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Original Article

Effect of phytase supplementation on mineral digestibility to *Catla catla* fingerlings fed *Moringa oleifera* leaf meal based test diets

Muhammad Mudassar Shahzad[1*], Syed Makhdoom Hussain[1], Farhat Jabeen[1], Abdullah Ijaz Hussain [2],Sajjad Ahmad[3], Abida Ashraf[4],Muhammad Zubair-ul-Hassan Arsalan[1]

[1]*Fish Nutrition Lab, Department of Zoology, Government College University, Faisalabad, Pakistan*
[2]*Department of Applied Chemistry and Biochemistry, Government College University, Faisalabad, Pakistan*
[3]*Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan*
[4]*Department of Chemistry, The Women University Multan, Pakistan*

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Abstract
Current study work was carried out to estimate the influence of phytase addition on mineral availability to fishfingerlings fed *Moringa oleifera* leaf meal (MOLM) based test diets. Anti-nutritional factors (phytate) are present in plant by-products reduces the bioavailability of minerals to fish, resulting in poor fish performance. Test ingredient was used as *M.oleifera*leaf meal (MOLM) to prepare six experimental diets that were supplemented with graded levels of phytase (0, 300, 600, 900, 1200 and 1500 FTU kg^{-1}). The fingerlings were fed at the rate of 4% of live wet weight twice a day and feces were collected from each tank. On the basis of results it was noted that addition of phytase showed clearly significant ($p<0.05$) enhancement in bioavailability of minerals. Maximum ADC% of minerals was noted at 900 FTU kg^{-1} level of phytase supplemented MOLM based test diet. By these results, we conclude that addition of phytase at 900 FTU kg^{-1} level is helpful to develop an eco-friendly and cost-effective fish feed by using MOLM based diet.
Keywords: MOLM, *Catla catla*, mineral absorption, phytase

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INTRODUCTION

The increased growth in human population has resulted in increasing demand for food and nutrition quality in under-developed countries throughout the world (Abdulkadir *et al.*, 2016; Murtaza *et al*., 2016). In human diet fish is being used as a quality protein supplement and to resolve the problem of food shortage (Sheikh and Sheikh, 2004). Demand for fish consumption is constantly increasing as a feed source and for health benefits (Tiamiyu *et al.*, 2016). As fish is the most important source of protein, it also needs a high amount of protein in its own diet. In past aquatic feed was usually dependent of fishmeal (FM) as major feed constituent. About 3.06 million tonnes (56%) of world FM production was consumed by aquaculture sector as a major protein source (FAO, 2008). In aquaculture, feed mainly accounts for 60% of overall budget of fish culture system (Essa *et al.*, 2004). Whereas, unstable supply, higher demand and increasing cost of the FM made it essential to explore substitute sources of protein that should be low in cost (Lim *et al.*, 2011; FAO, 2014). Use of different plant protein sources instead of fish meal utilization were suggested by many researchers (Barnes *et al.*, 2012; Dedeke *et al.*, 2013; Hussain *et al.*, 2015a).

The best alternatives to protein sources are plant by-products because of their low cost and easy availability throughout the year. By-products of plants are successfully being utilized as unconventional sources of protein in fish feed by researchers (Hussain *et al.*, 2014; 2015a, b; Liu *et al.*, 2015; Hussain *et al.*, 2016). But the availability of minerals especially cation minerals, protein and phosphorus (P) are adversely affected by the higher concentration of anti-nutritional factors (phytic acid or phytate)

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*Corresponding author: mudassarshahzad.uos@gmail.com

present in plant by-products based diets (Cao *et al.*, 2007; Hussain *et al.*, 2015a). Phosphorus (60-80%) is present in chelated form that is known as phytic acid and cannot be used by fish (Lei *et al.*, 2013), resulting in a nutrient discharge into water media causing aquatic pollution (NRC, 1993).

There was a need of a specific enzyme that can breakdown the phytate and decreases the problems of mineral absorption for fish. Mono-gastric fish species cannot hydrolyze the phytate because their body cannot produce phytase enzyme. Supplementation of phytase is an effective method to improve the minerals availability to fish. It also decreases water pollution by proper digestion and absorption of phosphorus in the fish body (Liu *et al.*, 2013; Hussain *et al.*, 2015a). Use of phytase can efficiently decrease the adverse effects of phytic acid that are present in plant by-products based diets (Hussain *et al.*, 2015b).

