Turk. J. Fish.& Aquat. Sci. 20(11), 795-805
http://doi.org/10.4194/1303-2712-v20_11_03

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Assessment of Immunostimulatory Characteristics of *Achyranthes aspera* Seeds and Leaves Supplemented Diets in *Labeo rohita* Fingerlings

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Article History
Received 27 November 2019
Accepted 13 October 2020
First Online 14 October 2020

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Keywords
Immunostimulant
Lysozyme
Hemagglutination
Lysozyme C and *G*
TNF-α

Abstract

The effect of dietary supplementation of leaves and seeds of *Achyranthes aspera* on rohu, *Labeo rohita* fingerlings was evaluated. Three experimental diets containing 0.25 (D1) and 0.5% (D2) leaves and 0.5% (D3) seeds of *A. aspera* and control diet (D4) without plant ingredients were formulated. After 45 days of feeding, rohu were immunized with 20% chicken RBC. Tissue and blood samples were collected on days-7, 14 and 21 after immunization. Dietary supplementation significantly ($P<0.05$) increased the average weight and specific growth rate of rohu. Serum lysozyme, myeloperoxidase, hemagglutination titer and nitric oxide synthase activities were significantly ($P<0.05$) higher in D3 compared to others. This group was followed by D2 and D1. Significantly ($P<0.05$) lower lipid and protein oxidations were found in D3 compared to others. This group was followed by D2 and D1. In hepatopancreas, lysozyme C, lysozyme G, tumor necrosis factor-α (*TNF-α*) and interleukin-1β (*IL-1β*) expressions were up-regulated while interleukin-10 (*IL-10*) expression was down-regulated. In anterior kidney, lysozyme C, lysozyme G and IL-10 expressions were up-regulated while *TNF-α* and *IL-1β* expressions were down-regulated. The dietary inclusion of leaves and seeds of *A. aspera* enhanced the growth, improved immune system and reduced oxidative stress of rohu.

Introduction

The occurrence of infectious diseases is a major challenge to the aquaculture industry as it is affecting sustainable aquaculture. Use of chemotherapeutants to control the disease problem has several disadvantages. It causes environmental pollution (Hektoen, Berge, Hormazabal, & Yndestad, 1995), helps in the development of antibiotic resistant bacteria (Huys *et al*., 2000) and has residual effect on fish (Jian & Wu, 2003). Hence, alternative strategies should be adopted to enhance fish resistance against diseases (Defoirdt, Sorgeloos, & Bossier, 2011). Applications of natural immunostimulants, probiotics, prebiotics etc. are gaining importance to control the fish diseases (Wang, Sun, Liu, & Xue, 2016) as these are eco-friendly and improve the immune system of fish. The non-specific immune response acts as a first line of defense and represents a wide range of immune responses (Dalmo, Ingebrigtsen, & Bøgwald, 1997). Spleen, anterior kidney and thymus are the main lymphoid organs of the fish. Fish has cellular as well as humoral immune responses. So, it is essential to improve the immune system of fish using natural immunostimulants as feed additives.

The member of the Amaranthaceae family, *Achyranthes aspera* L. is widely distributed in India and it has high medicinal value. Many studies show the compositions of different parts of the plant. The whole plant contains ecdysterone (Banerji, Chintalwar, Joshi, & Chadha, 1971); D-glucuronic acid and its ester are present in seeds (Hariharan & Rangaswami, 1970); the roots contain an important aliphatic acid *n*-hexacos-14-

enoic acid (Sharma, Vasudeva, & Ali, 2009); three triterpenoid glycosides, linolenic and oleic acids are also found in the seeds of *A. aspera* (Rashmi, Dayal, & Nagatsu, 2007; Chakrabarti, Srivastava, Kundu, Khare, & Banerjee, 2012). In methanolic extract of *A. aspera* leaves, carbohydrates, phenolic compounds, saponins, tannins, oils, fats, alkaloids, flavonoids etc. are found (Priya, Kumar, Karthik, & Rao, 2012). The mobile phases of 11 amino acids have been isolated from the leaves (Shendkar *et al*., 2013).

Many studies have documented that dietary inclusion of seeds and roots extract of *A. aspera* enhanced the innate immune system of catla *Catla catla*, common carp *Cyprinus carpio* and rohu *Labeo rohita* (Rao, Romesh, Singh, & Chakrabarti, 2004; Rao & Chakrabarti, 2004; Rao & Chakrabarti, 2005a, 2005b; Chakrabarti *et al*., 2012; Srivastava & Chakrabarti, 2012); seeds show antigen clearance properties in common carp (Chakrabarti & Rao, 2012). In UV-B irradiated rohu and catla larvae, dietary inclusion of *A. aspera* seeds has also modulated defense systems (Singh, Sharma, & Chakrabarti, 2013a, 2013b; Sharma, Singh, & Chakrabarti, 2015).

So far, impact of leaves of *A. aspera* in fish diet has not been studied. The mid-column feeder rohu *Labeo rohita* is a high valued carp and like other carps, disease problem is also reported in rohu. Therefore, an attempt has been made to assess the impact of the leaves and seeds enriched diets on the physiology of rohu; the impact on relative mRNA expressions of some immune related genes of rohu was also documented.

Materials and Methods

Diet Formulation

The leaves and seeds of *A. aspera* were harvested from the outdoor plant growing facility of Department of Zoology, University of Delhi. Leaves were collected; washed 3 times with tap water; 2 times with sterilized water and dried in sun light. The dried leaves were ground and passed through sieve (300 µ) to obtain fine crude powder. The ripen seeds were also collected, cleaned manually and ground for getting crude powder of seeds. The leaves were added at two concentrations: 2.5 (diet 1, D1) and 5.0 g/kg diet (diet 2, D2). The seeds powder was added at a rate of 5 g/kg diet (diet 3, D3). Control diet (diet 4, D4) was prepared without any supplementation (Table 1). In earlier study, the optimum dose of seeds was recommended as 0.5% in diet of fish (Chakrabarti & Srivastava, 2012). Based on this study, the present dose of 0.5% seed was selected. Pelleted diets (1 mm diameter) were prepared using Twin-screw extruder and pellets were oven dried. The composition of all four diets (AOAC, 1990) was analyzed.

