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Quantitative Analysis of Enzyme Parameters in *Cyprinus carpio* due to the Effect of Peanut (*Arachis hypogea*) Extracts as a Feed Additive

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Abstract: ***Aquaculture on a global scale increases at a growing rate of 6% annually and has been gaining importance over capture fisheries since 1990's (Reverter et al., 2014). A wide range of disease out-breaks in aquaculture are due to poor culture practices, overcrowding of fishes, poor quality of water etc. Hence it has resulted in vast loss of production (Gabriel et al., 2015). Plants are widely employed for conventional chemotherapeutics and antibiotics (Vasseharan and Thaya, 2014). Compared to vaccines, plant extracts act as better immunostimulants as they protect the fishes against a wide range of infectious agents (Anderson, 1992). In the present study, peanut or groundnut (Arachis hypogaea) has been considered not only as a highly nutritious food source for human beings but also an alternative to feed and as a potent immune stimulator. The specimens of Cyprinus carpio were obtained from a private fish farm, Chidambaram, Tamilnadu, India and used as experimental models. Hence the present study aimed at an evaluation of the presence of different phytochemicals along with antimicrobial activity obtained from stem, leaf and seed of Arachis hypogaea.***

Keywords: Arachis hypogaea, Cyprinus carpio, Aeromonas hydrophilia, enzyme assay

1. Introduction

Fish has been a prolonged source of protein for human nutrition. Generally, fish consumption is beyond ecological, socio-economical, cultural and religious boundaries and it continues to play a vital role in animal protein. Nowadays, fish is considered over 50% of total animal protein and is consumed by developing countries with the global estimate standing at 15.5% in 2003 (FAO, 2007). Fish acts as a cheap source of first-class high quality animal protein (Tidwell and Allan, 2001). Several new remedial compounds have been identified and isolated recently from higher plants which are of healing importance for specific diseases. A few plants contain secondary metabolites which is responsible for their use as herbs and influence biological activity in animals (Sofowora 1986). The present study was aimed at the complete exploration of phytoconstituents and immunostimulatory effect of *Arachis hypogaea*. The preliminary screening of this plant extract reveals that maximum classes of phytoconstituents were present. In this study, emphasis was laid on all the four extracts (Aqueous, Petroleum ether, Chloroform and Ethanol) which has its own alkaloids, glycosides, tannins, saponins, fixed oils & fats and phenol.

2. Materials and Methods

Plant sample

The stem, leaf and seed samples were collected from mature plants, washed with water and then chopped into small fragments. The materials were then shade dried at ambient temperature (32°C) for 10 to 15 days and the drying operation was carried out under controlled conditions to avoid chemical changes. The dried samples were crushed into fine powder using an electronic blender. The powdered samples were stored in polythene containers at room temperature. The powdered samples were extracted by using soxhlet apparatus at 47ºC along with petroleum ether, chloroform and ethanol which were used as solvents. After this, the extract was dried at 50ºC in a hot air oven. Extracted samples were stored for later use.

Animal maintenance

Specimens of *Cyprinus carpio* were obtained from a private fish farm, Chidambaram, Tamil Nadu, India and acclimatized to the laboratory conditions for 15 days inside a fish tank (4 X 3 X 3). During acclimatization period, fishes were fed *ad libitum* with rice bran and groundnut oil cake in the form of dough once daily. Water was replaced every 24 hours after feeding in order to maintain a healthy environment for the fish during both acclimatization as well as experimental period. This ensures sufficient oxygen supply for the fish and the environment is devoid of any accumulated metabolic waste. After acclimatization, fishes with an average length of 8.5 cm and average weight of 7.0 g were selected for the study.

Preparation of heat killed whole cell vaccine from *A. hydrophila*

Single colony of *A. hydrophila* from the agar plate was inoculated in tryptic soy broth. After 24 hours, the bacterial cells in the broth were subjected to 60ºC for one hour in a water bath. The sterility of the environment was checked by inoculating a sample on nutrient agar plate. The heat killed bacterial culture was centrifuged at 3000 rpm for 15 minutes Then, the packed cells were collected and required dose of (10^9 cells/fish) was prepared in PBS based on the enumeration of diluted samples in Neubauer counting chamber.

