Journal of Animal & Plant Sciences, 31(5): 2021, Page: 1485-1491
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2021.5.0350

ACIDIFICATION OF PHYTASE TREATED DIET IMPROVES THE BODY COMPOSITION AND BONE MINERALIZATION OF JUVENILE ROHU (*Labeo rohita*)

S. Z. H. Shah[1*], M. Fatima[2], M. Afzal[3], S. M. Hussain[4], H. Azmat[2], M. Bilal[5] and T. Ahmed[6]

[1]Department of Zoology, University of Gujrat, Gujrat, Pakistan; [2] Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore, Pakistan; [3]Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad; [4]Fish Nutrition Laboratory, Department of Zoology, Government College University, Faisalabad, Pakistan; [5]School of life Science and Food Engineering, Huaiyin Institute of Technology, Huaian, China; [6]Department of Life Sciences, Khwaja Fareed University of Engineering and Information Technology, Rahim Yar Khan, Pakistan.
[*] Corresponding author's E-mail: zakiruaf@gmail.com; dr.zakirhussain@uog.edu.pk

ABSTRACT

A 3×3 factorial feeding experimental was performed to find out the role of citric acid and phytase supplementations and interaction in improving body chemical composition and bone mineralization by adding them in plant based diet of rohu (*Labeo rohita)* juveniles. At first, the basal diet having citric acid at three different levels was supplemented *i.e*. 0%, 1.5% and 3%, and then three different phytase levels (0 FTU kg^{-1}, 750 FTU kg^{-1} and 1000 FTU kg^{-1}) were supplemented further with each level of citric acid. Hence, total 9 experimental diets were formulated in this feeding trial. The results revealed that the concentration of total ash, crude protein and dry matter content significantly increased ($p \leq 0.05$), when treated with citric acid and phytase. However, contents of crude fat showed a reduced level in the carcass of juveniles by feeding both additives. The supplementation of citric acid and phytase both significantly ($p \leq 0.05$) affected the bone mineralization of fish. An increasing trend was observed for the body proximate analysis and bone mineral concentrations with an increase of supplements level in the diet. Conclusively, the results of this experiment showed that the supplementation of citric acid and phytase in the diet of *L. rohita* improved body composition and bone mineralization.

Key words: Phytase, citric acid, rohu (*Labeo rohita)*, bone mineralization, body mineralization, body proximate composition

Abbreviations: Phytase (PHY); Citric Acid (CA); Labeo rohita (*L. rohita);* Analysis of variance (ANOVA)

Published first online January 24, 2021 Published Final August 07, 2021.

INTRODUCTION

Aquaculture is rapidly growing sector which acts as important component of food security (Ibrahem *et al.,* 2010). Success of aquaculture mainly depends upon the nutritional status as well as economy of feed for cultured species. In modern day intensive culture system feed cost accounts for 50% of the total culture economy (Ibrahem *et al.,* 2010). Fish nutritionists, now a day, are focusing on low cost feed production with a replacement of fishmeal with plant by-products (Carter and Hauler, 2000). Soybean meal is assumed to be best feed ingredient because of its balanced nutritional status and also used as feed source for a number of fish species (Gatlin *et al.,* 2007).

In the fish feed, the presence of anti-nutritional factors (phytic acid) causes major problems, when plant based proteins are used in the feed. Phytic acid is the main source of phosphorus and account for almost 80% of the total Phosphorus of plants (Pointillart *et al.,* 1987) and also restricts the digestibility of nutritionally significant nutrients including proteins (Liu *et al.,* 2012); and minerals, especially Ca, Mg, K, Zn, Cu and Fe (Sugiura *et al.,* 2001) leading to their biologically unavailability and growth depression.

Nutrients bound as phytic acid can only be made available to fish when are hydrolysed by phytase, an enzyme also known as myo-inositol hexaphosphate phosphohydrolase (Francis *et al.,* 2001; Cao *et al.,* 2007; Gatlin *et al.,* 2007). Phytase degrades phytate to mono-phosphate and di-phosphate compounds making them available to monogastric animals (Mitchell *et al.,* 1997). In monogastric animals, phytase is generally used as feed additive in the diet. In fish feed, phytase addition has shown increased utilization of phytate bound phosphorus in different fish species *i.e.* common carp (Schafer *et al.,* 1995), channel catfish (Li and Robinson, 1997), Nile tilapia (Furuya *et al.,* 2001; Tudkaew *et al.,* 2008; Verlhac-Trichet *et al.,* 2014), rainbow trout (Dalsgaard *et al.,* 2009; Verlhac-Trichet *et al.,* 2014). In yellow catfish, the P, Ca and Mg content of vertebrae and whole body was significantly enhanced (Zhu *et al.,* 2014) and a similar increase in Ca, P Mg and Zn contents of whole

Salmo salar (Denstadli *et al.,* 2007) has also been evident.

