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Efficacy of Dietary Supplementation with Phytase and Citric Acid on Whole-Body Composition and Hematological Parameters of *Cyprinus carpio* and *Cirrhinus mrigala* Fingerlings Fed on Canola Meal-Based Diet

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MZH conducted the study and wrote the manuscript. SMH acquired funds, administered and supervised the project. AIH and MH analyzed the data. MA, ZY and AAB edited and reviewed the manuscript.

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Citric acid, Phytase, Canola meal, Whole-body composition, Hematology

ABSTRACT

A 90-day feeding trial was designated to evaluate how canola meal (CM)-based diet supplemented with phytase (PHY) and citric acid (CA) affects whole-body composition and hematological parameters in *Cirrhinus mrigala* and *Cyprinus carpio* fingerlings. Sixteen experimental diets (T_1—T_{16}) with varied CA (0 and 2.5%) and PHY (0 and 750 FTU/kg) levels were prepared. The fingerlings were fed at the rate of 5.0% of live fish body mass. Fifteen fish were randomly stocked into triplicate tanks for each of sixteen test diets. Significant (p<0.05) increase in crude protein (CP) and crude fat (CF) was noticed in fish fed T_{12} (2.5% CA and 750 FTU/kg PHY) diets. Comparison of treatments showed maximum values of RBCs (3.57×106 mm^{-3} and 3.18×106 mm^{-3}), PLT (83.32 and 80.35), Hb (8.92g/100ml and 8.62g/100ml) and PCV (29.07% and 30.07%) in *C. mrigala* and *C. carpio* fingerlings, respectively, when fed with T_{12} diets. Conclusively, a 50% CM-based diet with 2.5% CA and 750 FTU/kg PHY supplementation, performed better in terms of whole-body composition and hematological parameters in *C. mrigala* and *C. carpio* fingerlings.

INTRODUCTION

Aquaculture has become the primary means of acquiring aquatic food due to a decline in wild fisheries sources (Gong *et al*., 2021). According to FAO's most recent global aquaculture statistics, the yield of farmed aquatic animals has reached about 82.1 million metric tons, with an average annual growth rate of 5.3% from 2011 to 2018 (FAO, 2020). This change is intimately related to the expansion of intensive aquaculture practice and the accessibility of high quality aqua-feeds. The significant protein source in aqua-feeds is fishmeal (FM), but due to the limited supply of natural resources, there are growing efforts to identify other substitutes, like phyto-proteins, to replace FM. Aquaculture producers and nutritionists now place a great deal of attention on plant proteins due to their accessibility, environment friendliness, sustainability and relative affordability compared to FM protein (Abdel-Latif *et al*., 2022).

Canola meal (CM) is a highly nutritious plant protein (33-45%) obtained from plant *Brassica napus* (Glencross, 2016), with well-balanced amino acids (methionine, lysine, and cysteine), minerals and vitamins (thiamine, choline, niacin, biotin, riboflavin) (Mohammadi *et al*., 2016). All over the world, it is cheaper than FM with easy availability and serves as the primary source of protein in feed formulations (Mushtaq *et al*., 2006), but one significant drawback is the existence of anti-nutritional factors like

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tannins, glucosinolates, and phytate that restrict its usage in fish feed (Von Danwitz and Schulz, 2020). Phytate, the primary phosphorus storage compound, has a significant chelating capability to make bonds with starch, protein, and minerals in intestine, reducing the bioavailability of these nutrients (Kies *et al.*, 2001). Therefore, for the efficient use of plant-based proteins in aqua-feed, it is crucial to identify and destroy anti-nutritional factors that prevent nutrient consumption.

Exogenous enzymes, i.e., phytase (PHY), may be employed in fish diets to boost the nutritional value of plant-based protein sources and inactivate anti-nutritional factors (Dalsgaard *et al*., 2012). PHY hydrolyzes phytate's phosphate group, lowers its affinity, and forces it to release nutrients in order to improve nutrient assimilation and decrease the nitrogen and phosphorus discharge (Kumar *et al*., 2010). Organic acids supplementation is another way to enhance plant protein quality (Reda *et al*., 2016; Hussain *et al*., 2017). Citric acid (CA) is an organic acid with strong buffering ability and particular taste that has widely been applied in aqua-diets (Hossain *et al*., 2007). By preserving a healthy gut pH, it increases the effectiveness of both exogenous and endogenous phytases. It also improves feed utilization and serves as an antimicrobial agent (Shah *et al*., 2015). CA inclusion has shown improved growth performance, feed intake and specific growth rate in many species such as, rainbow trout, beluga sturgeon, and tilapia (Pandey and Satoh, 2008; Li *et al*., 2009; Khajepour and Hosseini, 2012).