Experimental conditions

Four experimental diets were prepared: control, leaves, stem and seed. Fish meal, de-hulled soybean were used as protein sources. Wheat flour and fish oil were used as the

carbohydrate and lipid sources, respectively. The 0.5% of various sources (leaves, stem and seed) of *Arachis hypogaea* were included in the experimental diets at the expense of 0.5% rice bran. The *Arachis hypogaea* free feed was used as a control diet. Fishes were fed with these selected feed for one week. Water was not changed during this period. After the treatment period, water was changed and fishes were immunized with intra peritoneal injection of 10^9 cells of heat killed *A. hydrophila.* The immune parameters were assayed on different days based on the period of response.

Blood collection
A day after the final feeding, blood samples were obtained from the common cardinal vein of randomly chosen five fishes which were anesthetized with 100 mg tricaein methane sulfate by using a 1.0 mL heparinized syringe for every 7 days of the experiment in each tank for three weeks.

Antiserum Collection
The blood collected from the immunized fishes was kept at room temperature for 15 minutes. The clot was freed from the walls of the tube for efficient retraction and was left overnight at 4ºC. The serum was separated by spinning down the clot at 3000 rpm for 15 minutes and then collected in sterilized storage vials. The serum was decomplemented at 47°C for 30 min to inactivate complement (classical pathway) and then stored at - 20°C until use.

Enzyme parameters

Acid phosphatase
The acid phosphatase was assayed by the method of (Pattabiraman, 1988). The total volume of 5 mL of substrate solution was mixed with 10 µl of serum sample and the absorbance was noted at 420 nm immediately. Then, the mixture of substrate solution along with serum sample was incubated at 37℃ for 30 minutes and the absorbance was noted at 420 nm.

Alkaline phosphatase
In the serum, alkaline phosphatase activity was estimated by the method of Bergmeyer (1963). The total volume of 3mL mixture contained 1.5 mL of the buffer, 1.0 mL of substrate solution, 0.1 mL of magnesium chloride solution, 0.5 mL of water and 0.1 mL of the homogenate. The incubation was carried out at 37℃ for 20 minutes. By adding 1.0 mL of Folin-Ciocalteau reagent the reaction was controlled. The suspension was centrifuged and 2.0 mL of 15% sodium carbonate solution was added to the supernatant. The solution was incubated at 37℃ for 10 minutes. The standard phenol solution was also treated similarly with Folin-Ciocalteau reagent and alkali. The intensification of UV-Spectrophotometer was read at 640 nm in blue colour.

Serum peroxidase
Serum peroxidase activity was assayed by the method of Murugesan and Rajakumari (2005). The total volume of 3 mL mixture contained 1.4 mL of amino-antipyrine, 1.5 mL of hydrogen peroxide and 0.1 mL of serum sample. Absorption was measured at 510 nm for 5 minutes. In control group, the solution was served without serum and the standard solution containing different concentration of peroxidase in 0.66 m phosphate buffer were used to construct a standard curve. The results were expressed in IU/mL.

Statistical Analysis
All values were given as Mean ± Standard deviations with 95% confidence intervals. The statistical analyses were performed through the SPSS statistical software (Version 16.0).

3. Results and Discussion

Feed additive on acid phosphatase enzyme
The three different parts of the plant extracts such as Leaf, Seed and Root of *Arachis hypogaea* when exposed as a feed additive in three different concentration 250 ppm, 500 ppm,1000 ppm in *Cyprinus carpio* are tabulated in the Table 1 and illustrated in Figure 1.

It was observed that there was a significant stimulation of acid phosphatase enzyme in all the treatments. A dose dependent effect was observed with 1000ppm seed extract having the highest effect in 10 days (2.60±0.18) and root extract (250 ppm) with lowest stimulatory effect (0.44±0.07) in 30 days. The phosphatase values were significantly higher in experimental diet fed fishes. An overall increase in enzyme parameter has significantly increased in *Cyprinus carpio* when exposed to three different plant extracts of different concentrations when compared to control group of fishes, at regular intervals of 10, 20, 30 days. A comparison of acid phosphatase enzyme in fishes when exposed to higher concentration at different durations of exposure was also significantly higher ($p<0.05$).

