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Himalayan lapsi, *Choerospondias axillaris* (Roxb.) enhances concentration of vitamin C in tissues of rohu (*Labeo rohita H*) cultured at Chitwan (Nepal)

Abstract

Labeo rohita lacks the enzyme for endogenous synthesis of vitamin C and lapsi fruits are rich in vitamin C. A study was conducted to examine the concentration of vitamin C in the blood serum, brain and liver of *L. rohita* through lapsi fruits extract supplemented in the diets. Six groups of *L. rohita* were fed experimental diets containing lapsi fruits extract supplemented at 0 mg kg^{-1} (D1), 100 mg kg^{-1} (D2), 200 mg kg^{-1} (D3), 400 mg kg^{-1} (D4), 800 mg kg^{-1} (D5) and 1600 mg kg^{-1} (D6) for 90 days. Growth parameters (WG, SGR and FCR) and Vitamin C concentration in blood serum, brain and liver were evaluated during the experimental trial. Carps fed with a lapsi fruits extract supplemented diet showed higher specific growth rate (SGR) compared with control diet fed carps. Results from this study help to establish the beneficial effect of vitamin C rich lapsi fruits on growth and immunmodulation in rohu. It can be concluded that lapsi fruits extract supplemented diet can be used to improve the immune system of *L. rohita* as indicated by enhancement of vitamin C in the serum, brain and the liver.

Keywords: growth, vitamin c, brain, liver, serum, *choerospondias axillaris, labeo rohita*

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Introduction

Himalayan lapsi, *Choerospondias axillaris* (Roxb.) is native to Nepal and is also reported from south-east Asian countries.[1] Its fruits containing vitamin C,[2] Phenol and flavonoid compounds[3,4] are consumed to enhance the immunity[5] and neutralize free radicals formed in the body. Vitamin C is required to form collagen, growth, reproduction, resist diseases and for immunity in many fishes.[6] Oxygen present in air, high temperature, enzymes and multivalent cations destroy it. In the manufacturing process and storage of diet Vitamin C supplemented in it is lost.[7] Many structural and functional abnormalities result in fishes due to insufficient supply of vitamin C.[8] Teleost fishes like rohu lacking GLO enzyme[9] needs supply of vitamin C along with the diet.[10] Many researches on the effect of vitamin C on growth, its concentration in different tissues and stress overcome in fishes are available.[11] But the work on the effect of lapsi extract on growth and its concentration in brain, liver and blood in *L. rohita* is not available.

Materials and methods

Experimental design and set up

About four hundred farm-raised fingerlings of *Labeo rohita* (3.2 ±0.014 g) were selected from the nursery pond and transferred them to the stocking pond for their proper acclimatization. Altogether six test diets D1 (0), D2 (100), D3 (200), D4 (400), D5 (800) and D6 (1600) were prepared along with other standard ingredients (Table 1). Eighteen rectangular nylon happas (1m×1.5m×1m) were suspended in the experimental pond with ropes and bamboos. Two hundred seventy fingerlings of rohu (2.32±0.017 cm and 3.43±0.113 g) were selected and distributed in six treatment groups in triplicates. Fingerlings were fed with test and control diets at the rate of 3% of their body weight at 9 a.m. and 4 p.m for 90 days. Temperature was maintained between 25°C to 29°C and pH between 7.53 to 7.92 during experimental period. Every two weeks 5 fingerlings each happa were selected randomly and weighed to adjust the amount of feed to be given. The experiment was conducted in the ponds of Corona of Agriculture in Chitwan (Nepal).

Preparation of ethanol extract of lapsi fruits

The ethanol extract of the pulp of lapsi fruits was made by using 70% ethanol.[4] 10 g of lapsi fruit powder was mixed with 500 ml of 70 % ethanol in a conical flask. The flask was sealed by cotton plug and aluminum foil and then kept in orbital shaker for 48 hrs. The mixture was then filtered. The filtrate was centrifuged at 10,000 × g for 5 minutes. The supernatant obtained was concentrated at 70 °C in water bath. Finally, a greasy substance (crude extract) of the lapsi fruit pulp was obtained and transferred to screw-cap bottle and stored at 4°C for future use.

Formulation of feed and preparation of lapsi fruit extract supplemented diets

One control diet D1 and five treated diets D2, D3, D4, D5 and D6 were prepared. The treated diets were supplemented with 100, 200, 400, 800 and 1600 mg kg^{-1} lapsi fruit extracts respectively. Other standard ingredients were used during feed preparation (Table 1).

