ORIGINAL RESEARCH ARTICLE

Ethanol Extract of *Choerospondias axillaris* Fruit Pulp Enhances Haematological Parameters in *Oncorhynchus mykiss* Cultured in Nepal

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Abstract

The present outdoor experiment was carried out to evaluate the effect of dietary supplementation of lapsi *Choerospondias axillaris* (Roxburgh, 1832) on haematological parameters in rainbow trout *Oncorhynchus mykiss* in Nepal. The lapsi fruits were obtained from local market of Kathmandu. The feeding trail was conducted for 90 days. About 270 trout with similar body weight (5±1g) were distributed randomly at the rate of 15 fishes per cage ($1m^3$) into 18 cages placed in raceway pond. Six practical diets containing 40% protein were prepared as T1 (0.0 g kg^{-1}) T2 (0.1 g kg^{-1}), T3 (0.2 g kg^{-1}), T4 (0.4 g kg^{-1}), T5 (0.8 g kg^{-1}) and T6 (1.6 g kg^{-1}) supplemented by the ethanol extract of lapsi fruits along with other usual ingredients viz. fish meal, wheat flour and cod liver oil etc. At the end of the experiment the haematological parameters were measured. A significant difference ($P< 0.05$) in haematological parameters was observed between the treated diets fed groups to that of control diet fed group. Total erythrocytes count (RBC), Haematocrit (Hct), Haemoglobin concentration (Hb), and erythrocyte indexes (MCV, MCH and MCHC) were found significantly higher in T4 (0.4 %) diet fed trout as compared to the control. A minimum of 0.4 % (0.4g kg-1) lapsi fruit extract in fish feeds gave more increase in haematological parameters of *Oncorhynchus mykiss*. Inclusion of lapsi fruit extract at 0.4 % concentration is therefore could be used effectively in aquaculture.

Keywords: *Oncorhynchus mykiss,* growth, lapsi, haematocrit, haemoglobin

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Introduction

Aquaculture is one of the important sectors contributing significantly in the Nepalese economy. Aqua farmers are encouraged towards intensification of culture system to increase production and profit in the world [1]. In intensive aquaculture stress level in fish impairing immune responses against pathogens leads to disease outbreaks in fishes [2, 3]. Disease is one of the most important constraints of fish production both in culture system, as well as wild condition. Fish production is decreased due to the occurrence of disease caused by different pathogens in aquaculture. To overcome this problem and to achieve the sustainable development of aquaculture, control of infectious diseases, parasites and maintenance of good health of cultured fish concern the most.

Various chemical agents, herbal extracts, and nutritional factors which stimulate the non-specific defence mechanisms are used for growth performance and control of infectious diseases in fish [4] caused by various pathogens. Despite the use of a large number of antibiotics, drugs, pesticides, and chemotherapeutics to control diseases, they are not so effective and ecologically unsafe.

Several studies have confirmed the presence of active ingredients responsible for various biological activities [5] in plants and many of them have already been tested against various diseases to test its immunostimulant efficacy [6] in aquaculture. There are several medicinal plants used in fish aquaculture are *Cassia alata, Calophyllum inophyllum, Clinacanthus nutans, Clinacanthus sp., Glinus oppositifolius, Hura crepitan, Momordica charantia, Ocimum sanctum* (red), *Ocimum sanctum* (white), *Ochrocarpus siamensis, Phyllanthus acidus, Phyllanthus amarus, Phyllanthus debelis, Phyllanthus reticulatus, Phyllanthus urinaria, Psidium guajava, Tinospora crispa, Tinaspora cordifolia* [7]. Additionaly, many herbal extracts have been tested or its efficacy

against bacterial challenge in fish [8]. Dietary supplementation of *Anthra quinone* extract, *Zingiber officinale* and Curcuma longa stimulated immunity and enhanced resistance against pathogen aggravated stress in fish. Similarly, feed incorporated with natural herb enhanced the non specific immune responses in *C. mrigala* against the pathogen aggravated stress of *Pseudomonas aeruginosa* [9, 10].