Culture of Fish and Feeding Regime

Rohu fingerlings were obtained from a fish farm and acclimated in concrete tanks for 15 days. Then fingerlings (2.031 ± 0.481 g) were randomly distributed among four different feeding regimes. Twelve aquaria (60 L each) were used for the study (three aquaria per treatment) and the density was 10 fish/ aquaria. Fish were fed twice daily (9.00 a.m. and 5.00 p.m.) at the rate of 3% of body weight. The uneaten feed and feces were daily siphoned out from individual aquarium and uneaten feed was oven dried and measured with weighing balance.

A digital multi-parameter system (HQ 40d, Hach, USA) attached with specific probes for measurement of temperature, pH, dissolved oxygen, conductivity and nitrate-N was used to monitor water quality parameters. Ammonia-N was measured with ammonia-N electrode attached with electrochemistry meter (Orion VERSA STAR, Thermo Scientific, USA). The

Table 1. Test and control diets and proximate composition of prepared diets

Ingredient (g/kg diet)	Test diets			Control diet (D4)
	0.25% Leaves (D1)	0.5% Leaves (D2)	0.5% Seeds (D3)	
Fishmeal	651.6	651.6	651.6	651.6
Wheat flour	331.9	329.4	329.4	334.4
Cod liver oil	10.0	10.0	10.0	10.0
Vitamin and mineral complex	4.0	4.0	4.0	4.0
Leaves powder	2.5	5.00	-	-
Seeds powder	-	-	5.0	-
Proximate composition (g/100 g on dry matter basis)				
Moisture	11.09	10.03	9.43	7.14
Crude protein	37.05	36.98	37.02	36.96
Crude fat	9.24	7.82	8.31	8.64
Total carbohydrates	34.47	37.05	37.21	40.06
Ash	8.15	8.12	8.03	7.20
Crude fibre	6.8	6.5	4.4	4.1
Energy (kcal/100g)*	369.24	366.50	371.71	385.84

*Calculated digestible energy (Halver, 1976)

DE (kcal/100 g) = [(CP g/100 g × 4) + (CF g/100 g × 9) + (TC g/100 g × 4)]

standard methods were followed for nitrite and phosphate estimations (APHA, 2012). The water temperature, pH and conductivity were 27.16°C (27–30°C), 7.91 (7.80–8.13) and 629 µS/cm (565–667 µS/cm), respectively throughout the experiment. The level of dissolved oxygen was 6.2 mg/L (6.0–6.5 mg/L). The levels of ammonia-N, nitrate-N, nitrite-N and phosphate were 0.15 mg/L (0.08–0.27 mg/L), 1.25 mg/L (0.70–1.58 mg/L), 0.003 mg/L (0.001–0.008 mg/L) and 0.15 mg/L (0.02–0.21 mg/L) respectively, during the experimental period. The whole experiment was conducted following the guidelines of Animal Ethics Committee, Department of Zoology, University of Delhi (DU/ZOOL/IAEC-R/2015/08).

Preparation of Chicken RBC (c-RBC)

The chicken blood was collected in Alsever's solution (1:3) and stored at 4°C for 24 h. Then the settled c-RBC was washed thrice with phosphate buffer saline (1X PBS, pH 7.4). Then 20 mL of c-RBC was mixed with 80 mL of 1X PBS (pH 7.4) to make 100 mL of 20% (v/v) c-RBC solution. This solution was used for the immunization of fish.

Immunization of Fish and Sample Collection

After 45 days of initial feeding, fish from four treatments were anaesthetized using MS 222 (Sigma, USA); chicken RBC (c-RBC) solution (20 µL; 20%, v/v) was injected intraperitoneally using 26G × ½ syringe (0.45 × 13 mm). Fish were observed for any abnormal signs after immunization.

Rohu were sampled after immunization in the following sequence: on day-7, day-14 and day-21. On each sampling day, i.e. on days-7, 14 and 21, rohu (2 fish/ aquaria, 2 x 3 = 6/ treatment) were collected and anaesthetized with MS222. The weight of fish was measured individually; blood was collected through caudal vein puncture of each fish and kept at 4^{o}C overnight for clotting. The sample was centrifuged and the serum was stored at -20^{o}C for various assays. The tissue samples (hepatopancreas and anterior kidney) were collected after the collection of blood; tissues were used for enzymatic assays and gene expression study.

Growth Parameters

The weight of fish was measured on days-7, 14 and 21 after immunization using electronic balance (Shimadzu, AUX220, Japan). Specific growth rate (SGR) and condition factor (CF) were calculated using following formulae:

$$SGR\ \% = [\ln(\text{final weight}) - \ln(\text{initial weight})] \times 100/\text{experimental period (in days)}$$

$$CF = (\text{wet body weight} \times 100)/ (\text{total length})^{3}$$

Immunological Assays

The turbidimetric method was used for serum lysozyme assay (Siwicki, 1989). Briefly, in 10 µL serum, solution of *Micrococcus lysodeikticus* (100 µL) was added. The initial and final (after 60 min of incubation) absorbance were recorded at 450 nm in a Microplate reader (Synergy H1 Hybrid Reader, Biotek, USA). The enzyme activity was expressed as mg/mL. The standard was prepared with hen's egg lysozyme.

Myeloperoxidase activity was measured by mixing serum (10 µL) and Hank's balanced salt solution (90 µL) in a microplate (Quade & Roth, 1997). Then 35 µL of 3, 3',5,5'-tetramethyl benzidine hydrochloride and hydrogen peroxide (Genei, India) were added in each sample. After 2 min of incubation, 4 M H_2SO_4 (35 µL) was added to stop the reaction. The optical density was recorded at 450 nm.