Pakistan is one of the countries having freshwater and marine water resources that support a wide range of highly nutritious and economically valuable freshwater fishes (Hussain *et al*., 2011). *Cirrhinus mrigala,* also referred to as mori, is a freshwater species widely cultivated in Pakistan. It has high market value and is grown semi-intensively in polyculture systems for the better success of carp culture system (Abid and Ahmed, 2009). *Cyprinus carpio*, commonly known as gulfam, is cultured in Asia, Eastern Europe and other parts of the world (Khan *et al*., 2016). This freshwater fish tolerates a wide range of temperature, salinity and pH. It is one of the most commonly farmed fish of world with characteristics of fast growth rate and good protein source for human food (Sugiura *et al*., 2001). Therefore, this study was designed with the aim of evaluating the importance of PHY and CA supplementation in plant-based diets as well as their effects on body composition and hematology of *C. mrigala* and *C. carpio* fingerlings.

To observe the physiologic variations in fish in response to particular diets, the hematological parameters, mainly, hemoglobin (Hb), white blood cells (WBCs), red blood cells (RBCs), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), mean cell volume (MCV) and lymphocyte count are observed (Shah and Altindağ, 2004). Studies regarding the effects of exogenous enzyme supplementation on fish hematological status are not widely available. Thus, this study was designed to evaluate the interaction between exogenous enzyme supplementation and hematology of fish.

MATERIALS AND METHODS

Test diets formulation

Sixteen isoenergetic and isonitrogenous experimental diets were prepared. CM was used to substitute 45.77% of FM protein and CA and PHY were added at levels of 0 and 2.5% as well as 0 and 750 FTU/kg, respectively (Table I). All ingredients of feed were ground finely, mixed properly along with CA, pelleted via 3 mm die and sprayed with PHY dissolved in 50 ml distilled water as stated by Robinson *et al*. (2002). Pellets were dried before being placed in sealed plastic jars and kept at below 20°C for the period of the feeding trial.

Experimental fishes and rearing conditions

A 90-day feeding trial was organized. Fingerlings of *C. mrigala* and *C. carpio* were bought from the Government Fish Seed Hatchery, Faisalabad, Pakistan and were acclimated to laboratory environment. These were fed on basal diet round the clock. Following a two-week acclimation period, 15 fish were stocked randomly in experimental tanks, each tank with 70 L water capacity, having triplicates per test diet. These were fed twice a day, at 0900 and 1700 h. to apparent satiety. After 2 h. of feeding period, the tanks were washed and refilled with water. Quality of water was checked daily by observing parameters like dissolved oxygen, pH, and water temperature.

Sample collection and chemical analysis

After 90-day experimental period, fingerlings were fasted for 24 h. Five fingerlings were picked randomly from each experimental tank for whole-body proximate analysis by standard protocols of AOAC (2005). Feed and fish samples were completely oven dried at 105°C and homogenized using a mortar and pestle before being inspected. Moisture content estimation was done after drying. Micro Kjeldahl apparatus was used to assess crude protein (CP) (N 6.25) and petroleum ether extraction system was utilized to determine crude fat (CF) (Soxtec HT2 1045 system). Ash was assessed by sample ignition at 550 °C for about 12 h in an electric furnace (Eyela-TMF 3100).

Table I. Ingredient's composition (%) of test diets.

Test diets	Replacement levels of fishmeal protein (%)	Fish meal	Canola meal	Phytase (FTU /kg)	Citric acid (%)	Wheat flour	Rice polish	Fish oil	*Vitamins Premix	**Minerals Premix	Chromic oxide
T_1	0	48	18	0	0	22	3	6	1	1	1
T_2					2.5	19.5					
T_3				750	0	22					
T_4					2.5	19.5					
T_5	25	36	35	0	0	17					
T_6					2.5	14.5					
T_7				750	0	17					
T_8					2.5	14.5					
T_9	50	24	53	0	0	11					
T_{10}					2.5	8.5					
T_{11}				750	0	11					
T_{12}					2.5	8.5					
T_{13}	75	12	71	0	0	5					
T_{14}					2.5	2.5					
T_{15}				750	0	5					
T_{16}					2.5	2.5					

*Each 100Kg of Vitamins premix contains: Vit A 2000,000IU, Vit B6 600 mg, Vit D 400,000 IU, Vit B12 2,000 mg, Vit E 4000 mg, Vit C 2,000 mg, Vit K_3 900 mg, Folic acid 200 mg, Vit B_1 200 mg, Calcium pantothenate 2,000 mg, Vit B_2 3000 mg, Nicotinic acid 10,000 mg. **Each Kg mineral premix contains: Na 45g, Ca 155g, Zn 3000 mg, Fe 1000 mg,Cu, 600mg, Mg, 55g, P, 135g, Mn, 2000mg, I, 40mg, Co, 40mg, Se, 3mg.