Nowadays, the use of natural plants as growth promoter and immune-stimulator along with their compounds such as essential oils and herbal extracts has improved the non-specific immune system in fishes. Use of medicinal plants is a suitable alternative to synthetic antibiotics, chemical growth agents and synthetic immune-stimulants. Herbal drugs improve the immune system and increase the host's resistance to disease by increasing the number of white blood cells and production of antibodies [11]. Citarasu et al., (2006) reported that immune-stimulants are substances, which increase the non-specific defense mechanism and provide resistance against pathogenic organisms [12]. Thus the use of cheaper and effective natural herbal extracts in the diet of fishes is gaining momentum which is less toxic and reduces the residual load to the aquatic environment [13]. These herbal extracts in aqua feed are less toxic and reduce residual load in the aquatic environment.

Dietary natural herbs incorporation not only enhance growth but also lead to an augmentation in non-specific immunity, anti-oxidation, enzyme activity, and disease resistance in fish due to presence of active phytochemicals [14, 8,15].

Improving immune system in valuable fish species such as rainbow trout is the most important in cold water aquaculture. Food additives, growth stimulants and immune-stimulants, including chemical agents, nutritional factors, bacterial and probiotic components, and animal or plant extracts affect the immune system and body defenses against disease agents [16]. Increased use of antibiotics, growth stimulants and chemical compounds leads to increased resistance of microorganisms against antibiotics and drugs. Antibiotic resistance is one of the most fundamental problems in aquaculture industry [17]. Drug resistance, decreased meat quality and high cost lead to the use of natural stimulants in fish farming, especially herbal and traditional plants [18].

Nowadays, the use of natural plants as growth and immune stimulant factors along with their compounds such as essential oils and herbal extracts has improved the non-specific immune system in fish farming. Using traditional and medicinal plants as safety stimulants is a suitable alternative to synthetic antibiotics, chemical growth agents and immune stimulants. Immune stimulants, especially herbal medicine improve the immune system and increase the host's resistance to disease via increasing the number of white blood cells and production of antibodies [11].

Lapsi, *Choerospondias axillaris* (Roxburg, 1832) of family Anacardiaceae is grown in 301 Village Development Committees of 29 hill districts of Nepal [19]. Its fruit is rich in vitamin C content [20] and enhance the immune system [21] of the organisms. Phenolic and flavonoid compounds [22] present in lapsi fruit pulp are antioxidant agents which can inhibit free radicals, so they can be effective in preventing many oxidative diseases such as cancer. These compounds also have antibacterial and antifungal effects [23].

Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792),) is the most preferred cold water fish species in aquaculture industry of Nepal It is one of the commercially cultured cold water fish in Nepal. It is an exotic fish species introduced in 1968 and 1971 from India and in 1988 from Japan to substitute fish import in star hotels catering for tourists, use of cold-water resources for aquaculture and to promote of fishing tourism in hill and mountains of Nepal for uplifting the living standard of people [24]. It is an important commercial fish in Nepal with maximum market demand and acceptability as food by the consumers due to their taste and flesh. The "One Village One Product (OVOP)" program has selected rainbow trout as one of the products in Nepal from 2063/64. This program

was implemented in Nuwakot District in the same FY 2063/64 in order to promote and product cold water fish farming.

Haematological parameters are gradually becoming a routine practice for monitoring health status in fish [25, 26, 27] to interpret physiological responses to environmental stress [28, 29] and changes in the proportion of blood cells may be indicative of a disease or an exposure to chemicals [30]. Blood indices must be analyzed when animals are exposed to pollutants [31], stress [32], infections [33, 34], parasitism [35] and seasonality [30]. Blood parameters changes during diseases or any changes occurring in the organism as a result of injuries to organs or tissues related to infectious diseases [36, 37].

