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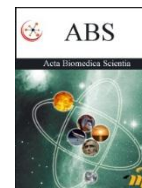

COMPARATIVE STUDY OF METHANOLIC LEAF EXTRACTS OF *Azadirachta indica* (Juss.) AND *Eichhornia crassipes* (Mart.) Solms ON SPECIFIC AND NON SPECIFIC IMMUNE RESPONSES IN *Labeo rohita.*(Ham)

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ABSTRACT

In Immune stimulant studies, herbal drugs are playing an important role in world wide fish care programmes and there is resurgence of interest in herbal medicines for treatment of various ailments of fish diseases. The present study was an attempt to identify the possible immunostimulatory activities of the medicinal plants such as *Azadirachta indica (*Neem*)* and *Eichhornia crassipes* (Water hyacinth) which can be applied to aquaculture industry for the maintenance of health in the cultivable fishes. The Crude extracts of both *A. indica* and *E. crassipes* exhibited specific and non-specific responses on *Labeo rohita* fishes. Among the two plants *A. indica* showed more immunostimulatory activities than *E. crassipes*.

INTRODUCTION

Aquaculture represents one of the fast growing food producing sectors of the world and aims to enhance the productivity per unit space. Among various kinds of cultivated organisms, fresh water fishes give high yield for aqua culturist which is namely *Labeo rohita* (Rohu). It is a freshwater fish of the carp family Cyprinidae and can be found throughout South Asia and South-East Asia in the weedy, slow flowing or standing waters of lakes and rivers. However, intensive fish stocking in ponds affects the health of fish and large scale mortalities of fish occurs due to infectious microbial and parasitic diseases caused due to high dense culture or by pollution mediated environmental stress. Protecting the fish from diseases can be done by through two ways. One is by strengthening the self-immune power of the organism and the second is through Medication [1]. Immunostimulants are substances which enhance the humoral and cellular immune response both in specific and non-specific way [2]. The use of plants as a productive system for immunostimulators facilitates a new and safe method of immunologically active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oils which can enhance immune function. Recently there has been an increased interest in the immune stimulating function of some herbs in aquaculture. These natural plant products have various activities like anti-stress, antioxidant, antimicrobials and immunostimulants.

The present communication reports on the immunostimulant activity of both plants used in the medicine as tonics for invigorating health and for a variety of ailments. At the beginning of human civilization the plant and plant products are used to treating bacterial diseases in aquaculture [3]. Hence, the main objective of the present study is to improve the immune power of

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Labeo rohita by using both plant leaf extract as immunostimulants. The haematological, immunological and enzymatic studies were conducted on the medicated fish infected with *P. fluorescens* pathogen.

MATERIAL AND METHODS

Collection of the experimental plants

The plant species of *A. indica* and *E. crassipes* were collected from Saliyamangalam, Thanjavur region of Tamil Nadu, India in June, 2013. The plant species were botanically identified.

Preparation of plant leaves extracts

The test plants leaves were cut into pieces and shade dried at room temperature. The dried leaves were subjected to size reduction to a coarse powder by using dry grinder and passed through sieve. 100 g of crushed leaves were continuously extracted with 95% methanol using soxhlet up to 48 h. The extract was filtered and concentrated in rotatory evaporator at 35-40 ºC under reduced pressure to obtain a semisolid material, which was then lyophilized to get a powder (28.5%, w/v).

Phytochemical analysis

The Methanolic leaves extract of *A. indica and E.crassipes* subjected to following test for the identification of its various active constitutions by standard method. Alkaloids were identified by Dragendroff's test, flavonoids and were identified by lead acetate test, carbohydrates were identified by Fehling's test, proteins were identified by Million's test, phenols were identified by Libermann's test and tannins were identified by Ferric chloride test. Saponins, Phytosterolterpenoids and Phlobatannins were identified by Harborne method.