Feed additive on alkaline phosphatase enzyme
The three different parts of the plant extracts such as Leaf, Seed and Root of *Arachis hypogaea* when exposed as a feed additive in three different concentrations 250 ppm, 500 ppm,1000 ppm in *Cyprinus carpio* are tabulated in the Table 2 and illustrated in Figure 2. There is significant stimulation of alkaline phosphatase enzyme in all the treatments. A dose dependent effect was observed with 1000ppm seed extract having the highest effect in 10 days (2.82±0.37) and root extract of 500 ppm with lowest stimulatory effect (0.81±0.06) in 30 days. Phosphatase values were significantly higher in experimental diet fed fishes. An overall increase in the enzyme parameter has significantly increased in *Cyprinus carpio* when exposed to three different plant extracts of varying concentration when compared to control group of fishes, at regular intervals of 10, 20, 30 days. A comparison of alkaline phosphatase enzyme in fishes when exposed to higher concentration at different duration of exposure was again significantly higher.($p<0.05$).

Feed additive on serum peroxidase enzyme
The three different parts of the plant extracts such as Leaf, Seed and Root of *Arachis hypogaea* when exposed as a feed additive in three different concentrations 250 ppm, 500 ppm,1000 ppm in *Cyprinus carpio* are tabulated in the Table 3 and illustrated in Figure 3. Again, there was a significant stimulation of serum peroxidase enzyme in all the treatments. A dose dependent effect was observed with 1000ppm of seed extract having the highest effect in 30 days

(37.4±1.0) and root extract of 250 ppm with lowest stimulatory effect (16.5±0.9) in 10 days. A comparison of serum peroxidase enzyme in fish is exposed to higher concentration at different duration of exposure was significantly higher ($p<0.05$).

The leaf extract of *Euphorbia hirta* in *Cyprinus carpio* showed a significant enhancement in the phosphatase activity when compared with the control fishes as reported by Sveinbjornsson & Selijelid (1994) and this enhancement of the phosphatase activity leads to the production of enzyme by the macrophage cells. Dalmo & Seljelid (1995) observed that the macrophages are stimulated by lipopolysaccharides for increase of acid phosphatase production. In *Labeo rohita*, the alkaline phosphatase activity was enhanced by the plant extract *Achyranthus aspera*. This study is in agreement with the effect of *Euphorbia hirta* plant leaf extract on immunostimulant response of *Aeromonas hydrophilia* infected *Cyprinus carpio* (Vijayakumari Pratheepha *et al*.,2014). The same study reveals that the different concentrations of the leaf extract also stimulated the serum peroxidase activity. When seabream was fed with lactoferrin, no significant difference was observed by Esteben *et al*., (2005). Whereas, in the present study enzyme activity has significantly increased within 10 days after treatment due to incorporation of *Arachis hypogaea* .

Table 1: Effect of *Arachis hypogaea* as a feed additive on acid phosphatase enzyme in *Cyprinus carpio*

Sample Concentration	Acid phosphatase IU/mL			
		10 days	20 days	30 days
Leaf	Control	1.32±0.06	0.88±0.04	0.60±0.07
	250 ppm	2.11±0.09	1.82±0.06	0.96±0.08
	500 ppm	2.25±0.25	1.34±0.11	0.64±0.05
	1000 ppm	2.37±0.37	1.57±0.30	0.83±0.27
Seed	250 ppm	20.1±0.25	1.27±0.08	0.69±0.05
	500 ppm	2.59±0.12	1.46±0.08	0.73±0.01
	1000 ppm	2.60±0.18	1.59±0.16	0.81±0.09
Root	250 ppm	1.02±0.03	0.69±0.03	0.44±0.07
	500 ppm	1.22±0.09	1.01±0.08	0.87±0.03
	1000 ppm	1.54±0.03	1.27±0.10	0.96±0.09