Examination procedures

Growth measurements and survival

Fingerlings were harvested after 24 hours of fasting. Final length and final weight of each individual carp were measured. Length gain (%), weight gain (%), specific growth rate (SGR), feed conversion ratio (FCR) and survival per cent were determined as follows:

$$LG(\%) = \text{Final length} - \text{Initial length} / \text{Initial length} \times 100$$

$$WG(\%) = \text{Final weight} - \text{Initial weight} / \text{Initial weight} \times 100$$

$$SGR = \left(\left(\text{In Wf} - \text{In Wi}\right)\right) / t \times 100$$

Where, 'Wi' and 'Wf' are the initial and final body weights and 't' the experimental days.

$$FCR = F / \left(Wf - Wo\right)$$

Where 'F' is the weight of food supplied to fish during the experimental period; 'W_o' is the weight of live fish at the beginning of the experimental period; 'Wf_{is}' the weight of live fish at the end of the experiment.

$$\text{Survival}\left(\%\right) = Nf \ / \ Ni \times 100$$

Where 'Nf 'is the number of fish harvested and 'Ni' the initial number of fish.

Blood and tissues collection

Three fish were collected at random from each replicate of control and treated groups on 90th day of the experiment. They were anaesthetized by 5 mg L^{-1} of MS-222 for 2-3 minutes. Blood samples were drawn from the caudal vein in sample collecting tubes (2 ml) without ethylene diamine tetra acetic acid (EDTA) by a syringe with 25 gauge needle. The tubes were centrifuged for 5 min at 3000 ×g and the supernatant serum was collected and stored at -20°C for future use. Fingerlings were dissected properly to obtain tissues of brain and liver. The collected tissues were place in Eppendort tubes containing buffer and stored at -4ºC for further use.

Vitamin C estimation in blood serum, brain and liver

Vitamin C in the blood serum, tissues of brain and liver were estimated according to the method described by.[12] Pre-weighed brain and liver tissues were homogenized in ice-cold 250 mM $HClO_4$ containing 5% trichloro acetic acid (TCA) and 0.08% EDTA. The homogenates were centrifuged at 27000 g for 30 min at 4ºC. 25 µl of 0.2% dichloro phenolindo phenol (DCIP) were added to the 250 µl of deproteinised samples. The same amount was added to a blank and then the mixtures were incubated at 37°C for 1 hour. After that 25 µl of 1% $KBrO_3$ were added and mixtures were incubated at 37°C for further 1 hour. Then 250 µl of 2% thiourea in 5% meta-phosphoric acid was added followed by an equal volume of 2% of 2, 4-dinitro phenyl hydrazine (DNPH) in 12 M H_2SO_4. All samples were incubated for 3 hour at 60°C after which 0.5 ml of ice-cold 18 M H_2SO_4 were added. The samples were transferred into Eppendorf tubes and centrifuged at 11300 g for 3 minutes. The absorbance was recorded at 524 nm with a spectrophotometer. Standard (20-200 µg/ml) were prepared with vitamin C (l-ascorbic acid, HiMedia).

Statistical analysis

Value for each parameter measured has been expressed as mean ± standard error of mean. One-way Analysis of Variance (ANOVA) was used to analyze the data followed by Duncan's Multiple Range Test[13] to find the difference at 5% ($P<0.05$) level.

Table 1 Composition of experimental diets (%) showing various ingredients

Ingredients (g/100g)	Experimental diets (% Inclusion) g/kg					
	D1	D2	D3	D4	D5	D6
Fish Meal†	29.31	29.31	29.31	29.31	29.31	29.31
Soya Meal‡	14.52	14.52	14.52	14.52	14.52	14.52
Groundnut oil cake†	9.17	9.17	9.17	9.17	9.17	9.17
Rice Powder†	14.16	14.16	14.16	14.16	14.16	14.16
Wheat Flour†	14.43	14.43	14.43	14.43	14.43	14.43
Corn flour†	11.37	11.37	11.37	11.37	11.37	11.37
Sunflower oil†	3	3	3	3	3	3
Cod liver oil†	2	2	2	2	2	2
Vitamin & Mineral Premix§	1	1	1	1	1	1
C. axillaris extract†	0	0.01	0.02	0.04	0.08	0.16
Betain Hydrochloride††	0.02	0.02	0.02	0.02	0.02	0.02
BHT(Butylated hydroxytoluene)††	0.02	0.02	0.02	0.02	0.02	0.02
CMC (Carboxymethyl cellulose) ††	1	0.99	0.98	0.96	0.92	0.84
Total	100	100	100	100	100	100

†Ingredients like fish meal, soya meal, groundnut oil cake, rice powder, wheat flour, corn flour, sunflower oil and Cod Liver Oil were procured from local market of Kathmandu Valley.

‡Ruchi Soya Industries, Raigad, India.

