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Effects of Lapsi *Choerospondias axillaris* on Growth and Immune-Related Genes in Silver Carp (*Hypophthalmichthys molitrix*)

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Abstract

This study investigates the effects of Lapsi Fruit Extracts (LFE) Choerospondias axillaris (Roxburg 1832) on growth performance, haematology, biochemical with some growth and immune-related genes in silver carp fingerlings (Hypophthalmichthys molitrix). For this purpose, three hundred and seventy-five fish (5.17±0.13g) divided into six groups into eighteen aquaria and fish were fed six purified diets supplemented with ethanol extract (80%) of lapsi fruits (LFE) as A (0.0 g control), B (0.2 g), C (0.4 g), D (0.8 g), E (1.6 g), and F (3.2 g /100 g) for 90 days. Significantly higher ($P<0.05$) weight gain, %weight gain, SGR, and lysozyme were recorded in D (0.8 g) diet-fed group, while FCR was higher in the control diet group. In haematology, no significant differences ($P>0.05$) were observed among the treated and control diet-fed group while reducing trends were observed in SOD, CAT, and GPx levels as compared to control. Glucose, triglyceride, and cholesterol level were higher in control and the decreasing trend observed in continuous feeding of the LFE supplemented diet. Protein albumin and globulin level were always higher ($P<0.05$) in all the treated groups and was better in D (0.8 g) diet-fed group. As compared to β-actin gene, growth hormone (GH), insulin-like growth factors I and II (IGF-I and IGF-II) in the liver were always higher and similar results were observed in immune - related genes interleukin - 8 (IL-8), interleukin-10 (IL-10) and tumor necrosis factor (TNF-α) in the head kidney of silver carp. The results suggest that lapsi fruit extracts (LFE) can be useful as a feed supplement for proper growth and enhance disease resistance by improving immunity in blood, liver, and head-kidney tissues.

Keywords: Immunity; Hematology; Glucose; Growth; C. Axillaris; Silver Carp; Gene Expression.

1. Introduction

Aquaculture is currently a fast-growing food-producing industry in the world. One of the most vulnerable points of aquaculture is diseases. In the last decade, diseases have caused extensive losses in cultured freshwater fish. The use of immunostimulants in the aquaculture industry has been essential in reducing antibiotics use and economic losses, which occur as a result of disease [1, 2]. There is a trend in the replacement of chemical drugs with herbal medicine and algal extracts, due to their potential health benefits with limited side effects [3, 4]. The silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844) (Cyprinidae) is one of the most versatile filter feeders that feed primarily on phytoplankton and zooplankton, but which also consume other food items such as vegetable detritus [5, 6]. Silver carp extends over most of eastern Asia, and significant Chinese rivers such as the Yangtze, Pearl, and Yellow rivers [7, 8]. Abdelghany and Ahmed [9] studied the effects of feeding rates on growth and production of Nile tilapia, *Oreochromis niloticus*, common carp, *Cyprinus carpio*, and silver carp poly-cultured in fertilized ponds. However, no available data is focusing on the ability of silver carp to consume a prepared diet.

Choerospondias axillaris, known as "Nepali plum," is a tree of the family Anacardiaceae, hails from a large part of Asia, from India to China and Japan [10]. The fruit is about 3 centimeters long and has a sour whitish flesh, green to

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yellow skin used in medicine. Therefore, the study about immunostimulants, especially those of plant origin, which have antioxidant and immune property, has remarkably increased in recent years [11, 12]. Recent studies have demonstrated the vital role played by nutritional antioxidants and immunostimulants in preventing the damage caused by toxic and chemical compounds in fish [13, 14]. Recently, lapsi fruits have highly studied due to their numerous and essential health benefits such as free radical scavenging, antioxidant, immunostimulant, antimicrobial, anti-inflammatory [11, 10]. Studies have focused on the effects of dietary administration of fruit extracts on the immune system of fish [15, 16]. Thus, this study has performed to evaluate the effects of ethanol extract of lapsi fruit on growth, immune response, and immune related genes in the *Hypophthalmichthys molitrix* (silver carp).

2. Material and Methods

2.1. Selection of Fish and Maintenance

The fingerlings of Silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844) (Cyprinidae) were procured locally from an authorized hatchery farm and transported to Aquaculture Research Laboratory of Amrit Campus, Tribhuvan University, Kathmandu, Nepal. After examination of its health status, around five hundred fingerlings (5.17±0.17g) distributed randomly into three stocking tanks for three weeks to acclimatize in laboratory conditions.

2.2. Preparation of Experimental Diets

Fruits of Lapsi (Figure 1) collected from the local nursery, and ethanol (80%) extract (LFE) prepared according to the method explained in Labh et al. (2015). Basal diets prepared as described in previous work [18] (Hoseinifar et al. 2015) with slight modification from Labh et al. [17]. Altogether six purified (40% protein) diets were prepared (Table 1) in which diet A (0.0 g kg^{-1}) controlled diet followed by diets B, C, D, E and F were supplemented by LFE with 0.2, 0.4, 0.8, 1.6 and 3.2 g kg^{-1} respectively. Other ingredients as per the standard norm of Fish Nutrition Lab, CIFE, Mumbai [17].