Values are expressed as mean ± SD ($p < 0.05$)

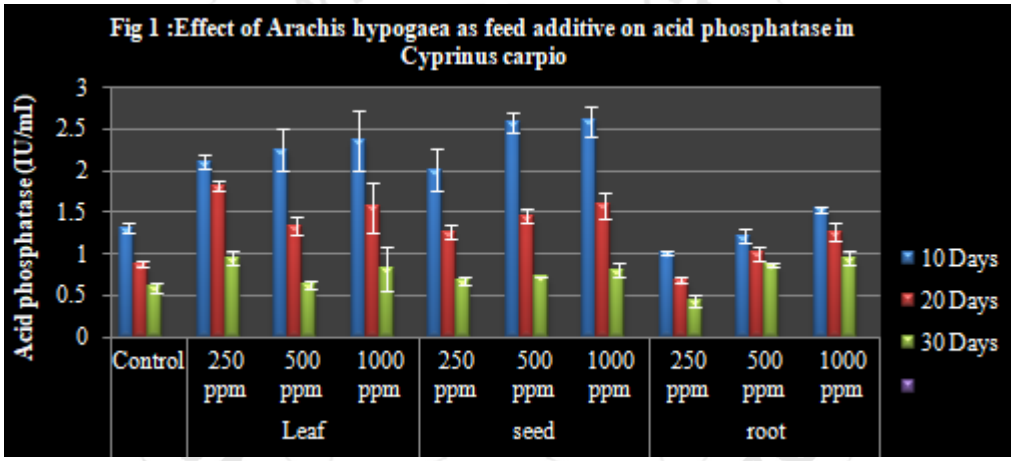

Figure 1: Acid phosphatase activity of *Arachis hypogaea* as feed additive in *Cyprinus carpio*. Data are expressed as mean ± SD at 5% Level of significance.

Table 2: Effect of *Arachis hypogaea* as a feed additive on alkaline phosphatase enzyme in *Cyprinus carpio*

Sample Concentration	Alkaline phosphatase IU/mL			
		10 days	20 days	30 days
Leaf	Control	1.95±0.10	1.35±0.09	0.84±0.08
	250 ppm	2.04±0.05	1.54±0.08	0.95±0.06
	500 ppm	2.52±0.08	1.63±0.02	1.04±0.09
	1000 ppm	2.68±0.06	1.85±0.08	1.22±0.02
Seed	250 ppm	2.50±0.06	1.60±0.04	0.99±0.07
	500 ppm	2.81±0.12	1.91±0.11	0.92±0.09
	1000 ppm	2.82±0.37	1.89±0.02	1.15±0.01
Root	250 ppm	2.05±0.08	1.10±0.05	0.82±0.09
	500 ppm	2.29±0.10	1.70±0.18	0.81±0.06
	1000 ppm	2.81±0.16	1.91±0.08	0.90±0.01

Values are expressed as mean ± SD ($p < 0.05$)

