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Potential of Phytase and Citric Acid Treated Canola Meal Based Diet to Enhance the Minerals Digestibility in *Labeo rohita* Fingerlings

Rana Zeeshan Habib[1], Muhammad Afzal[1], Syed Zakir Hussain Shah[2,*], Mahroze Fatima[3], Muhammad Bilal[4] and Syed Makhdoom Hussain[5]

[1]*Fish Nutrition Laboratory, Department of Zoology, Wildlife & Fisheries, University of Agriculture, Faisalabad*
[2]*Department of Zoology, University of Gujrat, Gujrat*
[3]*Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore*
[4]*Industrial Biotechnology Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad*
[5]*Department of Zoology, Government College University, Faisalabad*

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RZH performed the experiments, MA supervised the experiments. SZHS and MF wrote the manuscript. MB helped in experiment conduction while SMH statistically analyzed the data.

Key words
Phytase, Citric acid, Mineral digestibility, Rohu, Fingerlings.

ABSTRACT

Present trial was conducted to investigate the effect of phytase (PHY) and citric acid (CA) supplementation on minerals digestibility of *Labeo rohita* fingerlings fed on canola meal based diet. Nine experimental diets were prepared by supplementing CA (%) and PHY (FTU/kg) at the level of 0, 0 (control); 0, 1000; 0, 2000; 1.5, 0; 1.5, 1000; 1.5, 2000; 3, 0; 3, 1000; 3, 2000 respectively. Chromic oxide was used as inert marker in diets to estimate mineral digestibility. Fish fed on PHY supplemented diet showed higher apparent digestibility coefficient (ADC%) of Ca, P, Na, K, Mg, Fe, Cu, Mn, Zn. Similarly, CA addition also improved ($p<0.05$) ADC% of Ca, P, Na, K, Mg, Fe and Cu, however, ADC% of Mn and Zn remained unaffected. Also, acidification of PHY treated diet with CA significantly ($p<0.05$) improved the mineral digestibility performance of *L. rohita*. In conclusion, both the additives (CA and PHY) showed improved digestibility of minerals individually as well as in combination.

INTRODUCTION

Labeo rohita is a fish of prime importance in aquaculture industry of Pakistan due to its high quality flesh. Fish meal is being used as a prominent ingredient in fish feed, but its high price and limited supply has compelled the researchers to search for alternative feed sources, like plant protein based feed ingredients. Canola meal is one of the most promising fish feed source having 38% protein and is less in antithyroid factors, erucic acid and glucosinolates (Bell, 1993). The problem associated with its use is the presence of anti-nutritional factors like phytate. Canola meal constitutes 3.1% to 3.6% phytate which have deleterious effects on the fish gut that ultimately leads to poor growth performance of fish (Usmani and Jafri, 2002). Phytate represents 60 to 80% of the total phosphorus present in plant feed stuff which cannot be utilized by agastric or mono-gastric fish species like *L. rohita* due to lack of intrinsic phytase activity (Ogino *et al.*, 1979). Phytate is highly negative charged ion and have adverse effects on mineral absorption by making insoluble complexes with divalent and trivalent cations like Ca^{+2}, Zn^{+2}, Mn^{+2}, Cu^{+2} and $Fe^{+2/+3}$ (Kumar *et al.*, 2010). Poor phytate degradation also contribute to eutrophication which have detrimental effect on fresh water bodies (Persson *et al.*, 1998).

Microbial phytase (PHY) is enzyme capable of removing phosphate group from phytate and making it available to fish for utilization and absorption (Baruah *et al.*, 2007). Sugiura *et al.* (2001) reported increased mineral digestibility of rainbow trout as a result of PHY supplementation. Similarly, Laining *et al.* (2010) concluded that 2000 FTU/kg PHY enhanced mineral digestibility performance of juvenile tiger puffer (*Takifugu rubripes*) efficiently. A comparatively high level (8000 FTU/kg) of PHY supplementation was considered optimum to enhance the digestibility of Mg, P, Ca, Fe and Mn in tilapia when fed soybean meal-based diet (Nwanna, 2005). However, PHY activity is pH dependent and it is highly active at low pH of gut (Baruah *et al.*, 2007). Acid producing ability of carnivore fishes lower the pH of gut but monogastric fishes lack this ability (Ogino *et al.*, 1979). It is well documented that addition of organic acids such as citric acid (CA) in fish diet lowers the pH of digestive