It has been shown by various studies that the accessibility of biologically important minerals in fish is affected by the pH of gut (Sugiura *et al.,* 1998; Vielma *et al.,* 1999). Dietary supplementation of organic acids has the tendency to increase the minerals absorption by decreasing the intestinal pH (Jongbloed, 1987). Decreased intestinal pH enhances the absorption of phosphorus by increasing the phosphorus phytate solubility (Cross *et al.,* 1990; Ravindran and Kornegay, 1993). Along with its effect on intestinal pH, the dietary organic acid act as chelating agent which increases the absorption of certain minerals, that binds many cations present in the intestine (Wood and Serfaty-Lacrosniere, 1992). Organic acid has been proved an efficient antimicrobial agent which enhances disease resistance leading to improved growth, nutrient utilization (NRC, 2011). Moreover, studies have showed increased P and Ca level of *Huso huso* (Khajepour and Hosseini, 2011; 2012) and increased mineral contents of rohu (*L. rohita*) (Afzal *et al.,* 2020) in response to organic acid supplementation.

Phytase works optimally at 2.5 and 5.0-5.5 pH (Simons *et al.,* 1990) which can be achieved by additing acid in fish diet. Citric acid (CA) addition lowers gastrointestinal pH of fish stomach to optimum range of phytase action. Hence, considered as an efficient method to enhance phytase activity (Nourmohammadi *et al.,* 2011). Phromkunthong *et al.* (2010) reported that the citric acid and phytase interaction has improved bone mineral contents of *Cyprinus carpio*. Therefore, the present study was aimed at evaluation of phytase and citric acid efficacy for *L. rohita* composition.

MATERIALS AND METHODS

Experimental diets: Nine isonitrogenous (27.54±0.49), isolipidic (8.00±0.21) and isocaloric (3.67±0.48) experimental diets were formulated in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. Soybean being a major protein source contributed to about 57.77% crude protein of diet while fish meal (33.99%), rice polish (4.25%) and wheat flour (3.99%) also added share to total crude protein of diet. For lipid source as well as feed attractant fish oil was used. All experimental diets were similar in composition apart from the levels of citric acid and phytase supplementation. In experimental diets, the citric acid and phytase were added at levels of 0, 1.5 and 3% and 0, 750 and 1000 FTU/kg, respectively. Dry ingredients of feed were powder ground to a particle size of 0.05mm in cereal grinding machine (FFC-45, JIMO, China). Citric acid was added into dry ingredients and mixed electrically with gradual addition of fish oil. For the formation of dough, water was added upto 15%, which was further processed to make pallets (3mm) through hand palletizer. Afterwards, pellets of every level of citric acid were sprayed with three concentrations of phytase (Phyzme®xP 10000 $FTUg^{-1}$, Damisco Animal Nutrition. Fin-65 101 Vaasa Finland) in such a way that 0.5 g phytase gave 10000 FTU (Robinson *et al.,* 2002). Pellets were blow dried up to 10% moisture then sealed in airtight polythene bags and stored at -20°C throughout the feeding trial. Ingredients and proximate composition of experimental diets is given in Table 1. A 3^2 factorial experiment under completely randomized design was followed for feeding trial with three replicates of each experimental diet.

Rearing conditions and Experimental fish: Rohu (*L. rohita*) juveniles were taken from Govt. fish seed hatchery, Faisalabad, Pakistan. Upon arrival at laboratory fish were salt (5g NaCl/kg) bathed and stored for acclimation. The basal diet was given to fish one a day during acclimation period with round the clock aeration, to each experimental unit. Upon acclimation of two weeks 15 fish (average mean weight= 107.69±1.32g) were randomly distributed into each of 27 experimental tanks with 70 L water capacity. *L. rohita* were fed manually at 09:00 and 17:00 daily upto apparent satiation. After 3 hours feeding, the experimental tanks were cleaned and again filled with filtered, dechlorinated public utility water. Mean water temperature (24.9-28.7°C), pH (7.4-8.6) and dissolved oxygen (5.8-7.3 mg/L) were maintained throughout the feeding trial of 10 weeks within the recommended range for species culture.