Blood sample collection and hematological assay

Fingerlings were tranquilized with 150 mg tricane methane sulfonate solution to take blood samples from a fish's caudal vein (Wagner *et al*., 1997) after the 90-day experimental period. The samples of blood were transferred to the Molcare Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. Utilizing an automatic cell counter, the hematological indices, including Hb, WBCs, RBCs, MCH, MCV, and MCHC, were calculated. PLT and PCV were measured by following methods of Brahimi *et al*. (2009) and using a micro-hematocrit reader, PCV was calculated while being spun at 10,500 rpm. By following formulae, the MCH, MCV, and MCHC were estimated as well.

$$MCHC = Hb / PCV \times 100$$

$$MCH = Hb / RBC \times 10$$

$$MCV = PCV / RBC \times 10$$

Statistical analysis

The one-way analysis of variance was used to analyze the data on body composition and hematological indices (Steel *et al*., 1996). The Tukey's Honesty Significant Difference Test was utilized for comparison of differences between the samples, and considered significant at ($p<0.05$) (Snedecor and Cochran, 1989). The CoStat Computer Package (Version 6.303, CA, Monterey, PMB 320, 93940 USA) was utilized for statistical analysis.

RESULTS

Whole-body composition

Tables II and III showed the whole-body composition of *C. mrigala* and *C. carpio* fingerlings fed CM-based diets with varying supplemented levels of CA and PHY. There were significant ($p<0.05$) differences between body composition of fingerlings fed different test diets in case of CF, CP, ash, and moisture contents. The whole-body composition findings evaluated that the *C. mrigala* and *C. carpio* fingerlings fed T_{12} diet exhibited significantly ($p<0.05$) higher levels of CP (20.19% and 18.05%), ash (2.55% and 2.49%) and moisture (74.52% and 76.50%) while lower lipid levels (2.74% and 2.97%), respectively. It was noted that 50% CM level in diet T_{12} was adequate to improve the carcass protein compared to T_1 diet without CA and PHY supplementation.

Hematological parameters

The hematological indices of *C. mrigala* and *C. carpio* fingerlings fed diets with distinct replacement levels of CM and provided with varying CA and PHY levels are shown in Tables IV and V. The fingerlings of *C. mrigala* and *C. carpio* fed on test diet T_{12} gave the highest

Table II. Analyzed whole-body composition of *C. mrigala* fingerlings fed canola meal-based diet supplemented with citric acid (CA) and phytase (PHY).

Fishmeal replacement levels (%)	PHY	CA	Test diets	Pro-tein	Fat	Ash	Mois-ture
0	0	0	T_{1}	13.86^{p}	9.85^{a}	2.22^{e}	74.08^{d}
		2.5	T_{2}	14.49^{n}	9.09^{c}	2.29^{c}	74.14^{c}
	750	0	T_{3}	15.01^{m}	8.51^{d}	2.31^{c}	74.17^{c}
		2.5	T_{4}	17.78^{g}	5.49^{j}	2.44^{b}	74.30^{b}
25	0	0	T_{5}	14.29^{o}	9.35^{b}	2.25^{d}	74.11^{c}
		2.5	T_{6}	16.84^{i}	6.52^{h}	2.40^{b}	74.24^{c}
	750	0	T_{7}	17.37^{h}	5.93^{i}	2.42^{b}	74.28^{b}
		2.5	T_{8}	19.18^{d}	3.97^{m}	2.49^{b}	74.36^{b}
50	0	0	T_{9}	15.41^{l}	8.06^{e}	2.33^{c}	74.20^{c}
		2.5	T_{10}	16.45^{j}	6.96^{g}	2.38^{b}	74.22^{c}
	750	0	T_{11}	19.91^{b}	3.16^{o}	2.54^{a}	74.39^{b}
		2.5	T_{12}	20.19^{a}	2.74^{p}	2.55^{a}	74.52^{a}
75	0	0	T_{13}	15.88^{k}	7.57^{f}	2.35^{c}	74.21^{c}
		2.5	T_{14}	18.25^{f}	4.97^{k}	2.46^{b}	74.32^{b}
	750	0	T_{15}	18.87^{e}	4.32^{l}	2.47^{b}	74.33^{b}
		2.5	T_{16}	19.49^{c}	3.61^{m}	2.53^{a}	74.37^{b}
Pool Standard Error (PSE)				0.04	0.03	0.02	0.03

Means within columns having different superscripts are significantly different at $p<0.05$. Data are means of three replicates.

