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# Efficacy of phytase enzyme and citric acid on growth performance, nutrients and mineral digestibility of *Cirrhinus mrigala* fingerlings fed guar meal-based diet

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**Abstract**

The study was carried out to estimate the effects of phytase, and citric acid (CA) supplemented guar meal based diet on growth performance, nutrients and mineral digestibility in *Cirrhinus mrigala* fingerlings. The experiment consisted of nine test diets. Diets were formulated by spraying graded levels of phytase (0, 500 and 1000 FTU $kg^{-1}$) and 0% (0g), 2.5% (75g) and 5% (150g) citric acid supplementation to a guar meal-based diet. Chromic oxide was added as an indigestible marker. Fingerlings were fed at the rate of 5% of live wet weight. The maximum growth performance, minerals, and nutrients digestibility value were observed in fingerlings fed diet supplemented with 1000 FTU $kg^{-1}$ level of phytase and 2.5% CA. These values were significantly different from fish fed the control and other test diets. It was concluded that the phytase and CA supplementation to a guar meal based diet at 2.5% CA and 1000 FTU $kg^{-1}$ level is optimum to release sufficient amount of chelated minerals and nutrients for *C. mrigala* fingerlings. Our findings also suggested that phytase and CA supplementation can help in the development of sustainable aquaculture by reducing the feed costs and nutrient discharge through feces into the aquatic ecosystem.

**Keywords**: *Cirrhinus mrigala*, Phytase, Citric acid, Guar meal, Growth, Nutrient digestibility.

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## Introduction

*Cirrhinus mrigala* commonly known as "mori," one of the major carp species cultured in Pakistan, is a bottom feeder and feeds on vegetable debris and decaying organic matter. The aquaculture industry is expanding very rapidly to fulfill the need for high-quality fish protein for human nutritional requirements (Tacon and Metian, 2008; Naylor *et al.*, 2009; Yıldırım *et al.*, 2014). Both the feed costs and feed efficiency are primary factors which control the fish farm economy (Khajepour *et al.*, 2012a).

In the past fish feed was reliant on the use of fishmeal as a source of essential nutrients and growth factors because fish meal is enriched with essential nutrients such as indispensable fatty acids, essential amino acids, vitamins and many growth factors (NRC, 1993; Zhou *et al.*, 2004). Due to increasing demand, rising prices and uneven supply of fish meal made it necessary to search for alternative protein sources for the fish feed industry (Pham *et al.*, 2008; Lech and Reigh, 2012). Nowadays one of the most important challenges to the aquaculture industry is the formulation of cost-effective fish feed from better quality protein sources (Baruah *et al.*, 2004). The main objective for most fish farmers is to produce high-quality fish feed at low costs because feeds primarily account for 50 to 60% of the total cost in fish culture (Essa *et al.*, 2004).

Some researchers have found positive effects of plant meal on fish growth (Hussain *et al.*, 2011). So the use of plant meal has been recommended as an alternative protein source for fishmeal, as these are cheaper, easily available and environment-friendly because pollution causing contents of plant-based feeds are lower than that of the fishmeal (Dalsgaard *et al.*, 2009). Of these plant meals, guar meal is relatively inexpensive with high protein contents (33 to 45%). Guar meal is a good binding agent in feed formulation. It is also important that guar meal is free from salmonella, *E. coli*, and aflatoxin (Nagpal *et al.*, 1971). But unfortunately, it has low minerals availability due to the presence of the anti-nutritional factor i.e. phytate or phytic acid (Liu *et al.*, 2013). It has highly adverse effects on the fish digestive tract and results in poor growth performance (Baruah *et al.*, 2004). Phytic acid is the main form of storing phosphorus in plant seeds (Jorquera *et al.*, 2008) and usually, it causes minimum availability of minerals and decreases the apparent digestibility of nutrients and minerals (Gatlin *et al.*, 2007). Phytate has been historically considered an anti-nutritional factor because it is known as a strong chelator of divalent minerals such as Ca, Mg, Zn and Fe (Noureddini and Dang, 2008).

Bioavailability of dietary nutrients and minerals is also greatly influenced by acidification through organic acids in several ways. Firstly, they modify the mineral transport mechanism by altering the gastric acidity. Secondly, they decrease the chelating and complex forming ability of elements