Phytase deficiency may cause three main problems due to less availability of nutrients and minerals, 1) water pollution caused by nutrients discharge through fish feces 2) need of extra dietary P supplementation 3) as well as the depletion of rock P deposits (Lei *et al.*, 2013). Supplementation of microbial phytase is helpful for the breakdown of chelated phytate that can resolve some nutritional problems (Hassan *et al.*, 2009; Hussain *et al.*, 2015b).

It is an eco-friendly enzyme, as it reduces the amount of phosphorus that is discharged into the aquatic ecosystem through fish feces (Huynh and Nugegoda, 2011; Liu *et al.*, 2013; Hussain *et al.*, 2015b) by improving minerals (P, N, Mg, Ca, Cu, Zn, and Fe) digestibility (Christopher *et al.*, 2011; Hussain *et al.*, 2016). By-products of the plant were successfully used in fish feed without reducing the feed quality (Abo-State *et al.*, 2014). One of the better and cost effective plant protein sources is *M. oleifera* also known as the Miracle tree. Leaves of Moringa are an important source of the vital minerals such as Ca, Cu, Fe, Zn, K, P, Mg and Na (Bosh, 2004; Grubben and Denton, 2004).

Research is needed to improve the production of fish as well as to decrease the problems of expensive FM and aquatic pollution caused by P excretion by feces. Therefore, the purpose of the present research was to determine the impact of phytase addition on ADC% of minerals to *C. catla* fingerlings fed MOLM based test diets.

MATERIALS AND METHODS

The current experimental study was done to explore the phytase effect on ADC% of minerals to *C. catla* fingerlings fed MOLM based test diets. The study trial was performed in Fish Nutrition Lab, Department of Zoology, GC University Faisalabad, Pakistan.

Fish and experimental conditions

Fingerlings of *C. catla* were procured from the Govt. Fish Seed Hatchery, Satiana Road, Faisalabad. For fourteen days' fish fingerlings were adjusted to the laboratory conditions before the start of the experiment. Fingerlings were stocked in specially designed V-shaped water tanks having 70 L water capacity. Fish fingerlings were fed one time a day on basal diet during the acclimatization period (Allanand Rowland, 1992). Water quality parameters such as dissolved oxygen (DO), pH as well as temperature were observed on a daily basis. The air pump was used to supply air to the capillary system through-out the study period. Before the start of experimental work, fingerlings of fish were bathed for 1 to 2 minutes with 0.5% saline solution to kill the pathogens, if present (Rowland and Ingram, 1991).

Experimental Design

M. oleifera leaf meal (MOLM) was used as major experimental feed element to prepare six test diets and supplemented with different phytase levels (0, 300, 600, 900, 1200 and 1500 FTU kg^{-1}). Six fish groups were stocked in water tanks. They were fed on control diet (0 FTUkg^{-1}), and five phytases supplemented MOLM based experimental diets. Triplicate tanks were used for each treatment, and in each replicate, 15 fingerlings were stocked. Experimental duration of the current trial was 90-days. Each MOLM based diet supplemented with phytase was compared with other diets and control diet to determine ADC% of minerals and carcass composition by using Completely Randomized Design (CRD).

Processing of M. oleifera leaf meal (MOLM)

Fresh Moringa leaves were collected from the local garden, were washed to remove the dirt and dust particles. The leaves were drained appropriately and dried for six days under shady place to avoid the damage of vitamins by photo-dynamic oxidation or damage. Dried leaves of Moringa were separated from

the stalks to decrease crude fibers in the diet (Madalla *et al.*, 2013).

Formation of Feed Pellets

Ingredients used for the preparation of fish feed were procured from the market and were pressed by grinding method with the size of 0.3 mm. Before the experimental diets preparation, the chemical composition of feed components was analyzed (Table I) by following standard AOAC (1995) methods. Cr_2O_3 was used as an inert marker at the rate of one percent in all the experimental feeds. Feed mixer was used to mix all feed constituents for 5-10 minutes whereas code oil was also supplemented during this process. The suitably textured dough was prepared by slowly blending of feed ingredients in the mixer after adding 10-15% of tap water. The prepared dough was further processed through the pelleting machine to formulate feed pellets (Lovell, 1989). One control and five phytases supplemented test diets were prepared using Moringaleaf meal by spraying different phytase levels (0, 300, 600, 900, 1200 and 1500 FTU kg^{-1}). The required concentrations of phytase enzyme were formulated in 25 mL of distilled H_2O and sprayed on each experimental diet (Robinson *et al.*, 2002). Control diet (0 FTU kg^{-1} level) was also sprayed with a similar quantity of H_2O to conserve the equivalent amount of moistness. Sprayed diets were stored at 4°C until use after dehydration.

