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Physio-immunological responses of *Labeo rohita* fingerlings to commonly used phytogenic feed additives: A comparative evaluation

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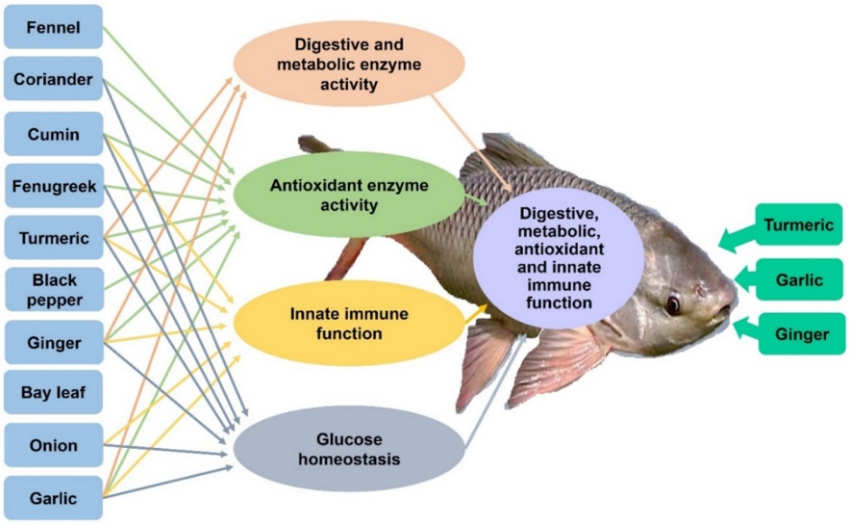
Abstract

Aim: The present experiment was undertaken to compare digestive, metabolic, antioxidant enzyme activities and immuno-biochemical responses in *Labeo rohita* fingerlings to some commonly used phytogenic feed additives.

Methodology: Eleven experimental diets were prepared by supplementing fennel, coriander, cumin, fenugreek seed, turmeric, black pepper, peeled ginger, bay leaf, peeled onion bulb or peeled garlic clove meal at 1% inclusion level along with a control diet. Four hundred and ninety-five fingerlings (average weight 6.45±0.01 g) were distributed randomly in eleven experimental groups in triplicates with a stocking density of 15 fish per tank (400 l of water).



Results: Turmeric, garlic or ginger meals appeared to be more effective than onion, fenugreek, cumin, coriander, fennel, black pepper and bay leaf meals for enhancing digestive, metabolic, antioxidant enzyme activities and innate immune functions. The physio-metabolic effects of phytogenic feed additives tested in *Labeo rohita* fingerlings were in the order of turmeric > garlic > ginger > onion > fenugreek > cumin > coriander > fennel > black pepper > bay leaf meal.

Interpretation: The enhanced digestive and metabolic enzyme activities, antioxidant function, glucose homeostasis and improved innate immune function through modulation of the haemato-biochemical profile of *Labeo rohita* due to feeding of specific functional compounds present in turmeric, ginger and garlic meals compare to other phytogenic additives.

Key words: Antioxidants, Digestion, Innate-immunity, Metabolism, Phytogenic additives.

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Introduction

Present-day aquaculture needs to be shifted from traditional extensive system to feed-based intensive system to enhance the productivity and production as the scope of horizontal expansion of aquaculture resources is squeezing day by day due to more urbanization and industrialization (Henriksson *et al.,* 2018). Moreover, technological advancement makes the aquaculture production more environment-friendly and sustainable. However, intensification causes stress in animals making them more susceptible to diseases. *Labeo rohita*, an Indian Major Carp, has more market demand and consumer preference in the Asian subcontinent. Recent trend of carp culture is mainly dependent on the plant-based feed. But, the anti-nutritional factors (ANFs) of plant-based ingredients adversely affects the beneficial gut microbes, reduce the digestibility of aquafeed either through making indigestible complexes with the nutrients or by forming enzyme-ANF complex leading to reduced activity of digestive enzymes and nutrient digestibility, poor growth, immuno-suppression and disease outbreak (Murashita *et al.,* 2015; Apines-Amar *et al.,* 2016; Encarnação, 2016). Hence, non-nutritive chemicals having several disadvantages are widely used to protect the fish from infectious diseases.

Concern have been raised to control the use of antibiotics, which are associated with the development of 'superbugs' (pathogens highly resistant to antibiotics) along with antibiotic residue in fish tissue, causing several human health hazards (Angulo and Griffin, 2000). Therefore, the use of non-therapeutic antibiotics in farm animals has been banned in Sweden and later Denmark, United Kingdom and various European countries (Vidya *et al.,* 2019). In this context, dietary use of organic acids, prebiotics, probiotics, phytogenic ingredients and immuno-stimulants are the options available to improve the fish gut health with the potential enhancement of nutrient digestion, growth and immunity of fish (Rico *et al.,* 2013; Encarnação, 2016). Intensive aquaculture often leads to infectious disease causing significant loss to the farmers (Harikrishnan *et al.,* 2011). This has created research interest in the use of phytogenic feed additives (PFAs) as digestive stimulants, growth promoters, anti-stress factors, immune-stimulants and bactericidal agent in aquafeed, which have no residual and side effects in fish (Lee *et al.,* 2015; Nair *et al.,* 2017). Published reports have confirmed that supplementation of PFAs in aquafeed has improved the health status in fish probably due to the action of bioactive compounds (polyphenols, flavonoids etc.) present in these plant ingredients (Awad and Awaad, 2017; Esmaeili *et al.,* 2017).