(Ravindran and Kornegay, 1993). Thus they ensure the increased absorption of phosphorus (P) and other trace elements. Thirdly, these organic acids stimulate the proliferation of the epithelial cells in gastrointestinal mucosa (Sakata *et al.*, 1995) thereby increasing the absorption area for nutrients and minerals (Baruah *et al.*, 2007a). Among these organic acids, citric acid (CA) has been extensively used for diet acidification due to its unique flavor and high buffering capacity (Hossain *et al.*, 2007). Chemically, it is known as 2-Hydroxy-1, 2, 3- Propane tri-carboxylic acid with the chemical formula $(COOH)_3(CH_2)_2C(OH)$. It has good water solubility, and its gross energy is 2460 kcal $kg^{-1}$. The diets supplemented with CA, increased the retention of Ca, Mg, Na, K, Zn, and Mn in Yellowtail (Sarker *et al.*, 2012 a,b). It also enhanced nitrogen retention in red sea bream with its different supply levels in the diet (Sarker *et al.*, 2006). Baruah *et al.* (2007a) found that the addition of CA improved the absorption of minerals (Na, P, K, Mn, Mg, Fe, Cu, Ca and N) and their concentration in plasma and the whole body of Rohu. The increment in carcass mineral deposition suggests that the organic acids and other supplements enhanced the mineral utilization of dietary fish meal and plant protein meal (Hossain *et al.*, 2007). Citric acid increases the bioavailability of minerals in several ways. It solubilizes the bones present in the meal and releases the chelated nutrients and minerals (Sarker *et al.*, 2006). Being a strong chelator of Ca and P, it removes these minerals from the phytate; making it less stable and more susceptible to endogenous phytases (Khajepour and Hosseini, 2010). Moreover, CA increases the bioavailability of minerals by competing with the dietary mineral inhibitors. The addition of microbial phytase and CA enhances the availability of phosphorus from plant sources, improves bone mineralization, growth and feed efficiency. Combining a low dose of CA to the phytase supplemented diets significantly increased the positive effects of the enzyme (Phromkunthong *et al.*, 2010). The objective of the present study was to investigate the effects of phytase, and citric acid supplementation on nutrients and mineral digestibility in *C. mrigala* fingerlings fed a guar meal based diet with the aims of growth estimation.

**Materials and methods**

The study was conducted in the Fish Nutrition Laboratory, Department of Zoology Government College University Faisalabad.

*Fish and experimental conditions*

*Cirrhinus mrigala* fingerlings were purchased from the Fish Seed Hatchery, Satiana Road, Faisalabad. The fingerlings were stocked in V-shaped fish tanks (GCUF system) that were specially designed for the collection of fecal material. Fingerlings were acclimatized in the laboratory to experimental conditions for 15 days. During the acclimatization period fish were fed twice daily to apparent satiation on the basal diet, used in

subsequent digestibility studies (Allan and Rowland, 1992). Water quality parameters particularly water temperature; pH and DO were monitored on a daily basis. Aeration was provided 24 hours a day to fish throughout the study period. NaCl (5 g $L^{-1}$) was used on *C. mrigala* fingerlings for the treatment of ectoparasites as well as to prevent fungal infection, before starting the experiment.

*Experimental design*

Guar meal was used as the test ingredient to formulate the experimental diets. This diet was divided into three groups, each of 3 kg weight. The Guar meal based diet was treated with different concentrations of CA and phytase to formulate nine test diets such as T1: 0% CA, 0 FTU $kg^{-1}$, T2: 0% CA, 500 FTU $kg^{-1}$, T3: 0% CA, 1000 FTU $kg^{-1}$, T4: 2.5% CA, 0 FTU $kg^{-1}$, T5: 2.5% CA, 500 FTU $kg^{-1}$ T6: 2.5% CA, 1000 FTU $kg^{-1}$, T7: 5% CA, 0 FTU $kg^{-1}$, T8: 5% CA, 500 FTU $kg^{-1}$ and T9: 5% CA, 1000 FTU $kg^{-1}$. These test diets supplemented with CA and phytase were fed to nine groups of fish stocked in the experimental tanks. Each of the treatments and control diets had three replicates with 15 fingerlings in each replicate. The total duration of the experiment was 90 days. Guar meal based CA and phytase supplemented diets were compared with each other to determine growth, nutrient and mineral digestibility parameters using Completely Randomized Design (CRD).

*Feed ingredients and experimental diets*

The feed ingredients (Table 1) were purchased from the local market and were analyzed for chemical composition following AOAC (1995) before the formulation of the experimental diets (Table 2). The feed ingredients were finely ground to pass through a 0.5 mm sieve. All ingredients were mixed in an electric mixer for 10 min, and fish oil was gradually added while mixing the ingredients. 10% water was also added to form a suitable dough and it was extruded using a SYSLG30-IV experimental extruder to produce pellets (3 mm). The above procedure was followed to formulate the 9 guar meal based test diets. 0g (0%), 75g (2.5%) and 150g (5%) CA was added to them respectively. The required concentrations (0, 500 and 1000 FTU $kg^{-1}$) of phytase (Phyzyme® XP 10000 FTU $g^{-1}$; Danisco Animal Nutrition, Fin-65101 Vaasa, Finland) were prepared in 25 mL distilled water and sprayed on 1 kg of test diets (Robinson *et al.*, 2002). The control diet (0FTU $kg^{-1}$) was sprayed with an equal volume of distilled water to maintain similar moisture contents. All the prepared diets were dried and stored at 4°C before use.