It has been observed that blood parameters such as haematocrit, haemoglobin concentration and RBC count are related to environmental factors such as water temperature and salinity [38]. The knowledge of the haematological parameters can be used as an effective and sensitive index to monitor physiological and pathological changes in fishes [39]. Normal ranges for various blood parameters in fish have been established by different researchers in fish physiology and pathology [40,41].These parameters are also closely related to the response of the fish to the environment, an indication that the environment where fish lives could exert some influence on the blood characteristics [42, 43]. Haematological parameters of Rainbow trout fingerlings fed with lapsi fruit extract supplemented diet have not studied. Thus, the present study was conducted to evaluate the efficacy of lapsi extract in different amount in diets on some haematological parameters of Rainbow trout in intensive aquaculture.

Table 1. Ingredients of experimental diets (%)

Ingredients	Experimental Diets (% inclusion) g kg^{-1}					
	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.

Table 2. Proximate composition of experimental diets (%)

Ingredients	Experimental Diets					
	T1	T2	T3	T4	T5	T6
Dry Matter (DM)	97.15	97.43	97.59	97.71	96.93	97.014
Moisture	2.85	2.57	2.41	2.29	3.07	2.986
Crude Protein (CP)	31.16	31.07	31.32	31.14	31.22	31.239
Ether Extract (EE)	6.56	6.37	6.11	6.98	6.755	6.855
Crude Fiber	8.32	8.32	8.43	8.79	8.845	8.997
Ash	9.23	8.73	9.53	7.69	7.84	7.458
NFE	44.73	45.51	44.61	45.4	45.34	45.451

The use of lapsi in diet in aquaculture is thus anticipated to be an excellent strategy for the prevention of infectious microbial diseases and to replace antibiotics and chemotherapeutic.

Materials and Methods

Study area and period

The experiment was carried out in Soodo Rainbow trout culture farm (Nepal) for 90 days. Rainbow trout were obtained from hatchery of Soodo Rainbow trout farm, private fish farm in Ranipauwa, Nuwakot district in Nepal.

Experimental design

The trout were acclimated for seven days feeding on control diet. A total of two hundred seventy acclimated trout of similar size (average weight 92.37±0.039 g) were randomly allocated to 18 wooden bordered plastic cages placed in race way pond, 15 fish in each cage. There were three replicates for each treatment. T2, T3, T4, T5, and T6 were treatment groups and T1 was control. Temperature ranged from 12 °C to 19 °C, dissolved oxygen ranged from 4.13 to 6.12. mg^{-1} and pH ranged from 7.33 to 7.67 throughout the study period.

Feed formulation and preparation of experimental diets

The crude extract of the pulp of lapsi fruits was prepared by using ethanol (70%) as described by [43]. Six experimental diets (40% protein) were prepared containing similar ingredient composition. Diet one was control diet (T1) without extract of lapsi fruits. Other five diets were supplements with lapsi fruit's extract T2 (0.1 g kg^{-1}), T3 (0.2 g kg^{-1}), T4 (0.4 g kg^{-1}), T5 (0.8g kg^{-1}) and T6 (1.6 g kg^{-1}). Table 1 shows the ingredients of the feeds.

Proximate analysis of feeds

The proximate composition of the experimental diets (**Table 1**) was analysed following the standard methods of the Association of Official Analytical Chemists [44]. The moisture content was determined by drying at 105 ^{o}C to a constant weight. Nitrogen content was estimated by automated Kjeldahl apparatus (2200 Kjeltec Auto distillation, Foss Tecator, Sweden) and crude protein was estimated by multiplying nitrogen percentage by 6.25. Ether extract (EE) was measured using a Soxtec system (1045 Soxtec extraction unit, Tecator, Sweden) using diethyl ether (boiling point, 40-60 ^{o}C) as a solvent and ash content was determined by incinerating the samples in a muffle furnace at 600 ^{o}C for 6 hours. Nitrogen free extract (NFE) was calculated by difference i.e., NFE = 100-(CP+EE+CF+Ash).

Nitrogen Free Extract (NFE) = 100-(CP+EE+CF+Ash)

Maintenance and feeding

Each cage was covered by mosquito net to prevent the fish from jumping out. All cages were kept in the same raceway pond under adjust the feeding status of trout. The fish were hand fed at 3 % of body weight two times a day at A randomly 5 trout were weighed from each cage biweekly interval to balance the diet.