Hemagglutination assay was performed with serum to check antigen-specific antibody titer. The 2% c-RBC solution (V/V), used for hemagglutination assay was prepared with phosphate buffer saline (pH 7.4). The 96-well plate (round bottom) was used for serial dilution of 50 µL serum with PBS (pH 7.4). The 50 µL of c-RBC solution (2%) was mixed to each sample. The whole mixture was allowed to stay for 1 h at room temperature and then kept at 4^{o}C. The hemagglutination reaction was observed in 96-well microplate and the reciprocal of highest dilution reflects the antigen specific antibody titer.

Nitric oxide synthase (NOS) activity of tissues was determined (Lee *et al*., 2003). The tissue (hepatopancreas/anterior kidney) was homogenized with 1 mL phosphate buffer (pH 7.4) and then centrifuged at 10,000 × *g* at 4^{o}C for 15 min. The supernatant (100 µL) was taken in a 96-well microplate, mixed with Griess reagent (100 µL). After 10 min, the optical density was measured at 540 nm and enzyme activity was given as µM/mg tissue.

Oxidation of Tissue Lipid and Protein

The oxidation of lipid and protein were assayed in both hepatopancreas and anterior kidney separately. The tissue (1 g) was washed with 9 mL of chilled potassium chloride (1.15%) and homogenized with 900 µL of chilled 1.15% KCl for lipid peroxidation assay (Ohkawa, Ohishi, & Yagi, 1979). Then 25 µL homogenate was taken in 2 mL micro-centrifuge tube and mixed with 25 µL of sodium dodecyl sulphate (8.1%), 187.5 µL of 20% acetic acid (pH 3.5) and 187.5 µL of thiobarbituric acid (0.8%). Then distilled water was added to make the total volume of reaction mixture 0.5 mL; it was incubated for 1 h at 95°C in water bath. The sample was centrifuged for 15 min at 800 x *g* at 4°C. The absorbance of supernatant was measured at 532 nm and the concentration of thiobarbituric acid reactive substances was given as µmol MDA/mg tissue.

Protein oxidation was measured in the form of carbonyl protein in tissues (Lenz, Costabel, Shaltiel, & Levine, 1989). The tissue (100 mg) was homogenized with 1 mL of 50 mM potassium phosphate buffer (pH 7.0) that contains Ethylene Diamine TetraAcetic Acid (0.5 mM) and phenyl methylsulfonyl fluoride (100 µM). The homogenate (250 µL) was mixed with 0.5 mL of trichloro acetic acid (10%); then centrifuged at 13,000 x *g* for 5 min. The pellet was mixed with 1 mL of 10 mM dinitrophenyl hydrazine and kept at room temperature for 1 h. Then centrifuged at 13,000 x *g* for 5 min and the pellet was washed thrice with 1 mL of ethanol-butylacetate (1/1, v/v). The pellet was completely dissolved with 6 M guanidine hydrochloride and again centrifuged. The absorbance of supernatant was recorded at 370 nm. The activity was quantified as nanomoles/mg protein using 22×10^3/M/cm molar extinction coefficient.

Relative mRNA Expression Study

The expressions of various immune related genes *viz.* lysozyme C, lysozyme G, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 10 (IL-10) were studied for both hepatopancreas and anterior kidney. The total RNA extraction was carried out in TRIzol™ (Invitrogen™, USA) using the protocols provided by manufacturer. The RNA concentration was assayed with Nanodrop Spectrophotometer (ND-1000, Thermo Scientific, USA). The RNA samples with 260/280 ratio of ~2.0 was used for cDNA preparation. RNA (1 µg) was mixed with DNase I enzyme (Amplification grade, 1 U, Sigma- Aldrich, USA). It removed the genomic DNA contamination if present in the RNA. In 1% agarose gel, the quality and purity of RNA was estimated. The cDNA Reverse Transcription kit (Applied Biosystems, USA) was used to reverse transcribe the RNA into cDNA as per manufacturer's protocol. The PCR amplification with β-actin primers and prepared cDNA template established the quality of cDNA synthesis.

The SYBR™ Green Master Mix (Applied Biosystems) was used for relative mRNA quantification of different genes in Quant Studio 6 Flex machine (Applied Biosystems). The primer-BLAST, an online tool of NCBI-NIH was used for designing specific primers (Table 2). Each primer was run with serially diluted cDNA template. Then standard curve was prepared and melt curve was analyzed to check the efficiency of primers. The reaction mixture (10 µL) consisted of 5 µL of 2 × SYBR™ Green Master Mix, 0.25 µL of each gene specific Forward primer as well as Reverse primer (Both 2.5 µmol), 1 µL cDNA template (dilution 1/3) and 3.5 µL of water (nuclease-free). The 40 cycles were run using software v1.1. The details of one cycle were as follows: pre-denaturation at 95°C for 10 min, followed by denaturation at 95°C for 15s and then annealing and extension at 60°C for 1 min. The reaction specificity was evaluated using melt curve analysis. The details of melt curve plot were: 95°C - 15s, 60°C - 1 min and 95°C - 15s. The gene expression results were estimated using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) after verification of 100% primer amplification efficiency. Duplicate samples were run in 96-well plate (Applied Biosystems) with each target gene. The efficiency of reaction was also checked with duplicate non-template control. The house keeping

Table 2. Real-time PCR primer pairs for reference and target genes and their efficiency and slope values calculated in Quant Studio 6 Flex Real Time PCR system (Applied Biosystems, USA)

Target gene	Primer sequence (5'-3')	Accession number/reference	Efficiency (%)	Slope
Lysozyme C	**F-** CGATGATGGCACTCCAGGT	EF203085.1	99.079	-3.344
	R- CATGCTTTCAGTCCTTCGGC			
Lysozyme G	**F-** CAATGGCTTTGGCCTCATGC	KC934746.1	95.477	-3.435
	R- CACGTGGGAAACTTTGCTCTG			
TNF-α	**F-** GGCGGCTTGAAAGTAGTGGA	FN543477.1	97.660	-3.379
	R- TATGCAGAACGTCGTGGTCC			
IL-1β	**F-** GTACCCCACAAAACATCGGC	AM932525.1	93.037	-3.501
	R- CAAGAGCAGTTTGGGCAAGG			
IL-10	**F-** GCTCAGTGCAGAAGAGTCGAC	Banerjee, Mitra, Purohit, Mohanty, & Mohanty, 2015	92.143	-3.526
	R- CCCGCTTGAGATCCTGAAATATA			
β-actin	**F-** GACTTCGAGCAGGAGATGG	Mohanty & Sahoo, 2010	94.160	-3.470
	R- CAAGAAGGATGGCTGGAACA			