Previous studies have reported that dietary supplementation of PFAs either as meal or crude extract results in improved growth and immune responses in Indian major carp (Sahu *et al.,* 2007; Behera *et al.,* 2011; Chakrabarti *et al.,* 2014; Giri *et al.,* 2015; Sukumaran *et al.,* 2016). However, physio-biochemical responses like digestive and metabolic enzyme activities, antioxidant status also need to be assessed for better understanding of mechanism of action of PFAs in fish. Therefore, the present study was carried out to compare the multi-functional effects of different individual phytogenic stimulants on the activities of digestive and metabolic enzymes, antioxidant status, and innate immune responses of *Labeo rohita* fingerlings.

Materials and Methods

Preparation of phytogenic ingredients and experimental diets: Ten herbs namely fennel seed (*Foeniculum vulgare*), coriander seed (*Coriandrum sativum*), cumin seed (*Cuminum cyminum*), fenugreek seed (*Trigonella foenum-graecum*), turmeric (*Curcuma longa*), black peppercorn (*Piper nigrum*), ginger (*Zingiber officinale*), bay leaf (*Laurus nobilis*), onion (*Allium cepa*) and garlic (*Allium sativum*) were procured from local market, Andheri West, Mumbai. Turmeric and ginger, onion bulb and garlic clove were cleaned properly with water to get rid of dust, peeled, chopped, sun-dried and grinded to make fine powder (<200µm), packed and stored at 4°C for further use. Eleven isonitrogenous (33.5% crude protein), isolipidic (7%) and iso-energetic (15 MJ DE/kg) experimental diets *viz*., C (control without herbal supplementation), FSM (fennel seed meal), CRSM (coriander seed meal), CSM (cumin seed meal), FGSM (fenugreek seed meal), TRM (turmeric root meal), BPM (black pepper meal), PGM (peeled ginger meal), BLM (bay leaf meal), POM (peeled onion meal) and PGCM (peeled garlic clove meal) at 1% inclusion level were used in the feed (Table 1). Ground ingredients and PFAs were weighed according to the formulation and mixed well to get a homogenous mixture, followed by mixing with water to prepare a dough, which was steam cooked for 20 min in a pressure cooker. The cooked dough was allowed to cool at room temperature. Pre-weighed phytase, vitamin premix, mineral premix and fish oil were then homogenously mixed with the dough, which was used for preparing pellets. The extruded pellets were then dried at 40°C, packed and stored at 4°C until further use.

Proximate composition: Proximate composition (Table 1) *viz.,* moisture, crude protein (CP), ether extract (EE), crude fiber (CF), total ash (TA) and nitrogen-free extract (NFE) of experimental diets were estimated as per standard methods (AOAC, 1995). Gross energy was estimated using bomb calorimeter (Changsha Kaiyuan Instruments Co. Ltd, Model No.5E-AC/PL) following the manufacturers manual. The gross energy (GE) value was expressed as MJ kg^{-1}. NFE and Digestible energy (DE) was calculated by the formula given by Halver (1976).

Experimental design and maintenance of experimental fish: *Labeo rohita* fingerlings were procured from Hans Aquaculture, Raigarh, Maharashtra, India and acclimatized in laboratory condition for 15 days. During this period, the fish was fed to satiation level three times a day. Four hundred and ninety five (495) acclimated fingerlings of *Labeo rohita* (average weight 6.45 ± 0.01 g) were distributed randomly into eleven experimental groups in triplicates with a stocking density of 15 fish per tank (400 l water capacity) following a completely randomized design (CRD). Round the clock aeration was provided in all the

experimental tanks. Before starting the experimental feeding, the fish were fed with control diet for 7 days. After that the fish of different experimental groups were fed to satiation level thrice daily with respective experimental diets for 15 days. Faecal matters were siphoned out daily and drained water was replenished with fresh borewell water to maintain the same water volume in the tanks throughout the experiment.

