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BIOCHEMICAL ESTIMATIONS OF VITAMIN C IN BRAIN AND LIVER OF RAINBOW TROUT *ONCORHYNCHUS MYKISS* FED ETHANOL EXTRACT OF LAPSI FRUIT (EELF) *CHOEROSPONDIAS AXILLARIS* (ROXB.) IN RACEWAYS FISHERIES AT NUWAKOT, NEPAL

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ABSTRACT

We found that vitamin C is an essential nutrient for cold water fish, the rainbow trout (*Oncorhynchus mykiss*). This was demonstrated by the absence of L-gulonolactone oxidase (GLO) activity, the enzyme responsible for the biosynthesis of vitamin C, in brain or liver of rainbow trout and by a feeding trial in which trout without vitamin C dietary supplementation developed poor growth rate. Vitamin C (Ascorbic acid or AA) is heat labile so directly cannot be given and it is found that lapsi (*Choerospondias axillaris*) contains rich amount of vitamin C in it. Thus, trout weighing 37.24±0.39 g were divided into six groups (T_1, T_2, T_3, T_4, T_5 and T_6), and each group was fed an ethanol extract of lapsi fruits (EELF) supplemented semi purified diet containing 0, 100, 200, 400, 800 and 1600 mg equivalent (EELF)/kg diet for 12 wk. The experiment was conducted in cold water raceways environment designed in recirculation system of water through nylon cages at SOSOD trout farm at Nuwakot, Nepal and vitamin C was supplemented in the diets as EELF which is found more stable to oxidation than AA. At the end of 90 days, trout fed no EELF had significantly lower weight gain than fish fed the EELF supplemented diets ($P < 0.05$). The concentration of AA in brain (51.95%) and in liver (21.63%) was higher in T4 diet group compared to trout fed with control.

KEYWORDS: Rainbow trout (*Oncorhynchus mykiss*), ascorbic acid deficiency, L-gulonolactone oxidase (GLO), lapsi (*Choerospondias axillaris*), ethanol extract.

1. INTRODUCTION

Vitamin C (Ascorbic acid) is an important nutrient in fish diets and especially in freshwater fish. The biosynthesis of Vitamin C does not occur in fish, and therefore must be supplied in the food. Vitamin C is a water soluble antioxidant vitamin.[1] involved in collagen formation, growth and reproductive processes as well as disease resistance and immune response in many fishes.[2] Fish show signs if they are fed diets deficient in vitamin C. Most commercial feed ingredients are almost completed devoid of vitamin C and it has to be supplemented. However, Vitamin C is very sensitive to oxidative destruction during processing and storage and that a significant amount is lost at that time. Lapsi *Choerospondias axillaris* (Roxburgh, 1832) fruits are rich in vitamin C content[3] and its use enhances the immune system of the body.[4,5]

Figure 1: Trout Village at Ranipauwa, Nuwakot where this experiment was conducted.

Fish are constantly in contact with pathogens inhabiting water. High population density as well as poor hydrodynamic conditions and feeding lead to an increased sensitivity towards infections.[6] In order to prevent major economic losses due to diseases, various medications are used for treatment and prevention of infections. The use of antimicrobial drugs in aquacultures could lead to emergence of resistance in pathogenic microorganisms.[7]

Alternatives are being sought over the last few years to replace antibiotics, and medicinal plants are one of available options for this purpose.[8] These plants are rich in secondary metabolites and phytochemical compounds, which have an effect against viral, bacterial, and parasitic diseases in fish. Their main advantage is their natural origin and most of these plants do not represent threat for human health, the fish, and the environment.[9]

Rainbow trout (*Oncorhychus mykiss*) is a fish belonging to the family of Salmonidae and native to the cold water rivers and lakes of the Pacific coasts of North America and Asia. It was introduced in Nepal for the first time during the late 1960s and early 1970s from UK, Japan and India. Now it is the best suited exotic fish for growing commercially in mid-hills of Nepal. Ranipauwa of Nuwakot is considered as the trout village of Nepal. Rainbow trout is an exotic carnivorous sport fish which can survive in water temperatures ranging from 0 to 25°C. The optimum temperature for growth is between 16 and 18°C, but the suitable water temperature range for feeding and growth is 13-18°C[10], and 9-14°C for the spawning and hatching of eggs. Pollution of pond water, high water temperature (>23°C), high water turbidity, high cultured stock density, overfeeding, rough handling, nutritional and vitamin deficiency and excessive nitrogen gas in water (>0.4 mg/L) are some of the primary causes of disease outbreak. The consistency of environmental conditions with regard to water temperature, volume and quality is very important for trout culture. Although a few data suggest that the use of dietary vitamin C in fish improves their natural resistance to infections, the possible mechanism and the doses are not well established. In the rainbow trout, in particular, the results are too scanty. Thus, to understand the vitamin C level in different tissues (brain and liver) of trout this experiment was conducted at Sosoda Trout Farm of Ranipauwa, Kakani-3, Nuwakot.