§Composition of vitamin mineral mix (EMIX PLUS) (quantity 2.5kg $^{-1}$)

Vitamin A 55,00,000 IU;Vitamin D_3 11,00,000 IU;Vitamin B_2 2,000 mg;Vitamin E 750 mg;Vitamin K 1,000 mg;Vitamin B_6 1,000 mg;Vitamin B_{12} 6 µg; Calcium Pantothenate 2,500 mg; Nicotinamide 10 g; Choline Chloride 150 g; Mn 27,000 mg; I 1,000 mg; Fe 7,500 mg; Zn 5,000 mg; Cu 2,000 mg; Co 450 mg; Ca 500 g; P 300g; L- lysine 10 g; DL-Methionine 10 g; Selenium 50 mgl^{-1}; Selenium 50 mgl^{-1}; Satwari 250 mgl^{-1}; (Lactobacillus 120 million units and Yeast Culture 3000 crore units).

†Fruits of C. Axillaris were obtained locally and then extracts were prepared from the pulp of lapsi fruits.

††Himedia Laboratories, Mumbai, India.

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Results

Growth measurements and Survival

After the completion of the experiment cent per cent survival rate was observed in D4 and D5 fed groups followed by 97.78±6.65 % in D2, D3 and D6 and 93.33±11.54 % in D1. Significant ($P < 0.05$) differences were observed in the treated groups (D2, D3, D4, D5 & D6) of rohu fed lapsi after 90 days of feeding trials as compared to control (D1) diet fed group. Average initial length of rohu was 2.32±0.017 cm in the beginning of the experiment while, highest 15.49±0.199 cm length was recorded in D4 diet fed group. In other treated groups the average final length recorded were 13.57±0.254 cm (D3), 13.13±0.412 cm (D5), 12.02±0.96 cm (D6) and 11.42±0.165 cm (D2) in control diet fed group the final length recorded was 10.68±0.375 cm (D1). The length gain in all the treated and control groups were 8.36±0.37cm (D1), 9.1±0.17cm (D2), 11.25±0.25 cm (D3), 13.17±0.19 cm (D4), 10.81±0.41cm (D5) and 9.71±0.96 cm (D6). The length gain (%) was found high in D4 (567.5±0.462) diet fed group followed by D3 (484.8±0.977), D5 (465.75± 0.794), D6 (418.01± 0.903), D2 (392.38 ±0.952) and D1 (360.47 ±0.902). Similarly, a higher 57.43 % increase in length was observed in D4 diet fed group as compared to control D1 diet fed group and the length gain increment in other treated groups were 34.5 (D3), 29.21 (D5), 15.96 (D6), and 8.85 (D2) respectively. Similar results were found in the average final weight and weight gain (%) of different diets fed groups. The average initial weight was 3.43 ± 0.11 g in the beginning of experiment which after 90 days of feeding trials, highest average final weight and average weight gain were recorded in D4 (22.42±0.23 g, 19.45±0.54 g) diet fed group. The average weight gain % was found high in D4 (567.5±0.88) diet fed group followed by D3 (484.82±0.01), D5 (465.75±0.77), D6 (418.13±0.71), D2 (392.38±0.31) and D1 (360.47±0.04). Similarly, a higher 67.5 % increase in weight was observed in D4 diet fed group as compared to control D1 diet fed group and the weight gain increment in other treated groups were 40.2 (D5), 35.64 (D3), 26.66 (D6), and 12.64 (D2) respectively. Significant results were observed in SGR among all the treated and control diet fed groups after 90 days of feeding trial. SGR level was found high inD4 (2.17±0.019) followed by D5 (2.00 ±0.03), D3 (1.97±0.05), D6 (1.91±0.15), D2 (1.80±0.021) and D1 (1.73±0.06) diet fed groups. FCR level was found decreased up to D4 diet fed group but in D5 and D6 it showed a bit increasing trend. The Highest FCR was recorded in control D1 (2.18 ± 0.03) diet fed group.

Vitamin C concentration in blood serum, brain and liver

Vitamin C (L-ascorbic acid) levels of blood serum, brain and liver were also estimated. Significant differences (P<0.05) were found in the vitamin C concentration in blood serum and tissues of brain and liver of all the treated diet fed groups in comparison to control diet fed group. The highest vitamin C concentration in blood serum was observed in D4 diet fed group (15.38 ± 0.329µg/mg). In liver also similar trend was recorded. The vitamin C concentration in D4 diet fed group was 191.83 ± 3.29 µg/mg followed by groups fed with diet D3 (148.51 ± 9.07µg/mg), D6 (142.63 ± 23.08 µg/mg), D2 (136.72 ± 1.11 µg/mg), D5 (135.49 ± 14.87 µg/mg) and the minimum was in D1 (127.52 ± 5.80 µg/mg) diet fed group. In the brain highest vitamin C concentration was 91.197 ± 3.59 µg/mg in D4 diet fed group followed by 81.86 ± 0.02 (D3), 78.93 ± 0.97 (D2), 65.86 ± 0.01(D5), 59.02±06 (D6) and 57.373 ± 3.318 (D1) (Table 2).

