

Research Article

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Effect of Peanut (*Arachis hypogea* L.) extracts as a feed additive on growth and hematological parameters of rohu fish (*Labeo rohita*) fingerlings

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Abstract

The main objective of the present study is to improve the immune power of *Labeo rohita* by using *Arachis hypogea* plant leaf, seed and root extracts as immunostimulants. The specific antibody response, immunological and host resistance test were conducted on the medicated fish infected with *Aeromonas hydrophila* pathogen. The results obtained from the hematological studies show that the RBC count, total WBC count, and lymphocyte content were increased in the infected fish at higher concentration of all three extract. The feeds with seed extract of *Arachis hypogea* were able to stimulate the specific antibody response by increasing the titre value of antibody. It was observed that fish have survival percentage significantly at higher concentration (1000 ppm) of *Arachis hypogea*, when compared with the control. This research work suggests that the *Arachis hypogea* has immunostimulant activity by stimulating both specific and non-specific immunity at higher concentrations.

Keywords

Arachis hypogea,
feed additive,
immunostimulatory,
Aeromonas hydrophila

Introduction

Aquaculture fish production has been increased significantly over the past few decades contributing fish protein with minerals such as zinc, magnesium, sodium to the consumers (Ravenhalt, 1982; and Barlas, 1986). The infectious diseases are a serious issue in aquaculture, causing heavy loss to fish farmers. *Aeromonas hydrophila* is one of the most vital bacteria associated with the diseases in marine and freshwater fishes. It can cause several health interferences in both fish and humans, including tail and skin rot and fatal hemorrhagic septicemias in fish and soft-tissue wound infection and diarrheic diseases in humans (Lu, 2002). However, these diseases are becoming ruthless with increasing culture and in recent days, development of intensive aquaculture practices has led to a growing

interest in understanding fish diseases. This helps the researchers for the development of medicines, which can treat or prevent the infectious diseases (Logambal *et al.*, 2000). Synthetic drugs and chemotherapeutics used to control these diseases can result in the development of a drug-resistant bacteria, environmental pollution and residues in fish (Jian and Wu, 2003). It shows that extensive research is needed for the development of environment friendly and highly active moiety for the treatment.

Similar to humans, fish depends on both specific and non-specific mechanisms to protect themselves against invading pathogens. In fish, the primary lines of non-specific defenses are the skin and mucus, when

pathogens enter into the body, cellular and humoral non-specific defences are mobilized (Dugenci *et al.*, 2003). The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, such as lysozymes or the complement system (Secombes and Fletcher, 1992; Galina *et al.*, 2009). The pathogen attacks the immune system in the fish and causes infectious diseases. The stimulation of the immune system of the fish can protect the fish from infectious diseases.

Now a days, increasingly more attention has been paid to the development of immunostimulants for both fish and other animals (Raa *et al.*, 1992; Jeney & Anderson, 1993; Sakai, 1999; Raa, 2000; Yin *et al.*, 2006). The use of natural products, like plant extracts in controlling fish diseases is new and emerging field which needs further researches to find out the most effective measures to replace chemotherapy (Sivaram, 2004). A number of plant extracts have been screened and used with encouraging results in controlling bacterial diseases in fishes (Pachanawan *et al.*, 2008; Subeenabegum and Navaraj, 2012; Yogananth *et al.* 2016). Hence, the present study focuses on the immunostimulant effect of *Arachis hypogea* on the common rohu (*Labeo rohita*) infected with *Aeromonas hydrophila*.

Materials and Methods

Plant sample

The plant parts of stem leaf and seed were collected from mature plants and 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 47°C petroleum ether, chloroform and ethanol were used as a solvent. After extraction, the extract was dried at 50°C in hot air oven. Extracted samples were stored at properly and can be used for immunostimulatory activity.