Sample collection and clinical examination

P.fluorescens infected skin of *L. rohita* fish samples were collected through sterile container and it was grinded with help of mortar and pestle, then centrifuged at 2,000 ×g (10 min), the supernatant was removed, and deposit was dissolved in 1 ml of PBS. A portion (50 µl) of the concentrate was inoculated in to the nutrient agar medium containing petridishes and incubated at 37°C for 24 h.

Growth and heat killing of *P. fluorescens*

*P. fluorescens*was cultured on Nutrient agar (Himedia) and harvested in Nutrient broth (Himedia). The broth was incubated overnight in a shaker for 12 h and centrifuged at 10,000 rpm for 20 min. The bacterial pellet was washed with milli-Q water thrice and kept in boiling water bath for 15 min at 80º C.

Route of administration of *P. fluorescens* to *L. rohita*

The most effective of administration of immunostimulants to fish by injection [4]. The fish was administrated with heat killed bacteria as an antigen to fish by injection through the intraperitoneal route. After 7 days of incubation, the plant extract was administrated to delineate the dose response relationship in immunomodulation. After three days of plant extract administration, the booster dose was given to stimulate the immune system of fish.

Antiserun collection

The blood collected from immunized fish was kept at room temperature for 15 minutes. The clot was freed from the wall of serology tube for efficient retraction and was kept overnight at 4oC.The serum was separated by spinning down the clot at 3000 rpm for 15to20 minutes and collected in sterilized storage vials. The serum was kept at47oC in a water bath for 30 minutes to inactivate complement (classical pathway) and stored at 200C until further use.

ANTIBODY TITRATION

Bacterial agglutination assay

Antibody titration was performed in 96 well "v" bottom microtitre plates.25µl of serum was added to the first well and two fold serial dilutions were made with PBS (0.85%). A volume of 25µl of heat killed bacterial cell suspension (108cells/ml) prestained with crystal violet were added to each well. The microtitre plate was hand shaken for effective mixing and incubated for overnight at 370c. The highest dilution of the serum sample which showed detectable macroscopic agglutination was recorded and expressed as log2 antibody titre of the serum [5].

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by reduction of ferricytochrome using Secombes, method [6].

Activity of acid and alkaline phosphatases

Both acid and alkaline phosphatase activities were determined following the method of [7] using p-nitrophenyl phosphate (PNPP) as substrate. These enzyme activities were measured against blank at 420 nm in UV-VIS spectrophotometer (*Synergy HT*) and compared from a standard curve drawn from serial dilution of 1 mM p-nitrophenol (PNP) solution.

Activity of Catalase

Catalase activity in supernatant was determined according to the method of [8] by monitoring the initial rate of disappearance of Hydrogen peroxide at 240 nm in UV (*SynergyHT*) -visible Spectrophotometer. Results were reported as rate constant per second (k) per milligram protein (i.e. k/ mg protein).

Hematological Analysis

The blood was collected into vacuum tubes

containing heparin as anticoagulant (Greiner). The levels of RBCs and WBCs were counted by hemocytometer; Hb concentrations were estimated by Cyanomethaemoglobin method [9]

Determination of total protein

The protein concentrations of enzyme samples were determined by using Lowary method [10].

Statistical Analysis

All the results are presented as mean ± SEM data were analysed by the standard deviation method with help of SPSS software. Results were considered statistically at P<0.001.

RESULTS

Phytochemical analysis of *A. indica* and *E. crassipes*

The qualitative phytochemical analysis of methanolic extracts in both plant extracts revealed the presence of alkaloids, flavonoids, carbohydrates, proteins, saponins, phenols, terpenoids, phytosterols.

Effects of *A. indica* and *E. crassipes* on Bacterial agglutination assay

The response of antibody elevation with reference to challenge of heat killed *P. fluorescens* challenge was significantly increased on 10th day and decreased later till the end of the treatment (30 days) in the positive control (PC) than negative control (NC). After seven days of exposure of heat killed pathogen, the administration of *A. indica* and *E. crassipes* to treatment groups T1, T2 and T3 (10, 20 and 30 ppm respectively) was done and showed an drastic increase as 30ppm > 20ppm > 10ppm in the antibody response on 10th day. The level of antibody decreases on 20th and 30th day of treatment groups of test plants (Table1).