Collection and preparation of sample: At the end of the experiment, nine fish from each tank were anesthetized with clove oil (50 µl l^{-1}). Blood samples were collected from the caudal vein with 1 ml hypodermic syringe and transferred to EDTA (2.7%) coated 1.5 ml vial to prevent coagulation. The whole blood was used for estimation of total leucocyte count (TLC) and total erythrocyte count (TEC). Whereas, blood collected from the other fish was transferred to an eppendorf tube without any anticoagulant and kept the tube in a tilted position for an hour at room temperature for allowing the blood to clot. Serum sample was carefully collected in an eppendorf tube after centrifugation at 6000 rpm for 15 min, and kept at -20°C for further analysis. After blood collection, the same fish (three fish per tank) were dissected and samples from the intestine, liver, muscle and gill were collected in centrifuge tubes to make 5% tissue homogenate using 0.25 M sucrose solution. The tissue homogenate was centrifuged at 6000 rpm for 10 min at 4°C in a refrigerated centrifuge. The supernatant was collected and transferred in a screw cap tube and stored at -20°C.

Nitrobluetetrazolium (NBT) assay: The method of Stasiak and Baumann (1996) were followed to assay the respiratory burst activity of leucocyte, in which the formation of blue precipitate and reduction of NBT was detected at 620 nm.

Hematological parameter: Blood haemoglobin level was assayed by cyanmethemoglobin method (Varley *et al.,* 1991). The standard method of Schalm *et al.* (1975) was used to determine total erythrocyte count (TEC), total leucocyte count (TLC) and differential count of leucocytes. The serum total protein, albumin and glucose were estimated using commercial kit (ERBA Kit, Transasia Bio-medicals Ltd., Mannheim) and albumin value was subtracted from total protein value to obtain serum globulin level. Albumin content was divided by globulin content to get albumin to globulin ratio (A:G).

Estimation of tissue protein: The protein contents of liver, intestine, gill and muscle tissues were analyzed following the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as standard and the absorbance was read at 660 nm. Tissue protein content was calculated from the standard graph and expressed as mg g^{-1} wet weight.

Assays of digestive and metabolic and antioxidant enzyme activities: Protease activity of intestine tissue was assayed according to the casein digestion method of Drapeau (1974). Amylase activity of intestinal tissue was assayed with 2% (w/v) starch solution as substrate (Rick, 1974). Lipase activity was assayed according to Cherry and Crandall (1932). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the liver and muscle tissues were assayed following the method of Wooten (1964). Superoxide dismutase (SOD) activity of liver and gill tissues was estimated by the method described by Misra and Frodovich (1972). Catalase activity of tissue homogenate was estimated following the method of Takhara *et al.* (1960).

Statistical analyses: One-way analysis of variance (ANOVA) was performed to analyze the data of the present study using SPSS 22.0 version for windows. Duncan's multiple range test (DMRT) in SPSS was used to evaluate significant difference among the mean values at 5% probability (p<0.05). All the analyzed data were presented as mean ± standard error. The assumption of homogeneity of variance was tested by Levene's test.

Results and Discussion

The digestive, metabolic and anti-oxidant enzymes along with immunological parameters in *Labeo rohita* were measured after the feeding trial. A significantly (p<0.05) higher protease activity was recorded in the TRM, BLM and PGCM groups and amylase activity in TRM and PGRM groups compared to control (Table 2). Similarly, lipase activity was found to be significantly (p<0.05) higher in TRM, PGRM, POBM and PGCM and lower in CRSM, CSM and BPFM groups in comparison to control. The AST activity of the liver was found to be significantly (p<0.05) higher in TRM, PGRM, POBM and PGCM groups while higher (p<0.05) AST activity of muscle was found only in TRM than the control (Table 2). A significantly (p<0.05) higher hepatic ALT activity was recorded in CRSM, FGSM, TRM, PGRM and PGCM groups, and lower activity in POBM group compared to the control. However, CRSM, CSM, FGSM, TRM and PGCM groups showed significantly (p<0.05) higher muscle ALT activity than the control.

Digestive enzymes hydrolyse the nutrients to increase their bioavailability. Moreover, there is a positive correlation exist with metabolic enzyme (AST and ALT) activities of liver and muscle tissue with the growth of fish (Jiang *et al.,* 2015). In the present study TRM, PGRM and PGCM were found to be more effective in increasing digestive and metabolic enzyme activities in comparison to other phytogenic feed additives in *Labeo rohita* fingerlings. Curcuminoid is the major bioactive compound of turmeric that probably could elicit the digestive function and health benefit in fish. In corroboration with the present findings, Jiang *et al.* (2016) reported that turmeric powder at 5 g kg^{-1} could increase digestive enzymes activities in crucian carp (*Carassius auratus*). Similar to our observation, Bhavan *et al.* (2013) also found increased protease, amylase and lipase activities in *Machrobrachium rosenbergii* due to feeding of ginger meal at 5% dietary inclusion level and enhanced enzyme activities probably mediated through the action of gingerol, gingibe and camphene present in ginger. Feeding of garlic meal could enhance the digestive enzyme activities of *Labeo rohita* in the present study,

Table 1: Formulation and proximate composition of different experimental diets fed to *Labeo rohita* fingerlings for a period of 15 days