**Table 1: Ingredients composition (%) of guar meal based diets.**

| Ingredients | Test diet-I | Test diet-II | Test diet-III | Test diet-IV | Test diet-V | Test diet-VI | Test diet-VII | Test diet-VIII | Test diet-IX |
|---|---|---|---|---|---|---|---|---|---|
| Citric acid | 0 | 2.5 | 5 | 0 | 2.5 | 5 | 0 | 2.5 | 5 |
| Fish meal | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Guar meal | 56 | 56 | 56 | 56 | 56 | 56 | 56 | 56 | 56 |
| Rice polish | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Wheat flour | 10 | 7.5 | 5 | 10 | 7.5 | 5 | 10 | 7.5 | 5 |
| 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 |
| Phytase* FTU $kg^{-1}$ | 0 | 0 | 0 | 500 | 500 | 500 | 1000 | 1000 | 1000 |

*Phytase enzyme was used at the expense of wheat flour

**Table 2: Chemical composition (%) of feed ingredients (Dry matter basis).**

| Ingredients | Dry matter (%) | Crude Protein (%) | Crude Fat (%) | Crude Fiber (%) | Ash (%) | Gross Energy (kcal $g^{-1}$) | Carbohydrates |
|---|---|---|---|---|---|---|---|
| Fish meal | 91.63 | 48.15 | 7.06 | 1.09 | 24.73 | 2.13 | 16.84 |
| Wheat flour | 92.45 | 10.10 | 2.35 | 2.65 | 2.08 | 2.96 | 79.86 |
| Guar meal | 89.00 | 36.50 | 3.50 | 11.00 | 11.10 | 2.00 | 35.90 |
| Rice polish | 94.09 | 12.25 | 13.44 | 12.60 | 10.15 | 3.33 | 48.23 |

*Feeding protocol and sample collection*

*Cirrhinus mrigala* fingerlings were fed two times a day (morning and afternoon). At the start of the experiment the fish fingerlings were fed at the rate of 5% of live wet weight on their prescribed diet and later on adjusted to a daily basis intake of feed by fish. For each test diet, three replicates were used, and in each replicate, 15 fingerlings (average weight: 8.02 g $fish^{-1}$) were stocked. From each tank, the uneaten diet was drained out after the feeding period of two hours. Before refilling the water, the tanks were washed completely to remove the particles of uneaten diets. Feces were collected from the fecal collection tube of each tank after two hours. Fecal material of each replicate treatment was dried in an oven and stored for further chemical analysis.

*Growth study*

Fish in each tank were bulk weighed every $15^{th}$ day during the experiment to assess growth performance of *C. mrigala* fingerlings. Weight gain (%) and feed conversion ratio (FCR) of fingerlings were evaluated based on standard formulae.

Weight gain % = (Final weight – Initial weight) × 100

Initial weight

FCR= Total dry feed intake (g)

Wet weight gain (g)

*Chemical analysis of feed and feces*

The samples of feed ingredients, experimental diets and feces were homogenized using a motor and pestle and then analyzed by standard methods (AOAC, 1995). Moisture was determined by oven drying at 105$^{o}$C for 12h; crude protein (N × 6.25) by micro-Kjeldahl apparatus; crude fat by petroleum ether extraction method through Soxtec HT2 1045 system; crude fiber as loss on ignition of dried lipid-free residues after digestion with 1.25% $H_2SO_4$ and 1.25% NaOH; Ash, by ignition at 650$^{o}$C for 12 h in electric furnace (Eyela-TMF 3100) to constant weight. Gross energy was determined with the help of oxygen bomb calorimeter.

*Chromic oxide estimation*

Chromic oxide was used as an inert marker in diets assuming that the amount of the marker in the feed and feces remains constant throughout the experimental period and the entire ingested marker appears in the feces. Chromic oxide contents in diets and feces were estimated after oxidation with molybdate reagent (Divakaran *et al.*, 2002) using UV-VIS 2001 spectrophotometer at 370nm absorbance.

*Minerals analysis of feed and feces*

For mineral estimation, the diets and feces samples were digested in boiling nitric acid and perchloric acid mixture (2:1) by following standard methods (AOAC, 1995). After appropriate dilution, mineral contents (calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn) and manganese, (Mn) were estimated using atomic absorption (Hitachi Polarized Atomic Absorption Spectrometer, Z-8200). Calibrated standards for mineral estimation were prepared from commercially available standards (AppliChem® Gmbh Ottoweg4, DE-64291 Darmstadt, Germany). The estimation of sodium (Na) and potassium (K) was done through flame photometer (Jenway PFP-7, UK). Phosphorus (P) was analyzed calorimetrically (UV/VIS spectrophotometer) using ammonium molybdate as a reagent at 720 nm absorbance using standard methods (AOAC, 1995).

