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RESEARCH PAPER

Effects of *Avicennia marina* extracts on *Labeo rohita* (Ham) challenged with *Pseudomonas fluorescens*

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Abstract The medicinal plant *Avicennia marina* was evaluated for their immunostimulatory activity on *Pseudomonas fluorescens* infested fish, *Labeo rohita*. The fish was dosed intraperitoneally at 10, 20 and 30 ppm concentrations of ethanolic leaves extract of *A. marina* and control. After 10, 20 and 30 days of treatments, the immunological, hematological and serum protein level of fish was assessed in control and treatments. All the concentration of plant leaves extract significantly enhanced the agglutination, hematological parameters and total serum protein on 30th days after treatment. The highest agglutination activity was observed in the group treated with 30 ppm concentration of *A. marina* on 20 days. The WBC, RBC and hemoglobin content was increased with increasing concentration of the treatments. The results, clearly indicates that *A. marina* leaves extract will be used as immunostimulatory agent to aquaculture for mass production of healthy fish.

Introduction

Fish provide a good protein source with some other nutrient for human health; hence, human beings consumed fish as a diet. Therefore, pressures on aquaculture industry to supply the fish for fast growing world populations by enhance the fish production. During the over production of fish in a pond, it may leads to microbial disease. It kills the entire population of fish in a pond (Elliott & Shotts Jr, 1980). In this connection, fisherist should take effort to maintain fish pond with hygiene for harvest good yield in order to obtain sustainable economic gains. The fish culture practice given an interest toward understanding fish diseases and sustainable aquaculture. When the fish is susceptible to bacterial infection; they were immunosuppressed conditions. Many fish farms were severely affect by bacterial diseases during culture period, this diseases may wash out entire population in the fish farm (Wang et al., 2015). Using antibiotics and other practices to control the bacterial diseases in aquaculture system were negative impact to fish and envi-

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ronment. Many vaccine used in fish farm for control bacterial diseases it was high cost, disease and pathogen specific and also, more income will be used for disease management (Robertsen, 1999). Therefore, the need for natural products has been rising with a particular focus on plant products to be used in aquaculture industry instead of antibiotics and vaccines. Recently, increasing attention has been given on the use of plants as immunostimulants for disease-control strategies in aquaculture (Talpur, Ikhwanuddin, & Bolong, 2013). The most important advantage of using plants as immunostimulants in aquaculture due to natural organic materials, they were safe to fish health and environment (Citarasu, 2010).

In India, herbs have been used for promotion of health, prevention and treatment of diseases. *Avicennia marina* (Forssk.) Vireh (Avicenniaceae) is a mangrove tree. Generally mangroves contain many chemical constituents with potential medicinal properties. The plant was used to cure ulcers and skin diseases (Bandaranayake, 1998; Kathiresan & Bingham, 2001). Organic compound from plants having antioxidant and immunostimulatory activities at low concentrations; they are very cost effective. It is biodegradable and environmental friendly (Logambal, Venkatalakshmi, & Micheal, 2000). Hence the present study was aimed to assess the effect of *A. marina* extracts on *Labeo rohita* (Ham) challenged with *P. fluorescens*.

Materials and methods

Plant materials

The *A. marina* was collected from Muthupet, Thiruvarur, Tamil Nadu, and was authenticated by Botanist at Department of Botany, St. Joseph's College, Tiruchirappalli and kept in herbarium (DK 001).

Plant sample extraction

The collected plant was washed and shade dried at room temperature. Then it was powdered by electric blender. At 100 g of powder was continuously extracted with ethanol using soxhlet apparatus. The extraction was done at 60 °C up to 6 h. The extract was stored at 4 °C until further use (Lee et al., 2017).

Sample collection

The collected *L. rohita* samples were grinded and centrifuged at 2000 × *g* (10 min) then the supernatant was dissolved in 1 ml of PBS, from that 50 μl was taken and inoculated in to nutrient agar medium; incubated at 37 °C for 48 h. The bacterial colony was identified as *P. fluorescens* (JQ247720) (Dineshkumar et al., 2014).

Growth and heat killing of *P. fluorescens*

The bacterial culture was maintained in agar and incubated in broth at over night. They were centrifuged at 10,000 rpm for a period of 20 min. The pellet was washed 3 times in milli-Q water and was kept in water bath for period of 15 min at 80 °C (Dineshkumar et al., 2014).

Route of administration of *P. fluorescens*

The *P. fluorescens* (1.5×10^4 cells/mL) was inserted in to fish by intra-peritoneally as antigen. Seven days after incubation, *A. marina* extract (100, 200 and 300 ppm/kg concentration) and control received 0.1 ml of distilled water were administrated for immunomodulation activity (Dineshkumar et al., 2014).