Ingredients (%)	Treatments (Diets)[1]										
	C	FSM	CRSM	CSM	FGSM	TRM	BPFM	PGRM	BLM	POBM	PGCM
Soybean meal (SBM)	20	20	20	20	20	20	20	20	20	20	20
Ground nut oil cake (GNOC)	30	30	30	30	30	30	30	30	30	30	30
Mustard oil cake (MOC)	15	15	15	15	15	15	15	15	15	15	15
De oiled rice bran (DORB)	25	25	25	25	25	25	25	25	25	25	25
Wheat flour (WF)	6.86	5.86	5.86	5.86	5.86	5.86	5.86	5.86	5.86	5.86	5.86
Fish oil	1	1	1	1	1	1	1	1	1	1	1
Vitamin premix[2]	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Mineral premix[3]	1	1	1	1	1	1	1	1	1	1	1
Phytase[4]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Carboxymethyle cellulose (CMC)	1	1	1	1	1	1	1	1	1	1	1
Phytogenic feed additives (PFAs)	0	1	1	1	1	1	1	1	1	1	1
Total	100	100	100	100	100	100	100	100	100	100	100
Proximate composition[5] (on dry matter basis)											
Dry matter (%)	91.34	91.53	91.50	91.62	91.46	91.41	91.44	91.49	91.57	91.42	91.51
Crude protein (%)	33.62	33.69	33.57	33.57	33.65	33.63	33.64	33.59	33.58	33.60	33.56
Ether extract (%)	7.23	7.35	7.29	7.25	7.20	7.26	7.35	7.53	7.25	7.47	7.42
Crude fiber (%)	10.47	10.56	10.66	10.40	10.34	10.49	10.67	10.72	10.68	10.69	10.67
Total ash (%)	9.37	9.23	9.20	9.28	9.29	9.38	9.29	9.38	9.35	9.32	9.33
Nitrogen free extract (%)	39.31	39.17	39.29	39.50	39.53	39.24	39.05	38.78	39.14	38.92	39.03
Gross energy (MJ/kg)	18.29	18.33	18.27	18.14	18.48	18.26	18.31	18.28	18.38	18.35	18.32

[1]C (Control without herbal meal), FSM (1% fennel seed meal), CRSM (1% coriander seed meal), CSM (1% Cumin seed meal), FGSM (1% fenugreek seed meal), TRM (1% turmeric root meal), BPFM (1% black pepper fruit meal), PGRM (1% peeled ginger root meal), BLM (1% bay leaf meal), POBM (1% peeled onion bulb meal) and PGCM (1% peeled garlic clove meal). [2]Composition vitamin premix (quantity/kg): Vitamin A, 55,00,000 IU; Vitamin D3, 20,00,000 IU; Vitamin B2, 6000 mg; Vitamin E, 40000 mg; Vitamin K, 4000 mg; Vitamin B6 3645 mg; Vitamin B7 200 mg; Vitamin B9 1500mg; Vitamin B12, 10 mcg; Pantothenic acid, 13000 mg; Niacin, 12000 mg; Vitamin C, 40000 mg. [3]Composition mineral premix (quantity/kg) Ca, 255 g; P, 127.5 g; Mn, 27,000 mg; I, 325 mg; Fe, 1500 mg; Mg, 6000 mg; K, 100 mg; Na, 5.9 mg;Mn, 1500 mg; S, 7.2 g, Zn, 9600 mg; Cu, 1200 mg; Co, 150 mg; DL-Methionine, 1000 mg; Selenium 50 ppm. [4]Microbial (*E. coli*) phytase (Quantum blue, 500U/ kg), AB Vista, Wiltshire, UK. [5]The proximate composition of the ingredients were estimated in triplicates

which can be well compared with the study of Esmaeili *et al.* (2017) in rain bow trout probably due to the action of allicin, the main bioactive compound present in garlic. Overall, dietary turmeric, ginger and garlic meal at 1% inclusion level could elevate the digestive and metabolic enzyme activities in *Labeo rohita* fingerlings other than phytogenic feed additives tested in the present study.

All the treatment groups, except BLM and POBM, showed a significantly (p<0.05) higher SOD and catalase activities in the liver and gill tissues as compared to control (Table 3). A wide range of bioactive compounds of different herbs are reported to inhibit the generation of reactive oxygen species (ROS) and scavenge oxidative stress induced free radicals in animals, including fish. Antioxidant properties of fennel, coriander and cumin seed had been reported by Pandey *et al.* (2012) to support the present findings. Similar to current observation, Awad *et al.* (2015) reported the antioxidant properties of fenugreek in gilthead seabream. The scavenging of superoxide anion and hydroxyl radical by dietary turmeric had been found in crucian carp, *Carassius auratus* (Jiang *et al.,* 2016). Therefore, significant improvement of antioxidant enzyme activities of *Labeo rohita* fingerlings of turmeric group in the present study suggests better antioxidant status of fish to prevent the ROS-mediated damage. The antioxidant efficacy of black pepper had been reported in the rat (Vijayakumar *et al.,* 2004) to support the present findings. Dietary ginger was found to alleviate ROS-mediated health problem in *Labeo rohita* fingerlings (Sukumaran *et al.,* 2016) through enhancing the antioxidant enzyme activities and up-regulating the antioxidant enzyme gene expression. In corroboration to our finding, dietary garlic at 4% inclusion level could induce to enhance SOD and catalase activities in Nile tilapia (Metwally, 2009).