Table I: Chemical composition (%) of feed ingredients (Dry matter basis)

Ingredients	MOLM	Fish meal	Rice polish	Wheat flour	Corn gluten 60%
Dry matter (%)	91.83	91.67	94.06	92.4	92.34
Crude Protein (%)	28.95	48.17	12.38	10.15	59.51
Crude Fat (%)	2.83	7.12	13.46	2.3	4.58
Gross Energy (kcal/g)	3.84	2.65	3.18	2.95	4.35
Crude Fiber (%)	19.45	1.12	12.74	2.67	1.23
Ash (%)	8.91	24.66	10.17	2.06	1.36
Carbohydrates	36.02	16.28	48.07	79.87	28.97

Table II: Ingredients composition (%) of control and test diets (Dry matter basis)

Ingredients	Test Diet-I (Control)	Test Diet-II	Test Diet-III	Test Diet-IV	Test Diet-V	Test Diet-VI
Phytase Level (FTU kg^{-1})	**0**	**300**	**600**	**900**	**1200**	**1500**
MOLM	35	35	35	35	35	35
Fish meal	15	15	15	15	15	15
Soybean meal	15	15	15	15	15	15
Wheat flour*	17	17	17	17	17	17
Rice polish	8	8	8	8	8	8
Fish oil	6	6	6	6	6	6
Vitamin Premix	1.0	1.0	1.0	1.0	1.0	1.0
Chromic oxide	1.0	1.0	1.0	1.0	1.0	1.0
Ascorbic acid	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mixture	1.0	1.0	1.0	1.0	1.0	1.0

MOLM= *M. oleifera* leaf meal *Phytase enzyme was used at the expense of wheat flour

Feeding protocol and sample collection

C. catla fingerlings were weighed and fed on their prescribed diet at the rate of 4% of body weight twice daily. Uneaten diet was collected and drained after two hours of the feeding period. The tanks were washed and were again filled with water to eliminate the uneaten diet particles. Feces were collected by opening fecal collecting tube of every replicated fish tank. Fecal material was collected carefully to avoid breakage of feces for minimizing the leaching of minerals in the water. In the oven,

feces were dried at 65^{o}C, stored for further analysis.

Chemical analysis of feed and feces

1g of the sample (experimental feed and feces) was weighed for mineral estimation. Weighted samples were taken in open mouth conical flask. Before putting on a hot plate, HNO_3 (20ml) was added to the flask. Ten milliliters of per-chloric acid was added before placing it on a hot plate. Heat the mixture until it left only 1ml than diluted by 50 mL of H_2O after removing from the hot plate.

Filter the solution to eliminate all particles remained in digestion solution before the minerals analysis. Atomic Absorption Spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan) was used to estimate minerals from the diluted mixture using AOAC (1995) methods. Na and K were estimated by a flame photometer (Jenway PFP-7, UK). UV/VIS spectrophotometer at 720 nm absorbance was used to determine phosphorous contents in the experimental diets and feces (AOAC, 1995). Chromic oxide (feeds and feces) was estimated after oxidation with molybdate reagent (Divakaran *et al.,* 2002) using UV-VIS 2001 spectrophotometer at 370nm absorbance. The apparent absorption of minerals such as Ca, P, Mg, Na, K, Fe, Cu, Zn and Mn was determined indirectly at the end of the experiment using chromic oxide as the inert marker.

Calculation of minerals ADC% (Apparent digestibility coefficient)

ADC% of minerals to fingerlings were estimated using following standard formula (NRC, 1993).

$$\text{ADC (\%)} = 100 - 100 \times \frac{\text{\% minerals in feces} \times \text{\% marker in diet}}{\text{\% minerals in diet} \times \text{\% marker in feces}}$$

Statistical analysis

Finally, data of ADC% of minerals (K, Ca, Fe, Na, Cu, Mn, Zn, P, Mg, Al, Cr, Sr, Pb, Ba, Cd, Co, Mo and Ni) were subjected to one-way ANOVA i.e. Analysis of Variance (Steel *et al.*, 1996).Tukey's Honesty Significant Difference Test was used to compare the differences among treatments. It was considered significant when $p<0.05$ (Snedecorand Cochran, 1991). For statistical analysis, CoStat Computer Package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used.

