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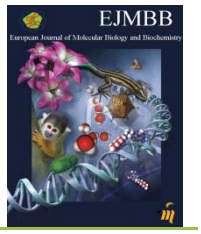

IMMUNOSTIMULANT EFFECT OF ON *Eichhornia crassipes* (MART.) SOLMS AGAINST *P. fluorescens* INFECTED INDIAN MAJOR CARP *Labeo rohita* (HAM.)

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ABSTRACT
Fish is one of the cheapest and promising sources of animal protein .People can easily digest 93.2% and 93.7% of fish protein and fat respectively .Now a days, the bacterial infection acts as an important limiting factor for fish culture. Especially, the infection of *Pseudomonas fluorescens* bacterium has lead to great economic loss for fish culture business. The uncontrolled and repeated use of antibiotics to treat bacterial infection lead to development of antibiotic-resistant pathogens. To overcome this problem, antibiotics from plants resources cannot produce resistant bacterium and it can be frequently used to control fish diseases. Medicinal plants are used for the formation of drugs and these plants are used traditionally to cure various diseases. These medicinal plants contain some phytochemical active compounds such as flavonoids, alkaloids, tannins, and phenols etc., which shows Immunostimulant activity against the pathogens. Hence, the present study investigated the immuno stimulatory effect of *Eichhornia crassipes* against *P. fluorescens* .The plant leaves extract *E. crassipes* treated fish group showed no mortality. The bacterial agglutination assay of the plant extract treated fish exhibited earlier and more bacterial agglutination and the respiratory burst activity was also high. Both acid and alkaline phosphatase was reduced at the end of the experiment and the catalase was increased. Heamatological parameters were also elevated. It is concluded that 30 ppm of *E. crassipes* plant leaves extract is more effective to defend *P. fluorescens*. Thus *E. crassipes* acts as an immunostimulant to enhance the activity of fish.

INTRODUCTION

Aquaculture is a rapid developing industry. However, unmanaged fish culture practices and adverse environmental conditions affect the fish health leading to production losses [1]. Diseases are major bottlenecks in the development and sustainability of aquaculture practices throughout the world [2]. The present trend of intensification in aquaculture is a major concern for the outbreak of disease in fishes are more prone to stress and subsequent infection by pathogen. Fish diseases are great threat to economic viability of any aquaculture practices [3]. The herbs are used not only against diseases but also as growth promoters, stress resistance boosters and preventatives of infections. Plants are rich in a wide variety of secondary metabolites of phytochemical constituents such as tannins, alkaloids and flavonoids, which act against different diseases [4,5]. Extracts of some plants can boost the humoral [6] and cell mediated immunity [7] against viruses [8], bacteria [9], Fungi, Protozoa and cancer [10]. Herbs can also act as immunostimulants, conferring the non-specific defence mechanisms of fish and elevating the

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specific immune response. Natural immunostimulant are biocompatible, biodegradable and safe for the environment and human health. Now a days, disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance. The use of natural immunostimulants in fish culture helps to prevent the diseases and amplify the specific immune response which plays an essential role in protection against fish diseases. Therefore, instead of antibiotics and chemotherapeutic agents, increasing attention is being paid to the use of immunostimulants for disease control measures in aquaculture. The immunostimulants mainly facilitate the function of phagocyte cells, increase their bactericidal activities and stimulate the natural killer cells, complement system, lysozyme activity and antibody responses in fish which confer enhanced protection from infectious diseases.

Pseudomonas fluorescens has been associated with diseases in fishes like carp, eel, catfish, tilapia and opportunist in stress related diseases in salmonids [11]. *Pseudomonas fluorescens* is a gram negative rod shaped bacteria. It causes Erythroderma, Inflammation and bleeding from the skin, loss of scales, necrosis of the terminal fins and red blotches around the upper and lower jaws. *P.fluorescens* is a major pathogen which affects the growth and causes severe diseases in Indian major carps. Hence, the present study was carried out to test the immunostimulant effects of *Eichhornia crassipes* on indian major carp *Labeo rohita* against *P. fluorescens.*

MATERIALS AND METHODS

Plant materials

The plant species of *Eichhornia crassipes* were collected from Saliyamangalam, Thanjavur region of Tamil Nadu, India and identified to confirm by the Taxonomist, Botanical Survey of India, Tamilnadu, India.

Plant sample extraction

The leaves were cut into pieces and shadow 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 *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, Phytosterol terpenoids and Phlobatannins were identified by Harborne method

Sample collection and clinical examination

Bacterial infected skin of *L. rohita* fish samples were collected through sterile container and it was grinded with help of morter and pistle, 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.

16s rDNA sequences and Phylogenetic analysis of *P. fluorescens*

Genomic DNA was isolated from the Bacterial infected skin of L. rohita fish culture using DNeasy® Blood and Tissue Kit and it was amplified with help of 16S r DNA primers such as forward primer (C GAATTCGTCGACAACAGAGTTTGATCCTGGCTCA) and Reverse Primer (CCCGGGATCC AAGC TTA CGGCTACCTTGTTACGACTT) by using PCR machine The PCR products were checked using agarose gel electrophoresis and it was sequenced in sequencer (Applied Biosystems, USA). The 16s rDNA sequences submitted to NCBI (JQ247720). The retrieved sequences were fed with ClustalW for multiple sequence alignment to observe sequence homology. Evolutionary tree was inferred by using the neighbour-joining method [12]. Dendrogram was constructed by neighbour-joining method using PHYLIP software package.