Bacterial agglutination assay

The antibody response, bacterial accumulation assay was performed (Karunasagar, Ali, & Otta, 1997). At 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 serum sample with highest dilution showed detectable macroscopic agglutination and expressed as $\log_2$ antibody titer of the serum.

Determination of serum protein

The protein concentration was estimated by the method of Bradford (1976).

Alkaline phosphatases

The alkaline phosphatase activities were determined by the method of Michell, Karnovsky, and Karnovsky (1970).

Hematology

The RBC and WBC were estimated by the method of Russia and Stood (1992). The hemoglobin (Hb) was estimated by Drabkin (1946).

Statistical analysis

The tabulated results were present as mean ± SD. All the values were subjected to one way ANOVA followed by Tukey's test.

Results

Bacterial agglutination assay

In the present study, *A. marina* induced the primary and secondary antibody production in fish after treated with of bacteria (*P. fluorescens*), when compared to control the bacterial accumulation was high (2.77) in 30 ppm concentration of *A. marina* on day 10, followed by 20 ppm concentration. When the day of exposure increased the bacterial accumulation was reduced in treatment. The lower value was recorded in fish infected with *P. fluorescens* (no treatment with plant extract) (Table 1). The reduction of bacterial

Table 1 Bacterial agglutination assay of *Avicennia marina* on blood serum, *Labeo rohita* post challenged with *Pseudomonas fluorescens*.

Concentration/days	Day after treatment		
	10	20	30
Control[+]	2.25 ± 0.08^{a}	2.24 ± 0.10^{a}	2.35 ± 0.10^{b}
Infected with *P. fluorescens*[*]	2.13 ± 0.06^{a}	2.15 ± 0.02^{a}	1.98 ± 0.04^{a}
10 ppm	2.48 ± 0.08^{b}	2.64 ± 0.13^{b}	2.54 ± 0.15^{c}
20 ppm	2.66 ± 0.03^{c}	2.79 ± 0.11^{c}	2.55 ± 0.72^{c}
30 ppm	2.77 ± 0.21^{c}	2.90 ± 0.09^{c}	2.52 ± 0.09^{c}

Within each column, means ± SD followed by the same letters do not differ significantly (ANOVA, Tukey's HSD test, $P > 0.05$).
[+] No treatment with *Avicennia mari*.
[*] No treatment with *P. fluorescens* and *Avicennia mari*.

Table 2 Alkaline phosphatase (IU/L) activity of *Avicennia marina* on *Labeo rohita* post challenged with *Pseudomonas fluorescens*.

Concentration/days	Day after treatment		
	10	20	30
Control[+]	0.230 ± 0.02^{b}	0.276 ± 0.011^{b}	0.294 ± 0.23^{b}
Infected with *P. fluorescens*[*]	0.207 ± 0.01^{a}	0.201 ± 0.014^{a}	0.206 ± 0.01^{a}
10 ppm	0.307 ± 0.01^{c}	0.340 ± 0.01^{c}	0.357 ± 0.01^{c}
20 ppm	0.327 ± 0.01^{d}	0.338 ± 0.01^{c}	0.347 ± 0.01^{c}
30 ppm	0.340 ± 0.01^{d}	0.367 ± 0.02^{d}	0.383 ± 0.01^{d}

Within each column, means ± SD followed by the same letters do not differ significantly (ANOVA, Tukey's HSD test, $P > 0.05$).
[+] No treatment with *Avicennia mari*.
[*] No treatment with *P. fluorescens* and *Avicennia mari*.

agglutination due to the treatment nullifies the bacterial activity.

Alkaline phosphatase

Table 2 shows alkaline phosphatase activity of fish after treatment of ethanol extract of *A. marina*. Alkaline phosphatase activity was significantly higher in 30 ppm treatment when compared to other treatment and fish infected with *P. fluorescens* (no treatment with plant extract). In all the day of observation, at 30 ppm treatment showed maximum activity when compared to other treatments and controls.

Serum protein

The bacterial infested fish was treated with *A. marina*. It induced the serum protein, the increasing concentration of the plant extract and duration of exposure the serum protein was increased. At 30 ppm concentration was statistically differ from other treatments and control's (Table 3). The maximum protein content of 2.556 g/dL was recorded in 30 ppm concentration of the treatments.