* Corresponding author: zakiruaf@gmail.com
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tract and enhances the PHY efficacy Baruah *et al*. (2005). Apart from providing the optimum conditions to the PHY, CA itself dephosphorylate phytate complex and enhance the availability of P and other minerals (Zyla *et al*., 1995). Khajepour and Hosseini (2011) reported that addition of CA in the feed of Beluga (*Huso huso*) efficiently enhanced the Ca and P contents of fish. Enhanced utilization of P and other minerals by supplementation of CA to fish feed was reported by Sugiura *et al*. (2001).

Thus, the objective of the present study was to investigate the main as well as synergetic effect of phytase and citric acid on mineral digestibility performance of *L. rohita* fingerlings fed canola meal based diet.

MATERIALS AND METHODS

The present 3×3 factorial experiment was conducted in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan.

Fish and experimental conditions

The fingerlings were obtained from Government Fish Seed Hatchery, Faisalabad and acclimatized to experimental conditions in laboratory for two weeks in V-shaped tanks (UA system) especially designed for fecal collection from water media. Twelve fingerlings (average weight of 7.61 g) were stocked in each tank having 70 L water capacity. During this period the fingerlings were fed once daily to apparent satiation on the basal diet (Allan and Rowland, 1992). Water quality variables, particularly temperature, pH and dissolved oxygen were monitored by the usage of Jenway pH meter (model 3510) and D.O. meter (model 970) respectively during the study period. Aeration was provided 24 h to all tanks through capillary system. Before starting the feeding trial, the fingerlings were treated with 5g/L NaCl solution to make fish free from fungal infection and ectoparasites (Rowland and Ingram, 1991).

Feed ingredients and experimental diets

The feed ingredients were bought from a commercial feed mill and analyzed for chemical composition following (AOAC, 1995) prior to the formulation of the experimental diet. The feed ingredients were ground and sieved to required particle size before formulation of experimental diets (Table I). All dry ingredients were mixed in electric mixer for 10-20 min, where-after fish oil was gradually added, while mixing constantly. Chromic oxide (1%) was used as an inert non digestible marker. Citric acid was added at the levels of (0, 1.5 and 3%) to dry mixed ingredients to make three test diets. Ten to fifteen percent water was added to prepare suitable dough of each test diet, and was further processed through lab extruder for making floating pellets. Three test diets were made based on 3 concentrations of CA, after pelleting which were further sprayed with three PHY levels (0, 1000 and 2000 FTU kg^{-1} diet) resulting in the formulation of nine test diets. The phytase (Phyzyme® XP 10000 FTU/g; Danisco Animal Nutrition, Fin-65101 Vaasa, Finland) solution was prepared by dissolving 2g of microbial phytase (powder form) into 1 L of distilled water (Robinson *et al*., 2002). One unit of phytase activity (FTU) is defined as "the enzyme activity that liberates 1 µmol of inorganic orthophosphates per min at pH 5.5 (37°C) at a substrate (sodium phosphate) concentration of 5.1 mmol/L" (Engelen *et al*., 1994). The proximate and mineral composition of experimental diets is given in Table II.

Table I.- Ingredient composition (%) of experimental diets.

CA level (%)	**0**			**1.5**			**3**		
PHY Level (FTU/kg)	**0**	**1000**	**2000**	**0**	**1000**	**2000**	**0**	**1000**	**2000**
Test Diets	**T1 (Control)**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**
Fish meal	12	12	12	12	12	12	12	12	12
Canola meal	56	56	56	56	56	56	56	56	56
Rice polish	12	12	12	12	12	12	12	12	12
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
Minerals	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**

Table II.- Analyzed proximate and mineral composition (%) of experimental diets.