Figure 1. Lapsi fruits are displayed in the market

Table 1. Percentage of the experimental diets containing supplement of different levels of Choerospondias axillaris (Lapsi fruit extract) (LFE) and proximate composition

Ingredients	Experimental diets (% inclusion)					
	A (0%)	**B (0.2%)**	**C (0.4%)**	**D (0.8)**	**E (1.6%)**	**F (3.2%)**
Fish Meal†	29.31	29.31	29.31	29.31	29.31	29.31
Soya meal‡	13.16	13.16	13.16	13.16	13.16	13.16
Groundnut oil cake†	9.17	9.17	9.17	9.17	9.17	9.17
Rice Powder†	15.52	15.32	15.12	14.72	13.92	12.32
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
Betain Hydrochloride††	0.02	0.02	0.02	0.02	0.02	0.02
BHT(Butylatedhydroxytoluene)††	0.02	0.02	0.02	0.02	0.02	0.02
Crude *C. axillaris Extract (LFE)*	0	0.2	0.4	0.8	1.6	3.2
CMC (Carboxymethylcellulose)††	1	1	1	1	1	1
Total	**100**	**100**	**100**	**100**	**100**	**100**

Proximate analyses						
Protein, (%DM)	39.87	39.76	39.91	39.64	39.83	39.92
Fat, (%DM)	7.68	7.68	7.72	7.83	7.75	7.62
Ash, (%DM)	5.63	5.64	5.94	5.32	5.45	5.92
NFE, (%DM)♯	47.16	47.16	47.16	47.17	47.18	47.17
Energy, (kj/g)♯♯	19.34	19.85	19.35	19.78	19.76	19.39

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

‡ Ruchi Soya Industries, Raigadh, India.

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

Vitamin A 55,00,000 IU; Vitamin D3 11,00,000 IU; Vitamin B2 2,000 mg; Vitamin E 750 mg; Vitamin K 1,000 mg; Vitamin B6 1,000 mg; Vitamin B12 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).

†† Himedia Laboratories, Mumbai, India.

♯ Nitrogen–free extracts (NFE) = dry matter – (crude lipid+crude ash+crude protein+crude fiber).

♯♯ The energy calculated according to 23.6 kJ g $^{-1}$ protein, 39.5 kJ g $^{-1}$ lipid, and 17.0 kJ g $^{-1}$ NFE

2.3. Experiment Design and Rearing System

Three hundred seventy-five fingerlings 5.17±0.17 g of Hypophthalmichthys molitrix (silver carp) distributed randomly into 18 plastic tanks (water volume 150 l) at the rate of 15 fish per tank into three replicate form (Figure 2). Fish sampled on the 90th day, and during sampling, the own weight of fish recorded following the collection of blood, head kidney, and liver tissues from each diet group. During sampling, 5 fishes from each tank sacrificed by an overdose of MS222 (100 mg L^{-1} Sandoz). The fish dissected, head kidney, and liver tissues collected according to the procedure described elsewhere previously (Zhang & Zhou 2014). At the end of the 90th day, five fishes from each treatment group challenged with a lethal concentration (3×108 CFU ml^{-1}) of A. hydrophila via intraperitoneal injection and mortalities recorded to understand the relative percent survival.

2.4. Examination Procedure

The entire examinations and analysis procedure are represented in the flow chart (Figure 3) and the details of examination procedures are as follows:

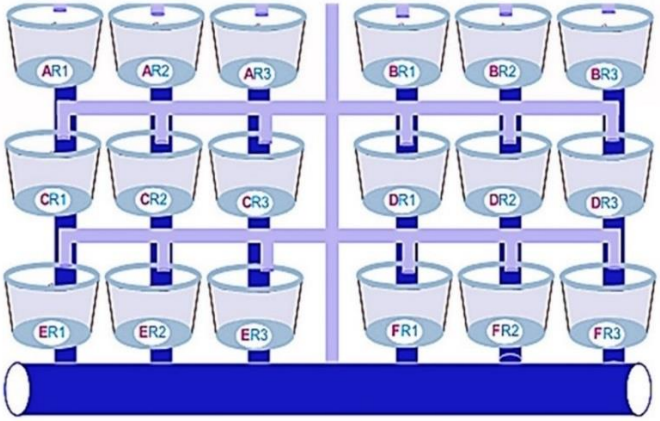

Figure 2. Experiment design and rearing system during the experiment

Growth performance

The percent weight gain, specific growth rate (SGR), feed conversion ratio (FCR)) and survival rate measured following standard formulae and excel worksheets. The calculation was as follows:

Weight gain % (%WG) = (final body weight-initial body weight) ×100/initial body weight;

Specific growth rate (SGR %) = (LnWt-LnW0) ×100/t; whereW0 and Wt are the initial and final body weights, and t is the culture period in days;

Feed conversion ratio (FCR) = total feed fed (g)/total wet weight gain (g) Survival (%) = (Number of fish survived / Number of fish leased) × 100.

