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Research Article

IMMUNE PROTECTION OF *SALVINIA MOLESTA* D.S. MITCHELL IN FRESHWATER CRAB *OZIOTELPHUSA SENEX SENEX* BACTERIALLY CHALLENGED WITH *AEROMONAS HYDROPHILA*

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ABSTRACT

Objective: This is aimed to study the immune protection parameters of freshwater weed *Salvinia molesta* in bacterially challenged freshwater crab *Oziotelphusa senex senex*.

Methods: In this present study, ethanolic extract of freshwater weed *S. molesta* was tested for its ability to induce immunity in bacterial challenged freshwater crab *O. senex senex.* Male and female crabs were challenged with *Aeromonas hydrophila* in relevant concentrations. The treated groups were allowed to withstand for 96 hrs. After relevant incubation time, the hemolymph of the treated crabs was subjected for various hematological, biochemical, and immunological assays.

Results: Total hemocyte count increased on infection at 96 hrs, whereas significantly reduced on treatment with *S. molesta* at 96 hrs. All the three cell types of differential hemocytes showed significant positive changes on treatment. Levels of prophenoloxidase decreased significantly on infection and showed a significant increase in treated groups at 96 hrs of treatment.

Conclusion: The present study elucidated the medicinal and pharmaceutical role of *S. molesta* weed which is been subjected to eradication in the recent days. Thus, the plant source can be utilized as an immunomodulatory agent and a better alternative to treat aquatic diseases.

Keywords: *Oziotelphusa senex senex*, *Aeromonas hydrophila, Salvinia molesta,* Immunostimulant, total hemocyte count, Differential hemocytes count, Prophenoloxidase.

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INTRODUCTION

The growth of aquaculture sector in the recent years and increased demand for marine products had provocated in-depth need for aquaculture farming in such sectors. Still fishes, prawns, and crabs are highly susceptible toward infectious diseases caused by pathogens, namely, bacteria, viruse, and fungal agents. Administration of synthetic chemotherapeutants and antibiotics to prevent and control infections are highly disapproved for their negative impacts such as resistance formation and immunosuppression, and hence, had ended up in reduced preference for antibiotics treated crab population [1]. Diseases caused by bacterial infection in crab causes great economical loss to the crab population. So as to maintain the animal health, several antibiotics have been used to overcome the bacterial diseases which had resulted in the development of resistance among pathogenic agents. There was a significant antibiotic resistance generated by pathogenic bacteria due to repeated uses of antibiotics, and hence, there is an urgent need for researchers to discover new drugs against such pathogenic bacteria. In the past decade, to control microbial diseases, a number of chemotherapeutic agents including synthetic antibiotics are used in crab farms. This had led to serious problems such as antibiotic resistance and decreased survival rates [2-4]. Hence, rather using such chemotherapeutics and synthetic antibiotics, a notable attention is generated for the use of natural immunostimulants from plants to control infectious diseases in aquaculture sectors. Immunostimulants are those groups of biological compounds that can effectively improve the non-specific cellular and humoral defense mechanism without destructing the base metabolism in animal systems [5]. Medicinal plants, since time immemorial, have been used as a source of medicinal agent altering the immune system. Medicinal plants chosen as immunomodulatory agent must serve as an alternative potential to conventional chemotherapy methods in curing diseases, especially in relation to host defense mechanism. Plant phytochemicals such as flavonoids, tannins, and saponins are responsible for stimulating immune responses in various *in vitro* animal models by virtue of its antioxidant ability [6]. *Salvinia molesta* D.S. Mitchell, also known as giant *Salvinia*, is a genus of floating ferns belonging to the family Salviniaceae, and it is a diversified plant with 10-14 species in world, particularly at the tropics. The plant could double its biomass in 2-3 days under favorable conditions and can stay functional even at dry conditions [7]. The potentially of *S. molesta* to accumulate certain metals and effluent treatment was recently studied and proven, whereas the medical or pharmaceutical potential of giant *S. molesta* remains still underexplored [8]. Hence, the present study was focused on screening the immunostimulant activity of *S. molesta* extract in freshwater crab *Oziotelphusa senex senex* challenged with *Aeromonas hydrophila.*