CA (%)	0			1.5			3		
PHY (FTU/kg)	**0**	**1000**	**2000**	**0**	**1000**	**2000**	**0**	**1000**	**2000**
Test Diets	**T1 (Control)**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**
DM	95.96±0.53	96.32±0.73	94.98±0.658	94.64±1.03	95.59±0.27	96.17±0.34	95.53±0.83	96.58±1.13	96.72±0.28
CP	32.17±0.05	32.19±0.02	32.21±0.06	32.22±0.04	32.18±0.06	32.19±0.06	32.17±0.06	32.19±0.07	32.19±0.074
CF	3.15±0.015	3.17±0.02	3.14±0.01	3.15±0.03	3.13±0.02	3.17±0.01	3.17±0.02	3.12±0.01	3.13±0.01
GE	3.18±0.01	3.20±0.02	3.21±0.02	3.20±0.02	3.21±0.02	3.21±0.02	3.20±0.02	3.19±0.02	3.20±0.01
Ca	0.14±0.002	0.14±0.002	0.14±0.002	0.14±0.002	0.14±0.002	0.14±0.003	0.14±0.002	0.14±0.003	0.14±0.001
P	1.97±0.01	1.98±0.01	1.97±0.02	1.98±0.02	1.97±0.02	1.99±0.01	1.98±0.01	1.97±002	1.97±0.01
Na	0.78±0.01	0.78±0.02	0.79±0.02	0.80±0.01	0.79±0.01	0.80±0.01	0.78±0.02	0.79±0.01	0.78±0.01
K	1.25±0.01	1.28±0.02	1.28±0.01	1.26±0.02	1.28±0.01	1.26±0.02	1.27±0.02	1.26±0.03	1.26±0.02
Mg	0.07±0.004	0.07±0.002	0.07±0.002	0.08±0.002	0.078±0.003	0.07±0.003	0.07±0.003	0.07±0.003	0.07±0.002
Fe	0.074±0.002	0.07±0.002	0.07±0.003	0.07±0.002	0.071±0.002	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.006
Cu	0.74±0.025	0.75±0.02	0.74±0.01	0.74±0.02	0.74±0.02	0.75±0.02	0.74±0.02	0.74±0.02	0.74±0.02
Mn	0.07±0.002	0.09±0.002	0.09±0.001	0.09±0.002	0.09±0.002	0.09±0.002	0.09±0.002	0.09±0.002	0.09±0.002
Zn	0.07±0.003	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.002

Data are means of three replications ± standard deviation.

Table III.- Apparent mineral digestibility coefficient (%) of *L. rohita* fingerlings fed experimental diets.