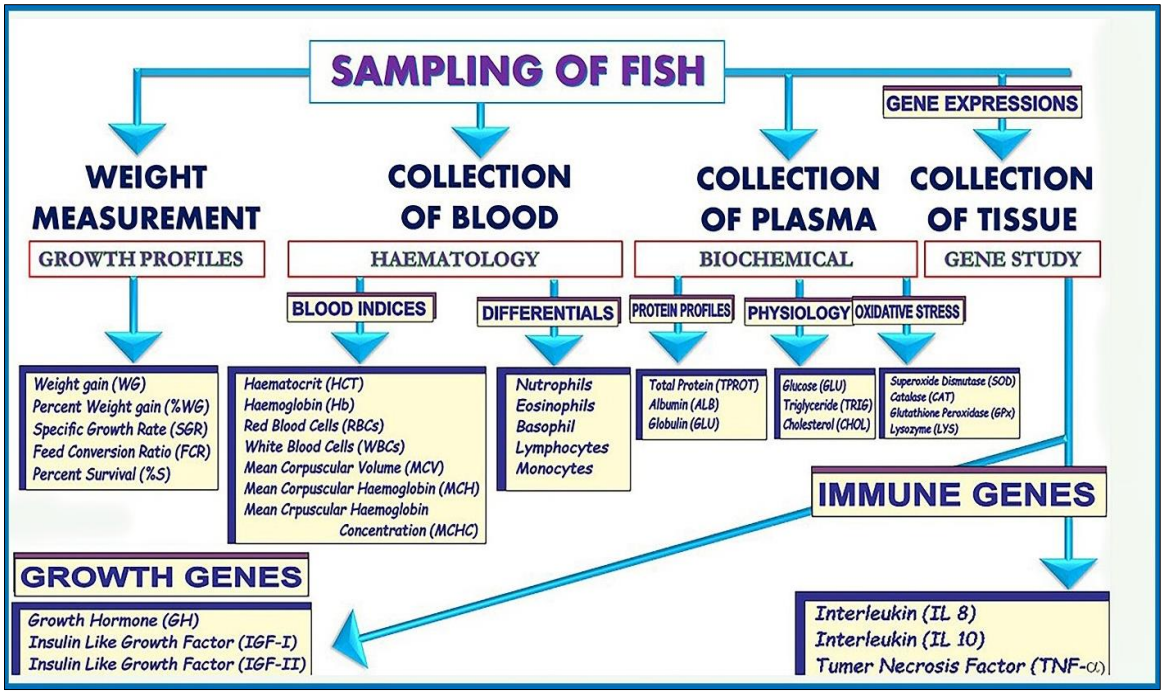

Figure 3. Flow chart representing the stepwise methodology during the 90 days of feeding trials with lapsi fruits

Hematological analysis

For haematological analysis, blood samples (1 ml) were taken from the caudal vein of fish using disposable syringes (3 ml) flushed with heparin (Sigma, UK). These divided into two aliquots, one sample for white and Red Blood Cell (RBC) counts and differential White Blood Cell (WBC) counts, and for plasma collection, blood was centrifuged at 3000x g for 5 min and once separated stored at -80 °C for further analysis.

Red Blood Cell (RBC) and White Blood Cell (WBC) counts were measured as described by Natt and Herrick (1952). Blood (20 µl) was added to 4 ml Natt-Herricks's solution and mixed thoroughly. A haemocytometer was filled with the blood suspension (10 µl), which allowed us to settle for 2-3 min before counting the RBCs and the WBC. For haematocrit values, capillary tubes were filled with blood and sealed at one end with clay. The filled tubes were then centrifuged at 15,000×g for 5 min in a *micro*-haematocrit centrifuge. The haematocrit (HCT) values expressed as a percentage of the packed cell volume (PCV). Mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentrations (MCHCs) calculated according to Bain et al. (2006).

Differential WBC counts were made, according to Nussey et al. [19]. Blood smears were prepared and allowed to air dry before fixing with methanol for 3-5 min. The slides were then stained with Giemsa (IVD, UK) for 30 min, rinsed two times with buffer solution for 1 min, before air drying the slides and mounting them with Pertex®. The cells examined under a light microscope (×100 magnification), and the number of different WBCs present in 200 cells determined.

Biochemical assay

The determination of plasma glucose (GLU), total protein (TP), albumin (ALB), globulin (GLO), triglyceride (TRIG), and cholesterol (CHO) were determined using a commercially diagnostic kit (Bioanalytic Diagnostic Industry, Co.). Plasma lysozyme (P-Lys) assessed using the turbidometric assay. A Micrococcus lysodeikticus suspension of 875 µl (Sigma, ATCC 4698) at a concentration of 0.2 mg/ml (in PBS) was added to 25 µl of blood samples and were measured spectrophotometrically at 530nm after 0.5 and 4.5 minutes at 25°C, with a spectrophotometer. A unit of lysozyme activity defined as the amount of serum caused a reduction in absorbance of 0.001/min.The Superoxide dismutase (SOD) enzyme assay performed by using the method described by Kakkar et al. [20], Catalase (CAT) activity determined according to the methodology described by Iwase et al. [21], and Glutathione peroxidase (*GPx*) activity measured by the method described by Rotruck et al. [22] following standard kits.

Real-time PCR

Relative gene expression of growth factor (GH, IGF-I, and IGF-II), and immune-related genes (IL-8, IL-10, and *TNF-α*) was measured from 3 fish per treatment using real-time PCR as described previously [18]. Briefly, total RNA extracted from the head kidney of tissues collected on the 90th day, using TRIzol® Reagent. It quantified by spectrophotometry, and purity calculated. Quality checked by agarose gel electrophoresis. The RNA treated with RNA-free DNase I (Promega, Fitchburg, WI, USA) to remove genomic DNA contamination. Complementary DNA

(cDNA) synthesized from 1mg of total RNA using a Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions with an oligo-dT18 primer. The expression of the selected genes analyzed by real-time PCR. It performed with an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 lL of 29 SYBR Green supermix, 5 lL of primers (0.6 mM each) and 5 lL of cDNA template) incubated for 10 min at 95°C. Then after followed by 40 cycles of 15 s at 95°C, 1 min at 60°C and finally, 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. For each mRNA, gene expression was corrected by the β-actin RNA content in each sample. The relative gene expressions determined according to the ΔΔCt method using IQ5 software (BIO-RAD). Primers used in this study shown in Table 2. In all cases, each PCR carried out with triplicate samples.

