Research Article

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EVALUATION OF IMMUNOSTIMULATORY POTENTIAL OF *PHYLLANTHUS AMARUS* IN *LABEO ROHITA* INFECTED WITH *AEROMONAS HYDROPHILA*: HAEMATOLOGICAL ASSESSMENT

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ABSTRACT

Outbreak of disease is an important limiting factor in aquaculture. Incorporation of herbal immunostimulants in diet is one of the widely followed methods to improve the general resistance in fish. The present experiment was carried out to evaluate the Immunostimulatory potential of the medicinal herb *Phyllanthus amarus* in fish. The experiment was carried out in two sets. In experiment-I, one group of *Labeo rohita* was fed with control diet ('C' diet) and the other group was fed with *Phyllanthus amarus* incorporated diet ('P' diet) for 14 days and the haematological parameters were analysed on 1st, 3rd, 7th and 14th day of the experiment. Significant increase in Total Erythrocyte Count (TEC), Haemoglobin (Hb g%), Total Leucocyte Count (TLC), lymphocytes, monocytes and neutrophils were observed in 'P' diet fed fishes with increase in the duration of the experiment. As there is positive response in the first experiment hence experiment-II was carried out. In experiment-II, One group of *Labeo rohita* were fed with 'C' diet and other group received 'P' diet for 14 days, post-infected with *Aeromonas hydrophila* and haematological parameters were analyzed on 1st, 3rd, 7th & 14th days after infection. Significant increase in TEC, Hb, TLC, lymphocytes and a steady population of monocytes and neutrophils were observed in 'P' diet fed fishes. On the other hand basophils and eosinophil count exhibited a fluctuating trend. Thus dietary incorporation of *Phyllanthus amarus* helps to improve the general health and resistance of the fish.

Key words: *Phyllanthus amarus*, *Labeo rohita*, *Aeromonas hydrophila*, haematology, Immunostimulants.

INTRODUCTION

The recent expansion of intensive aquaculture has led to a growing interest in understanding of fish disease so that they can be treated or prevented. Disease outbreaks are particularly prevalent in rapidly developing aquaculture industries, affecting the economic development of this sector.

The herbs are used not only against diseases but also as growth promoters, stress resistance boosters and preventatives of infections. Herbs can also act as immunostimulants, conferring the non-specific defence mechanisms of fish and elevating the specific immune response[1]. The use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development[2,3]. Natural immunostimulant are biocompatible, biodegradable and safe for the environment and human health[4]. Plants are rich in a wide variety of secondary metabolites of phytochemical constituents such as tannins, alkaloids and flavonoids, which act against different diseases[5,6]. Extracts of some plants are potentially curative, some of these extract can boost the humoral[7] and cell mediated immunity[8] against viruses[9], bacteria[10], Fungi[11], Protozoa[12] and cancer[13].

Phyllanthus amarus is a traditional herb belonging to Euphorbiaceae family, grows to 12-24 inches and blooms with many yellow flowers. The main phytochemical constituents include; lignins (Phyllanthine and Hypophyllanthine), alkaloids, bioflavonoids (Quercetin) and repandusinic acid. It cures kidney and gallbladder stones, hepatitis, colds, flu, tuberculosis and other viral infections. It has also been proven effective in liver diseases like jaundice and liver cancer, it is also used for bacterial infections such as cystitis, prostatis, venereal diseases and urinary tract infections, it has also been shown to have antiviral properties *In vitro*, inhibiting HIV and HTLV-1 replication[14], besides it contains minerals like calcium, potassium, sodium, manganese, magnesium, iron, copper and zinc. It also exhibits anti-microbial activity against *P.auregenosa, E.coli, S.aureus* and *C.albicans*[15].

Aeromonas hydrophila has been associated with diseases in fishes like carp, eel, milkfish, channel catfish, tilapia and opportunist in stress related diseases in salmonids[16]. Blood forms an integrated and inevitable part in all immune system and the changes in these parameters can be correlated to the response of the organism to the changing environmental conditions and therefore can be used to screen the health state of fish[17].

The present study was carried out to test the immunostimulant potential of this medicinally important herb *Phyllnathus amarus* in the fish *Labeo rohita* post infected with *Aeromonas hydrophila*.

MATERIALS AND METHODS

The experimental fish *Labeo rohita* (12g±2g) were collected from Subam fish farm (Kallidaikurichi) and they were acclimatized to laboratory condition for a week. Ambient temperature 29±1°C, pH 7.1±0.5, dissolved oxygen 6.4±0.5mg/l. Fishes were maintained in cement tanks at a stocking density of ten fishes in each container.

P.Amarus a medicinal herb (Herbarium Voucher No: XCH 25277,25280) tried as the immunostimulant in the present investigation.The leaves of *Phyllanthus amarus*

were collected and allowed to dry for few days, powdered and stored.