RESULTS AND DISCUSSION

Analysis showed that there was the balanced composition of all the minerals such as Fe, Ca, K, Zn, Na, Mn, Cu, P, Mg, Al, Cr, Sr and Pb in control and test diets formulated by MOLM based diet supplemented with phytase at different levels. However, Ba, Cd, Co, Mo, and Ni were found lowest from the range (<0.0001) in diets (Table III). Analysis of feces excreted by fingerlings showed that there was a significant ($p<0.05$) difference between some minerals when fingerlings fed on control and different levels of phytase supplemented MOLM based diets. According to these results, it was observed that highest amount of minerals was excreted through feces in water when fingerlings fed control diet, whereas overall minerals excretion was decreased with increment also of phytase till 900 FTU kg^{-1} level supplemented based diet (Table IV).

Phytate commonly exists in plant-based ingredients that usually binds with divalent cations and is known as a major anti-nutritional factor (Soetan and Oyewole, 2009). Phytate makes minerals unavailable to fish and decrease their absorption because of the binding between phytate complex and major minerals (Oh *et al.*, 2004; Nwanna *et al.*, 2007). Breakdown of the complex chelated structure of phytate enhances the release and utilization of essential minerals. Many researchers indicated that phytate present in plant by-products might chelate with some of the important minerals i.e. Fe, Ca, Mn, Cu, Ni, Cr, Na, K, P and Mg (Hussain *et al.*, 2014; Dersjant-Li *et al.*, 2015; Hussain *et al.*, 2014; 2015a,b).

Mineral absorption was recorded lowest at control diet (0 FTU kg^{-1} level) after which it started to increase (from 300 FTU kg^{-1} level) and reached to its highest level when fed at 900 FTU kg^{-1} level supplemented based diet followed by the 1200 FTU kg^{-1} level based diet. The further increase also of phytase at 1500 FTU kg^{-1} level, decreased the ADC% of minerals (Fig. I).

Digestibility data revealed that maximum values of minerals i.e. Ca (76%), Na (72%), K (75%), Fe (79%), P (74%) and Al (69%) were noted at test diet IV (900 FTU kg^{-1} level). However, highest absorption value of Cu (72%), Zn (77%), Mg (74%), Cr (63%) and Sr (52%) was noted at 1200 FTU kg^{-1} level supplemented based diet followed by fish fed at 900 FTU kg^{-1} level supplemented diet. Similar to our findings, Hussain *et al.* (2015a) also noted that anti-

nutritional factors in soybean meal based diets such as phytate played negative role in mineral absorption, whereas supplementation of phytase at level of 1000 FTU kg^{-1}, enhanced mineral absorption by breaking down the chelated phytate-minerals complex resulting in maximum utilization of essential minerals by fish and decreased mineral discharge in water through feces. Zhu *et al.* (2014) noted a dramatically decrease in minerals contents of feces after phytase supplementation.

Table III: Analyzed mineral composition (%) of MOLM based test and control diets

Minerals	Test Diet –I	Test Diet –II	Test Diet –III	Test Diet –IV	Test Diet –V	Test Diet –VI
	Phytase levels (FTU kg^{-1})					
	0	300	600	900	1200	1500
Ca	3.75±0.06	3.73±0.08	3.73±0.07	3.74±0.05	3.73±0.06	3.76±0.07
Na	0.34±0.03	0.35±0.06	0.35±0.04	0.34±0.05	0.34±0.06	0.34±0.05
K	0.53±0.05	0.52±0.07	0.53±0.04	0.53±0.06	0.54±0.05	0.53±0.04
Fe	0.016±0.0004	0.015±0.00	0.016±0.00	0.015±0.00	0.016±0.0004	0.016±0.001
Cu	0.017±0.0005	0.017±0.00	0.017±0.00	0.017±0.00	0.017±0.0005	0.017±0.000
Zn	0.035±0.001	0.035±0.00	0.034±0.00	0.034±0.00	0.035±0.001	0.035±0.001
Mn	0.049±0.001	0.049±0.00	0.049±0.00	0.049±0.001	0.049±0.0005	0.049±0.001
P	0.68±0.013	0.69±0.011	0.69±0.011	0.69±0.008	0.69±0.01	0.68±0.01
Mg	0.010±0.000	0.010±0.00	0.010±0.00	0.010±0.000	0.0103±0.001	0.0106±0.00
Al	0.0003±0.00	0.00032±0.00	0.00032±0.00	0.00032±0.00	0.00031±0.00	0.00032±0.00
Cr	0.097±0.006	0.097±0.00	0.097±0.004	0.097±0.007	0.096±0.005	0.097±0.004
Sr	0.0002±0.00	0.00022±0.00	0.00022±0.00	0.00022±0.00	0.00022±0.00	0.00022±0.00
Pb	0.0043±0.000	0.0044±0.000	0.0044±0.000	0.0044±0.000	0.0043±0.00	0.0043±0.000
Ba	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cd	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Co	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Mo	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Ni	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Data are means of three replicates