Table III. Analyzed whole-body composition of *C. carpio* fingerlings fed canola meal-based diet supplemented with citric acid (CA) and phytase (PHY).

Fishmeal replacement levels (%)	PHY	CA	Test diets	Pro-tein	Fat	Ash	Mois-ture
0	0	0	T_{1}	14.75^{m}	12.81^{a}	1.00^{p}	71.44^{p}
		2.5	T_{2}	15.38^{l}	10.82^{c}	1.05^{n}	72.74^{n}
	750	0	T_{3}	15.78^{k}	10.10^{d}	1.08^{m}	73.04^{m}
		2.5	T_{4}	16.87^{g}	6.13^{j}	2.03^{g}	74.97^{g}
25	0	0	T_{5}	14.75^{m}	6.13^{j}	1.03^{o}	72.04^{o}
		2.5	T_{6}	16.27^{i}	12.18^{b}	1.68^{j}	74.07^{j}
	750	0	T_{7}	16.57^{h}	7.98^{g}	1.98^{h}	74.77^{h}
		2.5	T_{8}	17.38^{d}	6.68^{i}	2.39^{c}	75.67^{d}
50	0	0	T_{9}	16.27^{i}	4.56^{l}	1.78^{i}	74.37^{i}
		2.5	T_{10}	16.03^{j}	7.58^{h}	1.48^{k}	73.74^{k}
	750	0	T_{11}	17.84^{b}	8.75^{f}	2.47^{b}	76.67^{a}
		2.5	T_{12}	18.05^{a}	3.03^{n}	2.49^{a}	76.50^{b}
75	0	0	T_{13}	16.08^{j}	2.97^{o}	1.28^{l}	73.34^{l}
		2.5	T_{14}	17.03^{f}	9.30^{e}	2.13^{f}	75.07^{f}
	750	0	T_{15}	17.18^{e}	5.22^{k}	2.33^{e}	75.27^{e}
		2.5	T_{16}	17.58^{c}	4.02^{m}	2.43^{d}	75.97^{c}
Pool Standard Error (PSE)				0.14	0.14	0.01	0.01

Means within columns having different superscripts are significantly different at $p<0.05$. Data are means of three replicates.

Table IV. Hematological parameters of *C. mrigala* fingerlings fed on canola meal-based test diets supplemented with Phytase and citric acid.