With respect to control, the hematological profiles (hemoglobin level, total erythrocyte count, total leucocyte count and differential count) were positively influenced by dietary PGCM, TRM, CSM, PGRM and POBM in *Labeo rohita* fingerlings (Table 4). Hemoglobin level was significantly (p<0.05) higher in the fish of TRM, PGRM and PGCM groups (p<0.05) might be associated with the bioactive compound induced protection of haemoglobin from oxidation (Unnikrishnan and Rao, 1995). Total erythrocyte count (TEC) was significantly (p<0.05) higher in the CSM, TRM, PGRM, BLM, POBM and PGCM groups in

Table 2: Effect of dietary phytogenic stimulant on digestive and metabolic enzyme activities of *Labeo rohita* fingerlings

Treatment	Digestive enzyme			Metabolic enzyme			
				AST[4]		ALT[5]	
	Protease[1]	Amylase[2]	Lipase[3]	Liver	Muscle	Liver	Muscle
C	15.82±0.02^{abc}	10.60±0.12^{abc}	1.27±0.07^{cd}	29.58±1.51^{ab}	32.70±0.57^{bcd}	14.13±0.44^{b}	17.26±0.22^{ab}
FSM	19.05±6.67^{cde}	11.08±0.80^{abcd}	1.20±0.12^{cd}	29.30±1.28^{ab}	31.53±1.02^{bc}	12.92±0.35^{b}	16.71±0.83^{ab}
CRSM	14.15±01.06^{abc}	12.06±0.81^{cde}	0.73±0.07^{a}	32.44±0.94^{bc}	33.79±0.45^{cde}	18.11±1.32^{d}	21.33±0.92^{de}
CSM	12.99±01.08^{a}	11.24±0.30^{abcd}	0.80±0.12^{ab}	26.77±1.57^{a}	31.41±1.19^{bc}	12.23±0.64^{b}	20.82±0.43^{de}
FGSM	17.53±01.07^{bc}	12.01±0.29^{cde}	1.40±0.12^{d}	29.60±1.11^{ab}	35.89±2.31^{de}	17.37±0.43^{d}	23.02±1.74^{ef}
TRM	22.61±1.67^{ef}	13.27±0.36^{e}	2.27±0.07^{f}	39.23±0.81^{d}	37.80±0.45^{e}	21.62±0.78^{e}	24.63±0.80^{f}
BPFM	15.47±1.19^{abc}	10.20±0.45^{ab}	0.76±0.08^{ab}	27.90±2.71^{ab}	33.47±1.06^{cd}	14.29±0.24^{bc}	16.15±0.59^{a}
PGRM	17.92±0.09^{bcd}	12.23±0.31^{de}	1.89±0.08^{e}	35.82±1.55^{cd}	35.96±2.48^{de}	16.71±0.37^{cd}	19.07±0.31^{bcd}
BLM	21.90±1.24^{def}	9.97±0.20^{a}	1.06±0.07^{bc}	28.89±1.15^{ab}	28.75±0.88^{ab}	13.58±0.96^{b}	17.86±0.37^{abc}
POBM	18.64±0.97^{cde}	10.43±0.26^{ab}	2.04±0.13^{ef}	36.22±1.66^{cd}	26.34±1.33^{a}	8.35±0.95^{a}	16.72±0.24^{ab}
PGCM	24.57±62.60^{f}	11.61±0.65^{bcd}	2.27±0.13^{ef}	36.24±0.19^{cd}	28.81±0.54^{ab}	18.80±1.68^{d}	19.73±0.12^{cd}
p value	<0.001	0.001	<0.001	0.001	0.001	<0.001	<0.001

Data is presented as Mean ± SE (n = 3), values with different superscripts in the same column differ significantly ($p<0.05$). [1]Protease activity is expressed as micromole tyrosine released min^{-1} g^{-1} protein; [2]Amylase activity is expressed as micromole of maltose released min^{-1} g^{-1} protein; [3]Lypase activity is expressed as units min^{-1} g^{-1} protein; [4]AST, Aspartate aminotransferase, the activity is expressed as micromoles of sodium pyruvate formed min^{-1} mg^{-1} protein.; [5]ALT, Alanine aminotransferase, the activity is expressed as micromoles of oxaloacetate formed min^{-1} mg^{-1} protein

Table 3: Changes of antioxidant enzyme activities of *Labeo rohita* fingerlings due to feeding of phytogenic stimulant for a period of 15 days