*Calculation of digestibility*

Apparent nutrient digestibility coefficients (ADC%) of experimental diets were calculated by the formula reported in NRC (1993).

ADC (%)=100-100 ×Percent marker in diet × Percent nutrient in feces

Percent marker in feces×Percent nutrient in diet

*Statistical analysis*

Finally, data of nutrient digestibility of experimental diets was subjected to two-way analysis of variance, ANOVA (Steel *et al.*, 1997). The differences among means were compared by Tukey's Honesty Significant Difference Test and considered significant at $p<0.05$ (Snedecor and Cochran, 1990).

The CoStat-computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analysis.

## Results

From the present results, it was found that phytase and CA supplementation increased the minerals and nutrient availability to fish resulted in higher growth performance. The highest digestibility of minerals and nutrients, as well as growth performance, was observed in fish fed test diets supplemented with 2.5% CA and 1000 FTU $kg^{-1}$ diet ($T_6$).

*Cirrhinus mrigala* fingerlings fed on phytase and CA supplemented guar meal based diets showed improved weight gain, weight gain (%) and FCR as compared to the control diet. The maximum weight gain (29 g), weight gain % (418 %) and the best FCR value (1.03) of *C. mrigala* fingerlings was noted on the guar meal based diet having 2.5% CA and 1000 FTU $kg^{-1}$ level supplementation ($T_6$). The second highest weight gain (28 g), weight gain % (403%) and the best FCR value (1.24) were observed when fingerlings were fed on the guar meal based diet supplemented with 5% CA and 1000 FTU $kg^{-1}$ level. These values were significantly ($p$<0.05) different from the control diet (weight gain (23 g), weight gain% (304 %) and FCR value (2.09) as shown in Table 3.

**Table 3: Growth performance of *Cirrhinus mrigala* fingerlings fed on CA and phytase supplemented guar meal based diet.**

| Treatments | CA (%) | Phytase level (FTU $kg^{-1}$) | Initial weight | Final weight | Weight gain | Weight gain % | Weight gain ($fish^{-1}day^{-1}$) g | Feed intake ($fish^{-1}day^{-1}$) g | FCR |
|---|---|---|---|---|---|---|---|---|---|
| $T_1$ | | 0 | 5.62 | 22.69 | 17.07 | 304.03 | 0.24395 | 0.51040 | 2.09 |
| $T_2$ | 0 | 500 | 5.63 | 24.07 | 18.44 | 327.83 | 0.26352 | 0.47267 | 1.79 |
| $T_3$ | | 1000 | 5.62 | 25.15 | 19.53 | 347.34 | 0.27905 | 0.40433 | 1.45 |
| $T_4$ | | 0 | 5.62 | 24.56 | 18.94 | 336.90 | 0.27052 | 0.42600 | 1.58 |
| $T_5$ | 2.5 | 500 | 5.62 | 25.65 | 20.03 | 356.55 | 0.28614 | 0.39967 | 1.40 |
| $T_6$ | | 1000 | 5.61 | 29.08 | 23.47 | 418.03 | 0.33524 | 0.34667 | 1.03 |
| $T_7$ | | 0 | 5.61 | 22.69 | 17.08 | 323.49 | 0.25943 | 0.49800 | 1.92 |
| $T_8$ | 5 | 500 | 5.61 | 27.52 | 21.91 | 354.94 | 0.28448 | 0.41833 | 1.47 |
| $T_9$ | | 1000 | 5.62 | 28.31 | 22.69 | 403.43 | 0.32410 | 0.40233 | 1.24 |
| | PSE | | 0.02 | 0.42 | 0.40 | 6.22 | 0.01 | 0.01 | 0.03 |

Data are means of three replicates.
PSE = pooled SE = √MSE/n (where MSE = mean-squared error).

Table 4 showed that there was a significant difference in nutrients discharge through feces. Maximum digestibility values of dry matter (30%), crude protein (70%), crude fat (75%) and gross energy (71%) of guar meal based diet were observed in the test diet ($T_6$) having 2.5% CA and 1000 FTU $kg^{-1}$ level. The next highest values of dry matter: 23%, crude protein: 66%, crude fat: 73% and gross energy: 67% were observed with 5% CA and 1000 FTU

$kg^{-1}$. It was found that these maximum values were significantly ($p$<0.0005) different from the values analyzed for the control diet (dry matter: 18%, crude protein: 47%, crude fat: 60% and gross energy: 40%) as represented in Table 6.