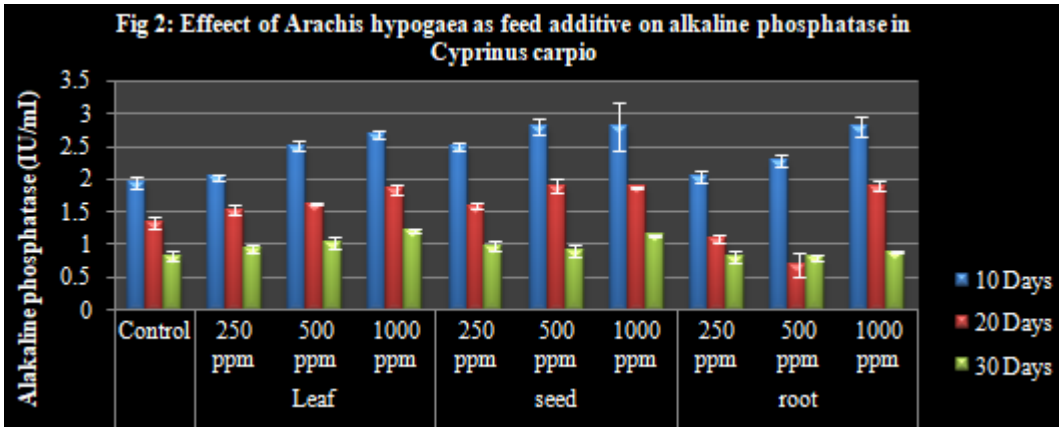

Figure 2: Alkaline phosphatase activity of *Arachis hypogaea* as feed additive in *Cyprinus carpio*
Data are expressed as mean ± SD at 5% Level of significance.

Table 3: Effect of *Arachis hypogaea* as feed additive on serum peroxidase enzyme in *Cyprinus carpio*

Sample Concentration		Serum peroxidase IU/mL		
		10 days	20 days	30 days
Leaf	Control	12.5±0.9	14.8±0.9	15.7±0.8
	250 ppm	19.1±0.3	25.4±0.8	26.8±0.2
	500 ppm	22.3±0.7	24.6±1.1	29.7±0.4
	1000 ppm	26.5±1.3	35.2±0.8	32.9±0.8
Seed	250 ppm	21.9±0.9	23.5±0.7	29.1±0.5
	500 ppm	27.0±0.4	30.2±0.3	31.8±1.1
	1000 ppm	31.5±1.7	34.6±1.1	37.4±1.0
Root	250 ppm	16.9±0.9	20.0±0.1	23.3±0.3
	500 ppm	20.7±1.0	22.0±0.8	24.7±0.9
	1000 ppm	22.2±0.8	24.5±0.5	28.1±1.0

Values are expressed as mean ± SD ($p < 0.05$)

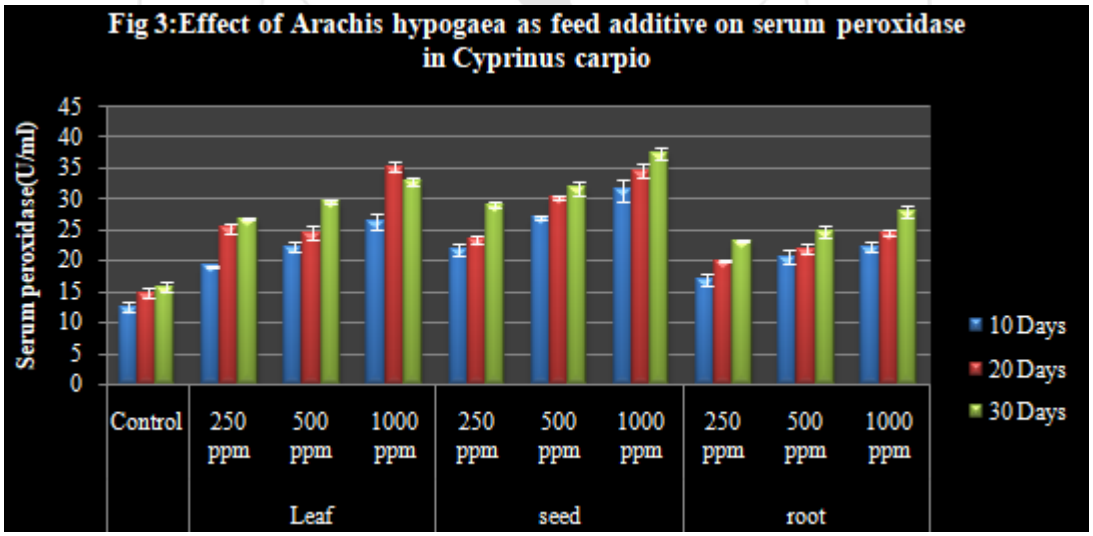

Figure 3: Serum peroxidase activity of *Arachis hypogaea* as feed additive in *Cyprinus carpio*.
Data are expressed as mean ± SD at 5% Level of significance

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