Collection of sample and analysis: On the termination of feeding trial, 24 hours after last feeding, fish were anesthetized using 3000mg/L clove oil solution for 40-60sec (Khajepour *et al.,* 2012) and sacrificed. Ten fish were randomly selected from each tank minced, dried at 60°C, pulverized and used for mineral and whole body proximate analysis. For the analysis of mineral contents of bones, five whole fish were boiled until stripping off flesh from bones (approximately 15 mins). Bones were slightly brushed to remove soft tissues, rinsed with distilled water and for 2 h oven dried the sample for 2 h at 110°C. Bones were defatted in anhydrous ethyl ether and crushed in mortar and pestle. For estimation of minerals dried fish samples were acid digested, and Mg, Ca, Fe, Mn, Cu and Zn contents were estimated by atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan) according to the official method 968.08 of the AOAC (AOAC, 1997). K and Na were estimated on flame photometer (Jenway PFP-7, UK) while P contents by UV-VIS spectrophotometer (U-2001, Hitachi) at 750 nm absorbance after oxidation with molybdate reagent. Standard AOAC (1997) methods were adopted for proximate composition (crude fats, dry matter and crude protein) of diet and whole body.

Statistical analysis: Using CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA), two-way analysis of variance (ANOVA) was applied on the experimental data to study the efficacy of phytase and citric acid supplementation and interaction between these two supplements. For the determination of differences among means Tukey`s Honestly Significant Difference Test was applied (Snedecor and Conhran, 1991). Significant differences were observed at $p \leq 0.05$.

RESULTS

Body Proximate Composition: Proximate composition of whole body of Rohu in response to dietary citric acid and phytase is given in Table 2. The results indicated that the phytase supplementation improved the proximate composition of juvenile's whole body. Improved crude protein, total ash and dry matter ($p \leq 0.05$), meanwhile reduced crude fat in the body of fish was observed in groups having phytase supplemented diet. In the same trend, dietary supplementation of CA also enhanced the whole body nutrient contents of juveniles. Except crude fat, which was decreased, all the other parameters (crude protein, dry matter and total ash) were increased significantly ($p \leq 0.05$) with dietary CA treatment response. Interaction plots of CA and PHY showed strong interaction between both the supplements for proximate composition of juvenile's body. An increasing trend was observed for the crude protein, dry matter and total ash concentrations with an increment in concentrations of the supplements. Maximum increase in these parameters (total ash, crude protein and dry matter) was recorded at highest tested levels of PHY (1000 FTU/kg) and CA (3%) while highly reduced crude fat level was also recorded on these same levels of both the supplements. Two-way ANOVA results also showed a significant positive interaction for crude fat and total ash concentrations in the juveniles.

Response of Whole-body Mineralization: Whole body mineral contents of rohu juveniles fed on CA and PHY supplemented diets is shown in Table 3. All the minerals (Ca, P, Mg, Zn, K, Na, Mn, Cu and Fe) which were observed showed higher contents in fish whole body as compare to control fish in PHY supplemented groups. Their concentration was affected significantly by the amount of PHY fed. Highest deposition of these minerals was detected in T3 fish diet (that was supplemented with 1000 FTU/kg diet). Main effect data of CA supplementation indicated a significantly enhanced mineralization of whole body. Concentration of minerals was also affected by the amount of CA given to the fish. Highest mineral deposition was recorded against to 3% CA level. Interactions of both supplements (PHY and CA), improved the amount of these minerals (except Zn) in the body. Test diets having 1000 FTU/kg PHY and 3% CA resulted in maximum deposition of minerals in the fish body.

***Bone Mineralization*:** Effect of CA and PHY on mineralization of bones of rohu juveniles is given in Table 4. PHY addition significantly enhanced the minerals contents in bones. Its supplementation showed dose dependent effect on bone mineralization. Highest amount of minerals deposition was observed in group having supplementation of 1000 FTU/kg PHY level in the diet. Like that supplementation of CA also improved mineral contents in fish bones. Maximum deposition of K, Na, Cu, Zn and Fe were recorded in fish having 1.5% CA while P, Ca and Mn showed their utmost deposition at 3% CA level. Interaction plots represent a strong interaction among CA and PHY for bone mineralization. Likewise, whole body proximate analysis, increasing trend in deposition of Na, K, Na, Mg, Zn, P, Fe, and Cu in bones of fish was observed with increasing the concentrations of PHY and CA.