Test diets	Fishmeal protein replacement levels (%)	Phytase	Citric acid	Red blood cells ($10^{6}mm^{-3}$)	White blood cells ($10^{3}mm^{-3}$)	Plate-lets	Hemo-globin (g/100ml)	Packed cell volume (%)	Mean corpuscular hemoglobin concentration (%)	Mean corpuscular hemoglobin (pg)	Mean corpuscular volume (fl)
T_{1}	0	0	0	3.14^{a}	8.09^{c}	67.93^{h}	8.20 ± 0.09^{c}	20.94^{f}	26.06^{k}	26.48^{i}	96.40^{j}
T_{2}			2.5	3.16^{a}	8.36^{c}	73.3^{e}	8.45 ± 0.56^{b}	26.00^{c}	30.60^{g}	25.74^{j}	84.50^{m}
T_{3}		750	0	3.25^{a}	8.43^{c}	75.28^{d}	8.54 ± 0.02^{b}	27.68^{b}	32.48^{e}	27.00^{h}	106.08^{g}
T_{4}			2.5	3.31^{a}	8.14^{c}	74.71^{d}	7.87 ± 0.01^{d}	24.68^{d}	28.42^{i}	50.70^{f}	139.17^{d}
T_{5}	25	0	0	3.28^{a}	8.20^{c}	78.91^{c}	7.22 ± 0.01^{e}	23.23^{e}	35.60^{c}	70.12^{c}	204.70^{b}
T_{6}			2.5	3.44^{a}	8.31^{c}	72.36^{f}	7.80 ± 0.01^{d}	28.77^{a}	27.33^{j}	28.48^{g}	93.40^{k}
T_{7}		750	0	3.27^{a}	8.24^{c}	66.54^{i}	8.59 ± 0.27^{b}	28.00^{b}	31.31^{f}	27.70^{g}	82.83^{n}
T_{8}			2.5	3.40^{a}	8.21^{c}	85.34^{a}	8.54 ± 0.02^{b}	29.00^{a}	33.32^{d}	29.00^{g}	102.08^{h}
T_{9}	50	0	0	3.51^{a}	8.24^{c}	74.46^{d}	7.73 ± 0.23^{d}	26.00^{c}	29.08^{h}	53.03^{e}	136.17^{e}
T_{10}			2.5	3.26^{a}	8.28^{c}	78.41^{c}	7.22 ± 0.01^{e}	25.45^{d}	36.32^{b}	74.59^{a}	209.69^{a}
T_{11}		750	0	3.36^{a}	8.44^{c}	77.83^{c}	7.80 ± 0.01^{d}	28.77^{a}	28.94^{i}	28.80^{g}	94.06^{k}
T_{12}			2.5	3.57^{a}	8.47^{c}	83.32^{b}	8.92 ± 0.01^{a}	29.07^{a}	32.60^{e}	28.17^{g}	88.49^{l}
T_{13}	75	0	0	3.20^{a}	8.29^{c}	75.56^{d}	8.54 ± 0.02^{b}	29.34^{a}	33.47^{d}	29.00^{g}	113.08^{f}
T_{14}			2.5	2.53^{b}	8.24^{c}	74.26^{d}	7.80 ± 0.11^{d}	27.34^{b}	30.44^{g}	55.37^{d}	140.84^{c}
T_{15}		750	0	2.32^{c}	8.85^{a}	78.11^{c}	7.22 ± 0.01^{e}	23.34^{e}	37.63^{a}	73.81^{b}	201.37^{a}
T_{16}			2.5	3.22^{a}	8.68^{b}	68.43^{g}	7.80 ± 0.01^{d}	25.10^{d}	28.73^{i}	29.48^{g}	99.40^{i}
Pool Standard Error (PSE)				0.15	0.08	0.86	0.10	0.51	0.34	0.54	0.01

Means within rows having different superscripts are significantly different at $p<0.05$.

Table V. Hematological parameters of *C. carpio* fingerlings fed on canola-meal based test diets supplemented with phytase and citric acid.

Test diets	Fishmeal protein replacement levels (%)	Phytase	Citric acid	Red blood cells ($10^6 mm^{-3}$)	White blood cells ($10^3 mm^{-3}$)	Plate-lets	Hemo-globin (g/100ml)	Packed cell vol-ume (%)	Mean corpus-cular hemoglo-bin concentra-tion (%)	Mean cor-puscular hemoglobin (pg)	Mean corpuscu-lar volume (fl)
T_1	0	0	0	2.37^b	6.94^g	53.58^o	4.53^m	19.93^k	21.72^m	24.47^c	93.72^c
T_2			2.5	2.55^a	7.15^f	55.80^m	4.94^l	20.00^k	23.59^l	26.73^c	81.26^c
T_3		750	0	2.65^a	7.22^f	75.27^b	4.99^l	26.91^b	32.47^b	27.37^c	104.12^c
T_4			2.5	2.90^a	7.92^a	62.03^g	6.16^g	23.67^f	27.97^f	49.53^b	141.87^b
T_5	25	0	0	2.52^b	7.13^f	54.66^n	4.87^k	22.22^g	24.59^k	69.87^a	206.36^a
T_6			2.5	2.80^b	7.68^d	59.68^j	5.69^h	21.76^h	27.32^g	27.25^c	93.49^c
T_7		750	0	2.86^b	7.81^c	61.53^h	5.96^h	23.33^f	31.31^c	26.60^c	85.12^c
T_8			2.5	3.02^b	8.09^a	65.33^d	7.19^d	25.34^d	27.01^h	28.00^c	103.18^c
T_9	50	0	0	2.83^b	7.70^d	60.45^i	5.72^i	21.34^i	25.08^j	51.16^b	139.40^b
T_{10}			2.5	2.79^b	7.40^e	57.70^k	5.21^j	20.78^j	24.31^k	72.41^a	209.79^a
T_{11}		750	0	3.11^b	8.13^a	74.96^b	7.89^b	29.76^a	30.27^d	27.60^c	97.42^c
T_{12}			2.5	3.18^b	8.19^a	80.35^a	8.68^a	30.07^a	33.59^a	27.06^c	87.05^c
T_{13}	75	0	0	2.68^b	7.28^f	56.55^l	5.09^k	20.67^j	24.97^j	28.33^c	113.39^c
T_{14}			2.5	2.92^b	7.96^a	63.67^f	6.67^f	22.00^g	26.44^i	54.30^b	141.17^b
T_{15}		750	0	2.96^b	8.07^a	64.43^e	6.99^e	24.00^e	28.01^f	70.80^a	201.13^a
T_{16}			2.5	3.06^b	8.12^a	67.75^c	7.46^c	26.00^c	29.72^e	27.80^c	99.491^c
Pool Standard Error (PSE)				0.11	0.10	0.69	0.11	0.37	0.29	3.80	10.03