Effect of *A. indica* and *E. crassipes* on respiratory Burst Activity

The effect of methanolic extracts of both plants leaves in relation to the concentrations on respiratory burts activities reveals that the respiratory burst activity was enhanced on 20th day rather than 10th and 30th day in, *Labeo rohita*. 20ppm concentration of methanolic extract significantly enhanced the respiratory burst activity than positive control. In contrast, the administration of *A. indica* and *E. crassipes* to treatment groups T1, T2 and T3 (10, 20 and 30 ppm respectively) decreased the respiratory burst activity (Table 1).

Acid and alkaline phosphatase

Acid and alkaline phosphatase activity was significantly decreased during 30th day of treatment (T1, T2 and T3) in *A. indica* and *E. crassipes* treated groups when compared to the 10 and 20 days. Among the two plants, *A. indica* was more effective in immunostimulants and antioxidant activity than *E. crassipes* (Table 2)

Catalase: The catalase level was increased in the higher concentration T3 group when compared to other two (T1 and T2) on 10th day of both methanolic plant extracts of *A. indica* and *E. crassipes*. Administration of *A. indica* showed excellent catalase activity than *E. crassipes* (Table 2).

Haematological parameters

At the end of the experiment, total RBC and WBC were counted. They were significantly increased in T3 when to compared to others (T1 and T2) and control. Serum protein and haemoglobin also increased in 30 ppm concentration when compared to others (T1 and T2) and control. Finally, the results exhibited that *A. indica* plant extracts significantly increased the RBC, WBC and platelets counts than *E. crassipes* treated groups (Table3).

Table 1. Activity of Bacterial Agglutination Assay and Respiratory Burst Activity in blood serum of infected fish *Labeorohita*

Concentration / days	10 ppm	20 ppm	30 ppm
Positive control (PC)	2.107±0.107	2.214±0.107	1.968±0.213
	0.305±0.000	0.691±0.002	0.453±0.005
Negative control (NC)	1.723±0.138	2.000±0.000	1.528±0.290
	0.302±0.003	0.660±0.002	0.412±0.005
*A. indica*10ppm (AT1)	2.409±0.087	2.302±0.169	2.194±0.194
	0.355±0.106	0.718±0.003	0.572±0.003
*A. indica*20ppm (AT2)	2.733±0.074	2.547±0.226	2.302±0.169
	0.360±0.001	0.777±0.002	0.608±0.009
*A. indica*30ppm (AT3)	2.871±0.064	2.709±0.201	2.483±0.161
	0.374±0.001	0.756±0.002	0.504±0.005
*E. crassipes*10ppm (ET1)	2.584±0.000	2.302±0.169	2.000±0.000
	0.359±0.001	0.718±0.002	0.459±0.004
*E. crassipes*20ppm (ET2)	2.635±0.197	2.483±0.161	2.194±0.194
	0.345±0.001	0.784±0.002	0.589±0.004
*E. crassipes*30ppm (ET3)	2.867±0.067	2.571±0.140	2.214±0.107
	0.347±0.001	0.760±0.002	0.615±0.004

Table 2. Activity of Acid and Alkaline Phosphatase and catalase in the blood serum of infected fish *Labeo rohita*