Table 1. Composition of experimental diets.

CA (%)		**0**			**1.5**			**3**	
PHY (FTU/kg)	**0**	**750**	**1000**	**0**	**750**	**1000**	**0**	**750**	**1000**
Soybean meal	43	43	43	43	43	43	43	43	43
Fish meal	24	24	24	24	24	24	24	24	24
Rice polish	13	13	13	13	13	13	13	13	13
Wheat flour	10	10	10	10	10	10	10	10	10
Fish oil	6	6	6	6	6	6	6	6	6
Vitamin premix	1	1	1	1	1	1	1	1	1
Mineral mixture	1	1	1	1	1	1	1	1	1
Ascorbic acid	1	1	1	1	1	1	1	1	1
Chromic oxide	1	1	1	1	1	1	1	1	1
Total	100	100	100	100	100	100	100	100	100
Proximate composition									
Dry matter	92.02	92.14	93.07	93.13	92.72	93.31	93.11	93.63	93.83
Crude protein	26.37	27.33	27.66	28.07	28.03	27.57	28.04	27.49	27.42
Crude fat	7.81	7.875	7.99	7.88	7.93	8.00	7.95	7.99	8.61
Gross energy	3.69	3.645	3.71	3.79	3.69	3.67	3.64	3.65	3.61

Table 2. The influence of citric acid and phytase supplementation on the whole-body proximate composition of (g/kg) of *L. rohita*

CA level (%)	0			1.5			3			PSE	ANOVA		
PHY Level (FTU/kg)	0	750	1000	0	750	1000	0	750	1000		CA	PHY	CA X PHY
Dry matter	232.1^{e}	253.25^{c}	244.20^{d}	244.7^{d}	256.7^{bc}	254.7^{c}	243.4^{d}	257.5^{b}	261.5^{a}	1.41	≤0.05	≤0.05	NS
Crude Protein	214.4^{f}	227.05^{e}	234.95^{d}	236.9^{cd}	247.05^{b}	248.95^{b}	238.4^{c}	247.45^{b}	253.6^{a}	1.55	≤0.05	≤0.05	NS
Crude fat	52.45^{a}	46.15^{b}	41.85^{c}	41.55^{c}	34.4^{e}	37.45^{d}	42.15^{c}	31.85^{f}	28.8^{g}	0.84	≤0.05	≤0.05	≤0.05
Total ash	33.00^{f}	38.50^{e}	39.65^{de}	42.1^{c}	41.5^{cd}	44.3^{b}	40.25^{d}	46.3^{a}	45.4^{ab}	0.86	≤0.05	≤0.05	≤0.05

Means within rows having different superscripts are significantly different at P ≤ 0.05
Data are means of three replicates
PSE = pooled SE = √MSE/n (where MSE= mean-square error)

Table 3. The influence of citric acid and phytase supplementation on whole body mineralization of *L. rohita.*