**Table 4: Analyzed nutrient composition in *Cirrhinus mrigala* fingerlings feces fed on CA and phytase supplemented guar meal based diets.**

| Treatments | CA (%) | Phytase level (FTU $kg^{-1}$) | Dry matter (%) | Crude protein (%) | Crude fat (%) | Gross energy (kcal $g^{-1}$) |
|---|---|---|---|---|---|---|
| $T_1$ | | 0 | 86.71 | 19.23 | 2.30 | 2.12 |
| $T_2$ | 0 | 500 | 82.96 | 17.66 | 1.99 | 1.93 |
| $T_3$ | | 1000 | 83.40 | 17.24 | 1.84 | 1.83 |
| $T_4$ | | 0 | 84.78 | 17.89 | 1.74 | 1.83 |
| $T_5$ | 2.5 | 500 | 80.94 | 14.70 | 1.64 | 1.28 |
| $T_6$ | | 1000 | 73.29 | 10.54 | 1.40 | 1.01 |
| $T_7$ | | 0 | 85.21 | 18.45 | 1.84 | 2.01 |
| $T_8$ | 5 | 500 | 81.97 | 14.02 | 1.65 | 1.23 |
| $T_9$ | | 1000 | 81.96 | 12.40 | 1.53 | 1.13 |
| **PSE** | | | 0.80 | 0.42 | 0.02 | 0.04 |

Data are means of three replicates.
PSE = pooled SE = √MSE/n (where MSE = mean-squared error).

**Table 5: Apparent digestibility coefficient (%) of nutrients to *Cirrhinus mrigala* fingerlings fed on CA and phytase supplemented guar meal based diet.**

| Treatments | CA (%) | Phytase level (FTU $kg^{-1}$) | Dry matter (%) | Crude protein (%) | Crude fat (%) | Gross energy (%) |
|---|---|---|---|---|---|---|
| $T_1$ | | 0 | 17.75 | 47.00 | 59.97 | 40.06 |
| $T_2$ | 0 | 500 | 19.24 | 49.77 | 64.26 | 43.91 |
| $T_3$ | | 1000 | 21.69 | 52.45 | 67.98 | 48.23 |
| $T_4$ | | 0 | 20.67 | 50.66 | 69.61 | 47.82 |
| $T_5$ | 2.5 | 500 | 22.83 | 58.97 | 71.19 | 62.75 |
| $T_6$ | | 1000 | 30.75 | 70.29 | 74.93 | 70.49 |
| $T_7$ | | 0 | 20.15 | 49.05 | 67.87 | 42.46 |
| $T_8$ | 5 | 500 | 21.80 | 60.55 | 70.64 | 64.12 |
| $T_9$ | | 1000 | 23.27 | 65.73 | 73.24 | 67.32 |
| **PSE** | | | 0.68 | 1.19 | 0.44 | 1.53 |

Data are means of three replicates.
PSE = pooled SE = √MSE/n (where MSE = mean-squared error).

Analysis of control and test diets revealed that there was an equal amount of minerals in all the diets but different in feces (Tables 6 and 7). Lowest minerals were noted in feces when fingerlings were fed on 2.5% CA and 1000 FTU $kg^{-1}$ level based diet. Maximum ADC% values of minerals (Ca: 69%, P: 77%, Na%: 65%, K: 62%, Mg: 54%, Fe: 66%, Cu: 58%, Mn: 70% and Zn 74%) were noted for *C. mrigala* fingerlings when fed on guar meal supplemented with 1000 FTU $kg^{-1}$ and 2.5% of CA. The next highest values of mineral digestibility were observed with 1000 FTU $kg^{-1}$ with 5% of CA. These values found with 2.5% CA and 1000 FTU $kg^{-1}$ level based diet, were significantly different from control and other test diets as shown in Table 8. Lowest mineral digestibility (Ca: 41%, P: 44%, Na%: 42%, K: 42%, Mg: 29%, Fe: 31%, Cu: 40%, Mn: 43% and Zn 44%) was observed in fish fed on the

control diet. From these results, it was noted that 1000 FTU $kg^{-1}$ and 2.5% of CA supplementation is necessary for maximum fish performance after high digestibility of nutrients and mineral. The digestibility values at the mentioned levels differed significantly ($p$<0.05) as compared to the control and other test diets. This revealed that both the above levels of phytase and CA in corresponding feeds were effective to break down the mineral-phytate complexes and thus increase the availability of nutrients and minerals resulting in increased fish growth performance. A significant interaction was also observed between phytase and CA for improving minerals digestibility.