Table 3. Effect of *A. aspera* leaves and seeds enriched diets on the growth performances of rohu fingerlings. Means (± SE) sharing different letters in the same column are significantly (P<0.05) different (n = 3)

	Final Weight (g)			Specific Growth Rate (%)			Condition Factor (CF)		
	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days
D1	5.30 ± 0.13^{c}	5.98 ± 0.03^{c}	6.57 ± 0.09^{c}	1.85 ± 0.05^{c}	1.78 ± 0.01^{c}	1.80 ± 0.02^{c}	1.77 ± 0.02^{c}	1.80 ± 0.01^{c}	1.83 ± 0.02^{c}
D2	5.98 ± 0.30^{b}	6.88 ± 0.05^{b}	7.48 ± 0.09^{b}	2.08 ± 0.10^{b}	2.08 ± 0.01^{b}	1.99 ± 0.02^{b}	1.89 ± 0.01^{b}	1.86 ± 0.02^{b}	1.91 ± 0.03^{b}
D3	6.57 ± 0.10^{a}	8.27 ± 0.19^{a}	9.23 ± 0.02^{a}	2.27 ± 0.03^{a}	2.38 ± 0.04^{a}	2.29 ± 0.01^{a}	1.94 ± 0.01^{a}	1.92 ± 0.01^{a}	1.97 ± 0.01^{a}
D4	4.32 ± 0.05^{d}	4.93 ± 0.08^{d}	5.50 ± 0.12^{d}	1.46 ± 0.02^{d}	1.51 ± 0.03^{d}	1.52 ± 0.03^{d}	1.82 ± 0.03^{c}	1.81 ± 0.02^{c}	1.80 ± 0.02^{c}

D1: 0.25% leaves, D2: 0.5% leaves, D3: 0.5% seeds and D4: control

The interactive effect of diets and sampling days is significant (P<0.05) for final weight and non-significant (P>0.05) for specific growth rate (%) and condition factor of rohu.

gene β-actin was also run with the same cDNA template and used for normalization of samples.

Statistical Analysis

The results were presented as means ± SE. The normality of the data was evaluated using Shapiro-Wilk test (1965). Data were analyzed using one-way analysis of variance with Duncan's multiple range test (Montgomery, 1984). The two-way analysis of variance was performed for growth parameters. The statistical software IBM SPSS version 24 was used for analysis. The significance was considered at P<0.05 level.

Results

Growth Performance

There was no mortality of fish cultured in different treatments. Rohu fed with D3 diet showed significantly (P<0.05) higher final weight and SGR% compared to rohu fed with other diets on days-7, 14 and 21after immunization (Table 3). The final weight was lowest in diet D4 fed rohu. The final weight of fish increased with the culture period regardless of treatment. Significantly (P<0.05) higher condition factor was observed in rohu fed with D3 diet compared to the other diets fed fish on days-7, 14 and 21 after immunization (Table 3). There was no significant (P>0.05) difference between D1 and D4 diets fed rohu on days-7, 14 and 21 after immunization.

Immunological Assays

Serum lysozyme level was significantly higher in D3 diet fed rohu on days-7 and 21, and on day-14, in D3 and D2 treatments (Table 4). There was no significant difference in lysozyme level in rohu fed with 0.5% leaves and seeds enriched diets on day-14 after immunization. The highest lysozyme level (P<0.05) was found on day-14 after immunization in all diets, except D4. In D3 diet fed rohu, myeloperoxidase level was significantly higher compared to rohu fed with other diets throughout the experimental period. Rohu fed with D2 and D3 diets showed the highest myeloperoxidase activity on day-21 after immunization; in D1 and D4 diets, the highest levels were observed on day-7 after immunization. Significantly higher (P<0.05) hemagglutination titer level was found in rohu fed with D3 diet compared to other groups. The hemagglutination titer level was maximum on day-14 after immunization regardless of feeding regimes.

Significantly higher (P<0.05) nitric oxide synthase level was found in hepatopancreas of rohu fed with 0.5% seeds (D3) and 0.5% leaves (D2) enriched diets compared to rohu fed with D1 and D4 diets on day-7

Table 4. Lysozyme, myeloperoxidase and hemagglutination titer levels found in rohu fingerlings fed with enriched diets. Means (±SE) sharing different letters in the same column are significantly (P<0.05) different (n = 3)

	Serum lysozyme (mg/mL)			Myeloperoxidase (OD at 540 nm)			Hemagglutination titer		
	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days
D1	1.55±0.66^{c}	11.51±3.30^{b}	3.88±1.49^{c}	2.06±0.15^{b}	1.72±0.13^{b}	2.03±0.06^{c}	3.33±0.67^{c}	21.33±5.33^{b}	13.33±2.67^{b}
D2	3.35±0.80^{b}	22.05±1.38^{a}	8.33±0.43^{b}	2.18±0.04^{b}	1.87±0.10^{b}	2.42±0.19^{b}	5.33±1.33^{b}	26.67±5.33^{b}	13.33±2.67^{b}
D3	4.36±1.14^{a}	23.44±2.54^{a}	14.05±1.93^{a}	2.35±0.13^{a}	2.55±0.13^{a}	2.70±0.22^{a}	10.67±2.67^{a}	53.33±10.67^{a}	26.67±5.33^{a}
D4	0.73±0.56^{d}	1.11±0.48^{c}	1.85±0.47^{d}	1.83±0.14^{c}	1.06±0.23^{c}	1.59±0.08^{d}	2.67±0.67^{c}	13.33±2.67^{c}	6.67±1.33^{c}

D1: 0.25% leaves, D2: 0.5% leaves, D3: 0.5% seeds and D4: control

The interactive effect of diets and sampling days is significant (P<0.05) for serum lysozyme and non-significant (P>0.05) for Myeloperoxidase and hemagglutination titer of rohu.