CA level (%)	0			1.5			3			PSE	ANOVA		
PHY Level (FTU/kg)	0	750	1000	0	750	1000	0	750	1000		CA	PHY	CA×PHY
P (%)	0.81^{f}	0.91^{f}	0.94^{e}	0.96^{d}	1.06^{c}	1.09^{b}	0.92^{f}	1.10^{b}	1.13^{a}	0.01	≤0.05	≤0.05	≤0.05
Ca (%)	0.73^{h}	0.805^{g}	0.851^{d}	0.83^{e}	0.93^{c}	0.92^{c}	0.83^{f}	0.95^{b}	0.98^{a}	0.01	≤0.05	≤0.05	≤0.05
Mg (%)	1.25^{g}	1.75^{f}	1.95^{e}	1.70^{f}	2.45^{d}	2.75^{c}	1.85^{ef}	2.95^{b}	3.25^{a}	0.05	≤0.05	≤0.05	≤0.05
Na (mg/g)	2.60^{e}	3.25^{d}	3.7^{c}	3.40^{d}	5.05^{b}	5.25^{b}	3.70^{c}	5.55^{a}	5.75^{a}	0.11	≤0.05	≤0.05	≤0.05
K (%)	5.65^{f}	6.05^{e}	6.25^{de}	6.40^{d}	7.85^{c}	8.05^{bc}	6.45^{d}	8.20^{b}	8.70^{a}	0.11	≤0.05	≤0.05	≤0.05
Mn (ug/g)	7.45^{d}	7.85^{c}	8.00^{c}	7.75^{c}	9.40^{b}	9.4^{b}	7.95^{c}	9.65^{b}	10.0^{a}	0.11	≤0.05	≤0.05	≤0.05
Fe (ug/g)	31.0e	36.0^{d}	37.5^{d}	36.0^{d}	51.0^{b}	52.0^{b}	38.5^{c}	51.5^{b}	55.0^{a}	1.01	≤0.05	≤0.05	≤0.05
Cu (ug/g)	1.40^{g}	1.75^{f}	2.05^{e}	2.10^{de}	3.45^{c}	3.65^{bc}	2.30^{d}	3.80^{b}	4.15^{a}	0.11	≤0.05	≤0.05	≤0.05
Zn (ug/g)	1.40^{b}	1.75^{b}	2.05^{b}	2.10^{ab}	3.45^{ab}	3.65^{ab}	2.30^{ab}	3.80^{ab}	4.15^{a}	0.90	≤0.05	≤0.05	NS

Means within rows having different superscripts are significantly different at P ≤ 0.05
Data are means of three replicates
PSE = pooled SE = √MSE/n (where MSE= mean-squared error)

Table 4. The influence of citric acid and phytase supplementation on bone mineralization of L. *rohita.*

CA level (%)	0			1.5			3			PSE	ANOVA		
PHY Level (FTU/kg)	0	750	1000	0	750	1000	0	750	1000		CA	PHY	CA×PHY
P (%)	10.15^{b}	10.85^{ab}	10.8^{ab}	10.75^{ab}	11.8^{ab}	11.05^{ab}	10.7^{a}	11.8^{ab}	12.05^{a}	0.10	≤0.05	≤0.05	≤0.05
Ca (%)	21.65^{e}	24.01^{d}	25.25^{c}	21.8^{e}	26.15^{b}	27.27^{a}	21.6^{e}	27.2^{a}	27.55^{a}	0.43	≤0.05	≤0.05	≤0.05
Mg (%)	0.32^{f}	0.44^{e}	0.45^{de}	0.45^{de}	0.54^{c}	0.54^{c}	0.46^{d}	0.56^{b}	0.62^{a}	0.83	≤0.05	≤0.05	≤0.05
Na (mg/g)	1.35^{g}	1.56^{d}	1.5^{e}	1.48^{ef}	1.63^{b}	1.60^{c}	1.46^{f}	1.69^{a}	1.68^{a}	1.59	≤0.05	≤0.05	≤0.05
K (%)	0.34^{g}	0.44^{e}	0.48^{cd}	0.41^{f}	0.49^{c}	0.54^{b}	0.47^{cd}	0.58^{a}	0.57^{a}	1.17	≤0.05	≤0.05	≤0.05
Mn (ug/g)	44.7^{e}	49.1^{cd}	50.15^{c}	48.45^{d}	53.15^{ab}	54.25^{a}	47.95^{d}	52.55^{b}	54.05^{a}	0.63	≤0.05	≤0.05	≤0.05
Fe (ug/g)	31.55^{e}	36.45^{d}	38.2^{cd}	36.65^{d}	41.5^{b}	42.7^{b}	38.95^{c}	42.2^{b}	45.7^{a}	1.20	≤0.05	≤0.05	≤0.05
Cu (ug/g)	11.4^{h}	11.75^{g}	12.05^{f}	12.1^{ef}	12.45^{cd}	12.65^{bc}	12.3^{de}	12.8^{b}	13.15^{a}	0.13	≤0.05	≤0.05	≤0.05
Zn (ug/g)	116.05^{h}	119.55^{g}	122.2^{ef}	120.35^{fg}	124.95^{cd}	125.7^{c}	123.45^{de}	130^{b}	132.7^{a}	1.20	≤0.05	≤0.05	NS

Means within rows having different superscripts are significantly different at P ≤ 0.05
Data are means of three replicates
PSE = pooled SE = √MSE/n (where MSE= mean-squared error)