Concentration / days	10 ppm	20 ppm	30 ppm
Positive control (PC)	1.881±0.055	1.053±0.013	0.623±0.012
	2.076±0.155	1.004±0.115	0.463±0.009
	0.002±0.007	0.020±0.004	0.030±0.004
Negative control (NC)	2.869±0.023	1.023±0.053	0.550±0.008
	2.088±0.071	0.911±0.049	0.481±0.006
	0.054±0.002	0.042±0.003	0.044±0.022
*A. indica*10ppm (AT1)	2.212±0.113	1.623±0.080	0.608±0.008
	2.245±0.161	1.103±0.020	0.426±0.017
	0.097±0.042	0.077±0.027	0.072±0.022
*A. indica*20ppm (AT2)	2.309±0.096	1.192±0.017	0.628±0.012
	2.684±0.219	1.275±0.228	0.455±0.009
	0.162±0.018	0.128±0.012	0.125±0.012
*A. indica*30ppm (AT3)	2.212±0.069	1.280±0.066	0.628±0.012
	2.812±0.379	1.050±0.023	0.418±0.010
	0.246±0.035	0.171±0.018	0.148±0.016
*E. crassipes*10ppm (ET1)	2.161±0.148	1.148±0.042	0.632±0.017
	2.087±0.054	1.235±0.103	0.452±0.009
	0.137±0.049	0.096±0.030	0.115±0.033
*E. crassipes*20ppm (ET2)	2.480±0.131	1.001±0.041	0.623±0.012
	2.291±0.107	1.070±0.181	0.481±0.006
	0.155±0.038	0.123±0.029	0.163±0.038
*E. crassipes*30ppm (ET3)	2.746±0.130	1.463±0.067	0.613±0.017
	2.815±0.169	1.193±0.080	0.500±0.016
	0.279±0.074	0.214±0.056	0.193±0.051

Table 3. Activity of Haematological parameters in the blood of infected fish *Labeorohita*

Concentration / days	RBC cells/ml	WBC cells/ml	Haemoglobin (gms %)	Serum protein (gms %)
Positive control (PC)	5000	3500	1.7	4.0
Negative control (NC)	3000	2500	1.1	3.0
*A. indica*10ppm (AT1)	7000	4200	2.3	4.8
*A. indica*20ppm (AT2)	5000	3200	1.7	4.2
*A. indica*30ppm (AT3)	9000	5200	2.8	5.1
*E. crassipes*10ppm (ET1)	3000	2500	1.1	3.7
*E. crassipes*20ppm (ET2)	5000	3100	1.7	4.1
*E. crassipes*30ppm (ET3)	7000	4000	2.3	4.6

Values are expressed as mean ± SE

DISCUSSION

Herbal product have a potential application as an immunostimulant in fish culture, primarily because they can be easily obtained, are not expensive and act against a broad spectrum of pathogens [11]. The World Health Organization estimated that about 80 per cent of the world's aqua culturist still relies on plant-based medicines for their primary health care [12]. Nowadays nearly 88% of the global populations turn to plant derived medicines as their first line of defence for maintaining health and combating fish diseases [13]. The use of such plant products as immunostimulants in fish culture systems may also be of environmental value because of their biodegradability. Due to their beneficiary attributes the present study concentrate on herbal extracts can be used in fish culture as alternatives to vaccines, antibiotics or chemotherapeutic agents. Immunostimulants seems to be a valuable for the control of fish diseases and can be useful in fish culture. The application of plant-derived immunostimulants in aquaculture for the prevention of diseases is a promising new development [14].

Agglutination is an important technique in diagnosis for specific bacterial antigens. The effects of Gram-negative bacteria are usually deleterious in chronic infections as compared to acute cases [15]. The existence of natural antibodies in fish has long been known, the exact role of these pre-existing, IgM like molecules is not clear, although it has been proposed that they are involved in trapping of pathogens, clearance of bacteria or damaged self-components and first line of resistance to

infection[16]. In our present experiment, specific immunity measured by antibody response heat killed *P. fluorescens* during all experimental period; the antibody immune response was higher in 30 ppm concentrated groups than 10 ppm, 20 ppm and control. In a recent study, [17]observed that a significant negative correlation of survival to erythroderma with bacterial agglutinin and haemagglutinin titres Within aquaculture, there are many studies reporting herbal medicine extracts can be used as immune-stimulants to enhance non-specific immune system of cultured fish species[18].In our present experiment The response of antibody elevation against heat killed *P. fluorescens* showed an drastic increase as 30ppm > 20ppm > 10ppm in the antibody response on 10th day. The level of antibody decreases on 20th and 30th day of treatment groups of test plants (Table 1)

