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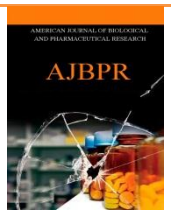

IMMUNOPROTECTIVE AND ANTIOXIDANT EFFECTS OF THE MEDICINAL PLANT *AZADIRACHTA INDICA* (JUSS.) ON INDIAN MAJOR CARP *LABEO ROHITA* (HAM.)

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ABSTRACT

Immunostimulatory can be used for fish disease prevention as it can enhance fish resistance by increasing the non specific defense mechanisms. The aim of this research was to examine the immunostimulatory effect of *Azadirachta indica* against *P. fluorescens*. .It contains alkaloids, flavonoids, carbohydrates, proteins, saponins, phenols, terpenoids and phytosterols. The plant leaves extract *A. indica* 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. Hematological parameters were also elevated. It is concluded that 30 ppm of *A. indica* plant leaves extract is more effective to defend *P. fluorescens*. Thus *A. indica* acts as an immunostimulant to enhance the activity of fish.

INTRODUCTION

Aquaculture represents one of the fast growing food producing sectors of the world and aims to increase productivity per unit space. Fish are palatable and proteinous food for human beings. India is now at the threshold of blue revolution and it has made a notable progress in the field of inland fisheries. Fishes not only play an important role in the demand of food for humans but they are widely used for various biological experiments [1]. The development of aquaculture industry worldwide is hindered by high mortality due to disease outbreaks. In order to combat this problem, antibiotics are rampantly used [2]. Such practices can cause pathogens, especially bacteria *P. fluorescens* which develop resistance against these antibiotics. In fact, the number of antibiotic resistant bacteria which are potentially pathogenic to fish has increased [3-7]. It has also noticed that these antibiotic-resistant bacteria can transfer their resistant genes to other bacteria which are capable of causing infection to animals and human [8]. Hence, the use non-chemotherapeutic methods for disease treatment in aquaculture are beneficial to the industry in the long run. These can include vaccine, probiotics, immunostimulants and natural therapeutics from plants [9]. In fact medicinal plants have already been used as remedy to many infectious diseases since the ancient time. According to Abutbul *et al*. [10,11], plants having antibacterial properties are potentially beneficial to aquaculture. Recently, a number of studies have been carried out to determine the immunostimulant properties of plants [12,13]. Many plants are known to have anti-stress, growth promoter, appetizing, tonic, immunostimulation,

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antimicrobials, antibacterial and aphrodisiac properties [14]. These properties, however, are linked to the presence of bioactive compounds such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and other essential oils [15-19]. Although many of the locally available medicinal plants have been investigated for their properties against human bacterial pathogens [20,21] few have been tested against aquaculture bacterial pathogens. Hence the present study focused on immunoprotective and antioxidant effects of the medicinal plant *Azadirachta indica* on indian major carp *Labeo rohita* against *P. fluorescens*

MATERIALS AND METHODS

Plant materials

The plant species of *A. indica* (Juss.) 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 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* 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

Pseudomonas fluorescens 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.

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 (Sakai, 1999). 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.

Bacterial agglutination assay

For detecting the effect of Plant extract on the antibody response by bacterial agglutination assay which was developed by[22]. 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 *Labeorohita*

The blood samples were used for determining respiratory burst activity (RBA) by nitrobluetetrazolium (NBT, Sigma) assay following the method of [23].

DETERMINATION OF TOTAL PROTEIN

The protein concentrations of enzyme samples were determined by using the standard method.

Activity of acid and alkaline phosphatases

Both acid and alkaline phosphatase activities were determined following the method of [24] 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 [25] 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).

Estimation of RBC and WBC Count

Red blood corpuscles were counted using haemocytometer. Total number of white blood corpuscles were counted and expressed in thousand per cubic .millimetre of blood [26].

Estimation of haemoglobin

Haemoglobin content of the blood was estimated by Shali's acid haematin method using Shali'shaemometer. 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 %.

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*

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

Effects of *A. indica*and 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*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 1).

Effect of *A. indica*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, *Labeorohita*. 20ppm concentration of aqueous and methanolic extract significantly enhanced the respiratory burst activity than positive control. In concontrast, the administration of *A. indica*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* treated groups when compared to the 10 and 20 days, the plant *A. indica* was more effective in immunostimulant and antioxidant activity (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 methanolic plant extracts of *A. indica* showed excellent catalase activity (Table 3).