Table IV: Analyzed mineral composition (%) in feces of *C. catla* fed phytase supplemented MOLM based test and control diets

Minerals	Test Diet –I	Test Diet –II	Test Diet –III	Test Diet –IV	Test Diet –V	Test Diet –VI
	Phytase levels (FTU kg^{-1})					
	0	300	600	900	1200	1500
Ca	$2.04±0.06^{a}$	$1.81±0.09^{b}$	$1.51±0.06^{c}$	$0.99±0.03^{e}$	$1.25±0.02^{d}$	$1.85±0.09^{b}$
Na	$0.21±0.02^{a}$	$0.17±0.02^{ab}$	$0.15±0.02^{bc}$	$0.10±0.01^{c}$	$0.13±0.02^{bc}$	$0.15±0.02^{bc}$
K	$0.28±0.03^{a}$	$0.23±0.02^{abc}$	$0.22±0.02^{bc}$	$0.15±0.01^{d}$	$0.19±0.01^{cd}$	$0.26±0.02^{ab}$
Fe	0.0091±0.000	0.0058±0.00	0.0045±0.00	0.0036±0.000	0.0057±0.000	$0.0067±0.0003^{b}$
Cu	$0.011±0.0005^{a}$	0.0097±0.00	0.0072±0.00	0.0063±0.000	0.0052±0.000	$0.0072±0.0004^{c}$
Zn	$0.019±0.001^{a}$	0.016±0.001	0.012±0.000	$0.011±0.0003^{c}$	0.00088±0.00	$0.015±0.001^{b}$
Mn	$0.03±0.0003^{a}$	0.026±0.001	0.012±0.000	$0.015±0.0003^{d}$	$0.020±0.001^{c}$	$0.025±0.001^{b}$
P	$0.38±0.005^{a}$	$0.32±0.009^{b}$	$0.26±0.01^{d}$	$0.19±0.008^{f}$	$0.23±0.010^{e}$	$0.29±0.012^{c}$
Mg	0.0076±0.000	0.0069±0.00	0.0057±0.00	0.0047±0.000	0.0042±0.000	$0.0056±0.001^{b}$
Al	0.00023±0.00	0.0002±0.00	0.00016±0.0	0.00011±0.00	0.00013±0.00	0.00015±0.0000
Cr	$0.067±0.002^{a}$	0.059±0.002	$0.051±0.001^{c}$	$0.042±0.002^{d}$	$0.039±0.002^{d}$	$0.051±0.004^{c}$
Sr	0.00017±0.00	0.00017±0.0	0.00015±0.0	0.00014±0.00	0.00011±0.00	0.00013±0.0000
Pb	0.0033±0.000	0.0031±0.00	0.0027±0.00	0.0025±0.000	0.0024±0.000	$0.0021±0.0004^{c}$

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

Almost similar with present results Van-Weerd*et al.* (1999) concluded that phytase addition at the level of 1000 FTU kg^{-1} in plant meal based diet (Soybean meal) showed maximum ADC% of P in *Clariasgariepinus*. Our results are also supported by the findings of Hussain *et al.* (2015b). They reported significantly ($p<0.05$) higher absorption values of minerals for *L. rohita* fingerlings fed plant meal (cottonseed and canola meal) based diet supplemented with 1000 and 750 FTU kg^{-1} level supplemented based diet as compared to other phytase supplemented diets as well as 0 FTU kg^{-1} level. On the other hand, maximum ADC% of P had also been claimed in tra cat fish when they fed plant meal i.e. soybean meal based diet supplemented at higher phytase supplementation level *i.e.* 1500 FTU kg^{-1} (Hung *et al.*, 2015).