Animal maintenance

Specimens of *Labeo rohita* were obtained from a private fish farm, Chidambaram, Tamil Nadu, India and acclimated to the laboratory conditions for 15 days in fish tank (4 X 3 X 3). During acclimation period, fish were fed ad libitum with rice bran and ground 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 acclimation and experimental period. This ensures sufficient oxygen supply for the fish and the environment is devoid of any accumulated metabolic waste. After acclimation, fish with an average length 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 the tryptic soy broth. After 24 hrs, the bacterial cells in the broth are subjected to 60°C for one hour in a water bath. The sterility was checked by inoculating a sample on nutrient agar plates. The heat killed bacterial culture was centrifuged at 3000 rpm for 15 minutes Then the packed cells were collected and required dose (10⁹ 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. Percentage inclusions of ingredients of the experimental diets are given in Table I. Fish meal, de-hulled soybean were used as the protein sources. Wheat flour and fish oil was used as the carbohydrate and lipid sources, respectively. The 0.5% of various sources (leaves, stem and seed) of *Arachis hypogea* were included in the experimental diets at the expense of 0.5% rice bran. The *Arachis hypogea* free feed was used as a control diet. Fish were fed with these selected feeds for one week. Water was not changed during this period. After the treatment period water was changed and fish were immunized with intra peritoneal injection of 10⁹ 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 fish anesthetized with 100 mg tricaine methane sulfate by using a 1.0 ml heparinized syringe for every 7 days of the experiment in each tank for three weeks.

Ingredients	Control (T1)	Stem extract (T2)	Leaf extract (T3)	Seed extract (T4)
Fish meal	45.7	45.7	45.7	45.7
Soyabean meal	16.6	16.6	16.6	16.6
Rice bran	11.5	11.0	11.0	11.0
CMC binder	2.0	2.0	2.0	2.0
Wheat flour	21.0	21.0	21.0	21.0
Vitamin mix	2.0	2.0	2.0	2.0
Fish oil	1.2	1.2	1.2	1.2
Ground nut extract	–	0.5	0.5	0.5
Total	100	100	100	100

Antiserum Collection

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

Specific antibody response:

The anti-*Aeromonas* antibody titre was determined by bacterial agglutination assay in 96 well ‘U’ bottom micro titre plates (Torson) following the method of Roberson, (1990). 25 µl of serum was added to the 1st well and two-fold dilution was made with PBS. A volume of heat killed bacterial cell suspension (10⁸ cells / fish pre stained with crystal violet) were added to each well. The micro titre plate was hand shaken for effective mixing and incubated for overnight at 37°C. The highest dilution of the serum sample, which showed detectable macroscopic agglutination, was recorded and expressed as log² antibody titre of the serum.

Total Red blood cells (RBC)

Blood was taken upto 0.5 mark in the RBC pipette and excess blood was wiped off from the tip. The pipette was then filled to 101 marks with RBC diluting fluid. The RBC pipette was horizontally shaken and a drop of resultant mixture was discharged under the cover glass of a Naubauer counting chamber (Naubauer, Feinoptic, Germany). Number of erythrocytes in 80 small squares was counted under the light microscope. The number of cells in 1 mm³ undiluted blood was calculated.

Total and Differential WBC count

Total WBC was counted in a Neubauer counting chamber using Natt-Herrings solution as the diluting fluid (Rowley, 1990) 0.1 ml of blood was diluted to eight times using Natt-Herrings solution and kept for five minutes. The stained cells are counted in four large squares of Neubauer counting chamber. Differential count was done using Leishman stained blood smears. Cover the smear with stain and leave for 1-3 minutes. Add PBS and allowed to mix on slide and leave for five minutes. Rinse in distilled water. Blot dry the slide and examine under 100x magnification of a binocular microscope. 100 cells were counted and the number of cells was expressed in percentage.

Host resistance test (Knittel, 1981):

A challenge dose of 2 x 10⁶ cells/fish of virulent *A. hydrophila* always resulted in less than 50% survival in control fish group and was used as the standard challenging dose in this experiment. An plant extracts treated and saline injected control group were maintained. Then all the groups of fish (n=25/group) were experimentally infected with the challenging dose of virulent *A. hydrophila*, four weeks after vaccination mortalities were recorded after 96 hrs and degree of protection was assessed by calculating the relative percent survival

$$RPS = 1 - n \frac{\% \text{ Experimental mortality}}{\% \text{ Control mortality}} \times 100$$

Statistical Analysis:

All values were given as mean \pm Standard deviation which were means with 95% confidence intervals. The statistical analyses were performed through the SPSS statistical software (Version 16.0).