Hematological parameters

All the concentration of *A. marina* induced the RBC count when compared to control and fish infected with *P. fluorescens* (no treatment with plant extract), in case, the fish infected with *P. fluorescens* (no treatment with plant extract), the duration increased the RBC count was decreased. At end of the observation, maximum RBC count of 2.001 million cells/mm^3 was recorded at 30 ppm concentration which was statistically differ from other treatments and control (Table 4). Table 5 shows post WBC of bacteria infested and post challenged fish. After treatment with *A. marina*, the extracts induced the WBC count of fish when compared to control. The maximum count was recorded at 30 ppm concentration on all the day of observations. The untreated RBC count was declined when day of the exposure increased. The maximum hemoglobin content was recorded at 30 ppm concentration of all the treatments. In all the day observation at 30 ppm concentration of *A. marina* treatment was statistically differ from control (Table 6).

Discussion

Many of the methods are applied in immunostimulants activity in fish i.e., injection, oral administration or dietary and contact (in the medium) can be applied to stimulate the fish from disease resistance. In fisheries many plant materials used as disease managing agents.

In the present study, *A. marina* exhibited the bacterial agglutination activity in *L. rohita* post challenged with *P. fluorescens*. When concentration was increased the activity was increased also found that the activity was high in 20 day after observation and was similar on to control on day 30. The similar finding was observed by Aathi,

Table 3 Effect of *Avicennia marina* on *Labeo rohita* (serum protein g/dl) post challenged with *Pseudomonas fluorescens*.

Concentration/days	Day after treatment		
	10	20	30
Control[+]	2.304 ± 0.11^{b}	2.325 ± 0.05^{b}	2.345 ± 0.06^{b}
Infected with *P. fluorescens*[*]	2.155 ± 0.05^{a}	1.975 ± 0.14^{a}	1.812 ± 0.06^{a}
10 ppm	2.325 ± 0.06^{bc}	2.382 ± 0.03^{bc}	2.419 ± 0.07^{bc}
20 ppm	2.414 ± 0.09^{cd}	2.444 ± 0.09^{cd}	2.477 ± 0.10^{cd}
30 ppm	2.502 ± 0.07^{d}	2.512 ± 0.11^{d}	2.556 ± 0.10^{d}

Within each column, means ± SD followed by the same letters do not differ significantly (ANOVA, Tukey's HSD test, $P > 0.05$).
[+] No treatment with *Avicennia mari*.
[*] No treatment with *P. fluorescens* and *Avicennia mari*.

Table 4 Effect of *Avicennia marina* on *Labeo rohita* (RBC – million cells/mm^3) post challenged with *Pseudomonas fluorescens*.

Concentration/days	Day after treatment		
	10	20	30
Control[+]	1.212 ± 0.05^{b}	1.329 ± 0.02^{b}	1.413 ± 0.03^{b}
Infected with *P. fluorescens*[*]	0.932 ± 0.03^{a}	0.950 ± 0.07^{a}	0.890 ± 0.01^{a}
10 ppm	1.279 ± 0.044^{b}	1.441 ± 0.15^{b}	1.508 ± 0.13^{b}
20 ppm	1.528 ± 0.05^{c}	1.700 ± 0.21^{d}	1.730 ± 0.12^{c}
30 ppm	1.677 ± 0.15^{d}	1.892 ± 0.08^{e}	2.001 ± 0.05^{d}

Within each column, means ± SD followed by the same letters do not differ significantly (ANOVA, Tukey's HSD test, $P > 0.05$).
[+] No treatment with *Avicennia mari*.
[*] No treatment with *P. fluorescens* and *Avicennia mari*.

Table 5 Effect of *Avicennia marina* on *Labeo rohita* (WBC 10^4/ml) post challenged with *Pseudomonas fluorescens*.

Concentration/days	Day after treatment		
	10	20	30
Control[+]	2.739 ± 0.21^{b}	2.883 ± 0.24^{b}	3.157 ± 0.08^{b}
Infected with *P. fluorescens*[*]	2.338 ± 0.26^{a}	2.070 ± 0.04^{a}	1.732 ± 0.32^{a}
10 ppm	2.883 ± 0.33^{b}	3.169 ± 0.13^{c}	3.288 ± 0.10^{bc}
20 ppm	3.198 ± 0.04^{c}	3.243 ± 0.06^{cd}	3.281 ± 0.09^{bc}
30 ppm	3.301 ± 0.09^{c}	3.372 ± 0.16^{d}	3.415 ± 0.11^{c}

Within each column, means ± SD followed by the same letters do not differ significantly (ANOVA, Tukey's HSD test, $P > 0.05$).
[+] No treatment with *Avicennia mari*.
[*] No treatment with *P. fluorescens* and *Avicennia mari*.