DISCUSSION

Phytase hydrolysed the phytate, which is present in plants related feed stuffs, and it ultimately improves the phytate P and other minerals utilization (Cheng and Hardy, 2002). In the present study, supplementation of phytase enhanced the dry matter content of whole fish body. In contrast to this study, Carter and Sajjadi (2011) observed non-significant changes in dry matter of Atlantic salmon fed on phytase supplemented diets. Denstadli *et al.* (2007) examined the non-significant changes in dry matter of *Salmo salar* by adding phytase in fish feed. Contents of crude protein in juvenile's body was improved in phytase supplemented groups. Similar results in *Cyprinus carpio* were also shown by Sardar *et al.* (2007). Conversely, many of works cleared that body content of crude protein was significantly affected by dietary phytase supplemented but a negative response on some species such as Gibel carp (Liu *et al.,* 2012), Atlantic salmon (Carter and Sajjadi, 2011), grass carp (Liu *et al.,* 2014) and *Salmo salar* (Denstadli *et al.,* 2007) has also been reported. Additionally, negative response to whole body crude protein to phytase addition was observed in red sea bream (Laining *et al.* 2012). In the current study, decrease in level of crude fat of juvenile's body was observed in against phytase addition. Same decrease in crude fat contents in whole body of grass carp (Liu *et al.,* 2014), *Salmo salar* (Denstadli *et al.,* 2007), and *Cyprinus carpio* (Sardar *et al.,* 2007) were also observed in response to phytase addition. Contrary to present study, in red sea bream (Laining *et al.,* 2012) and Gibel carp (Liu *et al.,* 2012) non-significant differences among control and PHY treated groups were reported. Enhanced ash content in the whole body of fish were observed, when fish were fed with phytase treated diets which indicate that phytase might enhanced the minerals content of fish body (Sarker *et al.,* 2012). Similar results have also been reported by many preceding studies (Denstadli *et al.,* 2007; Sardar *et al.,* 2007; Carter and Sajjadi, 2011; Liu *et al.,* 2014).

Whole body mineral contents of *L. rohita* were enhanced by citric acid addition ($P \leq 0.05$), which indicates phytate hydrolysis. Same results were observed in a study that showed an increased content of P, Ca, K, Mn, Fe and Cu in *Pagrus major* when citric acid (3%) was added in the diet of fish (Laining *et al*., 2012). While in another study contradicted results were observed, where organic acid supplementation showed non-significant variations to whole body mineral contents of yellow catfish (Zhu *et al.,* 2014), Yellow tail (Sarker *et al.,* 2012), rainbow trout (Pandey and Satoh, 2008) and red sea bream (Hossain *et al.,* 2007). Citric acid when added to phytase supplemented diets showed positive interaction and enhanced mineral contents of rohu. Citric acid reduced intestinal pH up to range of phytase activity as well as breaks phytate complex physically. Positive interaction was observed in citric acid and phytase in order to enhance mineral (Ca, P, Na, Mg, K, Fe, Mn, Zn and Cu) contents of whole rohu. Similar synergistically positive interaction was also reported by Afzal *et al.* (2020).

Increased bone mineral contents were observed in this study by adding phytase in fish feed. Phytase hydrolyzed phytate and released bound minerals enhancing their free availability and deposition in bones. Similarly, some other studies have reported improved bones Ca and P of rainbow trout (Fox and Davies, 2011) and bones P, Ca, Mg and Zn of red sea bream (Laining *et al.,* 2012) and *Salmo salar* (Denstadli *et al.,* 2007). However, in a recent study non-significant results of bone minerals were detected by addition of phytase in the feed of fish (Liu *et al.,* 2014). Fish fed citric acid supplemented diet, showed increased mineral contents of bones which may be due to the reason that decreased pH by acid addition broken down mineral compounds and formed citric acid chelated mineral complexes which were easily absorbed by fish. In agreement to our study, better Ca, P and Zn concentrations in bones of rainbow trout has also been reported (Pandey and Satoh, 2008). Furthermore, citric acid assisted phytase performance by lowering pH in optimum phytase activity range and enhanced phytate hydrolysis, which alternatively improved minerals availability. Also, Afzal *et al.* (2020) confirmed that citric acid positively interacted ($p \leq 0.05$) with phytase for enhancing mineral contents of bones.

Hence, the present study evidenced that that dietary citric acid and microbial phytase are considered efficient dietary supplements to improve the nutrient and mineral profile of rohu (*L. rohita)* juveniles.

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