Table 2 Concentrations of vitamin C in blood serum, liver and brain of rohu fed varied doses of lapsi up to 90 days of trial

S.N.	Parameters	D1	D2	D3	D4	D5	D6
1	Vit-C S	7.54 ± 0.661	12.16 ± 1.169	12.52 ± 0.617	15.38 ± 0.329	14.46 ± 0.320	13.34 ± 0.320
2	Vit-C L	110.52 ± 5.80	136.72 ± 1.11	148.51 ± 9.07	191.83 ± 3.29	135.49 ± 14.87	142.63 ± 23.08
3	Vit-C B	57.373 ± 3.318	78.930 ± 1.977	81.867 ± 3.241	91.197 ± 3.598	65.867 ± 9.111	59.020 ± 4.064

Vit-C S,Vitamin C in Blood serum;Vit-C ,Vitamin C in Brain;Vit-C L,Vitamin C in Liver

Values are provided as mean ± SE; n, 3

Discussion

Many herbs are used in aquatic animals including fish to promote growth.[14,15] All the rohu fed with diets supplemented with ethanol extract of lapsi fruit showed better growth than control group. The maximum growth was observed in group fed with D4 diet. These results indicates that rohu needs vitamin C supplemented diet for better growth which agreed well with the works of Gouillous-Coustans et al.,[8]; Shiau & Hus[16] and Wang et al.,[17] Several species of fish including rainbow trout and Korean rockfish fed with diet containing sufficient vitamin C showed better growth.[18,19] The recommended ascorbic acid need for optimum growth of channel catfish is 10 to 25 mg per kg of diet.[20] *Cyprinus carpio*[8] and for newly hatched *Cirrhinus mrigala*[21] is 650 to 700 mg per kg diet. Growth rates in fishes depend upon the amount of Vitamin C present in the diet. The fish fed with diet containing more vitamin C grew more and the fish fed with diet containing fewer vitamins C grew less. The fish feed with diet without vitamin C showed less growth.[22] The herbal drugs promote growth, boost stress resistance boosters and prevent infections. Most fishes, including rohu, cannot synthesis vitamin C[23] due to lack of L-gulonolactone oxidase.[24] Stickney et al.,[25] reported supplementation of 50 mg of ascorbic acid in one kilogram diet resulted in maximum weight gain without any deficiency signs in blue tilapia *(Oreochromis aureus)*. Similarly 79 mg ascorbic acid in one kilogram diet was the required level for maximum weight gain of hybrid tilapia *(Oreochromis niloticus x Oreochromis aureus*.[26] Many studies have shown that fish with high concentration of vitamin C in tissues can tolerate ambient pollution and are better resistant to

bacterial infections.[27] Tilapia exposed to sub lethal dose of mercury showed weight gain, increase specific growth rate and survival rate when fed with diet containing high level of ascorbic acid.[28] Increase in the amount of lapsi extract in the diets directly relates with the concentration of vitamin C in the blood serum, brain and liver of rohu. Fish with more vitamin C in tissues are healthier than with less vitamin C.[29] Vitamin C concentration in blood serum, brain and liver were significantly ($P<0.05$) higher in the rohu fed with D4 diet and other treated groups and minimum in control diet fed group. The diet without vitamin C supplementation decreased the specific growth rate (0.32 % day^{-1}) in juvenile *O. karongae* and this is in accordance with studies conducted by Ai et al.,[30] who also observed decreasing specific growth rate in sea bass (*Scophthalmus maximus*) fed with vitamin C deficient diet. The concentration of vitamin C in various tissues is related to the vitamin C taken along with diet. Vitamin C concentration is brain and liver is high concentrations of vitamin C.[31]

Conclusion

Labeo rohita, an indigenous major carp, has high market demand in Nepal. Lapsi is an indigenous Himalayan medicinal herb and pulp of its fruits having rich in antioxidant properties has high medicinal values. *L. rohita* cannot make vitamin C. The finding of this study along with other findings from different researchers generally recognized that the lapsi fruit extract in feed of rohu increase survival rate, power to resist stressful environmental situations and accelerate growth. For successful culture of *L.rohita* in ponds 400 mg lapsi extract in one kg feed is recommended. [32–34]

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None.

Conflict of interest

Author declares that there is no conflict of interest.

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