CA (%)	0			1.5			3		
PHY(FTU/kg)	**0**	**1000**	**2000**	**0**	**1000**	**2000**	**0**	**1000**	**2000**
Test Diets	**T1 (Control)**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**
Ca	37.97±1.32^{c}	44.33±2.62^{c}	40.09±3.79^{de}	39.15±3.06^{de}	54.97±3.58^{b}	49.59±3.45^{b}	46.77±3.36^{c}	64.62±3.69^{a}	61.70±3.75^{a}
P	35.35±3.39^{g}	56.30±0.98^{cd}	51.54±3.89^{e}	42.77±3.51^{f}	64.52±1.71^{b}	60.46±2.68^{b}	54.27±2.50^{de}	76.78±1.34^{a}	72.47±0.69^{b}
Na	39.94±2.42^{e}	47.76±3.83^{d}	40.83±3.35^{e}	43.18±2.83^{d}	59.73±3.09^{b}	55.34±2.43^{bc}	53.53±3.97^{c}	65.33±3.04^{a}	60.79±3.91^{ab}
K	39.67±2.81^{f}	44.77±2.56^{cde}	41.28±1.41^{ef}	42.21±3.07^{def}	48.27±2.87^{c}	46.61±3.39^{cd}	45.55±3.10^{a}	61.15±3.47^{a}	55.84±3.53^{b}
Mg	30.30±2.81^{e}	36.15±3.78^{de}	33.66±1.31^{de}	31.55±3.88^{ab}	44.60±3.59^{bc}	41.88±3.99^{cd}	36.74±1.20^{e}	48.36±3.91^{a}	46.53±3.88^{ab}
Fe	28.29±2.84^{d}	30.34±3.93^{cd}	35.46±3.31^{c}	30.44±3.33^{cd}	61.57±2.87^{ab}	63.65±2.15^{a}	35.32±2.95^{c}	57.20±5.02^{b}	60.62±2.65^{ab}
Cu	38.07±3.80^{g}	44.88±1.01^{ef}	46.33±3.74^{de}	40.36±0.58^{fg}	51.54±3.49^{bc}	50.09±3.66^{bcd}	48.33±3.53^{cde}	54.94±3.14^{ab}	57.92±2.16^{a}
Mn	44.50±3.85^{e}	48.80±3.92^{de}	52.61±3.98^{cd}	46.23±3.72^{e}	64.28±3.28^{a}	48.70±3.01^{de}	48.57±3.33^{de}	59.44±2.76^{ab}	55.30±3.72^{bc}
Zn	43.33±3.66^{e}	49.88±3.71^{d}	48.58±3.81^{de}	44.77±2.59^{de}	71.39±3.88^{a}	56.44±3.58^{c}	47.25±3.67^{de}	67.09±3.11^{ab}	62.92±3.86^{b}

Data are means of three replications ± standard deviation.

Feeding protocol and sample collection

The fish fingerlings were fed at the rate of 3% of live wet weight on their prescribed diet. For each test diet, three replicate tanks were assigned with stocking density of twelve fish in each tank. After the feeding session of 3 h, the uneaten diet was drained out from each tank by opening the valves of the tanks. The tanks were washed completely to remove the particles of diets and refilled with water. After that, the fish were restocked in tanks. The feces will be collected from the fecal collection tube of each tank after 2 h intervals, by opening the valve I and valve II subsequently. Care was taken to avoid breakage of the thin fecal strings in order to minimize nutrient leaching. Fecal material of each replicated treatment was dried in oven ground and stored for chemical analysis. The experiment lasted for 2 months.

Chemical analysis of feed and feces

The sample of feed ingredients, test diets and feces were homogenized using pestle and mortar, and analyzed by standard methods (AOAC, 1995). Moisture was determined by oven-drying at 105˚C for 12 h, crude protein by micro Kjeldahl apparatus, crude fat by petroleum ether extraction method through soxtec HT2 1045 system, crude fiber was determined as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH, crude ash by ignition at 650˚C for 12 h in electric furnace (Eyela-TMF 3100) to constant weight, and gross energy with the help of adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, USA). Total carbohydrates (N-free extract) were calculated by difference *i.e.* total carbohydrate (%) = 100- (moisture% + CP% + EE% + Ash% + CF%). Chromic oxide contents in experimental diets and feces were estimated after oxidation with molybdate reagent (Divakaran *et al*., 2002) using a UV-VIS 2001 Spectrophotometer at 370 nm absorbance.

Apparent nutrient digestibility coefficients (ADC) of test diets were calculated by the formula reported in NRC (1993):

$$ADC\% = 100 - 100 \times \frac{\text{Percent marker in diet} \times \text{Percent nutrient in feces}}{\text{Percent marker in feces} \times \text{Percent nutrient in diet}}$$

Statistical analysis

Data of mineral digestibility of experimental diets were subjected to two-way analysis of variance (Steel *et al*., 1996). The difference among means were compared by Tukey's Honestly Significant Difference Test and considered significant at $p<0.05$ (Snedecor and Cochran, 1991). The CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analysis.