Preparation of Feed

The control feed ('C' diet) was prepared by mixing soya bean-23g, Groundnut oil cake 23g, rice bran-10g, fish meal powder 24g, wheat bran 10g and tapioca flour 10g. All the ingredients were mixed, sterilized in pressure cooker and made into noodles, dried and broken into required sized pellets. The 'P' diet was prepared by mixing the same quantity of ingredients following the same procedure and the aqueous extract of *Phyllanthus* leaf (10g/100ml) was added before noodle formation to the sterilized ingredients and used as experimental feed.

Experiment I

The fishes were divided into two groups, one was fed with 'C' diet and the other group was fed with 'P' diet for 14 days. While feeding the haematological parameters were analysed after 1st, 3rd, 7th and 14th day. This experiment was performed to observe whether the *Phyllnathus* diet makes any change in haematological parameters. As the result was positive the second experiment was carried out.

Experiment II

The fishes were divided into two groups, one was fed with 'C' diet and the other group was fed with 'P' diet for 14 days. Then all the fishes of both groups were injected with 10^5cfu of *Aeromonas hydrophila* intra muscularly and the haematological parameters were studied after 1, 3, 7 14 days of infection. The experiments were run in triplicates.

Haematological Analysis

The blood was collected by cutting the caudal peduncle in a vial precoated with EDTA (anticoagulant), and from the pooled blood the following blood parameters were analysed. The total erythrocyte count (TEC) and total leucocyte count (TLC) were calculated using haemocytometer with improved Neubaurer ruling chamber (Weber and sons, England). Haemoglobin content was estimated by cyanomethomoglobin method and differential leucocyte counts were calculated by staining the blood smears using May Grunewald's Giemsa's stain. The results were statistically treated, significant changes were calculated using students 't' and the values were tabulated.

Table 1: Comparison of haematological parameters between 'C' & 'P' diets in Experiment-I

Duration (Days)	Groups	TEC (x 10^6)	TLC (x 10^3)	Hb (gm%)
1st	C	1.2±0.2	3.2±0.5	4.3±0.4
	P	2.0±0.6*	3.3±0.7	7.9±0.6**
3rd	C	1.6±0.4	3.5±0.6	7.6±0.7
	P	2.2±0.8*	4.4±0.7*	8.8±1.2
7th	C	2.1±0.4	2.9±0.4	7.8±0.9
	P	2.6±0.3*	7.5±1.7**	9.7±1.3*
14th	C	2.3±0.8	2.6±0.8	8.4±1.2
	P	2.9±0.7*	9.3±1.7**	11.5±2.5**

Values are Mean ± SD of three values. * = significant, ** = highly significant

Table 2: Comparison of haematological parameters between 'C' & 'P' diets in Experiment-II

Duration (Days)	Groups	TEC (x 10^6)	TLC (x 10^3)	Hb (gm%)
1st	C	3.8±0.6	3.4±0.5	3.6±0.4
	P	5.4±0.8*	4.1±0.7	8.4±0.5**
3rd	C	4.2±0.5	4.4±0.3	3.4±0.6
	P	6.2±0.4*	4.3±0.4	8.8±1.2**
7th	C	4.0±0.6	4.1±0.7	2.8±0.9
	P	7.2±1.2**	6.8±0.9**	9.9±1.5**
14th	C	3.9±0.8	3.8±0.8	2.6±0.6
	P	7.5±0.7**	7.1±1.1**	10.2±1.9**