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 increase the RBC, WBC and platelets counts treated groups. (Table 3).

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

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
*A. indica*10ppm (AT1)	2.409±0.087	2.302±0.169	2.194±0.194
*A. indica*20ppm (AT2)	2.733±0.074	2.547±0.226	2.302±0.169
*A. indica*30ppm (AT3)	2.871±0.064	2.709±0.201	2.483±0.161
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

*A. indica*10ppm (AT1)	0.355±0.106	0.718±0.003	0.572±0.003
*A. indica*20ppm (AT2)	0.360±0.001	0.777±0.002	0.608±0.009
*A. indica*30ppm (AT3)	0.374±0.001	0.756±0.002	0.504±0.005

Values are expressed as mean ± SE

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

Concentration / days	10 ppm	20 ppm	30 ppm
Acid Phosphatase/ Alkaline Phosphatase			
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
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
*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
*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
*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

Values are expressed as mean ± SE

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

Activity of catalase			
Concentration / days	**10 ppm**	**20 ppm**	**30 ppm**
Positive control (PC)	0.022±0.007	0.020±0.004	0.030±0.004
Negative control (NC)	0.054±0.002	0.042±0.003	0.044±0.002
A. indica 10ppm (AT1)	0.097±0.042	0.077±0.027	0.072±0.022
A. indica 20ppm (AT2)	0.162±0.018	0.128±0.012	0.125±0.012
A. indica 30ppm (AT3)	0.246±0.035	0.171±0.018	0.148±0.016

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
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

DISCUSSION

In Immune stimulant studies, herbal drugs are playing an important role in fish care programmes worldwide and there is resurgence of interest in herbal medicines for treatment of various ailments of fish diseases. 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 [27]. 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 [28]. Plants are rich in a wide variety of secondary metabolites such as tannins terpenoids, alkaloids, flavonoids, etc. which have been found in vitro to have medicinal properties. Pharmacological studies have accepted the value of medicinal plants as potential source of bioactive compounds. The plants have been used as source of medicine for all kind of fish diseases [29]. Over 50 per cent of all modern fish drugs are of natural product origin and natural products play an important role in drug development programmes in pharmaceutical industry [30]. Large numbers of plants belonging to different families have been studied for their therapeutic properties [31]. However, plants such as *A. indica* belonging to Meliaceae and which have many therapeutic properties, have not been studied for their photochemical constituents and pharmacological properties and hence the present study focused on those plants.

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 [32]. 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 [33]. In our present experiment, specific immunity measured by antibody response heat killed *P. fluorescens* . Duringall experimental period, the antibody immune response was higher in 30 ppm concentrated groups than 10 ppm, 20 ppm and control. In a recent study, [34] observed that a significant negative correlation of survival to erythroderma with bacterial agglutinin and haemagglutinintitres. Therefore, antibody titration was found increased in the plant extract treated group than the control. Similar results were observed by [35].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 [36-40].

The respiratory burst (NBT) activity can be quantified by the nitrobluetetrazolium (NBT) assay which measures the quantity of intracellular superoxide radicals produced by leukocytes [40]. 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. Measurements of enzyme activities thus could be a useful clinico-biological method for learning the course of the disease and certain enzymes may be of diagnostic value with some fish diseases [41]. This study further opens up research avenues to understand the complex nature of bacterial infections and effectual other physiological correlates.

During this short-term study, increased activity of this enzyme was observed in treated groups compared to control. In 10 days a highly significant difference ($P<0.01$) in mean both acid alkaline phosphatase activity was found among experimental groups at both exposure periods. However, an opposite trend was observed between control and treated groups during this period. Hematological characteristics, as a tool for screening pathological status, have shown to be good indicator of physiological responses [42].

Red Blood Cells, is composed mainly of haemoglobin surrounded by a flexible protein membrane and an outer lipid bilayer. And the energy required for the maintenance of red cell shape, flexibility and osmotic pressure is provided by adenosine triphosphate (ATP), generated by anaerobic glycolysis [43]. In this study, the increased percentage of neutrophils and monocytes and decrease percentage of lymphocyte in the circulating blood of *A. indica* agrees with the report of [44] in *Anguilla Anguilla* exposed to handling stress.

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. Further, this work suggests that it was a model experiment to recommend the *A. indica* have potent herbal immunostimulants for the benefits of aquaculture.

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