RESULTS

The results of main effect of PHY and CA and their interaction on ADC% of minerals in *L. rohita* fingerlings fed on canola meal based diet are summarized in Table III. The supplementation of PHY and CA showed positive effects on the growth performance of fish (data not shown). Main effect data indicate that dietary addition of PHY enhanced the minerals digestibility significantly ($p<0.05$) as compare to control group (D1). Highest ($p<0.05$) digestibility of Ca, P and Na was observed at 1000 FTU/kg PHY level while other minerals (K, Mg, Fe, Cu, Mn and Zn) showed non-significant difference among 1000 FTU/kg and 2000 FTU/kg PHY levels.

The main effect data of CA supplementation also showed improved ADC% of all the observed minerals compared to fish fed on diet without CA addition. Ca, P, Na, K, Mg, Fe and Cu showed significantly ($p<0.05$) enhanced ADC% while non-significant ($p<0.05$) increase was observed for Mn and Zn. Highest ADC% of Ca, P, Na, K, Mg, Fe, Cu, Mn and Zn were 46.77%, 54.27%, 53.53%, 45.55%, 36.74%, 35.32%, 48.33%, 48.57% and 47.25%, respectively in diet supplemented with 3% CA.

Acidification of PHY treated diet with CA significantly ($p<0.05$) improved the mineral digestibility performance. Ca, P, Na, K and Mg showed higher ADC% at the level of 3% CA and 1000 FTU/kg PHY. ADC% of Mn and Zn was higher at 1.5% and 1000 FTU/kg levels of CA and PHY, respectively. Digestibility of Fe and Cu was higher at 2000 FTU/kg PHY level with 1.5% and 3% CA levels, respectively.

DISCUSSION

In the current study, PHY supplementation increased ($p<0.05$) the minerals digestibility compared to the fish fed on control diet. This may attribute to phytate hydrolyzing tendency of PHY. Phytate, which is an anti-nutritional factor and interacts with various minerals directly or indirectly and reduces their availability to the fish (Sandberg *et al*., 1993). Thus, addition of PHY in plant ingredients based feed hydrolyzed the phytate and released bound minerals (Debnath *et al*., 2005; Baruah *et al*., 2007).

Results of present study showed clearly that CA acidification of diet also enhanced the absorption of minerals in the body of fingerlings. Citric acid enhances the absorption of minerals by solubilizing the bones present in fish meal (Sarker *et al*., 2005) and by chelating the Ca and P from phytate, which make the phytate more susceptible for hydrolysis (Khajepour and Hosseini, 2010). It also compete with various dietary inhibitors of minerals and increases their bioavailability to fish (Ashmead, 1993).

Similar increase in P absorption by supplementing 3% CA was also observed by Baruah *et al*. (2007) in rohu, *L. rohita* and by Khajepour and Hosseini (2012) in Beluga, *Huso huso.* However, Sarker *et al*. (2007) observed increased P absorption at 1% CA level in Red Sea bream, *Pagrus major.* Baruah *et al*. (2007) also observed improved digestibility of Na, P, K, Mn, Mg, Fe, N, Ca and Cu by supplementing 3% CA in plant based diet.

In the present study, interaction data revealed a positive effect of acidification of diet with CA on PHY effectiveness for mineral absorption. Citric acid along with phytate hydrolysis also provided favorable conditions to PHY action by lowering the gut PH of fish (Baruah *et al*., 2005). Similar synergistic effect was also shown by common carp, *Cyprinus carpio* (Phromkunthong *et al*., 2010) and rohu, *L. rohita* (Baruah *et al*., 2005) for P digestibility. Improved Zn absorption by PHY and CA supplementation was observed by Brenes *et al*. (2003) in chick. Baruah *et al*. (2007) also observed a positive interaction of both additives for Na, K, Zn, Mn, Mg, Cu, Fe, Ca and N in rohu, *L. rohita* fingerlings.

CONCLUSION

CA and PHY supplementation to plant protein based diet efficiently improved the mineral digestibility performance of *L. rohita* fingerlings by acting individually and further improved by acting synergistically. The supplements showed the potential to prepare cost effective and environment friendly feed by minimizing the mineral supplementation and their discharge into natural water bodies.

Statement of conflict of interest

Authors have declared no conflict of interest.

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