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Effects of Fruits of Lapsi (Nepali Hog Plum) *Choerospondias axillaris* (roxb.) B.l. Burtt & A.W. Hill on Some Blood Parameters of Major Carp rohu *Labeo rohita* (H) During Intensive Aquaculture

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Abstract

The complete blood cell count (CBC) is an important and powerful diagnostic tool to monitor the health status of fish in response to changes related to nutrition, water quality, and disease in response to therapy.Thus, the present study was about to know the effect of lapsi fruit pulp (*Choerospondias axillaris*) on some blood parameters in the fingerlings of rohu *Labeo rohita* cultured in Corona of Agriculture Hatchery farm, Chitwan, Nepal. Altogether eighteen hapas made of heavy-duty nylon net (1.5m^3) were kept and placed inside the pond, distributed linearly, and then 270 fingerlings (@15 fishes/hapa) were kept distributed randomly. Six practical diets like 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}) were prepared. Feed containing 40% protein was supplemented with the ethanol extract of lapsi fruits. At the end of 90 days of the feeding trial, a significant difference ($P< 0.05$) in blood parameters were observed between the treated and control diet-fed groups. Hemoglobin (Hb), white blood cells (WBCs), red blood cells (RBCs), packed cell volume (PCV), and other erythrocyte indices were recorded higher in the treated groups. RBC, WBC, Hct, and Hb were found significantly higher in the T4 (0.4 %) diet-fed group. The study showed a minimum of 0.4 % (0.4g kg^{-1}) lapsi fruit extract needed in fish feeds to increase blood parameters to enhance growth and immunity.

Keywords

Lapsi fruits, rohu, blood count, Aquaculture, RBCs.

Introduction

Fishes have been extensively consumed as the source of protein in Nepal with an annual production of 86,544 metric ton in which 21,000 metric ton comes from capture fisheries (DoFD, 2017-2018). The per capita fish production in Nepal is 3.01 kg (DoFD, 2017-18), which is very low in comparison to neighbor countries like India (9.0 kg), Bangladesh (18.0 kg), and China (35.0 kg) and most of the fish demand is fulfilled by import from India (Gurung, 2014; Mishra and Kunwar, 2014). Aquaculture has become the only option to meet the growing demand for fish worldwide (Bhujel, 2012). To increase production and profit, intensive aquaculture practices have become standard practice in the whole world (Mohapatra *et al*., 2013). This practice has stressed immune systems of cultured fishes by pathogens with disease outbreaks (Inendino *et al*., 2005; Small and Bilodeau, 2005).

Herbal extracts supplemented in feed were reported to stimulate the non-specific defense mechanisms (Raa, 1996). The presence of active phytochemicals in herbs was reported to enhance growth due to-specific immunity, antioxidation enzyme activity, and disease resistance in fish (Xie *et al.,* 2008; Yeh *et al.,* 2009; Liu *et al.,* 2010). Additionally, the efficacies of many herbal extracts have been tested against bacteria in fish (Yeh *et al.,* 2009). Similarly, feed incorporated with herb extracts enhanced the non-specific immune responses in *Cirrhinus mrigala* against *Pseudomonas aeruginosa* (Sivagurunathan *et al.,* 2011; Kumar *et al.,* 2015). Lapsi fruit pulp (*Choerospondias axillaris*) is rich in vitamin C content (Shah, 1978) and phenolic and flavonoid compounds (Zhou, 2003), which not only enhances growth, immune power (Chunmei *et al.,* 2013) against the diseases.

The blood parameters are used as health indicators against different stress conditions (Thrall, 2004; Pimpao *et al.,* 2007) and can be used as an effective and sensitive index to monitor physiological and pathological changes in fishes (Fernandes *et al.,* 2003). These parameters are also closely related to the response of the animal to the environment exerting some influence on the blood characteristics (Arnold 2005; Labh *et al.,* 2015). Thus, the present study focusses on the properties of lapsi fruits on fish health with the objective as the effects of fruits of lapsi on some blood parameters of major carp rohu *Labeo rohita* during intensive aquaculture.

Materials and Methods

Selection of experimental fish and study area

Fingerlings (3.43±0.13 g) of major rohu *Labeo rohita* Hamilton, 1822 (Cyprinidae), were brought from a local hatchery. The experiment was conducted in the pond of Corona of Agriculture (P) Ltd and selected for this experiment.