Results and Discussion

Specific antibody response

In this present study significant enhancement of specific anti *Aeromonas* antibodies by all three

treatments in a dose dependent manner was observed, when *A. hypogea* plant extracts were administered as feed additive (Table 2). Maximum stimulation was observed by 1000 ppm seed extracts (4.6 ± 0.14) at 30 days and minimum by 250 ppm root extracts (2.0 ± 0.09) at 10 days. The present study clearly shows a significant enhancement of specific anti *Aeromonas* antibodies in all three treatments groups that too in a dose dependent manner. The results of the present study are in agreement with earlier observations (Venkatalakshmi and Michal, 2000; Logambal *et al.*, 2000; Sudhakar *et al.*, 2006).

Table 2: Effect of *Arachis hypogea* as feed additive on specific antibody response in *Labeo rohita*

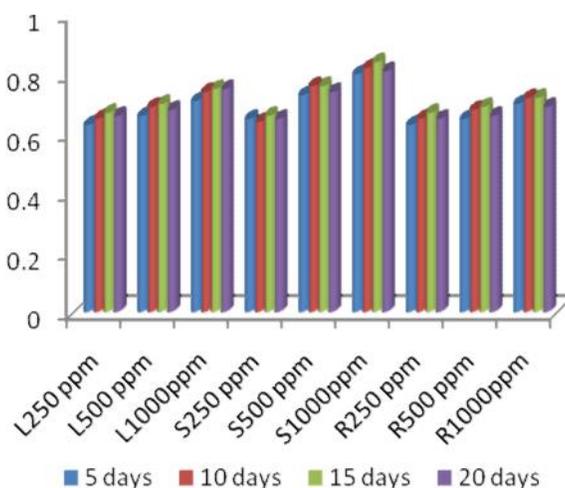
Sample conc		10 days	20 days	30 days
Leaf	250 ppm	2.6 \pm 0.12	3.0 \pm 0.08	3.2 \pm 0.05
	500 ppm	2.8 \pm 0.10	3.4 \pm 0.07	3.8 \pm 0.07
	1000ppm	3.1 \pm 0.09	3.7 \pm 0.14	4.1 \pm 0.12
Seed	250 ppm	2.7 \pm 0.07	3.1 \pm 0.06	3.8 \pm 0.09
	500 ppm	2.8 \pm 0.08	3.6 \pm 0.08	4.2 \pm 0.08
	1000ppm	3.3 \pm 0.11	3.9 \pm 0.16	4.6 \pm 0.14
Root	250 ppm	2.0 \pm 0.09	2.2 \pm 0.05	2.5 \pm 0.09
	500 ppm	2.3 \pm 0.03	2.6 \pm 0.03	2.9 \pm 0.06
	1000ppm	2.5 \pm 0.18	3.1 \pm 0.12	3.5 \pm 0.12

Total RBC

The haematological study on the fish shows that the maximum RBC count of $0.84 \pm 0.01 \times 10^6$ cells/mm³ was noticed on the fish fed with 1000ppm seed extract incorporated feed. The minimum count $0.63 \pm 0.03 \times 10^6$ cells/mm³ was found in 250 ppm leaf extract (Fig 1).

The RBC values were significantly higher in experimental diet fed fish. Sahu *et al.*, 2007 have also reported higher RBC counts in *Labeo rohita* fingerlings fed with *Mangifera indica*. They explained this increase as an indication of enhanced cellular immunity.