Values are Mean ± SD of three values. * = significant, ** = highly significant

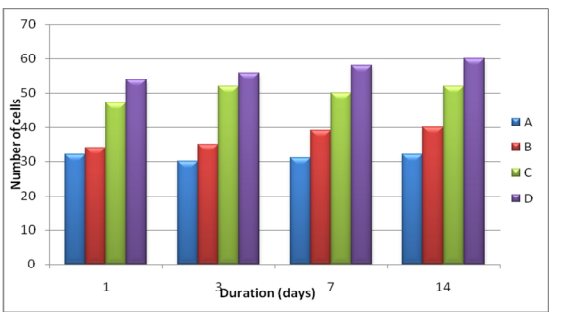

Figure 1: Comparison of Lymphocytes

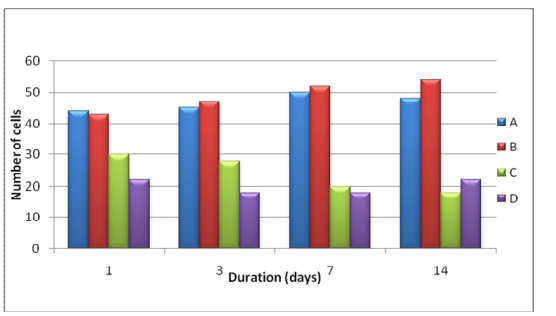

Figure 2: Comparison of Monocytes

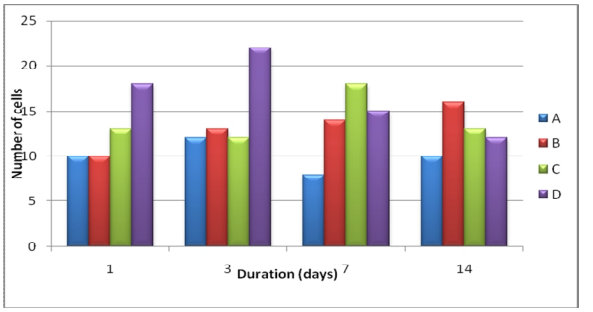

Figure 3: Comparison of Neutrophils

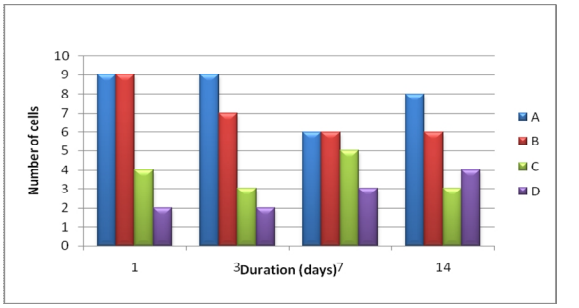

Figure 4: Comparison of Eosinophils

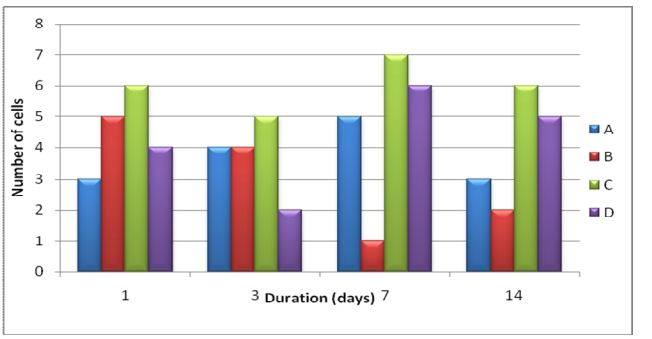

Figure 5: Comparison of Basophils

Experiment I: **A** = 'C' diet, Uninfected **B** = 'P' diet, Uninfected
Experiment II: **C** = 'C' diet, Infected **D** = 'P' diet, Infected

RESULTS

Experiment I

Total Erythrocyte Count (TEC)

Total Erythrocyte counts exhibited a progressive increase in both control ('C' diet) and Immunostimulant diet ('P' diet) fed fishes during the 14 day feeding trial. However the increase was significant in 'P' feed fed fishes throughout the experimental period (Table 1).

Haemoglobin (Hb gm%)

The haemoglobin level also increased significantly in both 'C' & 'P' diet fed fishes. However the increase was significantly higher in 'P' diet fed fishes only. On the other hand in 'C' diet fed fishes the increase was highly significant in the 3rd day of feeding and there after the increase was minimal, but steady increase in haemoglobin concentration was observed in 'P' diet fed fishes.

Total Leucocyte Count (TLC)

The Total Leucocyte Counts exhibited a marginal decline in 'C' diet fed fishes with increase in the duration of the experiment; whereas 'P' diet fed fishes exhibited a significant and steady increase in the counts.

Differential Leucocyte Counts (DLC)

Lymphocytes, Monocytes and Neutrophil populations were significantly higher in 'P' diet fed fishes over 'C' diet fed fishes and also exhibited a steady increase with the increase in experimental duration, however in 'C' diet fed fishes only marginal fluctuations were observed throughout the experimental period. Basophils and Eosinophils exhibited fluctuating changes in both 'C' & 'P' diet fed fishes (Figure 1-5).

TEC and Hb values increased in both 'C' & 'P' diet fed fishes throughout the experimental duration, The increase in 'C' diet fed fishes can be attributed to the administration of 'Formulated Balanced diet' and a significant increase in 'P' diet can be attributed to *Phyllanthus* induced effect. Steady increase in TLCs in 'P' diet fed fishes can be considered as *Phyllanthus* induced effect, which is further supported by significantly increased populations of lymphocytes, monocytes and neutrophils than their 'C' diet counterparts.

Experiment II

Total Erythrocyte Count (TEC)

TECs exhibited fluctuating trend in 'C' diet fed fishes throughout the 14 day experimental period but the counts increased significantly throughout the experimental period, further the increase was steep up to 3rd day of infection and thereafter the increase was marginal (Table 2). Further the counts were significantly higher in 'P' diet fed fishes during all sampling days. General increase in TECs and also steep increase up to 3rd day of infection can be considered as a stress induced immediate response to maintain general health.