METHODS

Collection of experimental animal and treatment

The male and female crabs, *O. senex senex,* were collected from paddy fields of Thirukazhukundram, Kanchipuram district, Tamil Nadu, and was brought to the laboratory and maintained in plastic tubs. Crabs were fed with beef mutton, and water was changed daily and was acclimatized for 15 days at existing room temperature. The crabs were divided into six groups of thirty crabs each and were subjected to treatment (Table 1). Group A and B are kept as saline-treated control. Groups C and D are infected with 0.1 ml of 10^9 cfu/ml (LD_{50}) standard concentration of *A. hydrophila*. Both the groups were allowed to withstand infection for 96 hrs. After 96 hrs of incubation, hemolymph was collected from ten crabs of each group for hematological and immunological assays. Remaining twenty infected crabs were treated

with 100 µl of ethanolic extract of *S. molesta*. Then, Groups E and F were allowed to incubate for 96 hrs. After 96 hrs, hematological and immunological assays were performed for the treated Groups E and F. Simultaneously, similar assays were performed for control Groups A and B.

Collection of hemolymph
Hemolymph of *O. senex senex* was collected aseptically from the base of one of the second walking legs using a sterile syringe with ice-cold citrate EDTA buffer (0.45 M NaCl; 0.1M glucose; 30 mM tri-sodium citrate; 20 mM citric acid; 100 mM EDTA, pH 4.6) as anticoagulant.

Total hemocyte count (THC)
THC was determined using standard methods of hemocytometer [9].

Differential hemocytes count (DHC)
DHC was performed using standard methods [10]. The smears were prepared carefully by streaking a drop of hemolymph and thoroughly mixed with hemocyte suspension on glass slides. These films were then air dried, incubated for 5 minutes in methanol. The films were washed in distilled water, washed with Giemsa stain solution for 20 minutes, and finally, rinsed with distilled water. The presence of large granule cells (LGC), small granule cells (SGC), and hyaline cells (HC) were determined.

Immunological assay
Prophenoloxidase (ProPO) assay
ProPO activity in hemolymph samples was determined using L- dihydroxyphenylalanine (L-DOPA) as a substrate. Tris-buffered saline (TBS) (30 µl) was added to the experimental cuvette containing 30 µl of hemolymph sample. Then, 60 µl L-Dopa solution (1.6 mg/ml in TMS) was added followed by immediate mixing and 200 µl of TBS was added as a diluent, and enzyme activity was measured by recording the absorbance of dopachrome at 490 nm against a blank containing 260 µl of TBS and 60 µl of L.DOPA. The absorbance value at 1 and 3 minutes after the addition of 200 µl of TBS was recorded. Enzyme activity was expressed in units, defined as the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per minutes/mg/protein [11]. All the obtained data were expressed as mean ± standard error of mean (SEM).

Statistical analysis
The statistical analysis system (SPSS version 17.0) software was used to analyze all the data. The data were expressed as mean ± SEM, and the data were analyzed using the Student's *t*-test and one-way analysis of variance. Differences were considered statistically significant at $p<0.05$ level.

Table 1: Grouping of male and female experimental groups.

Groups	Male/female groups and treatment groups
Group A	Control-male crab
Group B	Control-female crab
Group C	*A. hydrophila* infected male crab at 96 hrs
Group D	*A. hydrophila* infected female crab at 96 hrs
Group E	*S. molesta* injected to Group C male after 96 hrs
Group F	*S. molesta* injected to Group D female after 96 hrs

RESULTS

THC
When experimental Groups C and D infected with *A. hydrophila* were assayed for THC after 96 hrs, there were significant changes in hemocyte count. THC of control male and female crabs was 4231±31.83 cells/cu.mm and 3728±27.83 cells/cu.mm, respectively, whereas it increased to 6341±28.61 cells/cu.mm and 6039±26.41 cells/cu.mm in infected Groups C and D, respectively. There was an increase in hemocyte counts when compared to control Groups A and B which showed the response of immune defense mechanism against the pathogen. When Groups E and F (Groups treated with ethanol extract of *S. molesta*) were assayed for THC after 96 hrs of the incubation period, the THC levels decreased significantly to 4604±34.92 and 3556±22.14 in both male and female groups, respectively, depicting the immune protection of *S. molesta* (Table 2 and Fig. 1).