**Table 6: Analyzed minerals (%) composition in diets of *Cirrhinus mrigala* fingerlings fed on CA and phytase supplemented guar meal based diet.**

| Treatments | CA (%) | Phytase level (FTU $kg^{-1}$) | Ca | P | Na | K | Mg | Fe | Cu | Mn | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_1$ | | 0 | 0.143 | 1.980 | 0.784 | 1.282 | 0.077 | 0.072 | 0.754 | 0.091 | 0.071 |
| $T_2$ | 0 | 500 | 0.143 | 1.976 | 0.773 | 1.268 | 0.078 | 0.071 | 0.745 | 0.091 | 0.073 |
| $T_3$ | | 1000 | 0.143 | 1.982 | 0.798 | 1.278 | 0.080 | 0.070 | 0.754 | 0.094 | 0.073 |
| $T_4$ | | 0 | 0.143 | 1.975 | 0.795 | 1.264 | 0.078 | 0.072 | 0.745 | 0.092 | 0.072 |
| $T_5$ | 2.5 | 500 | 0.142 | 1.972 | 0.776 | 1.270 | 0.078 | 0.072 | 0.748 | 0.093 | 0.070 |
| $T_6$ | | 1000 | 0.143 | 1.989 | 0.788 | 1.260 | 0.078 | 0.073 | 0.750 | 0.094 | 0.075 |
| $T_7$ | | 0 | 0.142 | 1.973 | 0.771 | 1.278 | 0.081 | 0.074 | 0.751 | 0.090 | 0.072 |
| $T_8$ | 5 | 500 | 0.142 | 1.986 | 0.798 | 1.266 | 0.079 | 0.072 | 0.754 | 0.094 | 0.072 |
| $T_9$ | | 1000 | 0.142 | 1.979 | 0.793 | 1.271 | 0.079 | 0.074 | 0.745 | 0.095 | 0.072 |
| **PSE** | | | 0.0007 | 0.0011 | 0.0015 | 0.00175 | 0.00076 | 0.00082 | 0.0014 | 0.0011 | 0.0013 |

Data are means of three replicates.
PSE = pooled SE = √MSE/n (where MSE = mean-squared error)

**Table 7: Analyzed minerals composition (%) *Cirrhinus mrigala* fingerlings feces fed on CA and phytase supplemented guar meal based diets.**

| Treatments | CA (%) | Phytase level (FTU $kg^{-1}$) | Ca | P | Na | K | Mg | Fe | Cu | Mn | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_1$ | | 0 | $0.094^{a}$ | $1.233^{a}$ | $0.508^{a}$ | $0.828^{a}$ | $0.061^{a}$ | $0.056^{a}$ | $0.503^{a}$ | $0.058^{a}$ | $0.044^{a}$ |
| $T_2$ | 0 | 500 | $0.085^{b}$ | $0.991^{b}$ | $0.473^{b}$ | $0.753^{b}$ | $0.053^{b}$ | $0.047^{b}$ | $0.414^{b}$ | $0.052^{b}$ | $0.040^{b}$ |
| $T_3$ | | 1000 | $0.079^{d}$ | $0.889^{d}$ | $0.408^{d}$ | $0.728^{c}$ | $0.054^{b}$ | $0.043^{d}$ | $0.404^{d}$ | $0.051^{b}$ | $0.037^{c}$ |
| $T_4$ | | 0 | $0.070^{e}$ | $0.815^{e}$ | $0.390^{e}$ | $0.695^{d}$ | $0.052^{b}$ | $0.051^{e}$ | $0.451^{e}$ | $0.056^{a}$ | $0.043^{a}$ |
| $T_5$ | 2.5 | 500 | $0.066^{f}$ | $0.720^{f}$ | $0.366^{f}$ | $0.657^{e}$ | $0.051^{b}$ | $0.037^{f}$ | $0.380^{f}$ | $0.043^{c}$ | $0.027^{e}$ |
| $T_6$ | | 1000 | $0.048^{i}$ | $0.496^{i}$ | $0.297^{i}$ | $0.527^{h}$ | $0.039^{e}$ | $0.027^{i}$ | $0.340^{i}$ | $0.031^{e}$ | $0.021^{g}$ |
| $T_7$ | | 0 | $0.083^{c}$ | $0.948^{c}$ | $0.445^{c}$ | $0.756^{b}$ | $0.055^{b}$ | $0.053^{c}$ | $0.468^{c}$ | $0.057^{a}$ | $0.044^{a}$ |
| $T_8$ | 5 | 500 | $0.059^{g}$ | $0.656^{g}$ | $0.347^{g}$ | $0.626^{f}$ | $0.046^{c}$ | $0.040^{g}$ | $0.393^{g}$ | $0.038^{d}$ | $0.031^{d}$ |
| $T_9$ | | 1000 | $0.051^{h}$ | $0.540^{h}$ | $0.334^{h}$ | $0.584^{g}$ | $0.043^{d}$ | $0.032^{h}$ | $0.361^{h}$ | $0.037^{d}$ | $0.023^{f}$ |
| **PSE** | | | 0.00086 | 0.00198 | 0.0023 | 0.00185 | 0.00091 | 0.00115 | 0.0015 | 0.00143 | 0.0012 |