Growth and heat killing of *P. fluorescens*

P. fluorescens was seeded 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 .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.

Estimation of RBC and WBC Count

Red blood corpuscles were counted using haemacytometer. Total number of white blood corpuscles were counted and expressed in thousand per cubic milli meter of blood [13].

Estimation of haemoglobin

Haemoglobin content of the blood was estimated by Shali's acid haematin method using Shali's haemometer. Haemoglobin is converted into acid haematin the colour of which is compared with the colour of the standard haematin. Haemoglobin value was recorded and was expressed in gms %.

Bacterial agglutination assay

For detecting the effect of Plant extract on the antibody response by bacterial agglutination assay which was developed by Karunasagar *et al* [14]. Briefly, 50 µl of serum was added to the first well and twofold serial dilutions were made with PBS. A volume of 50 µl of heat killed *P. fluorescens* cell suspension was added to the plate which was incubated at 37 °C for 1 h. The highest dilution of serum sample that showed detectable macroscopic agglutination was recorded and expressed as log2 antibody titre of the serum.

Respiratory Burst Activity of *Labeo rohita*

The blood samples were used for determining respiratory burst activity (RBA) by Nitroblue Tetrazolium (NBT, Sigma) assay following the method of [15].

Determination of Total Protein

The protein concentrations of enzyme samples were determined by using the standard method of Lowry *et al*).

Activity of acid and alkaline phosphatases

Both acid and alkaline phosphatase activities were determined using the method of [16] 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 [17] 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).

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 *E. crassipes*

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

16S r DNA sequencing analysis

After the determination of colony colour, the morphological, physiological and biochemical characteristics, the isolate selected by the PCR analysis was tentatively identified as *P. fluorescens* and it was confirmed by the 16S r DNA sequencing with help of universal bacterial primers, 16s-UP-F and16s-UP-R. Sequence of the *P. fluorescens* isolate showed partial 16S r DNA sequences, consisting of 1441 nucleotides which was submitted to the GenBank (National Center for Biotechnology Information, USA) and an Accession Number (JQ247720) was obtained. Phylogenetic tree was deduced from species of *P. Fluorescens* using Neighbour-joining method. The sequences showed 100% similarity with the already established the species *P. fluorescens.* From this study, the isolate was confirmed as the isolate of the species *P. fluorescens.*

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 *E. crassipes* plant extracts significantly increase the RBC, WBC and platelets counts treated groups. (Table 1).

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

The response of antibody elevation with reference to challenge of heat killed *P. fluorescens* challenge was significantly increased on 10^{th} 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 *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 plant. (Table 2).

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

The effect of methanolic extracts of both plants leaves in relation to the concentrations on respiratory burst activities reaveals that the respiratory burst activity was enhanced on 20th day rather than 10th and 30th day in rohu, *Labeo rohita*. 20ppm concentration of aqueous and methanolic extract significantly enhanced the respiratory burst activity than positive control. In contrast, the administration to treatment groups T1, T2 and T3 (10, 20 and 30 ppm respectively) decreased the respiratory burst activity. (Table 2).

Acid and alkaline phosphatase

Acid and alkaline phosphatase activity was significantly decreased during 30th day of treatment (T1, T2 and T3) in *E. crassipes* treated groups when compared to the 10 and 20 days. The plant, *E. crassipes* was more effective in immunostimulants and antioxidant activity (Table 3).

Catalase

The catalase level was increased in the higher concentration T3 group when compared to other two (T1 and T2) on 10th day methanolic plant extracts of *E. crassipes* showed excellent catalase activity (Table 3).

Table 1. Activity of Haematological parameters in the blood serum of infected fish *Labeo rohita*

Haematological parameters				
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
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

Table 2. Activity of Bacterial Agglutination and Respiratory Burst Activity Assay in blood serum of infected fish *Labeo rohita*

Bacterial Agglutination Activity Assay			
Concentration / days	**10 ppm**	**20 ppm**	**30 ppm**
Positive control (PC)	2.107±0.107	2.214±0.107	1.968±0.213
Negative control (NC)	1.723±0.138	2.000±0.000	1.528±0.290
E. crassipes 10ppm (ET1)	2.584±0.000	2.302±0.169	2.000±0.000
E. crassipes 20ppm (ET2)	2.635±0.197	2.483±0.161	2.194±0.194
E. crassipes 30ppm (ET3)	2.867±0.067	2.571±0.140	2.214±0.107
Respiratory Burst Activity Assay			
Concentration / days	**10 ppm**	**20 ppm**	**30 ppm**
Positive control (PC)	0.305±0.000	0.691±0.002	0.453±0.005
Negative control (NC)	0.302±0.003	0.660±0.002	0.412±0.005
E. crassipes 10ppm (ET1)	0.359±0.001	0.718±0.002	0.459±0.004
E. crassipes 20ppm (ET2)	0.345±0.001	0.784±0.002	0.589±0.004
E. crassipes 30ppm (ET3)	0.347±0.001	0.760±0.002	0.615±0.004