Haemoglobin

In 'C' diet fed infected fishes the haemoglobin concentration gradually decreased with increase in the experimental duration. Whereas in 'P' diet fed infected fishes there is a significant increase in the haemoglobin concentration.

Total Leucocyte Count (TLC)

Marginal increase in TLCs was observed in 'C' diet fed fishes, whereas the increase was significant in 'P' diet fed fishes. It was also observed that the TLCs were always higher in 'P' diet fed fishes over their 'C' diet counterparts.

Differential Leucocyte Counts (DLC)

Lymphocyte counts exhibited an increasing trend in both 'C' & 'P' diet fed infected fishes, the increase was marginal in 'C' diet and it was significant in 'P' diet fed fishes and also it was observed that the population was always higher in 'P' diet fed fishes throughout the experimental period. The Monocytes and Neutrophil populations exhibited a declining trend in both diets whereas the decline was significant in 'C' diet fed fishes whereas it was only marginal in 'P' diet fed fishes. The counts of Basophils and Eosinophils exhibited fluctuating change in both diet fed fishes.

From the present experiment it was observed that in 'C' diet fed fishes the fluctuating change and decrease in haemoglobin concentration explains the inability of the fish to withstand and resist the infection induced stress whereas in 'P' diet fed fishes, the TLC and haemoglobin values increased significantly indicating the ability of the fish to maintain the general health. Erythropoiesis is the primary physiological response ie, maintaining/providing the energy producing mechanism which is vital to keep any cell including immune cell in viable state thus strengthening the First line of defence. Further increase in TLCs in infected fishes of both diets explains more the number of leucocyte better the resistance. In the present experiment also the significantly increased population of leucocyte in 'P' diet fed fishes can be considered as feed induced improved resistance. Increase in the lymphocyte count in 'P' diet fed fishes can be considered as improved specific resistance and quick increase in lymphocyte counts indicates earlier activation of humoral immune response. Maintenance of a minimal population of monocytes and neutrophils which are the mediators for specific immunity as they present the pathogen to the immune system. Thus more is the population of monocytes and neutrophils, quicker will be the humoral immune response.

DISCUSSION

From the 14 days feeding trial of Experiment I, it was observed that in *Phyllanthus* diet fed fishes, there was a general increase in TEC, Hb, TLC, lymphocytes, monocytes and neutrophils. Thus, *Phyllanthus* incorporated diet improves the general health and resistance as there was significant increase in the blood cell counts. In the experiment II, in *L.rohita* infected with *A.hydrophila* increase in TEC, Hb, TLC and lymphocytes and steady levels of monocyte and neutrophils were observed in 'P' diet fed fishes only.

Many recent experiments have shown that immunostimulants can be given to induce *In vitro* and *In vivo* responses[18], feed mediated immunization in fish is an effortless and stress free process which can be used for almost any age[19]. *Labeo rohita* fingerlings fed with *Magnifera indica* exhibited increase in RBC and WBC counts[20], *Catla catla* fed with *Corriandrum sativum* and *Plumbago rosea* exhibited an increase in TEC and TLC values[19], Garlic incorporated fed *C.carpio* exhibited an increased levels of RBC, WBC, Hb, lymphocytes, monocytes & neutrophils[21], *C.mrigala* fed with *Zingiber officinale* and *Curcuma longa* incorporated diet and infected with *Pseudomonas aeruginosa* exhibited an increase in TEC, TLC, Hb, PCV and MCHC[22], dietary supplementation of *Achyrarthus aspera* seed stimulated immunity and induced enhanced resistance to *A.hydrophila* in *L.rohita*[23], proliferation of WBC could be due to leucopoiesis, particularly lymphopoiesis as a response to enhanced immunity[24]. Increase in lymphocytes may be due to the presence of flavanoids and terpenoids found in the leaf extract[25,26]. Total and differential leucocyte counts are important indices of non-specific defence activities in fish[27] as leucocytes are centrally involved in phagocytic and immune responses to parasitic, bacterial, viral and similar challenges[28].

CONCLUSION

From the present study it was observed that dietary incorporation of *Phyllanthus amarus* in fish feed elevates the TEC, Hb, TLC & Lymphocytes and steady maintenance of Monocytes and Neutrophils. Anaemia can be correlated with sickness, whereas adequate population of TEC and Hb can be correlated with healthy state. Thus an increased level of TEC and Hb is an indication of healthy state. Similarly increased levels of TLC in *Phyllanthus* fed diet is an indicator of improved general health as both non-specific and specific immune response depends upon the population of leucocytes. Maintenance of steady population of neutrophils and monocytes is vital not only in resisting general infection but also to induce and elevate the specific immunity by increasing the lymphocytes. Thus from the present study it was evident that incorporation of *Phyllanthus amarus* in fish feeds helps in improving the general health and immunity.

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