Means within rows having different superscripts are significantly different at $p<0.05$.

value of RBCs (3.57×106 mm^{-3} and 3.18×106 mm^{-3}), respectively. In case of *C. mrigala* fingerlings, the highest value of WBCs (8.85×103 mm^{-3}) was found when fed with diet T_{15} (75% FM replacement with 0% CA and 750 FTU/kg PHY) while in *C. carpio* fingerlings, highest value of WBCs (8.19×103 mm^{-3}) was at T_{12} level. The fingerlings of *C. mrigala* and *C. carpio* fed with T_{12} diet showed the highest value of PLT (83.32 and 80.35), Hb (8.92 g/100ml and 8.68g/100ml) and PCV (29.07% and 30.07%), respectively. These all values were significantly ($p<0.05$) different from fishes given other test diets. The highest amount of MCH (74.59 pg and 72.41 pg) and MCV (209.69 fl and 209.79 fl) were found when fingerlings fed on test diet T_{10} (50% FM replacement level with 2.5% CA and 0 FTU/kg PHY), respectively whereas, the lowest amount of MCH (24.47 pg) and MCV (81.26 fl) were noted in *C. carpio* fingerlings fed T_1 (0 % CA and 0 PHY) and T_2 (2.5 % CA and 0 FTU/kg PHY).

DISCUSSION

The whole-body composition has a direct influence on the quality of aquatic food items. In order to use fish more effectively, it is helpful to have detailed understanding of its body composition. Body composition is undoubtedly regarded as a valuable method to estimate the physiological state and health of the fish, alongside hematological analysis (Fazio *et al*., 2019). Age, sex, nutritional status, digestive function, and other factors, all have an impact on the biochemical makeup of the fish's entire body (Abdel-Tawwab *et al*., 2006). Phytate contents are usually present in plant-based diets and make these diets non digestible. Basically, it chelates with important nutrients i.e., fat, protein, and minerals. Consequently, fish become unable to consume them, ensuing deficient fish body composition (Cao *et al*., 2007).

In this study, the body composition of both fishes was improved when fed with varying levels of CM-based diets supplied with PHY and CA. The results showed that CA and PHY addition is important for dissociation of phytate complexes ensuing higher retention of nutrients in body of fish as compared to those fed control diet (without CA and PHY supplementation). Best values of CP, CF and carcass ash were found in *C. mrigala* (20.19%), (2.74%) (2.55%), respectively and in case of *C. carpio* (18.05%), (2.97%), (2.49%), respectively when fed on T_{12} diet whereas the lowest values of CP (13.86%), CF (9.85%) and ash (2.22%) of *C. mrigala* and (14.75%), (12.81%) and (1%) of *C. carpio*, respectively was recorded when both fishes were fed on control diet (T_1). The results of body composition of this study were also corroborated by Ahmad *et al.* (2023) which suggested that when *Labeo rohita* fed on 50% replacement level of cottonseed meal (CSM)-based diets provided with 2.5% CA and 750 FTU/kg PHY, they showed highest amount of CP, CF, and ash contents in comparison to those fed on other test diets. Similarly, Khajepour *et al.* (2012) also found improved muscle proximate composition of *C. carpio* when fed on 3% CA and 500 FTU/kg PHY supplemented soybean meal (SBM)-based diet. On contrary, Afzal *et al.* (2019) noticed a non-significant interaction between both supplements i.e., CA and PHY, to improve fat and protein contents in fish body. They found that the SBM-based diets supplemented with CA and PHY separately, gave better results than control diet (with no supplement).