Table 2. Oligonucleotide primer sequences used in RT-PCR for gene expression analysis

Name of gene	Abbreviation	Primer used
Beta-actin	*β-actin*	F: AGACATCAGGGTGTCATGGTTGGT R: CTCAAACATGATCTGTGTCAT
Growth hormone	*GH*	F: TCTTCGCATCTCTTTTCACC R: AGTCGGCCAGCTTCTCA
Insulin-like growth factor-I	*IGF-I*	F: GGCATTGGTGTGATGTCTTT R: CATATCCTGTCGGTTTGCTG
Insulin-like growth factor-II	*IGF-II*	F: TGCCCAGAGGTTAAGGAGAG R: CTGAATGTGTGGAAGGATGG
Interleukin-8	*IL-8*	F: GTCTTAGAGGACTGGGTGTA R: ACAGTGTGAGCTTGGAGGGA
Interleukin-10	*IL-10*	F: CGCCAGCATAAAGAACTCGT R: TGCCAAATACTGCTCGATGT
Tumor Necrosis Factor-alpha	*TNF-α*	F: GGTGATGGTGTCGAGGAGGAA R: TGGAAAGACACCTGGCTGTA

2.5. Statistical Analysis

Data analysis was using SPSS software version 20 (SPSS, Michigan Avenue, Chicago, IL, USA). The significant difference between treatments was determined using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test [23]. All data presented in the text, figures, and tables are means ± standard error, and the significance level was $P< 0.05$.

3. Results

3.1. Growth Performance

The effects of dietary LFE on survival rate and growth performance of silver carp presented in Table 3. There was no mortality at the end of the study in all the groups. Growth performance parameters of silver carp feed with LFE supplemented diets shown in Table 2. WG, %WG, and SGR of carps fed with the D (0.8) diet were significantly higher ($p<0.05$) than fish fed with control diets ($p<0.05$). The FCR value was significantly higher ($p<0.05$) in control A (0%) group, and lower FCR ($p<0.05$) was recorded in D (0.8) diet-fed group (Table 3).

Table 3. Growth performance of silver carp fed experimental diets contain six levels of *Choerospondias axillaris (Lapsi fruit)* LFE for 90 days. Values presented as the mean ± SE

	A (0%)	B (0.2%)	C (0.4%)	D (0.8)	E (1.6%)	E (1.6%)
IW	5.17±0.13	5.17±0.13	5.17±0.13	5.17±0.13	5.17±0.13	5.17±0.13
FW	9.21±0.36^{a}	12.79±0.77^{ab}	15.92±0.59^{b}	15.88±0.46^{b}	16.98±0.87^{b}	16.09±0.11^{b}
WG	4.42±0.36^{a}	7.62±0.21^{ab}	10.75±0.51^{b}	10.71±0.43^{b}	11.81±0.45^{b}	10.92±0.78^{b}
% WG	77.96±0.35^{a}	147.55±0.69a^{b}	207.12±0.63^{b}	207.33±0.67^{b}	228.49±0.59^{b}	211.33±0.39^{b}
SGR	1.13±0.15^{a}	1.67±0.14^{ab}	2.08±0.23^{b}	2.02±0.31^{b}	2.19±0.77^{b}	2.09±0.51^{b}
FCR	0.551±0.038^{b}	0.274±0.019^{a}	0.192±0.087^{a}	0.154±0.027^{a}	0.169±0.036^{a}	0.174±0.014^{a}
%S	100	100	100	100	100	100

Values in a row with different superscripts denote a significant difference (Duncan's test P < 0.05).

IW=initial weight, FW=final weight, WG=weight gain, %WG=percent weight gain,
SGR= specific growth rate; FCR=feed conversion ratio; %S=percent survival

3.2. Blood Haemogram

Silver carp fed ethanol extract of lapsi fruit (LFE) supplemented diets, and complete hematological parameters evaluated (Table 4). There were no significant differences among the Hct, RBC, and MCV levels in any of the experimental groups or the control group ($p>0.05$). Besides, Hb, MCH, and MCHC also found to be significantly higher in the experimental groups ($p<0.05$). Similarly, WBC count and differential counts also did not vary significantly ($p > 0.05$) from the values observed in the treated groups to that from the control group (Table 4).

Table 4. Complete haemogram profile of silver carp fed experimental diets contain six levels of Choerospondias axillaris (Lapsi fruit) LFE for 90 days. Values presented as the mean ± SE

	A (0%)	B (0.2%)	C (0.4%)	D (0.8)	E (1.6%)	E (3.2%)
HCT (%)	24.11 ± 0.42a	24.00 ± 0.28a	24.11 ± 0.48a	25.33 ± 0.52a	24.11 ± 0.42a	24.00 ± 0.28a
Hb (g/dL)	8.88 ± 0.21c	14.47 ± 0.71ab	15.87 ± 0.32a	13.20 ± 0.72b	15.90 ± 0.59a	14.49 ± 0.79ab
RBC (10^6/µl)	4.93 ± 0.22a	5.11 ± 0.35a	4.66 ± 0.24a	5.27 ± 0.15a	4.97 ± 0.21a	5.17 ± 0.35a
WBC (10^3/µl)	78±0.18b	78.67±0.96e	73.43±0.89a	68.67±0.95c	72.61±0.57d	73.33±0.33cd
MCV (fl)	48.46 ± 1.97a	49.37 ± 2.17a	3 53.33 ± 4.08a	48.44 ± 1.89a	49.46 ± 2.92a	48.33 ± 3.57a
MCH (pg cell)	33.53 ± 1.15a	27.24 ± 1.53ab	39.58 ± 1.36c	35.23 ± 1.61b	32.57 ± 2.29a	28.52 ± 1.55ab
MCHC (%)	56.00 ± 1.11a	39.21 ± 2.17	57.02 ± 1.28c	62.02 ± 2.34b	66.00 ± 2.34a	60.42 ± 3.37
Differential Counts						
Neutrophils ($10^3\mu^{-1}$)	65.33±5.81	67.67±4.98	64.67±3.38	68.67±6.36	67.67±4.98	68.67±0.88
Eosinophils ($10^3\mu^{-1}$)	4.07±0.11	4.38±0.43	4.28±0.29	4.33±0.18	4.22±0.35	4.30±0.27
Basophil ($10^3\mu^{-1}$)	0.91±0.18abc	0.91±0.18abc	0.91±0.22abc	0.93±0.02ab	0.94±0.22abc	0.94±0.04bc
Lymphocytes ($10^3\mu^{-1}$)	27.26±0.57	27.85±0.12	24.98±0.74	27.27±0.46	27.73±0.04	25.63±0.35
Monocytes ($10^3\mu^{-1}$)	6.56±1.31	4.28±0.48	5.45±0.79	6.48±1.24	4.28±0.48	5.91±0.03