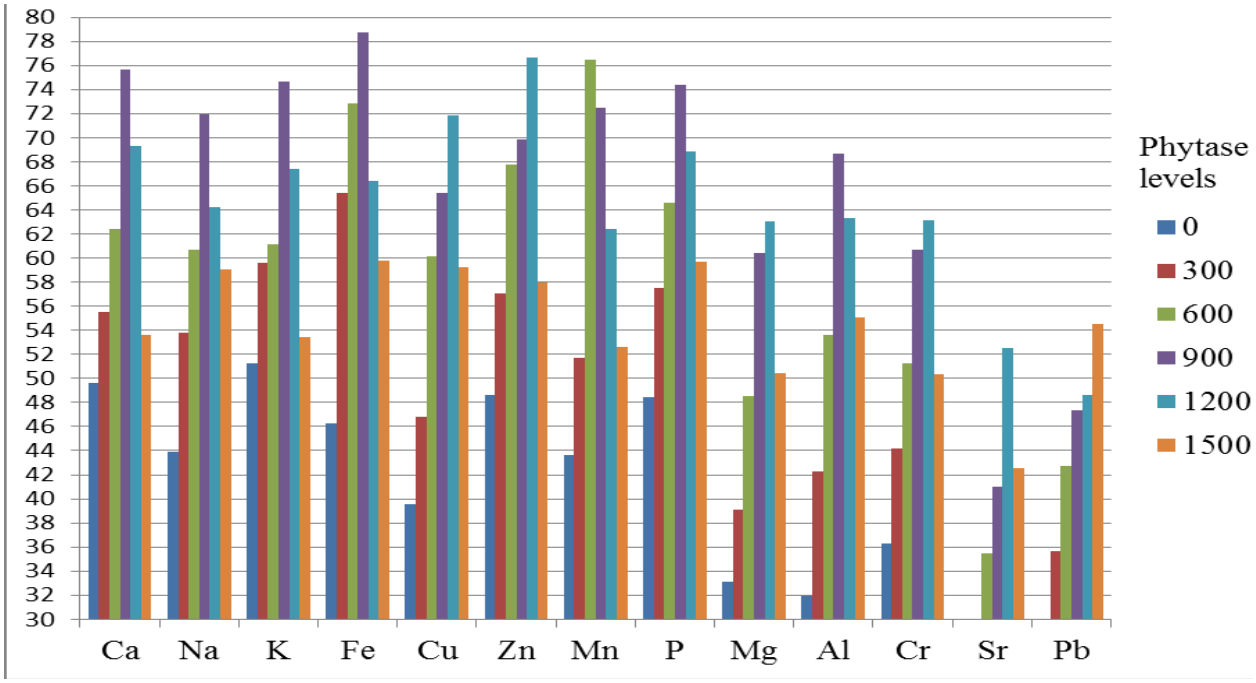

Figure I: Apparent mineral absorption (%) in *C. catla* fingerlings fed MOLM based test and control diet

On the other side, Mn (76.46%) was highly digested and absorbed at 600 FTU kg^{-1} level whereas Pb (54.49%) showed higher absorption values at 1500 FTU kg^{-1} level based diet. These values were statistically ($p<0.05$) different when compared with control and other phytase supplemented test diets. Whereas, Laining*et al.* (2010) observed highest mineral absorption and absorption in *Takifugurubripes*(tiger puffer) when fed at 2000 FTU kg^{-1} level in soybean meal based diet. In contrary to these findings and present results, Nwanna and Bello (2014) suggested that supplementation of phytase had a non-significant role on ADC% of mineral in *Oreochromis niloticus,* Nile tilapia fingerlings. They found little improvement in mineral absorption at a very high dose (8000 FTU kg^{-1}) of phytase supplementation. Their results were not in agreement of specific range of phytase supplementation (250 to 1500 FTU kg^{-1} level) as narrated by Cao *et al.* (2007). Reasons for these variations in results of different studies may be the use of different quality and quantity of phytase enzyme, feed formulation technology, feed drying methodology or process used for phytase supplementation (Baruah*et al.*, 2007; Dersjant-Li*et al.*, 2015). It was also observed that some of the minor minerals such as Ba, Cd, Co, Mo, and Ni were very low (<0.0001) in diet and could not be analysed when absorption was calculated.

According to this information, it was concluded that supplementation of phytase in Moringaby-product based diet played a significant ($p<0.05$) role in improving mineral

availability when it was compared with control diet. From these findings, it was suggested that 900 FTU kg^{-1} level supplemented MOLM based diet is most suitable for maximizing the mineral absorption in comparison with other phytase supplemented diets followed by 1200 FTU kg^{-1} level. This reduced mineral's discharge in water will also be helpful to control aquatic pollution.

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