Preparation of lapsi fruits supplemented artificial diets

Fresh lapsi fruits were obtained from the local market and processed to make powder from lapsi pulp were done according to the standard method. Extracts of lapsi fruit pulp were prepared using 70% ethanol (Labh *et al.,* 2015). Using standard calculation, altogether, six practical diets were prepared equally for each treatment (Table 1). The fish meal was dried well, ground in a grinder and sieved (mesh size: 500µ) to get a fine powder. Then the powdered fish meal was mixed, and lukewarm water was added in the required amount. Cod liver oil was added, mixed well, so that all the ingredients were spread homogeneously. The dough was prepared and passed through a feed maker using 1 mm die. The threads formed were dried and further chopped into small pieces of required sizes of pellets through a blender and then passed through a sieve to obtain equal-sized particles. Diet was kept in plastic containers and stored at 4ºC until used. Thus, six practical diets (40% protein) were prepared, along with different ingredients. The Diet one (T1) was a control diet (0.0 g kg^{-1}) without the extract of lapsi fruit pulp whereas the remaining five diets (T2 to T6) were supplemented with extract of lapsi fruit pulp (0.1 g kg^{-1}, 0.2 g kg^{-1}, 0.4 g kg^{-1}, 0.8g kg^{-1}, and 1.6 g kg^{-1}). These diets were stored at -20 C for further use.

Proximate analysis

For proximate analysis (Table 1), dry matter, crude protein, crude lipid, and ash were analyzed for experimental diets. Dry matter was investigated by drying the samples to constant weight at 105 °C. Crude protein was determined using the Kjeldahl method (AOAC, 1995) and estimated by multiplying nitrogen by 6.25. Crude lipid was measured by ether extraction using the Soxhlet method. Ash was examined by combustion in a muffle furnace at 550 °C for 6 h. Triple analyses were conducted for each sample.

Experimental design

Altogether eighteen hapas made of heavy-duty nylon net (1.5m^3) were suspended in the pond using a bamboo stick, arranged in such a way that six sets with replicate were observed. Then 270 fingerlings of Labeo rohita at the rate of 15 fishes per hapa of almost equal sizes were distributed equally and randomly. During the experiment, fingerlings were fed with test and control diets at the rate of 3% of their body weight twice daily at 9 a.m. and 4 p.m. Temperature, pH, and dissolved oxygen was monitored and recorded daily. The temperature was ranged between 26±0.13°C to 29±0.22 °C; pH was 7.33±0.93 to 7.67±0.14, and the dissolved oxygen was 5.83±0.94 to 6.83±0.94. The duration of the experiment was for 90 days.

Table 1: Ingredients and proximate composition of experimental diets (%).

Ingredients	Experimental diets (% inclusion)					
	T_1	T_2	T_3	T_4	T_5	T_6
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 (Carboxymethylcellulose) ††	1	0.99	0.98	0.96	0.92	0.84
Total	100	100	100	100	100	100
Proximate composition						
Dry Matter (DM)	97.15	97.43	97.59	97.71	96.93	97.14
Moisture	2.85	2.57	2.41	2.29	3.07	2.86
Crude Protein (CP)	31.16	31.07	31.32	31.14	31.22	31.39
Ether Extract (EE)	6.56	6.37	6.11	6.98	6.55	6.55
Crude Fibre	8.32	8.32	8.43	8.79	8.45	8.97
Ash	9.23	8.73	9.53	7.69	7.84	7.58
NFE♯	44.73	45.51	44.61	45.4	45.34	45.51

†Ingredients like fish meal, soya meal, groundnut oil cake, rice powder, wheat flour, cornflour, 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 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).
††Himedia Laboratories, Mumbai, India.
♯Nitrogen Free Extract (NFE)=100-(CP+EE+CF+Ash)

Collection of blood for analysis

At the end of the experiment, 3 fingerlings from each replicate from each treatment were collected and anesthetized with (5 mg/l) tricaine methanesulfonate (MS-222; Sigma Chemical Co. St. Louis, MO, USA) for 2-3 minutes. For blood collections, 1 to 2 ml of blood was drawn from the venaecaudalis with a disposable hypodermic needle (26 gauge). A blood sample was transferred to sterile Eppendorf tubes and kept at 4°C for hematological profiles.