Fig 1: Effect of *Arachis hypogea* as feed additive on total RBC in *Labeo rohita*

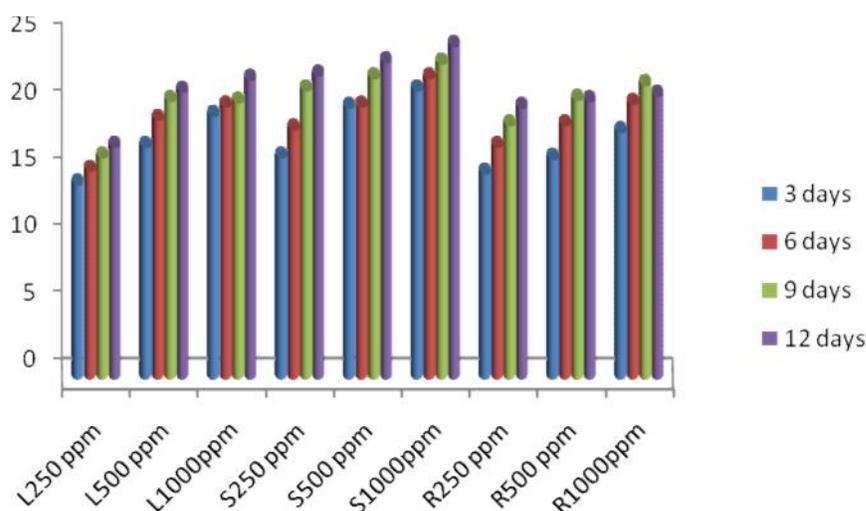


Total WBC

Figure 2 reveals that Peanut plant extracts increased the number of white blood cells when administered as feed additive. The maximum WBC count of $24.8 \pm 0.5 \times 10^6$ cells/mm³ was noticed on the fish fed with 1000ppm seed extract incorporated feed. The minimum count $14.5 \pm 0.3 \times 10^6$ cells/mm³ was found in 250 ppm leaf extract. The increase in WBC count observed in the present study might be due to the

direct stimulatory effect of Peanut plant extracts on B cells and T cells or indirectly stimulating interleukins, and monokines secretions (Cross *et al.*, 2002, Kitazawa *et al.*, 2001). Chukwudi *et al.*, 2011 observed that WBC counts in rats administered with *Mucuna pruriens* increased significantly in comparison to control. This increase in WBC total count likely had been triggered off by the metabolic assault from phenol content in *Arachis hypogea*.

Fig 2: Effect of *Arachis hypogea* as feed additive on total WBC in *Labeo rohita*



Differential WBC count

The fish immune system is well developed and is comparable to the mammalian immune system, as it consists of both T cell and B cell mediated immunity constituting both the cellular and humoral components (Iwana and Nakanishi, 1996). Harikrishnan *et al.*, (2005) observed that changes in differential count occur in response to an infection. Differential counts therefore were used as one of the tools for assaying the immune status of fish (Nussey *et al* 1995; Logambal, 1996, Misra *et al.*, 2006).

Total lymphocytes

There is significant stimulation of lymphocytes by all the treatments (Table 3). A dose dependent effect was observed with 1000 ppm seed extract having the highest effect in 9 days ($74.7 \pm 1.09\%$) and leaf extract 250ppm with lowest stimulatory effect ($57.4 \pm 1.00\%$) in 12 days. The number of lymphocytes was maximum on day 6 and 9 days post immunization. Observations of Kitazawa *et al.*, (2001) that plant extracts enhanced multiplication of B lymphocytes by stimulating the expression of CD molecules (Cluster of Differentiation system), could be a possible mechanism for the enhancement observed in the present study.

Table 3: Effect of *Arachis hypogea* as feed additive on total lymphocytes in *Labeo rohita*

Sample conc		Total lymphocytes (%)			
		3days	6 days	9 days	12 days
Control		53.0±0.2			
Leaf T1	250 ppm	60.2±1.12	61.9±1.13	62.5±0.65	57.4±1.00
	500 ppm	63.5±0.14	65.6±0.54	66.6±1.07	59.9±1.27
	1000ppm	65.9±0.99	69.5±0.72	70.1±0.78	65.7±0.00
Seed T2	250 ppm	62.2±0.56	66.2±1.28	68.1±0.84	63.0±0.00
	500 ppm	63.8±0.98	67.9±0.90	70.3±1.11	66.5±1.06
	1000ppm	65.9±1.12	72.2±0.58	74.7±1.09	69.0±0.77
Root T3	250 ppm	60.2±0.74	64.9±0.73	65.2±1.21	62.1±0.00
	500 ppm	61.3±1.02	65.2±0.78	67.5±1.11	63.7±0.98
	1000ppm	62.3±0.33	68.3±0.94	69.6±1.16	64.8±1.16

Total Monocytes

Maximum number of monocytes was observed on day 6 days post immunization. On that day an inverse dose dependent stimulation was observed. (table 4). When the overall response was analyzed, the effect was

seemed to be suppressive by all three treatments compared to control. A decrease in monocyte count could therefore be an indication of the stimulation of the specific and non specific defense mechanism (Roitt *et al.*, 1993).