Table 5. Nitric oxide synthase, thiobarbituric acid reactive substances (TBARS) and carbonyl protein levels in hepatopancreas and anterior kidney of rohu fingerlings fed with enriched diets. Means (± SE) sharing different letters in the same column are significantly (P<0.05) different (n = 3)

	Hepatopancreas								
	Nitric oxide Synthase (µmol/mg tissue)			TBARS (mmol MDA/mg protein)			Carbonyl protein (nmol/mg protein)		
	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days
D1	1.61±0.06^{b}	3.93±0.24^{c}	1.29±0.11^{c}	8.15±1.25^{b}	12.84±1.21^{b}	8.15±0.48^{b}	15.59±0.69^{b}	8.29±0.56^{b}	11.53±1.35^{b}
D2	5.66±0.70^{a}	5.89±0.27^{b}	2.14±0.33^{b}	7.11±1.49^{b}	5.28±1.43^{c}	6.91±0.52^{c}	3.48±0.23^{c}	5.10±1.27^{c}	7.23±0.60^{c}
D3	5.88±0.89^{a}	12.40±2.53^{a}	3.53±0.19^{a}	4.79±0.26^{c}	1.43±0.53^{d}	5.98±0.90^{c}	2.80±0.59^{c}	3.17±0.25^{d}	2.69±0.36^{d}
D4	1.47±0.16^{b}	2.43±0.38^{d}	0.95±0.01^{d}	10.82±0.49^{a}	25.93±1.06^{a}	11.51±0.94^{a}	28.37±4.41^{a}	22.02±3.39^{a}	20.68±0.38^{a}
	Anterior Kidney								
	Nitric oxide synthase (µmol/mg tissue)			TBARS (mmol MDA/mg protein)			Carbonyl protein (nmol/mg protein)		
	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days
D1	0.72±0.06^{c}	1.23±0.07^{c}	0.68±0.05^{c}	13.48±0.40^{b}	24.99±2.02^{b}	7.80±0.60^{b}	7.82±1.67^{b}	14.83±0.67^{b}	15.20±1.27^{b}
D2	1.05±0.04^{b}	1.71±0.34^{b}	0.96±0.02^{b}	8.59±1.42^{c}	15.85±0.81^{c}	5.48±0.52^{c}	4.80±0.35^{c}	9.65±0.30^{c}	11.09±0.55^{c}
D3	2.01±0.05^{a}	2.24±0.06^{a}	2.19±0.08^{a}	5.43±0.65^{d}	10.77±1.29^{d}	4.54±0.71^{c}	3.14±0.24^{d}	6.41±0.18^{d}	3.12±0.60^{d}
D4	0.40±0.06^{d}	0.90±0.04^{d}	0.46±0.05^{d}	22.72±1.25^{a}	42.77±1.27^{a}	9.28±0.84^{a}	19.47±1.86^{a}	22.55±0.29^{a}	37.33±2.97^{a}

D1: 0.25% leaves, D2: 0.5% leaves, D3: 0.5% seeds and D4: control

The interactive effect of diets and sampling days is significant (P<0.05) for nitric oxide synthase in hepatopancreas, TBARS and carbonyl protein in hepatopancreas and anterior kidney, and non-significant (P>0.05) for nitric oxide synthase in anterior kidney.

after immunization (Table 5). There was no significant difference (P>0.05) between D3 and D2 diets, and between D1 and D4 diets on this day. The level increased significantly (P<0.05) on day-14 after immunization and then decreased on day-21 after immunization in all diets. In anterior kidney, significantly (P<0.05) higher nitric oxide synthase level was found in rohu fed with D3 diet compared to other diets fed rohu on days- 7, 14 and 21 after immunization.

Oxidation of Tissue Lipid and Protein

The study of thiobarbituric acid reactive substances (TBARS) of hepatopancreas and anterior kidney represented the tissues lipid peroxidation status (Table 5). On day-7 and day-14 after immunization, the TBARS level was significantly (P<0.05) lower in hepatopancreas and anterior kidney of rohu fed with D3 diet as compared to other diets. Whereas on day-21 after immunization, significantly (P<0.05) lower level of TBARS was observed in hepatopancreas and anterior kidney of rohu fed with both D2 and D3 diets as compared to other diets fed fish. In hepatopancreas and anterior kidney, carbonyl protein levels were significantly (P<0.05) lower in rohu fed with D3 diet compared to other diets throughout the experimental period, except on day-7 after immunization in hepatopancreas of fish. The highest carbonyl protein levels were recorded in hepatopancreas and anterior kidney of D4 diet fed rohu on days-7 and after immunization, respectively.

Relative mRNA Expression

In the hepatopancreas and anterior kidney, the expressions of various immune related genes were recorded on days-7, 14 and 21 after immunization. Lysozyme C and G expressions were significantly