2. MATERIALS AND METHODS

Sosod Trout Farm (Figure 1) of Ranipauwa (known for trout farming village), Kakani-3, Nuwakot is located at the distance of about 22 Km. from Balaju bypass (Machhapokhari) and on the way to Trishuli. The culture farm is 30 minutes up and down walk in the hill area from the National High Way to Trishuli Hydro Power Station. There were all together seven cemented race way ponds with one hatchery. The main source of water was the stream which was perennial with clean and cold water suitable for trout culture. Altogether eighteen square nylon cages (1m × 1m × 1m capacity) were placed in the cemented race way pond (1.5 m x 3 m x 1 m) with an inlet and an outlet for flow of water. The cages were placed in three parallel rows with six in each row representing one treatment.

The crude extract of the pulp of lapsi fruits was prepared by using ethanol (70%) as described by Arabshahi-Delouee and Urooj (2007).[11] Altogether six practical diets (40% protein) were prepared for this experiment. Diet one (T1) was control diet without the lapsi extract and the remaining five diets T2, T3, T4, T5 and T6 were supplemented with 100, 200, 400, 800 and

1600 mg kg-1 ethanol extract of lapsi fruits along with dry fish powder, wheat flour, cod liver oil and other ingredients (Table 1). About 450 trouts (37.24±0.39 g) were selected and distributed randomly into 18 cages at the rate of 25 trout in each cage. Before starting the feeding trial trout were acclimatized for 14 days and fed with control diet. They were hand-fed with control and experimental diets at the rate of 3% of their body weight twice daily at 9 a.m. and 4 p.m. for 90 days.

Table 1: Composition of experimental diets (%).

Ingredients (g/100g)	Experimental diets (% Inclusion) g/kg					
	T1	T2	T3	T4	T5	T6
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.

2.1 Statistical Analysis

Value for each parameter measured has been expressed as mean ± standard error of mean. The results were analyzed by one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test. Significance was tested at $P<0.05$ level.

3. RESULTS AND DISCUSSION

3.1 Concentrations of vitamin C in brain

Concentration of vitamin C in brain was significantly (P<0.05) higher in rainbow trout fed with diet T4 (42.32±1.68c µg/mg) followed by trout fed with diets T3 (38.54±0.68d µg/mg), T6 (35.57±0.77c µg/mg), T5 (35.11±0.43c µg/mg) and T2 (29.82±1.18b µg/mg). The control diet (T1) fed group had the lowest vitamin C concentration in the brain (27.84±1.27a µg/mg). Vitamin C concentration was 51.95% higher in trout fed with T4 diet compared to trout fed with control (T1) diet (Fig. 2).

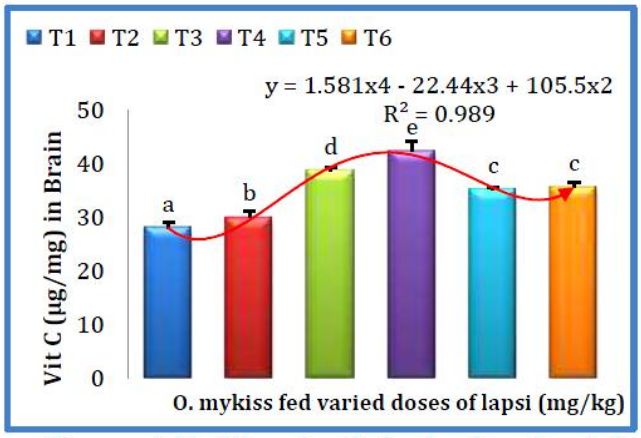

Figure 2: Vitamin C in brain of *O. mykiss* fed six different doses of lapsi fruits.