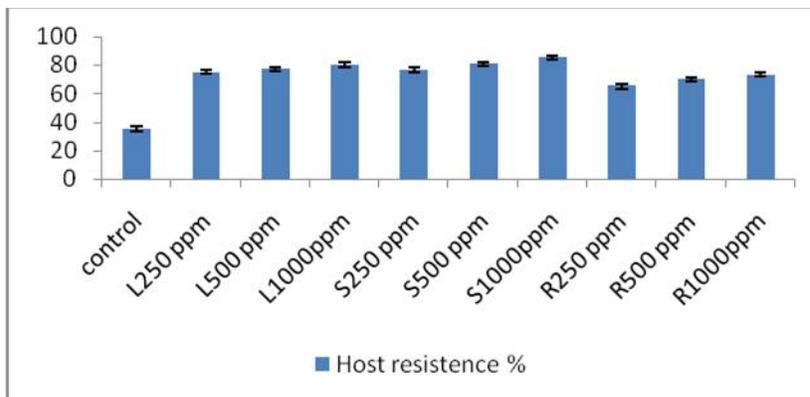
Table 4: Effect of *Arachis hypogea* as feed additive on monocytes in *Labeo rohita*

Sample conc		Total monocytes (%)			
		3days	6 days	9 days	12 days
Control		5.4±0.6			
Leaf	250 ppm	3.96±0.18	4.15±0.00	4.10±0.10	3.84±0.37
	500 ppm	4.02±0.33	4.35±0.14	4.15±0.16	3.95±0.25
	1000ppm	4.30±0.173	4.67±0.25	4.26±0.09	4.00±0.19
Seed	250 ppm	4.05±0.44	4.64±0.10	4.32±0.41	4.03±0.15
	500 ppm	4.49±0.21	4.87±0.72	4.40±0.14	4.11±0.34
	1000ppm	4.88±0.16	4.92±0.24	4.49±0.33	4.26±0.18
Root	250 ppm	3.75±0.00	4.08±0.14	3.95±0.28	3.51±0.00
	500 ppm	3.84±0.41	4.12±0.09	4.05±0.21	3.81±0.00
	1000ppm	4.02±0.00	4.22±0.15	4.14±0.20	3.97±0.43

Host resistance test:

In the present study, Pea nut plant extracts reduced the mortality of fish challenged with virulent strain of *A. hydrophila*. Relative percent survival was significantly increased by all the doses examined (Fig.3). 1000 ppm of Seed and leaf of feed additive method assured above 80% protection, while root extracts gave 65-73% protection. The overall protection efficiency by

the *Arachis hypogea* plant extracts treated groups was highly significant. The mechanism by which the survival was augmented appears to be positively correlated with increased phagocytosis, neutrophil activity, lysozyme activity, etc. Earlier studies revealed that dietary supplementation of *Ocimum sanctum* leaf extract enhanced disease resistance against *Aeromonas hydrophila* in *Oreochromis mossambicus* (Logambal *et al.*, 2000).

Fig 3: Effect of *Arachis hypogea* as feed additive on host resistance in *Labeo rohita*

In general, the immunostimulant (plant extract) was found to stimulate antibody response, RBC, WBC, phagocytosis and other immunological function in fish at higher concentrations. The disease resistance study indicates that the fish fed with plant leaf, seed and root extracts were able to increase their survival percentage significantly. This study also reveals that the scope of using extract of *Arachis hypogea* as an immunoprophylactic in the health management in culture of fishes. Finally appropriate field trials are necessary before using the aqueous extract as immunoprophylactics to prevent infectious diseases in fin fish aquaculture.

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