Hematological parameter

Estimation of Hemoglobin (Hb)

Estimation of Hemoglobin (Hb) was done by the Cyanomethanoglobin method. 0.02 ml of blood was transferred into a tube containing 5.0 ml of Drabkin's reagent. The pipette was rinsed several times with the reagent. The diluted hemoglobin solution was kept for 10 minutes to achieve full-color development. The absorbance was measured at 530-550 nm of the unknown sample (A blank) and that of a standard of known hemoglobin content (A standard) against a reagent blank. Final calculation was done with the formula:

$$\text{Hb content (Unknown) (g dl}^{-1}) = \frac{\text{A blank} \times \text{Conccentration of Hb stamdard (g/dl)}}{\text{4A standard}}$$

Total red blood cell (RBC) count

Counting of red blood cells was done following the standard procedure. Blood was drawn in a dry erythrocyte pipette to the 0.5 graduations and then Hayem's solution (Qualigens Fine Chemicals, India) to the 101 marks (dilution 1:200). The pipette was then shaken for 1 minute, and counting of the cells was done under ×250 magnification. The erythrocytes were counted in 5 group squares (1 group square = 16 small squares) the number of small squares being 80 in 1/400 sq. mm. All cells lying inside the group squares and also the erythrocytes lying to the left and below the demarcation line were counted. Final erythrocyte count was calculated with the formula given below:

$$\text{Total RBC mm}^{-3} = \frac{\text{No.of erythrocytes is 80 small squares}}{80 \times \frac{1}{400} \times \frac{1}{10} \times 1/200}$$

Total white blood cell (WBC) count

White blood cells were counted following the standard procedure. Blood was drawn up to the 0.5 marks, and then dilution mixture (WBC diluting fluid, Qualigens Fine Chemicals, Mumbai) was drawn up to 101 marks into the WBC pipette. The full WBC pipette was gently revolved for 2-3 minutes to mix with the diluting fluid; after efficient mixing, the counting chamber was filled with a small drop of diluting the blood. The cells were allowed to settle for 3 minutes, so counting was done in the large square of the four corners of the chamber & are demarcated by triple line ($1mm^3$). Final white blood cell count was calculated with the formula as follows:

Total WBC mm^{-3} = = Total number

$$\frac{\text{No.of leucocytes in four square of 1mm}^2}{4 \times \frac{1}{10} \times 1/200}$$

of cells × 50 cell mm^{-3}

Packed Cell Volume (PCV)

Anderson and Siwicki (1995) method was followed for Packed Cell Volume. In brief, blood was drawn into the graduation mark 100 on the heparinized hematocrit pipette. Both the openings of the pipette were closed with rubber stoppers and centrifuged for 3 minutes. After centrifuging, the capillary tubes were placed on a reading device, and the volume was recorded. The hematocrit value was expressed as the percentage fraction of blood cells in the total volume (volume %). Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC) were calculated using standard formulae.

Statistical Analysis

All the data were expressed as arithmetic mean ± Standard Error. Statistical analysis of data involved a one-way analysis of variance (ANOVA) followed by the comparison of means following Duncan's Multiple Range Test (DMRT) with available SPSS windows 20.0 software. Significance was tested at the P<0.05 level.

Results

At the end of 90 days of the feeding trial, a significant relationship ((P<0.05) was observed between the treated group and the control group. Hemoglobin concentration (Fig. 3.1) in blood was significantly (P<0.05) higher in rohu fed with diet T4 (25.02±0.42e mg/dl) compared to other treated and control diets fed rohu. The concentration of hemoglobin in T5, T6, T3, T2, and T1 diets fed rohu were 23.04±0.36de, 20.71±0.47cd, 18.27±0.33bc, 15.58 ±0.15ab and 12.95±0.91a mg/dl respectively. The concentration of hemoglobin in the T4 diet-fed rohu was 1.9 times higher compared to rohu fed with control diet T1 (Fig. 3.1).

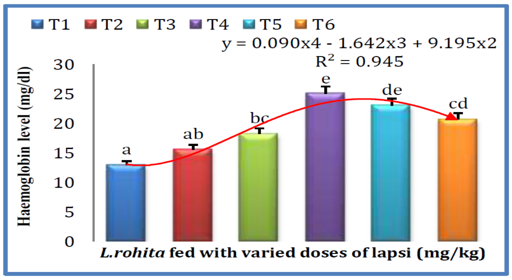

Fig. 3.1 Haemoglobin Concentration in Blood Level in Rohu Fed with Diet Containing Six Different Doses of Lapsi Fruits.