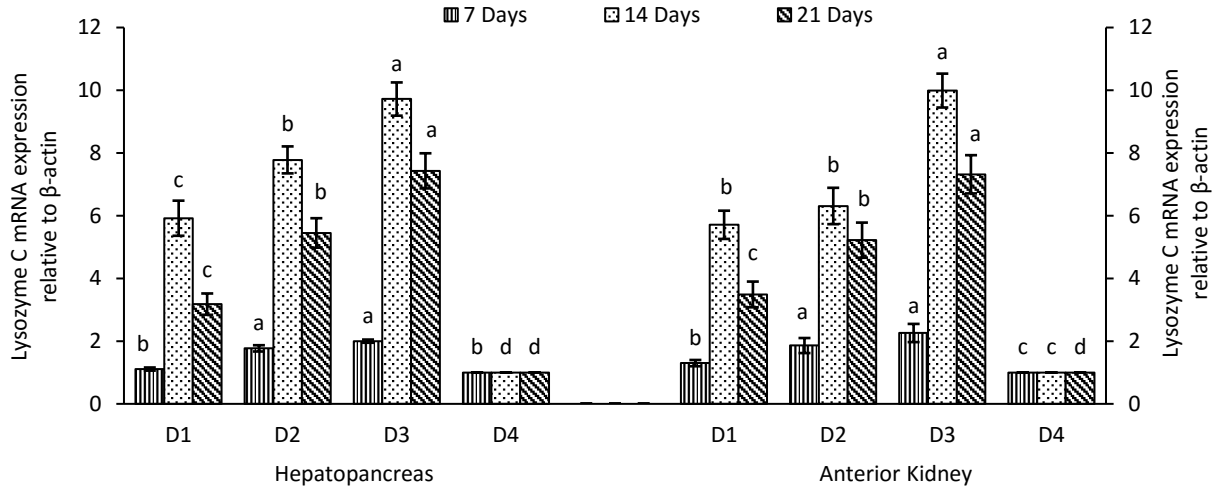

Figure 1. The relative mRNA expressions of lysozyme C in hepatopancreas and anterior kidney of rohu fingerlings fed with enriched diets. Bars with different superscripts are significantly (P<0.05) different (n = 3).

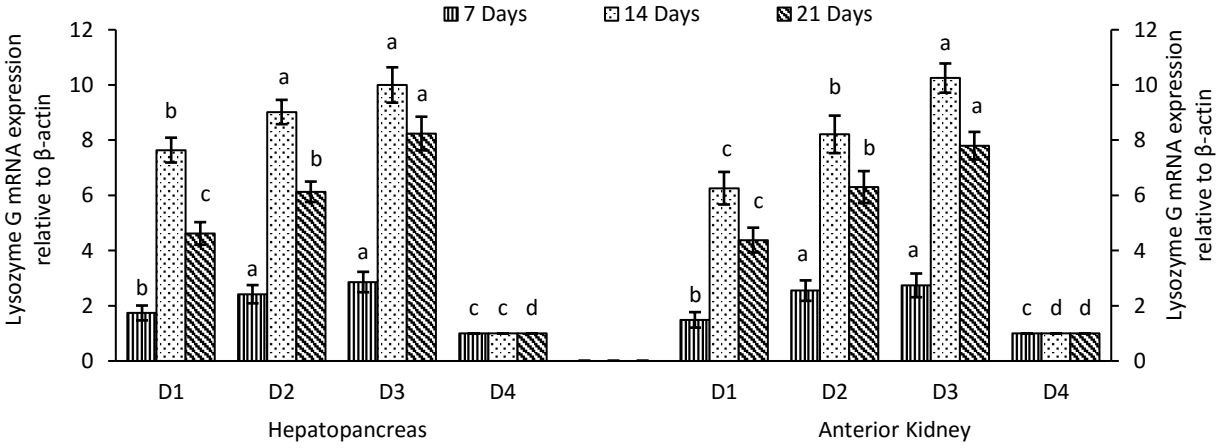

Figure 2. The relative mRNA expressions of lysozyme G in hepatopancreas and anterior kidney of rohu fingerlings fed with enriched diets. Bars with different superscripts are significantly (P<0.05) different (n = 3).

(P<0.05) higher in hepatopancreas and anterior kidney of rohu fed with enriched diets compared to the rohu fed with control diet (D4) on days-7, 14 and 21 after immunization (Figures 1 & 2). The expression was significantly (P<0.05) higher in D3 diet fed rohu compared to others. The highest expression was found on days-14 after immunization in all diets.

In hepatopancreas, TNF-α and IL-1β expressions were significantly up-regulated (P<0.05) in rohu fed with leaves and seeds supplemented diets compared to the control diet (D4) fed rohu on days-7, 14 and 21 after immunization. The expression was significantly (P<0.05) higher in D3 diet fed rohu on days-7 after immunization. The level of expression gradually decreased with the duration of trial (Figures 3 & 4). In anterior kidney, the expressions of TNF-α and IL-1β genes were down-regulated in rohu fed with leaves and seeds supplemented diets compared to the control diet (D4) fed rohu on days-7, 14 and 21 after immunization.

In leaves and seeds enriched diets fed rohu, significant (P<0.05) down-regulation and up-regulation of IL-10 gene were recorded in hepatopancreas and anterior kidney, respectively compared to the rohu fed with D4 diet on days-7, 14 and 21 after immunization

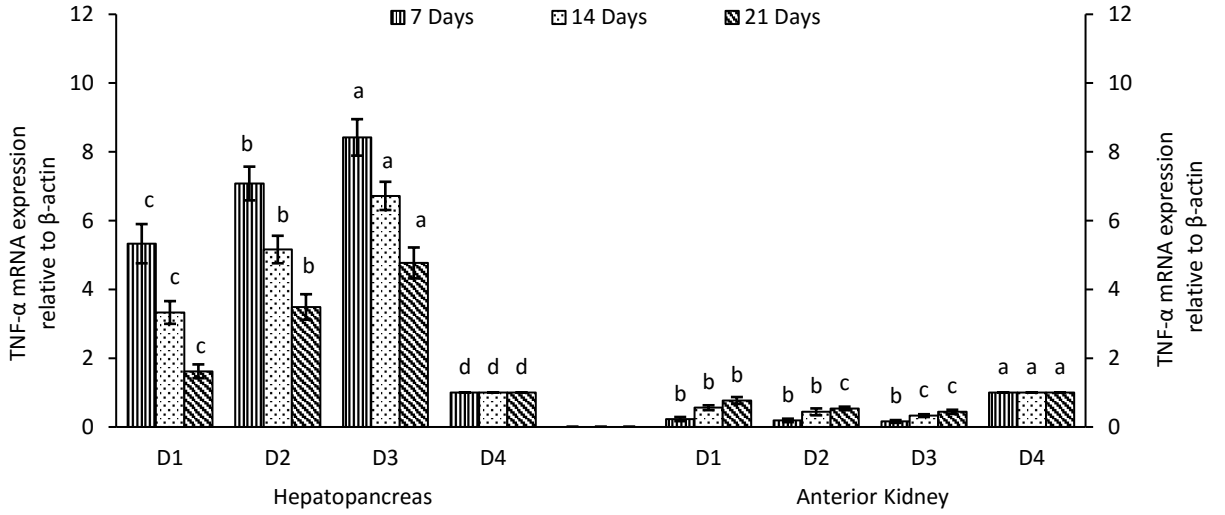

Figure 3. The relative mRNA expression level of TNF-α in hepatopancreas and anterior kidney of rohu fingerlings fed with enriched diets. Bars with different superscripts are significantly (P<0.05) different (n = 3).