The respiratory burst (NBT) activity can be quantified by the nitroblue tetrazolium (NBT) assay which measures the quantity of intracellular superoxide radicals produced by leukocytes [19]. Similarly, in the present study, a significant increase was observed in respiratory burst (NBT) activity in all experimental groups after treated the fish with plant extract. The respiratory burst activity, there are many studies reporting herbal medicine extracts can be used as immune-stimulants to enhance the super dioxide of cultured fish species [20].

Phosphatase enzyme is considered a member of lyzosomal enzyme and is widely considered a valuable parameter of macrophage activation. The results of the acid and alkaline phosphatase activities indicate that the fish, *Cyprinus carpio* fed with feed having leaf extract of *Euphorbia hirta* showed significant enhancement in the phosphatase activity when compared with the control fish. The enhancement of serum phosphatase activity in fish may caused by the increased production of enzyme by the macrophage cells. [21] reported that the lipopolysaccharide (LPS) stimulated the macrophage cells for the higher enhancement of acid phosphatase, when compared to the control macrophage cells. [22] reported that *Achyranthes aspera* enhanced the serum alkaline phosphatase activity in *Labeo rohita*. In the present study. Acid and alkaline phosphatase activity was significantly increased in the *A. indica* and *E. crassipes* treated groups .Among the two plants, *A. indica* was more effective in immunostimulants and antioxidant activity than *E. crassipes.*

Haematological parameters have been recognized as valuable tools for monitoring fish health. Knowledge of the haematological characteristics is an important parameter that can be used as an effective and sensitive index to monitor physiological and pathological changes in fishes [23]. The analysis of blood indices has proven to be a valuable approach for analysing the health status of farmed animals as these indices provide reliable information on metabolic disorders, deficiencies and chronic stress status before they are present in a clinical setting [24]. Blood biochemistry parameters including erythrocyte count, leucocyte, and haemoglobin concentration count has provided valuable information for fishery biologists in the assessment of fish health [25].[26] indicated that total and differential WBC counts are important indices of non-specific defense activities in fish. Also, they are centrally involved in phagocytic and immune responses to bacterial, viral and parasitic challenges The present study indicates that Methanolic leaves extract of *A. indica* and *E. crassipes* showed increased, RBC,WBC and haemoglobin, percentage in comparison to the control group ($p<0.05$). The haematological results of the present study reveal that the both leaf extract was able to reduce the immunosuppression caused by the pathogen through increasing the haematological response.

Certain herbal immunostimulants have been reported to enhance the total protein as well as total globulin in fish [26]. The serum protein level is an important indicator of humoral defence system. [27]In this study, a significant increase was seen in total immunoglobulin levels of experimental fish groups. Serum protein was increased in 30 ppm concentration in both test plants. Among the two test plants, *A. indica* is more effective to increase the total protein as well as total globulin in fish the activities when compared to *E. crassipes*. This research work was the first report in the *A. indica* and *E. crassipes* which shows highest efficacy of immunomodulation in fish, *L. rohita* leads to the benefits of animal wealth and aquaculturist.

CONCLUSION

Finally, the present study suggested that, among the two plants, *A. indica* have significant activity than *E. crassipes*. These Findings Provide enough Scientific evidence to Support traditional medicinal uses and indicate a promising potential for the development of immunostimulative and antioxidant agents from these plants. So these plants can be used to discover bioactive natural products that may serve as feeds for the development of new pharmaceuticals. Development of immunostimulants from these plants leaves is relatively inexpensive and less time consuming moreover, it is suitable to our economic conditions. Further, this work suggests that it was a model experiment to recommend the *A. indica* and *E. crassipes* have potent herbal immunostimulants for the benefits of aquaculture.

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