Treatment	SOD[1]		CAT[2]	
	Liver	Gill	Liver	Gill
C	27.46±0.31^{a}	8.69±0.21^{a}	17.78±0.41^{a}	4.66±0.33^{a}
FSM	30.64±0.99^{bcd}	12.00±0.78^{c}	22.51±0.51^{bc}	7.15±0.46^{b}
CRSM	32.51±0.23^{cd}	10.50±0.16^{b}	25.78±0.29^{de}	8.65±0.66^{cd}
CSM	29.86±0.98^{b}	11.17±0.72^{c}	22.99±1.40^{cd}	8.88±0.38^{cd}
FGSM	32.74±0.35^{d}	10.04±0.13^{bc}	24.61±0.96^{cd}	9.11±0.51^{de}
TRM	36.17±0.95^{e}	12.14±0.15^{c}	27.89±0.96^{ef}	10.57±0.47^{f}
BPFM	29.83±0.77^{b}	10.31±0.49^{b}	24.45±0.55^{cd}	7.74±0.21^{bc}
PGRM	30.53±0.37^{bcd}	12.18±0.14^{c}	28.79±1.20^{f}	10.21±0.30^{ef}
BLM	25.49±1.04^{a}	8.40±0.36^{a}	19.17±0.98^{a}	4.77±0.24^{a}
POBM	27.58±0.59^{a}	8.58±0.46^{a}	19.95±1.2^{ab}	5.07±0.34^{a}
PGCM	30.30±0.32^{bc}	10.09±0.12^{b}	27.47±2.06^{ef}	9.14±0.34^{de}
p value	0.001	0.001	0.001	<0.001

Data is presented as Mean ± SE (n = 3), values with different superscripts in the same column differ significantly ($p<0.05$). [1]SOD, Superoxide dismutase, the activity is expressed as 50% inhibition of epinephrine auto-oxidation mg^{-1} protein min^{-1}; [2]CAT, Catalase, the activity expressed as nanomoles H_2O_2 decomposed min^{-1} mg^{-1} protein

comparison to control ($p<0.05$) might be due to bioactive compound mediated erythropoiesis. Enhanced haemoglobin and TEC could be indicative of improved oxygen transport for better nutrient metabolism in fish. In agreement with the present result, dietary curcumin improved TEC in *Labeo rohita* (Sahu *et al.,* 2008). Similarly, enhanced TEC was also recorded in Asian sea bass (Talpur *et al.,* 2013) and rainbow trout (Nya and Austin, 2009) due to feeding of 0.1-1% ginger powder and 0.5-1.0% garlic powder, respectively to indicate better health status of fish. White blood cell (WBC) or leucocytes play an important role in first-line defense to protect the fish from invaded pathogens. In the preset study, the significant increase of TLC in *Labeo rohita* fingerlings due to feeding of CSM, TRM, PGRM, POBM and PGCM at 1% level improved the innate immune function to keep the fish healthy. There is no information available on the effect of dietary CSM on the TLC in fish. However, the present finding

Table 4: Dietary phytogenic stimulant affected the haematological profile of *Labeo rohita* fingerlings

Treatment	Hb (g dl^{-1})	TLC (×10^3 mm^{-3})	TEC (×10^6 mm^{-3})	Differential count			
				Lymphocyte (×10^3 mm^{-3})	Monocyte (Nos mm^{-3})	Neutrophil (Nos mm^{-3})	Eosinophil (Nos mm^{-3})
C	6.03±0.12^{bc}	1.12±0.05^{ab}	89.20±3.59^{b}	87.93±2.04^{b}	299.33±32.84^{c}	926.67±41.87^{bc}	478.67±38.95^{c}
FSM	5.40±0.17^{ab}	0.98±0.08^{a}	88.43±4.85^{b}	87.48±4.72^{b}	176.87±9.69^{abc}	592.63±59.61^{ab}	182.33±60.05^{ab}
CRSM	5.43±0.23^{ab}	1.24±0.03^{bc}	77.33±3.26^{b}	76.66±3.24^{b}	77.33±3.26^{a}	515.27±32.29^{ab}	77.33±3.48^{ab}
CSM	6.47±0.15^{cd}	1.34±0.10^{cd}	134.00±2.47^{d}	129.94±2.41^{d}	314.03±50.20^{c}	3615.80±406.85^{g}	134.00±2.31^{ab}
FGSM	4.87±0.15^{a}	1.09±0.08^{ab}	62.37±7.52^{a}	60.02±7.55^{a}	188.00±48.42^{abc}	2063.23±108.99^{f}	94.00±24.01^{ab}
TRM	6.77±0.20^{de}	1.45±0.04^{d}	133.10±3.40^{d}	130.48±3.31^{d}	533.10±82.36^{d}	1823.57±188.39^{ef}	262.67±69.70^{abc}
BPFM	5.43±0.20^{ab}	1.14±0.05^{ab}	88.41±4.80^{b}	87.61±4.77^{b}	88.33±4.98^{ab}	561.00±46.36^{ab}	150.33±35.26^{ab}
PGRM	7.87±0.15^{f}	1.94±0.05^{e}	107.45±4.18^{c}	107.03±4.26^{c}	142.00±29.54^{ab}	1224.33±63.39^{cd}	108.67±4.10^{ab}
BLM	5.43±0.38^{ab}	1.41±0.09^{cd}	63.57±4.40^{a}	63.11±4.38^{a}	63.67±4.33^{a}	323.00±58.97^{ab}	63.00±4.62^{a}
POBM	6.03±0.15^{bc}	1.50±0.04^{d}	103.20±5.61^{c}	102.20±5.75^{c}	240.00±22.90^{bc}	1480.67±54.77^{de}	309.00±55.02^{bc}
PGCM	7.10±0.17^{e}	1.82±0.05^{e}	125.73±4.68^{d}	123.98±4.40^{d}	471.67±104.26^{d}	1733.00±179.06^{ef}	816.33±203.96^{d}
p value	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data is presented as Mean ± SE (n = 3), values with different superscripts in the same column differ significantly (p<0.05). Abbreviation: Hb = Hemoglobin; TEC = Total erythrocyte count; TLC = Total leucocyte count