Means within columns 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 8: ADC% of minerals for *Cirrhinus mrigala* fingerlings fed on CA and phytase supplemented guar meal based diet.**

| Treatments | CA (%) | Phytase level (FTU $kg^{-1}$) | Ca | P | Na | K | Mg | Fe | Cu | Mn | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_1$ | | 0 | $40.79^{i}$ | $44.12^{i}$ | $41.91^{i}$ | $42.03^{i}$ | $29.21^{i}$ | $30.60^{g}$ | $40.01^{i}$ | $42.80^{g}$ | $44.17^{f}$ |
| $T_2$ | 0 | 500 | $44.99^{h}$ | $53.59^{h}$ | $43.38^{h}$ | $45.00^{h}$ | $36.45^{h}$ | $38.241^{f}$ | $48.360^{h}$ | $47.281^{f}$ | $48.85^{e}$ |
| $T_3$ | | 1000 | $50.52^{f}$ | $59.74^{f}$ | $54.10^{f}$ | $48.89^{f}$ | $39.16^{f}$ | $45.25^{e}$ | $51.86^{f}$ | $50.836^{e}$ | $54.69^{d}$ |
| $T_4$ | | 0 | $56.11^{e}$ | $62.92^{e}$ | $55.91^{e}$ | $50.59^{e}$ | $33.52^{e}$ | $36.367^{f}$ | $45.640^{e}$ | $45.831^{f}$ | $46.504^{f}$ |
| $T_5$ | 2.5 | 500 | $57.78^{d}$ | $66.84^{d}$ | $57.14^{d}$ | $53.03^{d}$ | $40.85^{d}$ | $52.71^{c}$ | $54.16^{d}$ | $58.185^{d}$ | $64.674^{d}$ |
| $T_6$ | | 1000 | $69.16^{a}$ | $77.16^{a}$ | $65.41^{a}$ | $61.67^{a}$ | $54.34^{a}$ | $65.95^{a}$ | $58.18^{a}$ | $69.628^{a}$ | $74.20^{a}$ |
| $T_7$ | | 0 | $47.49^{g}$ | $56.82^{g}$ | $48.10^{g}$ | $46.88^{g}$ | $38.75^{g}$ | $35.526^{f}$ | $43.789^{g}$ | $43.429^{g}$ | $45.270^{f}$ |
| $T_8$ | 5 | 500 | $62.25^{c}$ | $69.76^{c}$ | $60.26^{c}$ | $54.76^{c}$ | $47.04^{c}$ | $48.926^{d}$ | $52.027^{c}$ | $62.773^{c}$ | $60.175^{c}$ |
| $T_9$ | | 1000 | $67.72^{b}$ | $75.41^{b}$ | $62.11^{b}$ | $58.62^{b}$ | $51.57^{b}$ | $61.052^{b}$ | $56.650^{b}$ | $65.375^{b}$ | $70.81^{b}$ |
| **PSE** | | | 0.7333 | 0.3578 | 0.5382 | 0.4556 | 1.4263 | 1.5671 | 0.4950 | 1.4159 | 1.5778 |

Means within columns 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

The addition of CA to the diet reduces the pH of the stomach and enhances the phytase activity to break down the phytate complexes (Baruah *et al.*, 2005). In addition to this, the epithelial cell proliferation in the GIT mucosa is also stimulated by CA (Sakata *et al.*, 1995) and thus the digestibility of nutrients increases. In the current study the maximum values in term of growth, nutrients and mineral digestibility for *C. mrigala* fingerlings fed on guar meal based test diets were noted at 1000 FTU $kg^{-1}$ with 2.5% CA supplemented diet significantly different from control and other test diets. Baruah *et al.* (2007a) found maximum growth performance at 3% CA and 500 FTU $kg^{-1}$. Similarly, Zhu *et al.* (2014) reported that when 2 g of CA was combined with 500 FTU $kg^{-1}$ level of phytase, juveniles of yellow cat fish showed maximum growth. Similar results of higher growth were reported by Hussain *et al.* (2015), but the level was a little different from the present results. They reported that supplementation of 5 % CA and 500 FTU $Kg^{-1}$, resulted in the maximum growth of *C. mrigala* fingerlings fed corn gluten meal (30%) based diet. In literature, it was found that for fish feeds the optimal levels of CA are 2.5% to 5 % (Khajepour *et al.*, 2012a; Baruah *et al.*, 2007b) and optimal levels of phytase supplementation ranged between 250 and 1500 FTU$kg^{-1}$ levels (Cao *et al.*, 2007). These variations in findings depend upon the sources for origin of phytases, experimental fish species, diet making technology and studied response parameters.