Erythrocytes number in blood was significantly ($P<0.05$) higher in rohu fed with diet T6 (5.16±0.15e million/mm^3) compared to treated and control diets fed rohu. The number of erythrocytes in T5, T4, T3, T3, and T1 diets fed rohu were 4.75±0.21de, 4.41±0.16cd, 3.84± 0.32bc, 3.35±0.16b, and 2.40±0.06a million/m^3 (Fig.3.2).

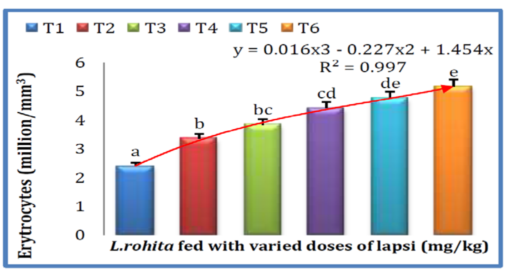

Fig. 3. 2 Erythrocytes (RBCs) in rohu fed with diet containing six different doses of lapsi fruits

Leucocyte number in blood was significantly ($P<0.05$) higher (45.95±0.70f 103/m^3) in rohu fed with diet T1 compared to other treated and control diet-fed rohu. The numbers of WBCs in T2, T6, T3, T5 and T4 diets fed rohu were 42.56±0.48e, 38.69±0.69d, 34.65± 0.04c, 31.42±0.06b, and 27.40±0.11a 103/m^3 respectively. Leucocytes' number in blood was 1.67 times higher in rohu fed with T1 diet-fed compared to rohu fed with T4 diet (Fig. 3.3).

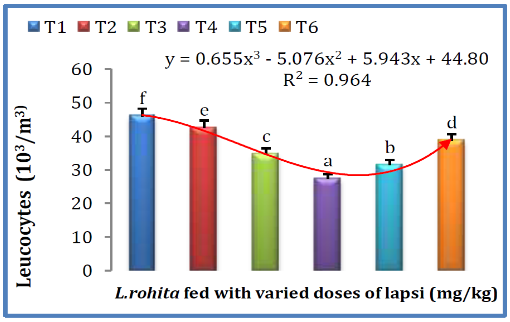

Fig. 3.3 Leucocytes (wbcs) in Rohu Fed with DIET Containing Six Different Doses of Lapsi Fruits

PCV level in blood was significantly ($P<0.05$) higher in rohu fed with diet T4 (55.18±0.51b %) compared to other treated and control diet-fed rohu. PCV level in T5, T3, T6, T1and T2 diets fed rohu were 47.04±0.27b, 46.49±0.13b, 45.62±0.27ab, 42.92±0.84ab, and 41.05±0.91a % respectively. PCV level in blood was 1.31 times higher in rohu fed with a T4 diet compared to rohu fed with control diet T1 (Fig. 3.4).

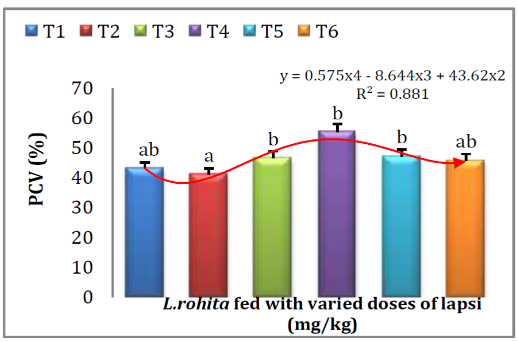

Fig. 3.4 Haematocrit (hct) or Packed Cell Volume (pcv) Level in Rohu Fed with Diecontaining Six Different Doses of Lapsi Fruits

Significantly ($P<0.05$) higher MCV level in the blood was recorded in rohu fed with diet T1 (179.24±1.62c µm^3). MCV levels in rohu fed with diets T4, T2, T3, T5, and T6 diets were 125.08±1.53b, 123.15±1.79b, 121.96±1.57b, 99.38±1.75a, and 88.57±1.06a µm^3 respectively.

MCV level in the blood of the T1 diet-fed rohu was 2.1 times higher compared to the rohu fed with the T6 diet (Fig. 3.5).