Table 5: Haemato-biochemical parameters and innate immune status of *Labeo rohita* fingerlings due to feeding of different phytogenic stimulant for a period of 15 days

Treatment[1]	Serum total protein (g dl^{-1})	Serum albumin (g dl^{-1})	Serum globulin (g dl^{-1})	Serum A:G[2]	NBT[3] ($OD_{620\ nm}$)	Serum glucose (mg dl^{-1})
C	3.37±0.06^{c}	1.30±0.02^{c}	1.97±0.04^{c}	0.72±0.01^{a}	0.29±0.01^{a}	117.62±2.93^{e}
FSM	3.11±0.03^{b}	1.18±0.01^{b}	1.82±0.02^{b}	0.70±0.01^{a}	0.26±0.02^{a}	112.19±2.64^{de}
CRSM	2.81±0.02^{a}	1.05±0.02^{a}	1.68±0.02^{a}	0.68±0.01^{a}	0.26±0.06^{a}	104.48±2.55^{cd}
CSM	2.65±0.04^{a}	0.98±0.02^{a}	1.58±0.02^{a}	0.67±0.01^{a}	0.31±0.03^{ab}	105.73±1.90^{cd}
FGSM	2.70±0.03^{a}	1.00±0.01^{a}	1.61±0.04^{a}	0.68±0.02^{a}	0.39±0.04^{bc}	97.65±2.90^{bc}
TRM	4.03±0.06^{e}	1.65±0.02^{de}	2.23±0.04^{d}	0.80±0.01^{b}	0.42±0.02^{c}	88.25±1.98^{a}
BPFM	3.55±0.05^{c}	1.58±0.03^{d}	1.97±0.06^{c}	0.80±0.04^{b}	0.29±0.01^{a}	117.99±2.10^{e}
PGRM	4.84±0.10^{f}	2.27±0.06^{f}	2.57±0.05^{e}	0.89±0.01^{c}	0.40±0.01^{c}	89.94±3.82^{ab}
BLM	3.81±0.09^{d}	1.73±0.04^{e}	2.08±0.06^{c}	0.84±0.01^{b}	0.25±0.01^{a}	116.01±2.75^{e}
POBM	3.81±0.07^{d}	1.72±0.05^{e}	2.09±0.02^{c}	0.82±0.02^{b}	0.41±0.02^{c}	105.95±3.29^{cd}
PGCM	5.16±0.06^{g}	2.44±0.03^{g}	2.72±0.03^{f}	0.90±0.01^{c}	0.42±0.02^{c}	91.56±3.95^{ab}
p value	<0.001	<0.001	<0.001	0.001	0.001	<0.001

Data is presented as Mean ± SE (n = 3), values with different superscripts in the same column differ significantly (p<0.05). Abbreviation: A:G = Albumin-globulin ratio; NBT =Nitrobluetetrazolium

corroborated the TLC value in *Labeo rohita* due to turmeric feeding (Sahu *et al.,* 2008), in Asian sea bass due to ginger feeding (Talpur *et al.,* 2013), in sea bass due to feeding onion (Saleh *et al.,* 2015) and in rainbow trout due to feeding garlic (Nya and Austin, 2009) might be owing to the action of respective bioactive compounds present in these phytogenic additives. Differential count of leukocytes provides a clearer picture of phagocytic activity of WBC in animals including fish. In the present study, lymphocyte was found to be the major part of TLC followed by neutrophil in the blood of *Labeo rohita* fingerlings, in which significantly higher (p<0.05) lymphocyte count and TLC was observed due to feeding of different PFAs. The significant improvement of lymphocyte, monocyte and neutrophilin the CSM, FGSM, TRM, POBM and PGCM fed groups might be attributed to enhance innate immune function in *Labeo rohita* fingerlings. Cumin contains immune-stimulatory bioactive compound like cuminoside A and B, and two alkyl glycosides, which can enhance the T lymphocytes, Th1 cytokines secretion in the animals (Chauhan *et al.,* 2010). In the present study, increased lymphocyte and neutrophil in cumin supplemented dietary group indicate a better innate immune function in fish. In relation to present observation, the enhanced lymphocyte and monocyte could be found in African catfish (*Clarias gariepinus*) due to feeding of turmeric powder at inclusion level of 2.5-7.5%