In current study, CA supplementation alone significantly enhanced (p<0.05) the muscle CP, ash and dry matter, while reduced the CF level. The positive impact of CA on reduction of body fat might be due to its impact on phosphorous retention. This decreased fat content in fish body due to utilization of CA supplemented plant-based diet depicts that more phosphorous is available to improve fat oxidation, hence providing more energy for better growth performance of fish (Shirmohammad *et al.*, 2003). Our results were also in line with Khajepour and Hosseini (2012) who detected increase in muscle CP and ash levels in *Huso huso* when fed on 2 and 3% CA supplemented test diets. CA may decrease the gut pH helping in the dissociation of chelated phytate nutrient complexes that leads to improved retention of these nutrients in fish body (Afzal *et al.*, 2019).

Like CA, PHY supplementation also individually improves (p<0.05) the proximate composition of muscle of fish. In accordance with this study, Sardar *et al.* (2007) also found increase in CP and decrease in CF levels in body of *C. carpio* due to PHY supplementation in feed. It is due to PHY enzyme's ability to hydrolyze phytate and release the chelated nutrients making them more accessible to fish (Afzal *et al.*, 2019). In another study, Shirmohammad *et al.* (2003) also found that SBM-based diet provided with 3% CA and 1000 FTU/kg level of PHY synergistically improved ash contents but could not increase protein and lipid contents in *C. carpio*. The combination of CA and PHY resulted in a non-significant reduction in body fat content. This could be due to change in pH of digestive tract, which improved PHY performance. A combination of PHY and CA is more numerically effective than either one alone.

In present study, the moisture level in body composition of *C. mrigala* and *C. carpio* was 74.52% and 76.50%, respectively. PHY supplemented diets can improve fish body mineralization by increasing nutrient retention. It is especially during the developing stage (Cao *et al.*, 2007). Nuez-Ortin (2011) and Reda *et al.* (2016) also reported that Nile tilapia fed on acidified diet significantly improved the chemical composition of fish body. When Nile tilapia were fed acidified diets, they retained significantly higher protein contents. Because crude proteins are more digestible at greater CA levels, they retained longer in the fish body. The rate of pepsinogen conversion to pepsin was raised when CA supplementation in diet was increased, resulting in higher pepsin activity (Kirchgessner and Roth, 1985). The outcomes of multiple research experiments are variable due to changes in diet ingredients, feed formulation processes, ecological variations and species differences, thus demanding more exploration. This study granted the valuable effects of dietary supplementation of both CA and PHY in *C. mrigala* and *C. carpio* diet and their role in improving the body composition and fish health status. Hence, 50% CM-based diet with CA and PHY supplementation could be added to aqua-feeds to improve body composition of cultured *C. mrigala* and *C. carpio* through dietary management.

Variations in hematological indices are key signs, utilized for evaluation of stress, nutritional and health status (Yilgmaz *et al.*, 2016; Khalafalla *et al.*, 2020) as well as physiological changes in fishes (Ranjan *et al.*, 2020). Therefore, monitoring hematological indices may reveal the fish's immune status in response to changes especially related to nutrition (Fazio *et al.*, 2019). In fish, hematological studies are necessary to check the health status of fish and to ensure that the formulated diets are of good quality (Shahzad *et al.*, 2016).

According to the current study, highest values of blood parameters were found when both fishes fed on test diet T_{12} having 50% replacement level of CM with 2.5% CA and 750 FTU/kg PHY showing that PHY and CA supplementation in plant-based diets synergistically improved the hematological status of the fishes. In a parallel study, Baruah *et al.* (2007) also found that 3% CA and 500 FTU/kg PHY supplementation in SBM-based diet synergistically improved the RBCs, WBCs, Hb hematocrit volume of *L. rohita* juveniles.

The chelated phytate complex in plant by-products has an impact on overall fish performance, including hematology (Ehsani and Torki, 2010). Because phytate chelates with iron, found in RBCs, it may reduce overall oxygen carrying capacity of RBCs (Spinelli *et al.*, 1983). Well-known features of PHY are that, it showed better nutrient digestibility and hematology of Indian major carps when fed plant-based diets (Hussain *et al*., 2011, 2016; Shahzad *et al*., 2017). PHY supplementation has been suggested as an effective immune system stimulant in monogastric animals that results in a higher number of monocytes and a higher production of blood cells (Ehsani and Torki, 2010). Shahzad *et al*. (2020) found that *C. catla* fingerlings showed improved values of RBCs, WBCs and Hb when fed on moringa-based diet having 900 FTU/kg PHY whereas the poorest values were noted when fed on control diet. Hb level in olive flounder (*Paralichthys olivaceus*) fed on PHY supplemented SBM diet was greater than the fish fed on control diet (Yoo and Bai, 2014).

