rainbow trout (*Oncorhynchus mykiss*) treated with chitosan by injection or immersion showed increased immunoglobulin concentrations [24].

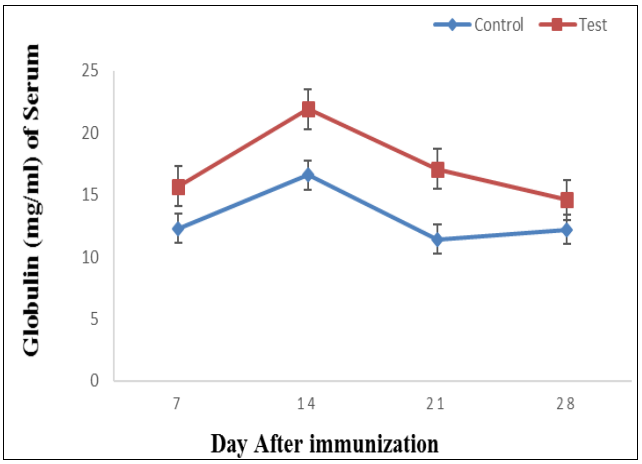

Fig 3: Effect of the experimental diet on the serum globulin levels in *L. rohita*. The values represented were the mean ± E.S of four fishes.

3.4 Total serum protein
There was a significant increase in total serum protein level from days 7 to 14. The increased level was maintained up to day 21, then decreased by day 28 (Fig. 4). However, there was no significant difference of total serum protein level between the test group and control group. All these results indicate that both the specific immunity and nonspecific immunity were enhanced in the fish fed with the experimental diet. The protein level was found high on day 14 [25] found elevated serum anti-protease levels in rohu fed with *A. aspera* incorporated diet in the present study These results confirm the stimulation of immunity in *L. rohita* by the experimental diet containing *A. aspera* as an ingredient.

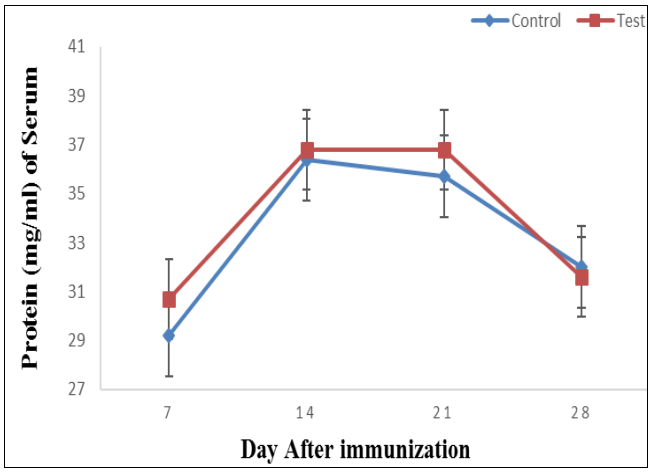

Fig 4: Effect of the experimental diet on total serum protein levels in *L. rohita*. The values represented were the mean ± E.S of four fishes.

3.6 RNA/DNA ratio

The RNA/DNA ratio in spleen was highest on day 7 and then gradually decreased on days 14 and 21 (of post immunization) (Fig. 5). The RNA/DNA ratio was significantly (Pt-test=0.024; 0.019) higher in the test group than control one on days 7 and 14. The sequential relationship between RNA/DNA ratio and, as the RNA/DNA ratio was maximum on day 7. The efficiency of antigen clearance was also enhanced in Catla treated with *Achyranthes* [26]. *Withania somnifera* root powder have a stimulatory effect on immunological parameters and increases disease resistance in *L. rohita* fingerlings against *Aeromonas hydrophila* infection [27].

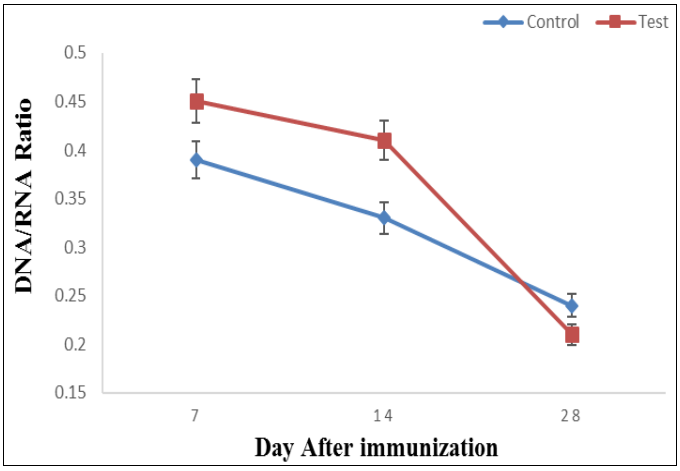

Fig 5: RNA/DNA ratios of spleen in *L. rohita* after immunization. The RNA/DNA values represented were the mean ± E.S of four fishes.

3.7 Growth Performance

All Rohu fed with different percentages of *A. aspera* supplemented diet test group showed significant growth as compared to the control group (Table 3). The specific growth rate exhibited an increased trend in all the test groups, however it was significantly higher in test group *A. aspera* added diet fed fishes. Sayeed *et al.*, [28] worked on Thai pangus polyculture with carps has been increasing for its high potential, however very few attempts were made to compare its growth using different types of feed. This study result shown that herbal growth promoter effects of feed additive in fish meal on the performance of *Oreochromis niloticus* [29].

Table 3: Growth parameters of *L. rohita* fed with different doses of *A. aspera* supplemented diets against *P. fluorescens.*

Growth parameters	Group	7 Days	14 Days	21 Days	28 Days
WG	Control	32.02±1.2	32.09±1.3	33.03±1.5	35.05±1.5
	Test	34±1.4	35.88±1.4	36.12±1.7	41.1±1.7
SGR	Control	1.12±0.14	1.00±0.6	1.69±0.4	2.00±0.14
	Test	1.14±0.3	1.70±0.6	2.20±0.5	2.45±0.5
FCR	Control	1.5±0.2	1.6±0.1	1.7±0.3	1.7±0.3
	Test	1.2±0.3	1.3±0.2	1.3±0.3	1.3±0.3

4. Conclusion

The results of the present study indicate the beneficial role of *A. aspera* in augmenting the immunity mediated through specific and non-specific immune responses, as evident from the enhanced hematological and immunological parameters such as total serum protein, Serum globulin level, Hemagglutination assay, growth and RNA/DNA ratio against *P. fluorescent* and no toxic effect were observed. The *A. aspera* extract modulation of immune response elicited by the extract in experimental fish which may act directly as immune-stimulant against pathogens. The overall outcomes of the result shown that test group of *A. aspera* extract induced highest positive response against *P. fluorescent* and exhibited the more optimum features in all most all the immunological parameters to a significant level.

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