Results of the present study are also supported by Yu and Wang (2000) who reported that addition of phytase 1000 FTU$kg^{-1}$ enhanced average weight gain of fish by 25%. Similar results were found by Nwanna and Schwarz (2008) in which they noted an increase in growth performance at 750 and 1000 FTU $kg^{-1}$ levels of phytase supplementation in diets of *Cyprinus*

*carpio*. However, Hussain *et al.* (2011) found that phytase supplementation with plant-meal based diet at 750 FTU $kg^{-1}$ level is optimal for highest growth performance of *L. rohita* fingerlings. In other studies, Hossain *et al.* (2007) and Sarker *et al* (2007) in red sea bream, *P. major*, Pandey and Satoh (2008) in rainbow trout, *O. mykiss* and Sarker *et al.* (2012b) in yellow tail found that 1% level of CA is optimum for maximum growth performance. Variations have been seen in the results of different researchers related to unpredictable factors such as water quality parameters, feed ingredients and citric acid sources (Liu *et al.*, 2013).

The dosage of CA and phytase supplementation differs in many reported studies for enhancing nutrient digestibility in fish. According to the present results, it was found that supplementation of CA (2.5%) and phytase (1000 FTU $kg^{-1}$) enhances fish growth as compared to the control diet. Nearly similar to our results, Baruah *et al.* (2005) found the maximum crude protein digestibility at 3% CA and 500 FTU $kg^{-1}$ phytase. Whereas, Saeed (2012) observed highest protein digestibility in *L. rohita* fingerlings fed on 5% CA with 750 FTU $kg^{-1}$ level. The findings of the current study are also supported by Ashraf and Goda (2007). They observed higher crude fat digestibility at 1000 FTU $kg^{-1}$ level after which it decreased. Saeed (2012) noted the highest crude fat digestibility at 5% CA and 750 FTU $kg^{-1}$ level of phytase supplementation. Contrary to the current results, Khajepour *et al.* (2011) reported that addition of CA in the fish diet reduced the digestibility of nutrients. Phromkunthong *et al.* (2010) reported an improvement in nutrient digestibility in Common carp fed plant meal based diet supplemented with CA (0.22%) and phytase (750 FTU $kg^{-1}$). Dry matter, crude protein, crude fat and gross energy digestibility were higher in *L. rohita* fed on canola meal based diets supplemented with 3% CA and 500 FTU $kg^{-1}$ (Arshad, 2013) and 1000 FTU $kg^{-1}$ level of phytase (Iqbal, 2012). On the contrary, Baruah *et al.* (2007b) did not observe any effect on crude protein and dry matter digestibility in response to the interaction of phytase and CA.

CA enhances the mineral availability for fish when fed on plant meal and mitigate the inhibitory effect of anti-nutritional component (phytate) on mineral digestibility (Khajepour and Hosseini, 2012b). The results of the current study proved the synergetic effects of CA and phytase because the availability of minerals was significantly improved after acidification of phytase supplemented diets. The digestibility values of Ca, P, Na, K, Mg, Cu, Mn, Zn, and Fe were highest at 2.5% CA and 1000 FTU $kg^{-1}$ in the case of guar meal based test diets. Similarly, the synergetic effect of CA and phytase on digestibility of minerals was noted by Baruah *et al.* (2007a). They reported a significant interaction effect between CA (3%) and phytase (500 FTU $kg^{-1}$) on the absorption of P, Na, K, Mg, Mn, and Fe in *Labeo rohita* fingerlings fed on a plant based diet. Saeed (2012) reported maximum digestibility values of minerals at 5%

CA and 750 FTU $kg^{-1}$ level. Among the minerals, their synergistic effect has a special influence on P availability, (Phromkunthong *et al.*, 2010) and retention in fish (Baruah *et al.*, 2005). On the contrary, non-significant interactions were observed between phytase and CA on the mineral utilization of juvenile yellow catfish (Zhu *et al.*, 2014). Soybean meal treated with phytase and CA had no effects on the calcium and phosphorus of muscle, scute and serum in beluga *Huso huso* (Khajepour et al., 2011). So the results of the current work are supported by the above studies and variations in some findings might be associated with a difference in diet composition, fish species, and rearing conditions.

In conclusion, this study provided evidence that acidification of phytase treated guar meal-based diets increased the growth performance of *C. mrigala* fingerlings by improving nutrient digestibility and mineral availability, as well as reduced the nutrients discharge into the water. It also showed a great interaction between CA and phytase regarding increase mineral digestibility in fish when fed on a plant meal based diet. The optimum levels of CA and phytase supplementation for guar meal are 2.5% and 1000 FTU $kg^{-1}$.

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