(Sodamola *et al.,* 2016). In agreement with present results, Apines-Amar *et al.* (2012) also reported improved lymphocytes and neutrophil count in grouper, *Epinephelus fuscoguttatus* fed with onion meal supplemented diet. In corroboration with present investigation, there was significant increase of different leucocyte cells in Asian sea bass due to feeding of 0.5-2.0% garlic powder (Talpur and Khwanuddin, 2012). In this study, the highest neutrophil and eosinophil counts were recorded in CSM and PGCM groups, respectively. Enhancement of neutrophil and eosinophil are usually linked with innate immune function, indicating better health status, although these levels can also be enhanced against the allergic response and inflammatory reactions to protect the cells. The health stimulating effect of cumin at dietary inclusion level of 1.14% in *Oreochromis mossumbicus* (Yilmaz *et al.,* 2012) support our present finding in *Labeo rohita* fingerlings.

Serum total protein and albumin are the basic index of health status of fish. Globulin, being the integral part of serum protein, contains all immunoglobulin, which play pivotal role in enhancing immune functions and protection of health of fish. In current finding, the significant enhancement of serum total protein, globulin and A:G ratio in TRM, PGRM and PGCM groups could be the indicative of better health status with improved innate immunity in fish. In relation with current observation, increased serum total protein, albumin, globulin and A:G ratio were recorded in *Labeo rohita* (Sahu *et al.,* 2008) due to feeding of turmeric powder at 0.1% levels. Similarly, Arulvasu *et al.* (2013) stated the efficacy of dietary ginger at 0.01-0.1% inclusion level to improve the total serum protein in *Catla catla*. In agreement with the current finding, Sahu *et al.* (2007) reported increased serum total protein in *Labeo rohita* due to feeding of garlic powder at 0.5-1.0% level.

The fish of FGSM, TRM, PGRM, POBM and PGCM groups exhibited significantly (p<0.05) higher respiratory burst activity of phagocytes or NBT value than the control. This is an indication of enhanced innate immune function and might be associated with increased monocyte and neutrophil as proved in this study. In corroboration with the present findings, Sahu *et al.* (2008) explained the ability of curcumin powder at 0.5% dietary inclusion level to enhance NBT activities in *Labeo rohita*. In agreement with the present findings, Nya and Austin (2009) observed that dietary ginger at 0.1% inclusion level could enhance the NBT value in rainbow trout. Sahu *et al.* (2007) also reported enhanced NBT value in *Labeo rohita* due to feeding of garlic powder at 1.0% level. Similarly, increased NBT activity was found in common carp due to feeding dietary fenugreek seed meal at 1% inclusion level (Kumar *et al.,* 2017). The serum glucose content of FSM, CRSM, CSM, FGSM, TRM, BPSM, PGRM, BLM, POBM and PGCM groups was significantly (p<0.05) lower than the control with convincingly lower level in TRM, PGRM and PGCM groups. The lowering blood glucose level in fish might be mediated through the action of bioactive compounds of phytogenic feed additives, which might stimulate pancreatic β-cells to secrete more insulin for improving peripheral glucose metabolism with decreased gluconeogenic enzyme activities (Thota *et al.,* 2019). Reduced serum glucose levels in CRSM, CSM, FGSM, TRM, PGRM, BLM, POBM and PGCM groups might be the indicator of maintaining glucose homeostasis in *Labeo rohita* fingerlings in the present study.

In support of current finding, dietary supplementation of turmeric at 5-10% could decrease serum glucose in *Clarius gariepinus* (Sodamola *et al.,* 2016). Similarly, the present findings in *Labeo rohita* fingerlings corroborate the observation of Talpur *et al.* (2013) who reported decreased blood glucose level in Asian sea bass due to feeding of ginger supplemented diet. In connection with the present study, onion supplemented diet significantly decreased the blood glucose level in juvenile common carp (Mousavi *et al.,* 2016). Sahu *et al.* (2007) documented lower blood glucose level in *Labeo rohita* fed with garlic supplemented diet to corroborate the results of the present study. Overall, the reduction in PFAs mediated serum glucose might be the indicative of better carbohydrate metabolism for energy supply in fish. It is concluded that out of ten PFAs, turmeric, garlic and ginger meals at 1% dietary inclusion level could result better physio-metabolic status and innate immune function in *Labeo rohita* fingerlings.

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