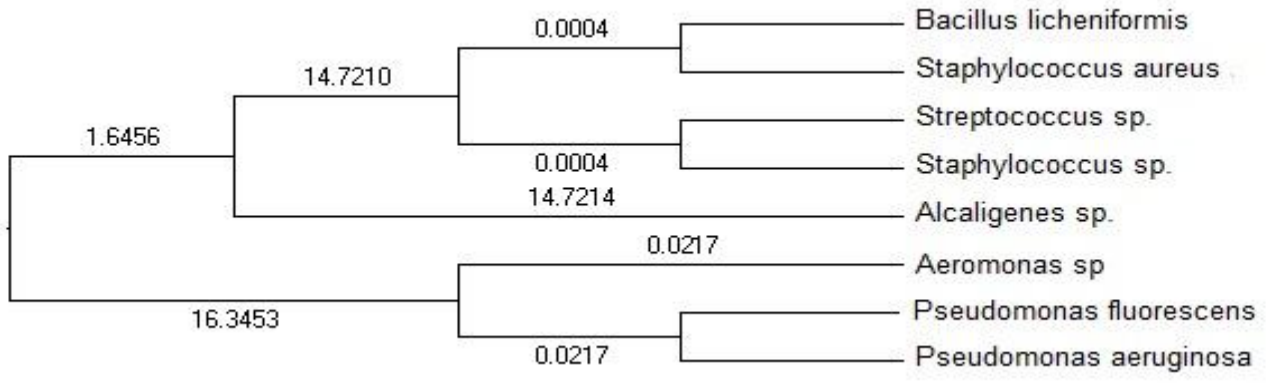
Values are expressed as mean ± SE

Table 3. 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**
Acid Phosphatase/ Alkaline Phosphatase/Catalase			
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
	0.022±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±0.002
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

Values are expressed as mean ± SE

Figure 1. Phylogenetic tree of *P. fluorescens*



DISCUSSION

In the recent years, herbs and herbal products plays significant role in immunostimulatory effect on fish culture research [18]. In aquaculture, the usage of heavy antibiotic needs to be reduced and alternatively the herbal plants used as potential and promising source of pharmaceutical agents against fish pathogens [19]. The application of medicinal herbs as immunostimulants can elevate the innate defense mechanisms of fish against pathogens during periods of stress, such as, intensive farming practices, grading, sea transfer, vaccination and reproduction. Hematological assays may provide an index of the physiological status of fish. RBC count, WBC count, and hemoglobin are particularly recommended as tests that could be performed on a routine basis in fish farms to monitor the health of the stock. The present study indicates that Methanolic leaves extract of *E. crassipes* showed increased, RBC,WBC and haemoglobin, percentage in comparison to the control group ($p<0.05$). De Pedro et al [20] 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.

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 [21]. 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 [22]. 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, [23] observed that a significant negative correlation of survival to erythroderma with bacterial agglutinin and haemagglutinin titres. Therefore, antibody titration was found increased in the plant extract treated group than the control. Similar results were observed by [24]. 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 [25-29].

Phagocytosis and the respiratory burst response by phagocytes in blood and tissues present a major antibacterial defense mechanism in fish [30] Respiratory burst activity measured by NBT is one of the most important bactericidal mechanisms in fish [31]. Staining the neutrophils with the NBT dye helps to confirm their activity. The soluble NBT dye, taken in by pinocytosis into the neutrophils, is reduced to dark blue formazan granules. that are distinctive on microscopic examination. A variety of medicinal herbs are known to stimulate phagocyte cells including ginger, garlic, curcumin and turmeric (*Curcuma longa*), etc, [32-36].

In the present study, the respiratory burst activity of blood leukocytes was significantly higher than in the control group after 12 weeks ($p<0.05$). This result is consistent with data obtained by [32], who reported that extracellular and intracellular respiratory burst activity and phagocytic activity of leukocytes were enhanced by Methanolic leaves extract of *E. crassipes.* The results show that the plant extracts stimulated enzymatic activity and increased pathogen clearance, improving the survial rate of the infected fish. Further, These Findings suggests that it was a model experiment to recommend the Methanolic leaves extract of *E. crassipes* have potent herbal immunostimulants for the benefits of aquaculture.

CONCLUSION

Herbal extracts and its product have a potential application as an immunostimulant in fish culture, because they can be easily obtained, are not expensive and act against a broad spectrum of pathogens. Most of the herbs and herbal extracts can be given orally, which is the most convenient method of immunostimulation. However, the

effect is dose-dependent, and there is always a potential for overdosing consequently, dosage optimization is strongly recommended. 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, we conclude that herbal extracts product can be used in fish culture as alternatives to vaccines, antibiotics or chemotherapeutic agents.

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