3.2 Concentrations of vitamin C in Liver

Vitamin C concentration in liver was significantly (P<0.05) higher in rainbow trout fed with T4 diet (86.71±1.32e µg/mg) compared to trout fed with all other treatment and control diets. Concentration of vitamin C in rainbow trout fed with diets T5, T3, T6 and T2 were 81.6±1.04d, 79.95±1.32c, 78.45±1.32c and 76.35±1.53b µg/mg respectively. The lowest concentration of vitamin C in liver was recorded in trout fed with T1 diet (71.29±0.68a µg/mg). Vitamin C concentration was 21.63% higher in trout fed with T4 diet compared to trout fed with T1 diet (Fig. 3).

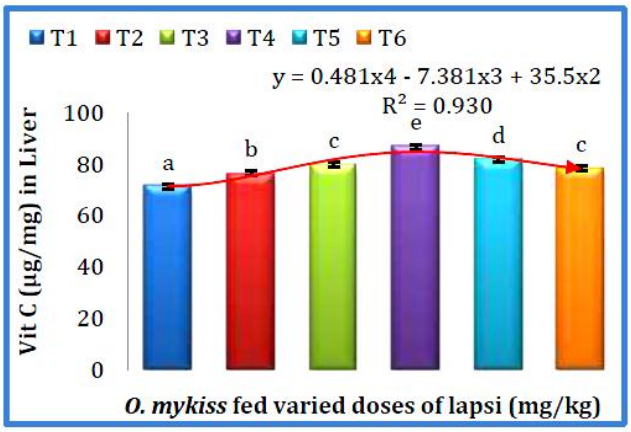

Figure 3: Vitamin C in liver of *O. mykiss* fed six different doses of lapsi fruits.

In this study fish feed included vitamin C considered to be present in lapsi incorporated diet as explained by Labh *et al* 2015[4], which is thought to be useful for normal growth of fish. After the 90-days of feeding trial a continuous trend was observed in both brain and liver tissues as the dose of lapsi fruits increased in the diets vitamin C level found significantly increased in the brain and liver of treated trout groups. Vitamin C not only influences growth and feed utilization but also has ability to be an immunomodulating agent. It affects the fish immune system, especially non-specific immunity, because fish seem to rely much more heavily on that immune system for protection.[12,13,14] Vitamin C (ascorbic acid, AA) is an important antioxidant vitamin and intake of vitamin C has been correlated with health in humans, animals, and cultured cells. In fish also it is assumed that vitamin C is an essential nutrient for optimum growth and maintenance.[15,16,17,18] With the exception of, perhaps, two or three species, vitamin C biosynthesis does not occur in fish due to the lack of the last enzyme of the biosynthetic pathway L-gulonolactone oxidase. The major signs of ascorbate deficiency include reduced growth, scoliosis, lordosis, internal and fin haemorrhage, fin erosion, and increased mortality. Another beneficial effect of vitamin C, which has been established in a number of animal species including fishes, is to stimulate the non-specific immune response.

The dose of vitamin C used was based on earlier reports that high levels of vitamin C are able to stimulate the immune system of fish and also act as antioxidant vitamins; it has a high safety margin for dietary incorporation.[19,20] In this experiment vitamin C in the form of lapsi fruits was supplied to trout, in feed, at a dose known to result in immunomodulation.[21] Similar results were also observed by Mitra and Mukhopadhyay (2003)[22] and Sahoo and Mukherjee (2003)[21], who gave vitamin C to healthy treated fishes. Vitamin C has been

demonstrated to modulate the immune response of fish when fed at a high level.[23,19] Some studies have showed a positive influence of vitamin C on macrophage activity.[24,25,26,27] Others have reported that phagocytosis, respiratory burst by macrophage and bactericidal activity are unaffected by dietary vitamin C.[28,29] Under the current experimental conditions, the results suggested that vitamin C is able to enhance some non-specific immune parameters, such as bactericidal activity, phagocytic ratio, and respiratory burst activity, when compared to fish fed a basal diet, i.e. without vitamin C supplementation. Dietary vitamin C has been reported to have a positive effect on disease resistance.[30,31,28,32]

To conclude, these results suggest that lapsi fruits incorporated diets having rich concentration of vitamin C diet may increase growth and dietary utilization, and modulate the non-specific immune response (by enhancing phagocytosis-mediated leucocyte functions, bactericidal activity, etc.) and increase survivability against pathogenic agents. The results of this study indicate that high levels of vitamin C may be useful in field situations to counteract damage caused by pathogenic bacteria and other strains and thereby increase the general disease resistance of the animal.

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