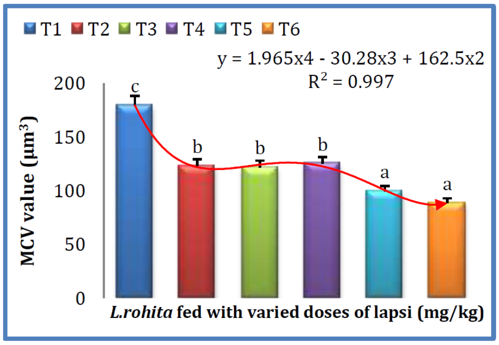

Fig. 3.5 Mean Corpuscular Volume (MCV) level in rohu fed with diet containing six different doses of Lapsi Fruits

Significantly (P<0.05) higher level of MCH (56.90±1.77b pg) was recorded in rohu fed with diet (T4). MCH levels in rohu fed with T1, T5, T3, T2, and T6 diets were 53.93±1.31ab, 48.73±1.78ab, 48.41±1.96ab, 46.96±1.35ab, and 40.24±1.96a pg, respectively (Fig.3.6).

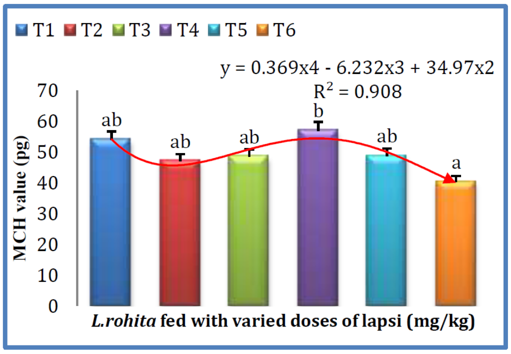

Fig. 3.6 mean corpuscular haemoglobin (mch) level in rohu fed with diet containing six different doses of lapsi fruits

Significantly (P<0.05), a higher percentage of MCHC was recorded (Fig. 3.7) in rohu fed with diet T5 (48.99±0.51c %) compared to other treated and control groups. The percentage of MCHC in rohu fed with diets T4, T6, T3, and T2 were 45.61±1.97bc, 45.38±0.76c, and 39.51±1.46b and 38.10±1.40ab % respectively. The lowest MCHC level was recorded in rohu fed with diet T1 (30.12±1.57a %). MCHC level in T5 diet-fed rohu was 1.6 times higher compared to the control diet T1 fed rohu (Fig. 3.7).

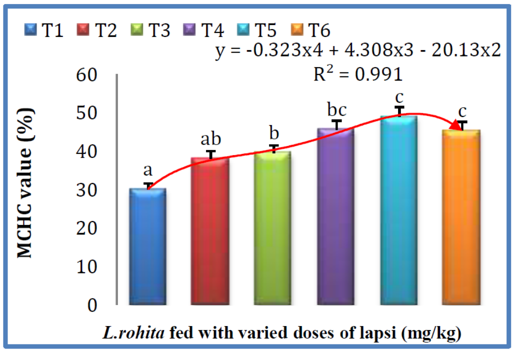

Fig. 3.7 Mean Corpuscular Haemoglobin Concentration (MCHC) Level in Rohu Fed with Diet

Discussions

Hematological parameters indicate the health status of fish species to detect physiological changes as a result of stress conditions such as transportation, handling, hypoxia, and acclimation (Alwan *et al.,* 2009; Gabriel *et al.,* 2004). Their changes depend on the fish species, age, the life cycle of sexual maturity, and health condition (Blaxhall, 1973; Hrubec *et al.*, 2001). Fish infected by microbes and exposure to toxicants also have profoundly influenced on the hematological parameters (Harikrishnana *et al.,* 2003).In the present study, blood parameters such as RBC and WBC count and hemoglobin obtained in the present study almost agree with earlier workers (Goel and Sharma 1987). The current result indicated that the inclusion of *C. axillaries* extracts in the fish diet significantly increased the RBC counts with increasing dietary inclusion levels of lapsi extract.

Hematological parameters act as physiological indicators to the changing external environment (Gill and Pant, 1981) as a result of their relationship with energetics (metabolic levels), respiration (hemoglobin levels) and defense mechanisms (leucocyte levels), as these parameters provide an integrated measure of the health status of an organism which overtime manifest in changes in weight (growth) (Gbem *et* *al.,* 2003).