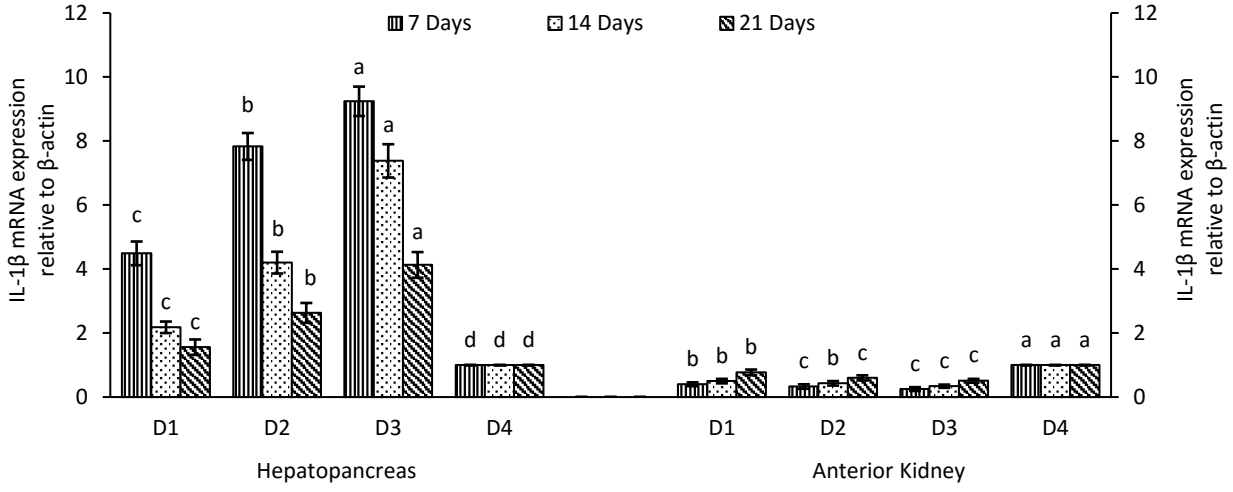

Figure 4. The relative mRNA expression of IL-1β in hepatopancreas and anterior kidney of rohu fingerlings fed with enriched diets. Bars with different superscripts are significantly (P<0.05) different (n = 3).

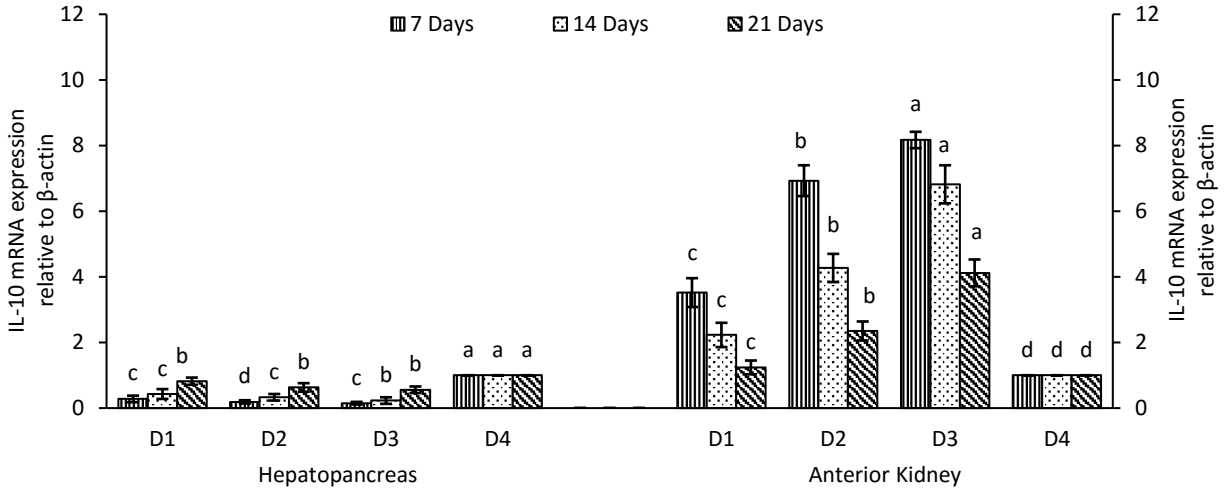

Figure 5. The relative expression of IL-10 in hepatopancreas and anterior kidney of rohu fed with enriched diets. Bars with different superscripts are significantly (P<0.05) different (n = 3).

(Figure 5). In anterior kidney, the IL-10 expression was maximum on day-7 after immunization and then decreased in all diets.

Discussion

The enrichment of diets with leaves and seeds of *A. aspera* influenced the physiology and enhanced the growth of rohu fingerlings. The positive impact of both leaves and seeds at 0.5% level was found in the present study with rohu. Growth rate was higher in all enriched diets fed rohu compared to the control diet fed rohu. Earlier study reported the increased the growth in seeds and different fractions of seeds supplemented diets fed common carp (Chakrabarti *et al.,* 2012). The enhanced growth was also observed in Nile tilapia; *Oreochromis niloticus* fed with *Basella alba* leaf extract supplemented diets (Chakraborty, Molnár, Ardó, Jeney, & Hancz, 2015). The improved condition factor was also observed in rohu fed with *A. aspera* leaves and seeds incorporated diets. Similar improved condition factor was also observed in rainbow trout (*Oncorhynchus mykiss*) when fed with *Coriandrum sativum* extract incorporated diets (Farsani *et al.,* 2019).