Blood Collection

Three fish were randomly sampled from each cage and were anaesthetized with buffered tricaine methane suffocate (MS-222; 5 mg l^{-1}). Blood was drawn from the caudal vein of treated and control fish using 2 ml heparinized plastic syringes and gauge hypodermic needles. The syringe is flushed with ethylenediamine tetraacetic acid (EDTA) as the anticoagulant.

Table 3. Haematological parameters of rainbow trout (*Oncorhynchus mykiss*) fed with diets of different levels (mean ± SD) of lapsi fruit' pulp extract for 90 days of the experiment

Treatments	Haematological Parameters					
	RBC ($x10^6$ mm^{-3})	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
T1	4.15±0.396	9.52±1.313	30.57±1.06	184.57±11.29	38.50±11.85	21.27±6.62
T2	4.59±1.523	12.07±3.444	31.73±7.62	177.87±4.00	36.23±2.15	22.05±6.34
T3	4.61±0.912	13.94±2.327	32.65±0.53	142.16±8.03	35.56±7.25	24.94±22.83
T4	5.86±0.737	15.24±3.204	36.83±12.42	130.94±4.00	30.77±5.84	29.99 ±0.07
T5	5.81±0.36	14.57±2.082	35.58±9.94	157.58±13.65	32.96±3.46	28.49±6.80
T6	4.88±0.66	14.22±1.151	33.75±9.23	165.51±26.03	34.97±6.17	25.41±9.54

The collected blood was transferred to eppendorfs of 1.5 ml capacity and stored in refrigerator for further analysis. These blood samples were used for determining erythrocyte count [45] and hemoglobin concentration [46]. Heamatocrit values (Hct) were calculated according to the formulae mentioned by Britton [47).

Haematological examination

Total Red blood cells were determined in a 1:100 dilution of blood sample in Shaw solution [48] with an improved Neubauer haemocytometer according to the procedure described by [49].The total numbers were reported as 10^6 per mm^3 for RBC and 10^3 per mm^3 for WBC [50]. Haemoglobin concentration was estimated by standard cyanmethemoglobin method [51]. Heamatocrit value (Hct) was determined after blood centrifugation in micro-haematocrit capillary tube, using a micro-haematocrit centrifuge and micro-haematocrit reader [52]. The mean cell volume (MCV), mean cell haemoglobin (MCH), and mean cell haemoglobin concentration (MCHC) were calculated [45] using the formulae:

MCV (fl) = (Hct / RBC) x 10

MCH (pg) = (Hb / RBC) x 10

MCHC (g/dl) = (Hb / Hct) x 100

Statistical analysis

The results are presented as means ± SE, difference between haematological parameters among control and treatment groups were analyzed by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test.

Results

The haematological parameter of rainbow trout (*Oncorhynchus mykiss*) fed with different doses of lapsi fruit extract was shown in Table 4. The Red blood cells count was significantly higher in treated groups compared to the control group (T1). Overall, a higher RBC count was found in the group fed with T4 diet (5.86±0.737) compared with group fed with T1 diet (4.15±0.396). Haemoglobin increased significantly in trout fed with treated diets compared with control ($P < 0.05$). The maximum concentration of haemoglobin was recorded in the group fed with T4 diet (15.24±3.204) followed by T5, T6, T3, T2 and the T1 (**Table 3**).

Haemocrit (Hct) was increased significantly in treated groups compared with control ($P < 0.05$). The maximum Hct value was recorded in T4 (36.83±12.42) group and minimum in control group (30.57±1.06). The minimum MCV value was observed in T4 group (130.94±4.00) and maximum values was recorded in control group (184.57±11.29) and MCH maximum value was recorded in control group (38.50±11.85) and minimum in T4 (30.77±5.84). MCHC increased significantly in treated groups compared with control ($P< 0.05$). MCHC maximum value was recorded in T4 (29.99 ±0.07) and minimum in control group (21.27±6.62).

RBC- Red blood cell count, Hb- Haemoglobin, Hct- haematocrit value, MCV- mean corpuscular volume, MCH-mean corpuscular haemoglobin, MCHC-mean corpuscular haemoglobin concentration.