DHC
Control Groups A and B when assayed for DHC and the following results were obtained. In that way, the LGC, SGC, and HC ranged as 23±0.31%, 51±0.21%, and 28±0.23% for male control Group A, whereas LGC, SGC, and HC for female control group ranged as 25±0.43%, 42±0.93%, and 20±0.36%, respectively (Table 1). After 96 hrs of exposure to *A. hydrophila*, the differential hemocyte count, LGC and SGC counts significantly increased, but HC gradually decreased in treated groups (E and F), whereas in *S. molesta*-treated groups, the levels of all the three cell types retained to closer control group values (Table 2).

ProPO activity
In hemolymph of control crabs of Groups A and B, the ProPO enzyme activity level recorded was 0.798±0.021 and 0.898±0.016 (mg/minutes/protein), respectively. After 96 hrs of exposure to *A. hydrophila*, the ProPO level gradually reduced in infected Group C, namely, 0.321±0.036 and Group D, namely, 0.491±0.026 (mg/minutes/protein). When treated Groups E and F are assayed after 96 hrs, the ProPO level increased significantly elucidating the role of *S. molesta* in immune induction (Table 2 and Fig. 2).

DISCUSSION

In the recent times, modern aquaculture farming practices are developed at rapid rate. Still, infectious diseases are posing a major problem in such industry, thus causing heavy loss to farmers [12]. There are numerous naturally occurring herbs that have been reported to possess high antioxidant and anticancer role and have proven to be a potent therapeutic agent [13,14]. Hence, herbal immunostimulants can be an effective alternative strategy in *lieu* of antibiotics and chemotherapies in marine disease management [15]. Ethanolic extract of *S. molesta*, an abundantly available freshwater weed, was proven for its cytotoxic activity against human non-small cell lung cells but not toxic to normal

Table 2: Levels of THC / DHC & ProPO in experimental groups.

Groups	THC cells/cu.mm	Differential hemocytes counts (DHC)	SGC %	HC %	ProPO minutes/mg/protein
		LGC %			
A	4231±31.83	23±0.31	51±0.21	28±0.23	0.798±0.021
B	3728±27.83	25±0.43	42±0.93	20±0.36	0.898±0.016
C	6341±28.61*	25±0.65**	70±0.32*	15±0.36*	0.321±0.036*
D	6039±26.41*	32±0.19*	52±0.34*	12±0.65*	0.491±0.026*
E	4604±34.92*	21±0.73*	53±0.21*	24±0.46*	0.801±0.023*
F	3556±22.14*	23±0.54*	45±0.46*	23±0.44*	0.853±0.012*

Each value represents mean±SEM of three individual observations. Group-A versus C versus E *p<0.05, **insignificant. Group-B versus D versus F *p<0.05, **insignificant