The present study showed that feeding of enriched diets improved the immune system of rohu. Lower levels of lysozyme, myeloperoxidase, nitric oxide synthase and hemagglutination titer were found in control group compared to the enriched diets fed rohu. *A. aspera* seeds showed better result compared to the leaves. The biochemical composition, especially presence of different amino acids and poly unsaturated fatty acids (PUFA) improved the health status of the fish. The supplementation of 0.5% leaves gave better results compared to 0.25% in rohu; the former dose may be optimum for this size group of rohu. *A. aspera* seeds containing phenolic compounds, linolenic and oleic acids and leaves containing flavonoids may influence the immune system and resulted in better performances of rohu (Chakrabarti *et al*., 2012; Priya, Kumar, Karthik, & Rao, 2012). Many studies showed the influence of various herbs and their extracts in fishes, *viz*. ethanolic extract of grass, *Cynodon dactylon* (Kaleeswaran, Ilavenil, & Ravikumar, 2011) in catla, *Euphorbia hirta* leaf extract in common carp (Pratheepa & Sukumaran, 2014) and *Azadirachta indica* leaf extract in Asian seabass *Lates calcarifer* (Talpur & Ikhwanuddin, 2013) enhanced lysozyme levels. Lysozyme shows bactericidal activity and thereby plays significant role in fish innate immune system. Higher myeloperoxidase activity in enriched diets fed rohu confirmed the immunostimulatory property of the ingredients. The dietary supplementation of essential oil of *Citrus limon* peels (Baba, Acar, Öntaş, Kesbiç, & Yılmaz, 2016) and *Nyctanthes arbortristis* seeds (Kirubakaran, Subramani, & Michael, 2016) enhanced myeloperoxidase levels in tilapia *O. mossambicus*. Myeloperoxidase is a specific hem containing enzyme and has significant role in the defense of organisms (Yeh & Klesius, 2013).

Lysins, C-reactive proteins and agglutinins are important molecules and are very effective before the onset of specific immune responses. Agglutinins help in removing bacterial infections. Enhanced hemagglutination titer in enriched diets fed rohu was recorded in the present investigation. According to Sheikhzadeh, Nofouzi, Delazar, and Oushani (2011), green tea *Camellia sinensis* extract influenced the hemagglutination titer level in rainbow trout. The nitric oxide synthase plays an important role in inflammatory process. The higher nitric oxide synthase level was always recorded in enriched diets fed rohu. Earlier study also reported that feeding of *A. aspera* seeds incorporated diets enhanced the nitric oxide synthase level in catla (Chakrabarti, Srivastava, Verma, & Sharma, 2014).

This study showed that feeding of rohu with enriched diets gave protection against oxidation of lipid and protein as lower levels of TBARS and carbonyl

protein were found in the anterior kidney and hepatopancreas. Seeds provided better protection against oxidation of lipid and protein. Addition of *Aloe vera* in diet of GIFT-tilapia reduced lipid peroxidation (Gabriel *et al*., 2015). Proteins are also susceptible for reactive oxygen species attack. The accumulation of carbonyl groups is an early indication of tissue damage (Stadtman & Oliver, 1991). The lower level of carbonyl protein in enriched diets fed rohu showed the role of plant in the prevention of oxidative stress. Reyes-Cerpa *et al*. (2018) found that plant extract (Saint John's wort, lemon balm, and rosemary) supplemented diet reduces the proteins carbonylation in Atlantic salmon *Salmo salar*.

Besides enhancing the innate immunity of rohu, this study also demonstrated that enriched diets played significant role in immune-related gene expression. The up-regulation of lysozyme C and lysozyme G genes was recorded in enriched diets fed rohu. The up-regulation of lysozyme genes also supported the increased activity of serum lysozyme in this study. Similar up-regulation of lysozyme C was observed in leucine enriched diet fed rohu (Giri *et al*., 2015a); lysozyme G up-regulation was found in polysaccharide enriched diet fed *L. bata* (Sahoo, Parhi, Debnath, & Prasad, 2017).

The expression of pro-inflammatory cytokine, TNF-α is an indicator of occurrence of inflammatory process (Secombes *et al*., 2001). According to Low, Wadsworth, Burrells, and Secombes (2003), IL-1β played significant role in tissue injury and inflammation; it induced a chain of reactions through lymphocyte activation and induced release of other cytokines. The up-regulation of TNF-α and IL-1β genes were found in hepatopancreas of rohu fed with enriched diets and both these genes down-regulated in anterior kidney. Co-expression of both these cytokines TNF-α and IL-1β is possible because these have similar functions of immune response initiation (Mulder, Wadsworth, & Secombes, 2007). Earlier study showed similar results, like levan (Gupta *et al*., 2018) and guava leaves (Giri *et al*., 2015b) supplemented diets in rohu influenced pro-inflammatory cytokines.

In fish, IL-10s mainly inhibit the synthesis of pro-inflammatory cytokines (Evenhuis & Cleveland, 2012). The down-regulation of IL-10 gene was observed in hepatopancreas and up-regulation was recorded in anterior kidney of rohu. In anterior kidney, TNF-α and IL-1β genes were down-regulated and IL-10 gene was up-regulated i.e. inverse relationship was observed between hepatopancreas and anterior kidney. This showed the specificity of tissue. Feeding of rohu with banana peels supplemented diets showed the similar responses of genes (Giri, Jun, Sukumaran, & Park, 2016).

Conclusions

The present study provides information on growth promoting, immunostimulatory and stress reducing properties of *A. aspera* leaves and seeds supplemented diets. The mRNA expression of various immune related genes also supports the physiological study. A dietary inclusion of leaves/seeds at 0.5% level is recommended.

Acknowledgement

This study was supported by Department of Biotechnology (DBT), Ministry of Science & Technology, New Delhi in the form of a research project to RC and JGS. AS is thankful to Department of Science and Technology (DST), Ministry of Science & Technology, New Delhi for providing him INSPIRE fellowship (IF150474).

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