Values in a row with different superscripts denote a significant difference (Duncan's test $P < 0.05$).

HCT=haematocrit, Hb=haemoglobin, RBC=red blood corpuscles, WBC=white blood corpuscles, MCV= mean corpuscular volume;

MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

3.3. Biochemical Performances

The effects of different concentrations of LFE on biochemical parameters of silver carp summarized in Table 5. Total protein, albumin, globulin level found higher ($p<0.05$) in all the treated diet-fed group as compared to control. Glucose, triglyceride, and cholesterol level were higher ($p>0.05$) in control diet-fed group. For oxidative stress studies in liver, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (*GPx*) activity level increased significantly (($p<0.05$) in control diet and as the dose of LFE increased in the diet the SOD, CAT, and GPx level gradually decreased in treated diets. It was a minimum in D (0.8) diet-fed group. Significant differences in Lysozyme levels observed among the treated and control groups. The lysozyme level was high ($p<0.05$) in D (0.8) diet-fed group.

Table 5. Biochemical performances of silver carp fed experimental diets contain six levels of *Choerospondias axillaris* (Lapsi fruit) LFE for 90 days. Values presented as the mean ± SE

	A (0%)	B (0.2%)	C (0.4%)	D (0.8%)	E (1.6%)	E (3.2%)
TPROT	1.96 ± 0.18b	2.07 ± 0.26b	2.96 ± 0.39a	2.96 ± 0.39a	2.24 ± 0.29b	2.74 ± 0.31b
ALB (g/dL)	1.36 ± 0.15b	1.43 ± 0.33b	1.40 ± 0.22b	1.99 ± 0.35a	1.99 ± 0.35a	1.40 ± 0.22b
GLO (d/dL)	0.60 ± 0.27a	0.64 ± 0.39a	0.85 ± 0.46a	0.97 ± 0.24a	0.95 ± 0.44a	0.91 ± 0.33a
GLU (mg/dL)	65.90 ± 12.19a	57.77 ± 12.19a	53.14 ± 3.15b	50.84 ± 9.65a	55.84 ± 3.73a	54.25 ± 6.42b
TRIG (mg/dL)	72.72 ± 11.15a	68.72 ± 10.75a	66.41 ± 13.09a	64.10 ± 13.54a	56.41 ± 8.03a	66.41 ± 13.09a
CHOL (mg/dL)	65.64 ± 13.33a	61.48 ± 9.73a	60.48 ± 9.73a	62.93 ± 18.01a	47.91 ± 14.15b	48.39 ± 14.15b
SOD (U/mg prot)	6.4667±0.17e	4.4467±0.19c	3.33±0.17c	1.5233±0.18a	2.43±0.17b	5.4167±0.21d
CAT (U/mg prot)	9.5967±0.17d	8.5467±0.21e	7.5767±0.19b	6.46±0.17a	6.56±0.17a	7.6533±0.18b
GPx (U/mg prot)	15.46±0.13g	14.36±0.13f	13.7±0.13e	8.1567±0.36a	10.62±0.18c	9.5133±0.2b
LYS	533.3 ± 6.66d	933.3 ± 6.66c	1200.00 ± 8.64b	1770.77 ± 9.68a	1763.21 ± 7.19a	1180.00 ± 7.31b

Values in a row with different superscripts denote a significant difference (Duncan's test $P < 0.05$).

TPROT=total protein, ALB=albumin. GLO=globulin; GLU=glucose, TRIG=triglyceride, CHOL=cholesterol

SOD=superoxide dismutase, CAT=catalase, GPx = glutathione peroxidase, LYS=lysozyme

3.4. Expression Analysis

Expression of growth-related genes

Silver carp fed ethanol extract of lapsi fruit (LFE) at six different levels for 90 days, and the expression analysis of growth-related three genes (GH, IGF-I, and IGF-II) evaluated (Figure 1) followed by β-actin gene as a control in the liver tissues. Compared to control β-actin gene (28.15±0.51), growth hormone (39.51±0.76), insulin-like growth factor IGF-I (45.91±0.35) and IGF-II 30.24±0.45 gene expressions were significantly higher ($P<0.05$) in the liver of D (0.8%) diet-fed group (Figure 4).