Table 6 Effect of *Avicennia marina* on *Labeo rohita* (hemoglobin content – g/dL) post challenged with *Pseudomonas fluorescens*.

Concentration	Day after treatment		
	10	20	30
Control[+]	6.198 ± 0.10^{a}	6.340 ± 0.12^{b}	6.376 ± 0.13^{b}
Infected with *P. fluorescens*[*]	6.075 ± 0.09^{a}	5.833 ± 0.21^{a}	5.862 ± 0.12^{a}
10 ppm	6.182 ± 0.05^{a}	6.355 ± 0.08^{b}	6.405 ± 0.19^{bc}
20 ppm	6.417 ± 0.11^{b}	6.551 ± 0.26^{bc}	6.597 ± 0.14^{d}
30 ppm	6.457 ± 0.15^{b}	6.617 ± 0.16^{c}	6.685 ± 0.14^{d}

Within each column, means ± SD followed by the same letters do not differ significantly (ANOVA, Tukey's HSD test, $P > 0.05$).
[+] No treatment with *Avicennia mari*.
[*] No treatment with *P. fluorescens* and *Avicennia mari*.

Ramasubramanian, Uthayakumar, and Munirasu (2013), who stated that chitosan incorporated diet which enhanced the agglutination activity of *L. rohita* post challenged with *Aeromonas hydrophila*. *Achyranthes aspera* extracts induced the immunological activity of agglutination of *Pangasius pangasius* infested with *P. fluorescens*; increasing concentration of the extracts agglutination activity was increased (Alam et al., 2016).

In the present study, *A. marina* extracts induced the alkaline phosphate activity *L. rohita* post challenged with *P. fluorescens*. Similarly, Gobi et al. (2016) reported that ethanolic extract of *Psidium guajava* induced the alkaline phosphate activity in *Oreochromis mossambicus* challenge with *A. hydrophila*.

In the present study, serum protein concentration of *L. rohita* was increased after treatment of *A. marina* extracts. Similarly, Das, Pradhan, and Sahu (2009) reported that serum protein concentration was increased in *L. rohita* post challenged with *A. hydrophila*, when it was consumed *E. viridis* as diet. The serum protein concentration was increased when *A. hydrophila* infested fish common carp injected with *Lawsonia inermis* and found that protein concentration was higher than control fish (2016).

A. marina extracts induced the RBC, WBC and Hemoglobin content of *L. rohita* post challenged with *P. fluorescens*. Our report corroborated with earlier findings of Ngugi et al. (2015) who stated that *Urticadioica* induced the RBC, WBC and Hemoglobin content of *L. victorianus* challenged with *A. hydrophila*. *Allium sativum* extracts enhanced the RBC, WBC and hemoglobin content of the *L. rohita* challenged with *A. hydrophila* when provide dietary administration (Sahu, Das, Mishra, Pradhan, & Sarangi, 2007a). Das et al. (2009) stated that supplementary diet of *Euglena viridis* consumed *L. rohita* post challenged with *A. hydrophila*; the hematological parameter of RBC, WBC and hemoglobin content was increased significantly when compared to control. Post infection of *A. hydrophila* in *L. rohita*, hematological parameter of RBC, WBC and Hb content was increased significantly when it was consumed *Ocimum sanctum* diet (Das, Raman, Saha, & Singh, 2013). Also Sahu, Das, Pradhan (2007) stated that dietary feed of *Magnifera indica* induced the RBC, WBC and Hb content of *L. rohita*, infected with *A. hydrophila*. Natural product from *Terminalia catappa* induced the hematological parameter like WBC, RBC and Hb in fish when it was infested with *A. hydrophila* (Nugroho, Manurung, Nur, & Prahastika, 2017). Uluköy et al. (2018) stated that plant extracts induced as immunostimulant to rainbow trout fish.

Conclusion

Fifty percent mortality of *L. rohita* was found in *P. fluorescens* infested fish without treatment of plant extracts within 12 days. There was no mortality found at 10, 20 and 30 ppm concentration of *A. marina* treatments. In the present study *A. marina* extracts, induced the bacterial agglutination, alkaline phosphatase, serum protein, RBC, WBC and hemoglobin content of *L. rohita* when compared to control and infected with *P. fluorescens* without treatment. The extract act as immunostimulatory to fish, which could be used for fish farm as immunostimulatory, before that further detail study needed to use in aqua culture industries.

Conflict of interest

We declare that we have no conflict of interest.

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