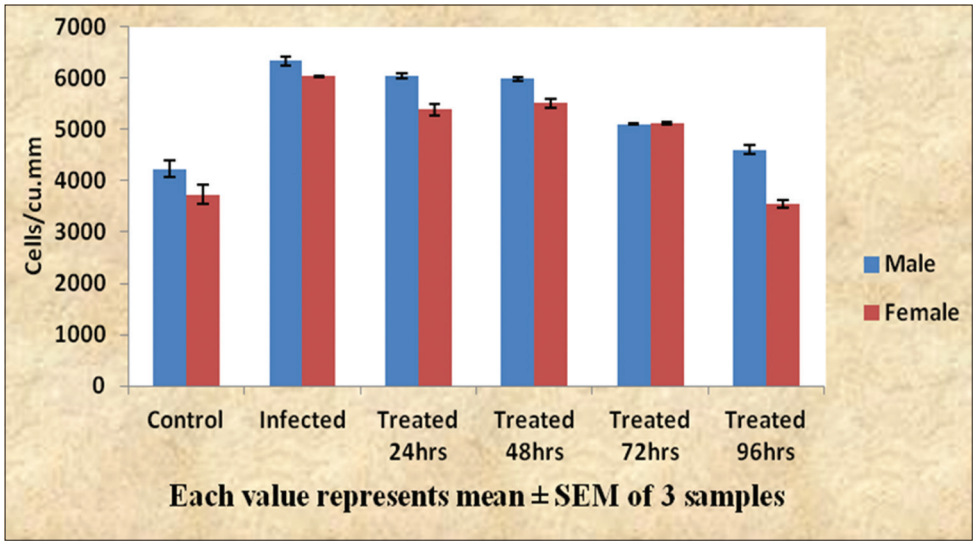

Fig. 1: Total hemocyte count

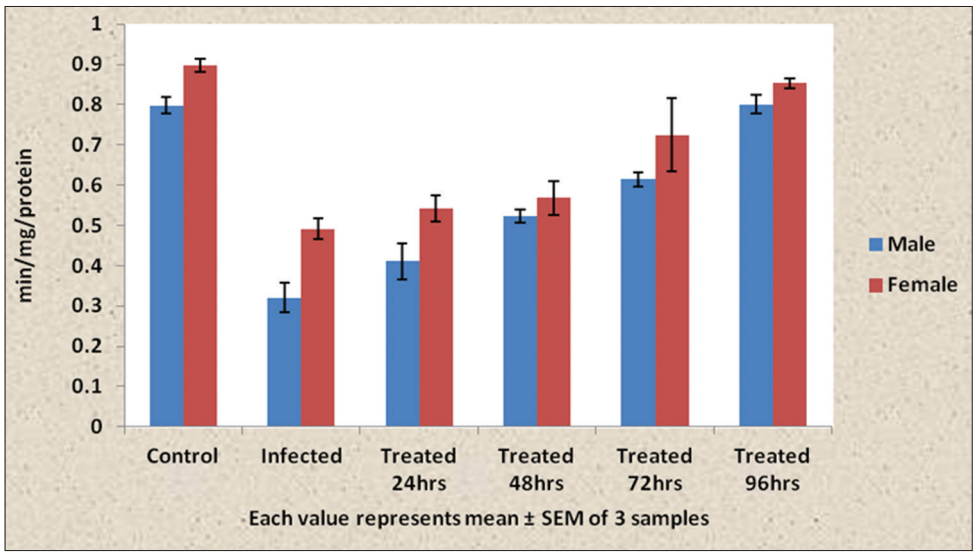

Fig. 2: Prophenoloxidase activity

human lung fibroblasts cells [8]. The previous studies also have proven that *S. molesta* has a potent antioxidant and antibacterial activity against *A. hydrophila* and is rich in significant phytochemicals [16]. In this way, the present study revealed that administration of ethanolic extract of *S. molesta* in standard concentrations to *O. senex senex* significantly increased innate humoral and cellular responses and disease resistance against *A. hydrophila* bacterial infections. In crustaceans, the vital function of circulating hemocytes in host defense mechanism is the elimination of any foreign particles that can gain access to the hemocoel. This mechanism is exhibited by a combination of phagocytosis, nodule formation, and encapsulation reaction, that depends on the dimension of the foreign bodies [17]. In this study, the hemocyte counts decreased significantly in the 96 hrs infected groups when treated with *S. molesta* which evidenced the immune alteration by the plant supplement. Crustaceans display relatively simple hemocytes that are mainly subdivided into three cell types: Hyaline, semi granular, and granular. These cell types vary not only in morphological features but also in biochemical characteristics and *in vitro* behavior. The levels of hyaline, semi granular, and granular cells vary according to the taxonomic grouping of the host [18]. In this study, there were significant changes in differential hemocyte count in treated groups after treatment with *S. molesta*, elaborating its immunostimulant role. ProPO is an inactive precursor of phenoloxidase (PO), and it converts tyrosine to DOPA, as well as DOPA-quinone. The end product of the non-enzymatic reactions that spontaneously formed is melanin, a brown or yellowish pigment. In arthropods, this pigment is produced in the cuticle as a result of a wound or parasitic attack [19]. Thus, infected groups showed a higher level of ProPO activity, whereas, in treated groups, there was observed a significant reduction in ProPO activity. Thus the present study revealed the potential immune protection efficacy of ethanolic extract of *S. molesta* in treated groups (E and F) of *O. senex senex*.

CONCLUSION

Since ancient times, medicinal plants have been used in traditional medicine for treatment and control of many diseases. Natural plant products are reported to possess antimicrobial, antibacterial, antioxidant, immunostimulation, and aphrodisiac properties due to the presence of active principle components such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids, and essential oils. Thus, plant products with significant and rich sources of immune-enhancing substances are used across developing countries to promote health, to increase the body's natural resistance to infection, and in prevention and treatment of various diseases. Plant products are an inexpensive and economical source of therapeutic medicine, possessing greater

accuracy than chemotherapeutic agents, and hence, offer a viable solution for all problems which aquaculture face currently. In this way, *S. molesta* D.S. Mitchell can be utilized as a potent immunostimulant to face the microbial infection challenges in crab farming.

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