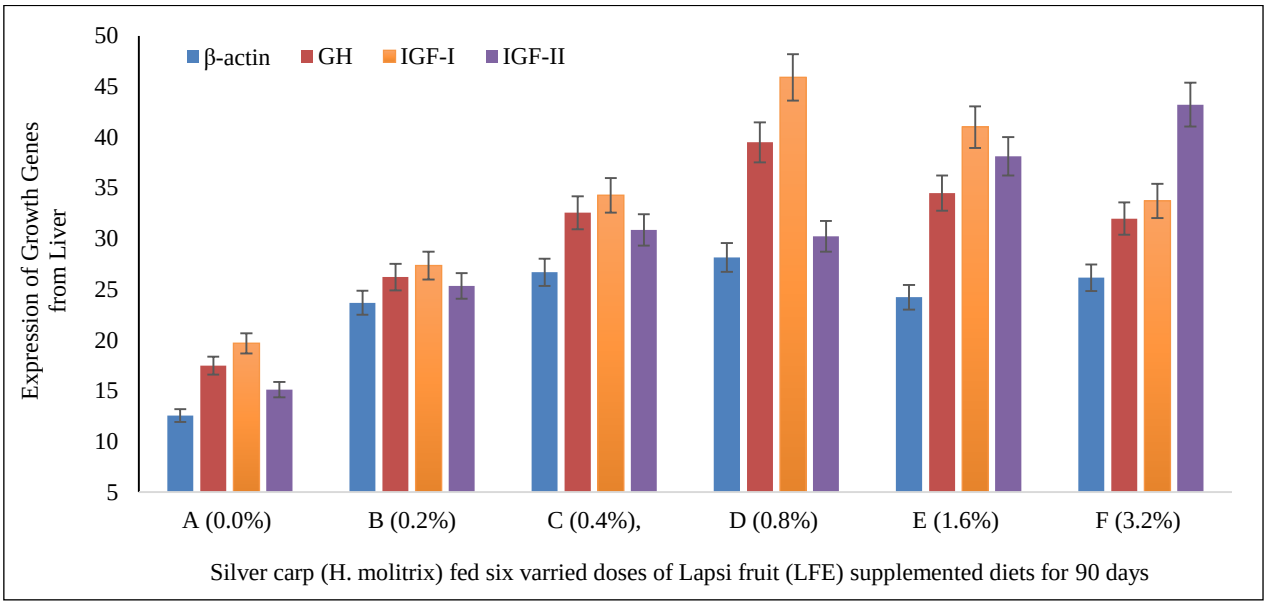

Figure 4. Growth hormone (GH) and insulin-like growth factor (IGF-I and IGF-II) genes relative expression levels to β-actin determined by real-time PCR in the head-kidney of silver carp fed six varied LFE supplemented diets for 90 days. Data represent as means ± standard error (n = 10).

Expression of immune-related genes

Three immune-related genes (IL-8, IL-10, and TNF-α) studied in the head kidney of silver carp fed six different levels of LFE diets for 90 days. Compared to the β-actin gene (28.15±0.13) as control, the gene expression levels were significantly higher ($P<0.05$) in IL-8 (34.14±0.71), IL-10 (38.07±0.11) and TNF-α (35.198±0.45) genes in the head-kidney of D (0.8%) diet-fed group (Figure 5).

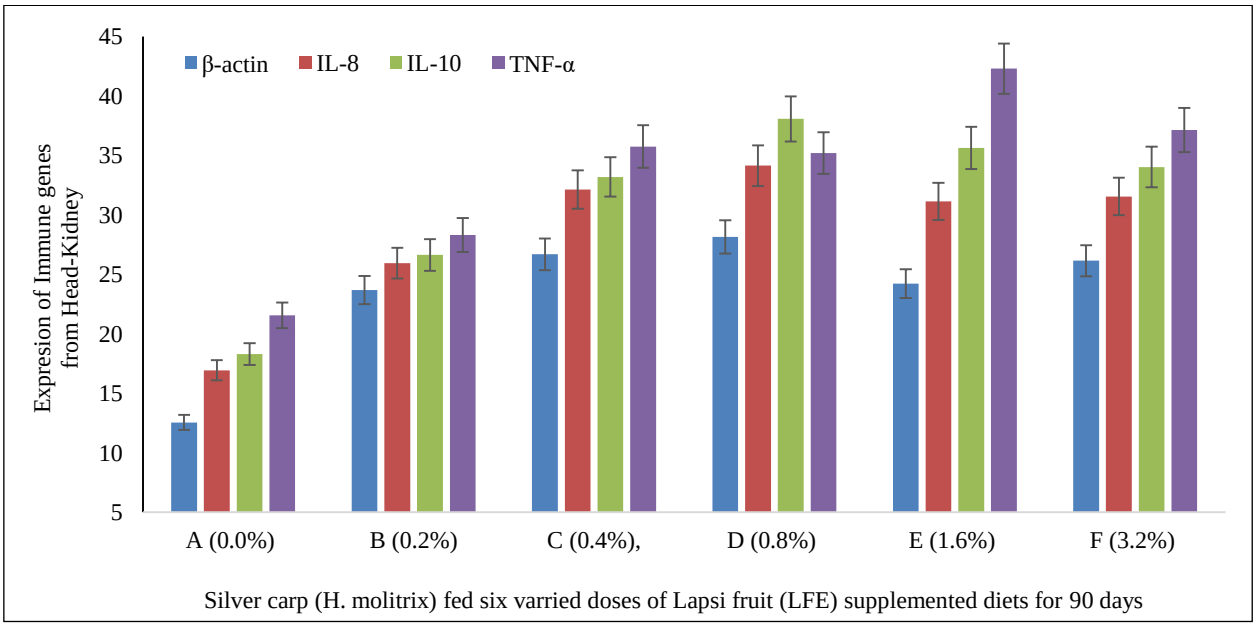

Figure 5. Interleukin (IL-8), Interleukin (IL-10), and Tumor Necrosis Factor (TNF-*α*) genes relative expression levels to β-actin determined by real-time PCR in the head-kidney of silver carp fed six varied LFE supplemented diets for 90 days. Data represent as means ± standard error (n = 10).