CA is already approved for use in animal diets, and previous studies have shown that it is safe for the target fish species (EFSA, 2015). Various hematological parameters in fish blood were improved as a result of diet acidification. Dietary acidification had a beneficial effect on fish blood parameters (Hb, RBCs, WBCs, PLT, MCH and MCV) as noted by Reda *et al*. (2016). In present study, WBCs in *C. mrigala* showed increasing trend as CM level increased but in the case of *C. carpio*, WBC count decreased when CM increased in the experimental diet. High WBC count is generally related to microbial invasion or due to foreign bodies or antigens found in blood circulation. Similar findings were also noted in other research studies (Bello *et al*., 2013; Hussain *et al*., 2018). In the current research, significant increases in WBCs number were observed in fishes fed with PHY supplemented diets as also noted by Ranjan *et al*. (2020) indicating the improved levels of immunity in fish. Significant increase in number of RBCs and WBCs along with Hb value might be the result of increased haemo-concentration (Ranjan *et al*., 2020). The possible reason for this might be due to water movement from blood plasma to the muscle cell (Wilson and Taylor, 1993). In current findings, the increased number of RBCs was found in fingerlings fed with enzyme supplemented CM-based diets. These improved RBCs could deduce a good immune response (Jiang *et al*., 2007). As there is not much insight into developed relation between enzyme supplementation in fish feed and fish hematological state, so more research is necessary to demonstrate the mode of action (Ranjan *et al*., 2020).

In the current study, PHY alone improved numbers of WBCs and MCHC of *C. mrigala* fish when fed test diets $T_{15,}$ having 75% replacement levels of CM with 0% CA and 750 FTU/kg of PHY enzyme. In contrast, Baruah *et al*. (2007) noticed non-significant impacts ($p>0.05$) on WBCs in *L. rohita* fed SBM-based diet with 500 FTU/kg PHY. Similar to our results, WBCs were maximum in *C. carpio* fed on soy-protein based diet with 500 FTU/kg PHY supplementation (Sardar *et al*., 2007). Maximum WBCs count was found in Nile tilapia fed on SBM-based diet with 500 FTU/kg PHY in comparison with fish fed on similar level of PHY supplementation with jatropha meal-based diet (Kumar *et al*., 2011).

The findings of present research demonstrated that CA individually enhanced the MCH and MCV values of both fishes *C. mrigala* and *C. carpio* when fed on test diet $T_{10,}$ having 50% CM-based diet with 2.5% CA and without PHY. Hussain *et al*. (2021) also observed that 3% CA significantly improved the hematological parameters of *C. mrigala* fingerlings fed *Moringa oleifera*-based diets. In contrary to our results, acidification of diet with CA had no significant ($p>0.05$) impact on RBCs, WBCs, MCH, MCV, and MCHC. Thus, at 3% CA level, concentration of Hb and Hct were significantly ($p<0.05$) greater than the control level (Khajepour *et al*., 2011). The information about MCHC and MCH has a key role in anemia analysis in many species and are recognized to be symptomatic of RBCs count and blood oxygen carrying capacity in fish body (Houston, 1997). These indices exhibited no distinction between different groups of this trial, showing that blood oxygen carrying capacity for different metabolic processes was not influenced.

The fish hematology is influenced by a number of aspects, including fish species, age, size, environmental conditions, physiological state and dietary practice i.e., quantity and quality of feed, protein sources, dietary ingredients, vitamins and probiotics etc. (Barnhart, 1969; Osuigwe *et al*., 2005). This important part of hematological study is the minimum explored area by researchers and it needs a rigorous research work to find out the probable interaction of PHY enzyme with hematological parameters of fish (Shahzad *et al*., 2016; Ranjan *et al*., 2020).

CONCLUSION

It is concluded that a 50% CM-based diet with 2.5% CA and 750 FTU/kg PHY supplementation, improved the whole-body composition and hematological parameters in *C. mrigala* and *C. carpio* fingerlings. Thus, dietary supplementation of CA and PHY in plant-based diets is effective to increase their use in aqua-feeds.

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IRB approval

The procedures and methods used in the study followed the ethical guidelines of Government College University Faisalabad.

Ethical statement

All applicable institutional, national and international guidelines for the care and use of animals were followed.

Statement of conflict of interest

The authors have declared no conflict of interest.

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