The current result is in agreement with observed increase in RBCs in *Cyprinus carpio* fed extract of *Eurphobia hirta* (Pratheepa and Sukumaran, 2014) and *Aeglemarmellos* (Pratheepa *et al.,2014)*, *Clarias gariepinus* fed *Morus alba* extract (Sheikhlar *et al.,* 2014), and juvenile *Huso huso* fed diets supplemented with stinging nettle (*Urtica diocia*) (Binaii *et al.,* 2014). Nya and Austin (2009) also reported higher RBC counts in rainbow trout fed with the garlic-added and ginger-added diets. The apparent increase in RBC after dietary supplementation with *C. axillaries* may be related to the presence of vitamin C, which is required for the production of RBC (Dugenci *et al.,* 2003). In agreement with the present findings, Sahu *et al.,* (2007) reported that RBC counts were higher in *L. rohita* fingerlings fed with *Mangifera indica* kernel when compared to control. The WBC count was increased in fish fed with treated diets compared to fish fed with the control diet. This result was supported by Sahoo and Mukherjee (2001), who found that WBC count was increased in *L. rohita* fingerlings treated with immunostimulants such as levamisole and ascorbic acid. Jah *et al.*, (2007) also reported increased WBC count in *C. cattl*e administered with yeast RNA, omega-3 fatty acid, and Beta-carotene. Nya and Austin (2009) also reported significantly higher WBC counts in rainbow trout fed with the garlic-added and ginger-added diets. The WBC counts also appeared to increase with increasing dietary inclusion levels of *C.axillaries* extract is due to the presence of flavonoids in its fruits, which are known to act as an antioxidant to neutralize highly unstable and extremely reactive free radicals, which attach the healthy cells of the body.

Fish with low WBCs are exposed to a high risk of disease infection. In contrast, those with high counts are capable of generating antibodies and have a high degree of resistance to diseases (Soetan *et al.,* 2013) and enhance adaptability to local environmental and prevalent disease conditions (Isaac *et al.,* 2013). The erythrocyte indices (PCV, MCV, and MCHC) increased with increasing inclusion levels of *C. axillaries* in the diets may be attributed to the activation of the non-specific immunity mechanism. Packed cell volume (PCV), also known as hematocrits (Hct), is involved in the transport of oxygen and absorbed nutrients. Increased PCV shows better transport of oxygen. The increase in total RBC, total WBC, Hb, MCV, MCH, MCHC following dietary inclusion of C. *axillares* in diets indicate the immunostimulant effects and anti-infection properties or the plant. Gopalakannan and Arul (2006) also reported that there was an increase in the WBC count after feeding the common carp with immunostimulants like chitin.

Hemoglobin has the physiological function of transporting oxygen to tissues for the oxidation of the glucose to release energy for the other body functions as well as transport carbon dioxide out of the body. The hemoglobin (Hb) levels increased with increasing doses of *C. axillaris* in rohu may be due to the increase in oxygen demand, as indicated by Verma *et al.*, (2007a) in *Cyprinus carpio*. According to the results, *C. axillaris* extract supplemented diets could increase hemoglobin content, WBC, and RBC levels in experimental groups compared to the control group. In agreement with the present findings, Ngugi *et al.*, (2015) showed increased hematological parameters with increasing dietary inclusion of stinging nettle (*Urtica dioica*) in *Labeo victorianus*. Enhancement of the immune system is the most promising method of preventing diseases in fish. The first line of defense against invading pathogens is the innate (non-specific) immune system. Supplementation of immunostimulants in feed can improve fish health and thereby reduce management costs. Among immunostimulants, *C. axillares* can also be used as a feed additive to replace antibiotics and enhance sustainable aquaculture.

Conclusion

The results of this research contribute to the knowledge of the blood parameters of the rohu, L. rohita, under the supplementation of C. axillaris fruits extracts. This investigation may be helpful as a tool to monitor the health status of fish. The incorporation of ethanol extract of C. axillaris fruits in rohu diets would improve blood parameters as evidence in the study by increasing RBC, WBC, Hb, and erythrocyte indexes in the treated fish. It is recommended to include 0.4% lapsi pulp extract in the diet of L. rohita for the enhancement of fish health. Acknowledgments

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