4. Discussion

The contribution of fisheries is very promising and essential for creating job opportunities for unemployed people, earning foreign exchange, alleviating poverty, and improving the nutritional status of the people. Today's world population is estimated to be about 7 billion, which by 2030, 2 billion more people added in the world population mean that aquaculture needs to produce nearly double that, eighty-five million tons of fish per year just to maintain current consumption levels (Corsin and Yamamoto 2014). In recent years, beneficial effects of immunostimulant have demonstrated in many aquatic species to improve growth performance, stimulate immunology, and disease resistance [18, 19, 24, 25].

Dietary supplementation of plants has reported stimulating immunity and disease resistance of several aquatic animals [26-30]. This result supported by previous studies on *Pangasius catfish*, *P. bocourti* [31, 32] rainbow trout, *O. mykiss* [33], rohu, *Labeo rohita* [34, 35]; white shrimp, L. vannamei [36]; freshwater prawn, *Macrobrachium rosenbergii* (Dash et al. 2014, 2015 [37]); grouper, E. coioides (Son et al. 2009 [38]); red sea bream, Pagrus major (Dawood et al. 2015 [39]); olive flounder, Paralichthys olivaceus (Beck et al. 2015); and blue swimming crab, Portunus pelagicus (Talpur et al. 2013). Several herbs tested for their growth, promoting activity in aquatic animals [40]. Wang et al. observed that dietary supplementation of Rehmannia glutinosa increased the growth rate [41].

Positive effects of herbal extracts on the growth performance of different fish have reported by other authors (MacLennan et al. 2002 [42]; Immanuel et al. 2009 [43]; Talpur and Ikhwanuddin 2013 [44]; Kanani et al. 2014 [45]). In the present study, the growth of silver carp increased as the dose of lapsi fruit extract (LFE) increased in fish diets. Silver carp fed 0.8% LFE showed significantly improved percent weight gain, SGR, and FCR compared with the control. The results have disagreed with studies on kelp grouper, E. brneus (Harikrishnan et al. 2012a [46]) and common carp, Cyprinus carpio (Dobsıkova´ et al. 2013 [47]) but complete agreement with results from studies on kelp grouper, Epinephelus brneus (Harikrishnan et al. 2012b [48]); Amur Catfish, Silurus asotus [30]; and tilapia (Abd Rahman et al. 2012 [49]).

A blood haemogram is a pathophysiological reflector and the counts of hematological parameters in fishes and indicates the health status by determining any abnormality [50]. The hematocrit value is an essential tool of the health status of fish in aquaculture [51]. In the present study, no significant changes observed in the hematocrit level. Many authors reported that there was no enhancement of the hematocrit level after using immunostimulant compounds in fishes Binaii et al. 2014 [52]). In our study, the Hb, RBC, MCH, and MCHC levels significantly increased in the group fed with especially 0.8% LFE supplemented diet of D group silver carp. The work reflected the similar works that have documented in different fish species such as common carp (Harikrishnan et al. 2005 [48]), juvenile beluga, Huso huso, (Binaii et al. 2014 [52]). An increase in the levels of serum protein, albumin, and globulins in fish thought to be associated with a more robust innate immune response [53].

The present study showed an enhancement of total protein in the carp group fed with 0.08% LFE supplemented diet, which recorded the highest values compared to the other groups. However, the total plasma protein, albumin, and globulin level were always higher in LFE treated group compared to control. This support with previous studies conducted using Astragalus membranaceus, Polygonum multiflorum, Isatis tinctorial and Glycyrrhiza glabra (Yuan et al. 2007 [54]), garlic (Nya and Austin 2009, 2011 [55, 56]), Nigella sativa and quercetin (Awad et al. 2013 [57]) and Choerospondias axillaris (Shakya and Labh 2019) in the sense that they have all enhanced serum total protein level in different fishes. Also, Binaii et al. recorded increases in total protein levels in juvenile beluga fed with nettle [52].

These studies suggested that a high concentration of total protein in fish serum was likely to be a result of the enhancement of non-specific immune response. Albumin and globulin are the primary plasma proteins in fishes [58]. The present results indicate that the albumin and globulin values increased along with the use of lapsi fruit extract (LFE) enriched diets. Similar results in globulin reported in rainbow trout fed with garlic enriched diets (Nya and Austin 2009 [56], Labh et al. 2017 [17]). Increasing albumin level was reported by Jagruthi et al. (2014) in carp fed with astaxanthin supplemented diet for four weeks.

An increase in glucose level was one of the stress indicators in fishes [59]. In this study, the LFE supplemented diet decreased glucose values in silver carp compared to the control group. As the value of LFE increased in the diet, the level of glucose decreased. The reports of Citarasu et al. [60], Sahu et al. [61], and Abasali and Mohamad [62] also explained the glucose levels reduced in different fish fed with herbal immunostimulant diets. In the present study, cholesterol level had significantly decreased in the 0.8% LFE/kg of diet group D compared to the control group A. The triglyceride levels were slightly higher in control diet (without LFE) fed group, but there was a significant difference ($P<0.05$) among the treated groups to that of control diet-fed group. Similar results observed in cholesterol estimation in which cholesterol levels found decreasing in LFE supplemented diet treated groups, which compared with various fish species fed with enhanced herbal diets [43, 63].