T1= Control (Without lapsi extract), T2= 100 mg Lapsi extract/kg, T3= 200 mg Lapsi extract/kg,

T4= 400 mg Lapsi extract/kg, T5 = 400 mg Lapsi extract/kg and T6 = 1600 mg Lapsi extract/kg.

Discussion

In fishes, blood is a patho-physiological reflector of the entire body and the haematological parameters such as leucocyte count, erythrocyte count, hematocrit and hemoglobin are routinely tested to monitor the health status of fish and any deviation from the normal ranges may indicate a disturbance in the physiological process. [53,54]. The changes in the blood parameters of *C. gariepinus* caused by stress due to exposure to environmental pollutant, diseases or by pathogens have been studied by many researchers especially in capture fisheries [55,56].

Hematological parameters may provide an index of the physiology status of fish which are directly influenced by sex, size, seasons, slow or fast movement and age of fishes [57] and indirectly influenced by quality of water, temperature, food availability physiological status of fish [58], microbial infection of fish and toxicants [59]. Ologhobo (1992) [60] reported that the most common blood variables consistently influenced by diet are the haematocrit (Hct) and haemoglobin (Hb) levels. The changes in RBC count, Hb concentration, Hct, MCV, MCH and MCHC show health status of fish [61]. Haemoglobin, RBC, Hct, MCV, MCH and MCHC values obtained in the experiment almost agreed with earlier workers. According to the results, lapsi extract supplemented diets could increase Hemoglobin content and RBC numbers in treated groups compared to control group. This agreed with the work of [62] who also reported similar increase in haematological parameters in Nile tilapia.

In agreement with the present findings, Sahu *et al.,* (2007) [63] found higher RBC counts in *Labeo rohita* fingerlings fed with *Magnifera indica* kernel when compared to control. Shalaby *et al.,* (2006) [64] reported significant increases in RBC, Hb and Htc of Nile tilapia fed with garlic that support the present study. Faisal (2003) [65] reported significant decrease of MCH and MCV in *Clarias garepins* fed with garlic that is also similar to the findings of the present study. *C. gariepinus* fed A. *barbadensis* leaves paste showed significant ($p < 0.05$) increase in haematocrit (Hct), haemoglobin, RBC, WBC in comparison to the control group. This was in agreement with the work of Haghigi and Rohani (2013) [66] who observed similar increase in the haematological parameters in rainbow trout fed ginger powder. These were also similar findings of Farahi *et al.* (2012) [67] who reported significant enhancement (higher values) of WBC and PCV in diet supplemented with *M. officinalis* and *Aloe vera.*

Concerning the effect of the lapsi supplemented diet on haematological parameters of rainbow trout (*Oncorhynchus mykiss*) indicated increased haematocrit, haemo-globbin, erythrocyte, MCH, and MCHC in comparison to the control group ($p<0.05$) (Table. 3). These could be attributed to the fact that, the lapsi in the diet has increased the haematological parameter values as a result of hematopoietic stimulation [68, 69, 70].

Conclusion

Number of chemicals and drugs used in aquaculture cause severe environmental problems. Most of the antibiotics and drugs are banned because of their negative impact and effluent remittance in the fish muscle which may cause side effect to the consumer. Herbal drugs, safer to fish and the environment are the viable alternative to antibiotics and other banned drugs. Most of the herbs and herbal extracts are given orally. The farmer does not know about the importance of herbal drugs. So it is necessary make aware on the use of biological and eco-friendly approach of herbs and their products in aquaculture to the farmers. It will reduce the production risk, negative impact and production cost and also increasing fish production with sustainable way. In conclusion, the present study revealed that lapsi, *Choerospondias axillaris* incorporated diet fed to rainbow trout, *Oncorhynchus mykiss* improved haematological parameters. Hence, 0.4 mg kg^{-1} lapsi extract could be used as a supplement in the diet of fish for better health. However, more research should be carried out on the uses of

Choerospondias axillaris in order to obtain optimal health.

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