The functional state of the liver determined by estimating the biochemical parameters such as SOD, CAT, and GPx in liver tissues (U¨ner et al., 2006) and know that the antioxidant defense system includes enzymes such as SOD, CAT and GPx act as ROS scavengers protecting cell membrane lipids from oxidative damage [64, 65]. In this experiment, the level of SOD, CAT, and GPx in liver tissues was always high in the control diet-fed group, and as the dose of LFE increased in the treated diets, the SOD, CAT, and GPx exhibited decreasing trends. The minimum SOD, CAT, and GPx were in D group 0.8% LFE diet-fed group. SOD, CAT, and GPx involve in cellular defenses against uncontrolled oxidative processes and catalyze the dismutation of superoxide radical and H2O2 (Otto and Moon1996). A few reports exist concerning the effect of feed components on SOD, GPx, and CAT activity in fish [66-68].

Lysozyme activity is another ingredient in the first line of a barrier in the innate immune system. Biological and synthetic immunostimulant products considered to increase serum lysozyme activity [68]. In the present study, fish fed diets supplemented with different levels of oat extract showed significantly higher lysozyme activities when compared to the control group. Similar results have also reported in common carp fed with herbal immunostimulant diets [41, 62, 69, and 70]. The increased lysozyme activity observed in this study supported a higher non-specific immune response in the common carp fed with oat extract supplemented diets.

Administration of immunostimulants is one of the most important methods of controlling diseases in aquaculture by strengthening the defense mechanism of fish (Chakrabarti 2005 [70]), which considered as a promising alternative to chemotherapy and vaccines [28, 70]. In the present work, three growth-related genes (GH, IGF-I, and IGF-II) and three immune-related genes (IL-8, IL-10, and TNF-α) studied after 90 days of feeding trial with LFE supplemented diets. β-actin gene used as a control gene and compared to all the genes. It has observed that growth and immune-related gene expressions were always high in silver carp fed LFE supplemented group. These effects can be due to active principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids, and essential oils [71-73].

The study revealed, for the first time, the effects of herbal immunostimulants like lapsi fruit on the growth-related genes followed by immune-related genes in silver carp fingerlings. The modulation of non-specific defense mechanisms using herbal extracts has received increasing attention during the last decade [12, 15, and 74]. In comparison with this study, the effects of lapsi fruit extracts mainly attributed to the increase in growth-related gene expression. The present study indicated that fish fed with lapsi fruit extracts significantly increased immune-related genes (IL-8, IL-10, and TNF-α). Similar results obtained in gilthead sea bream, rainbow trout, and carp fry (Nootash et al. 2013; Esteban et al. 2014; Hoseinifar et al. 2015; Cerezuela et al. 2016 [75, 14]). Accordingly, in the last decades, interest has grown in the role and administration of herbal antioxidants as an alternative way to hinder oxidative damage in various health disorders [48].

In this study, a significant increase in IL-8 gene expression level explored in head-kidney from fish administrated with 800 mg kg^{-1} lapsi fruit extract (LFE). IL-10, an anti-inflammatory cytokine, is a multifunctional cytokine and shows immunosuppressive function. The primary function of IL-10 seems to be regulation of the inflammatory response, thereby minimizing damage to the host induced by an excessive response or self-immune system [76, 77]. Thus, the blocking of chemokine receptors can lead to inhibition of the effects of pro-inflammatory cytokines and restriction of activating of macrophages/monocytes induced by IL-10 [74, 78]. In the head kidney of silver carp fingerlings, lapsi fruit extracts caused the high expression of the IL-10 gene. This finding was contrary to lower expression levels of IL-10, which found following the administration of different stimulants in common carp [41, 79] and the head kidney of rainbow trout [26, 57, 80, 81].

5. Conclusion

Infectious diseases are major constraints of the aquaculture industry which frequently come from the stress factors resulting from intensive aquaculture practices. Using chemotherapeutants and antibiotics to control fish diseases has led to unfavourable effects such as the development of antibiotic resistant strains and accumulation of those compounds in environment and/or fish tissue which ultimately reflect on human health. Therefore, medicinal plants mainly come as a promising and alternative safer and cheap method for prevention and/or control of fish disease in aquaculture. Medicinal plants and their derivatives (extract and active compound) are rich with many active principle components such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, and flavonoids that possess various biological activities. In fish, several studies reported the biological activities of medicinal plants including growth promotion, appetite stimulation, immune stimulation, antimicrobial, and anti-stress. Application is in several forms, either as crude, or extract or active compound from the plant. Several medicinal plants used as immunostimulants at various concentrations though injection, or immersion, or oral, has shown evidence of enhancement of the innate and adaptive immune system of fish against many bacterial and viral diseases.

A native to Nepal fruit of Lapsi Choerospondias axillaris is opulent source of essential amino acids, minerals and ascorbic acid and is commonly used for the treatment of cardiovascular diseases in Vietnam, Mongolia and China etc. The phytochemical constituents of lapsi fruit extracts are phenol and flavonoid compounds which exhibit potent antioxidant activity to scavenge various free radicals and thus protect from toxic and harmful. Thus, in conclusion,

herbal immunostimulants such as lapsi fruit extract can be considered as a promising way in fish nutrition, aiming to improve fish growth, health status, immune responses, and disease resistance. The present results demonstrate that dietary fruit extract improves silver carp antioxidant defence, growth-related genes and immune system, which could help to maintain a sufficient antioxidant balance and contribute to the host health, reducing the risk of disease and well-being status of carp fingerlings. These findings are of great importance and interest for aquaculture research and the fish farming industry. Medicinal plants can act as promising antibiotics to resist microbial diseases and as an alternative to the use of chemicals and synthetic drugs. Further investigations are required to confirm their efficacy in fish farms with special concern about their influence on the physiological and health condition of fish. Working in medicinal plants is a huge field and many investigations remain necessary to discover the secrets behind this field.

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8. Data Availability

The PRIMARY data used in this work to support the findings of this study included in the article.

9. Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Nepal Health Research Council (NHRC) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

10. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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