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Immunomodulatory activity of *Salvinia molesta* D.S. Mitchell in fresh water crab *Oziotelphusa senex senex* bacterially challenged with *Pseudomonas aeruginosa*

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1. Introduction

Marine products especially seafood business in India is booming in recent days. In 2014–15, Indian seafood export business has crossed $2-billion mark (US$ 2.1 billion) by exporting about 663, 603 tonnes of seafood. Presently seafood export is one of the major economic activities in India and it is the fourth largest contributor of net foreign exchange to the country (Handbook of Fisheries and Aquaculture, 2015). Crab species are one among the emerging aquaculture sector as they yield good economical benefits for the vendors when exported. It was estimated that the potential resource of crabs particularly from the estuaries and backwaters of south Indian coastal areas are 13,209 tonnes and has proven that southern part of the coasts are potentially richer than the northern part of Indian coastal region (Shelley and Lovatelli, 2011).

Hence the reason, scientific fish farming commenced into the country since 1980's with traditional, extensive, semi-intensive and intensive farming techniques. Increase in demand for crab products also had further opened path for intensive farming, where the animals are at high risk of pathogenic attack under multiple stress conditions. Major pathogens that are affecting aquaculture industry include bacteria (Wang, 2011), fungi, viruses and parasites (Ramaiah, 2006). Diseases caused by bacterial infections in crabs are observed to cause a higher percentage of mortality and economical loss when compared to fungal and viral attacks (Bagum et al., 2013). Among numerous bacterial pathogens studied for their pathogenic role in aquatic animals, *Aeromonas* spp. and *Pseudomonas* spp. are known to cause rapid mortalities in fishes and crabs by rapid tissue invasion and multiplication causing severe hemorrhagic septicaemia condition (Ponnerassery Sudheesh et al., 2012).

Repeated use of antibiotics can develop resistance in disease causing bacteria and can render life-saving antimicrobial compounds ineffective permanently. Moreover, diseases caused by antibiotic-resistant bacteria are difficult to treat and therefore alternates drugs to antimicrobial compounds which can provide an enhanced protection to aquatic animals are needed to treat diseases effectively. Plant products are recently explored for their potent role against fish diseases which would be cheaper and safer without serious side effects (Sivagurunathan et al., 2012). Medicinal plants are the significant source of drugs since time immemorial, holding the scenario of the Indian system of medicine (Sharma et al., 2009) and they are rich sources of bioactive compounds serving as an important raw materials for drug production. They are used for its therapeutic purposes since ancient times and their use is of a greater demand nowadays.

Immune-modulation is the process of modifying or regulating an immune response in a positive or negative manner by administration of a drug or compound. In short, they are biological or synthetic substances, which can stimulate, suppress or modulate any of the immune system including both adaptive and innate arms of the immune response (Nagarathna et al., 2013).

Oziotelphusa is a genus of freshwater crabs in the family *Gecarcinucidae*. It belongs to the crustacean family. These crabs are abundantly available in the paddy fields. The crabs are rarely

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seen in the open fields and they were studied for their nutraceutical and pharmaceutical potentials in previous studies. As per literature it is understood that *Oziotelphusa senex senex* serve as a significant animal model representing the crab species and these animal models are frequently used for experimental studies since they are easy to handle and also available in abundant.

Salvinia molesta D S. Mitchell, also known as giant *Salvinia*, is a genus of floating ferns belonging to the family *Salviniaceae* and has 10–14 species in the world, particularly in the tropics. Recently, it was found that the bioactive compounds present in *Salvinia molesta* are potent cytotoxic agent against human tumour cells (Shiyou Li et al., 2013) and also were utilised for successful waste management and effluent treatment. Though the invasive weed *Salvinia molesta* has multiple potentialities, their medical and pharmaceutical ability still remains underexplored. Hence the present study was conducted to assess the Immmunomodulatory efficacy of *Salvinia molesta* leaf extracts in *P. aeruginosa* bacterially challenged freshwater crab, *Oziotelphusa senex senex*.

2. Materials and methods

2.1. Collection of specimens (Salvinia molesta)

The whole plant of fresh *Salvinia molesta* was collected from the lakes of Kaliyakkavilai, Kanyakumari district. The collected leaves were cleaned and cut into small pieces before being dried under shade at room temperature. The dried material were ground to fine powder using a mechanical blender and passed through 24 mesh sieve. The powdered sample was further used to make ethanolic solvent extraction.

2.2. Collection of experimental animal and treatment

Male and female freshwater crabs, *Oziotelphusa senex senex* were collected from paddy fields in Tirukazhukundram village near Chengalpatu, Kanchipuram district, Tamil Nadu and were brought to the laboratory and maintained in plastic tubs. Crabs were fed with beef mutton and the 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. Group A and B are kept as saline treated control. Group C and D are infected with 0.1 ml of 10^7 CFU/ml standard concentration of *P. aeruginosa*. Both the groups are allowed to withstand infection for 96 hrs. After 96 hrs hemolymph was collected from ten crabs of each group for haematological and Immunological assays. Remaining twenty bacterial infected crabs were treated with 100 μl of ethanolic leaf extract of *S. molesta*. The treated groups are maintained as group E, E_1, E_2 and E_3 (male) and F, F_1, F_2 and F_3 (female) in respective time interval of 24 hrs, 48 hrs, 72 hrs and 96 hrs. After 96 hrs, the haematological and immunological assays were performed for infected and treated groups (Table 1).

2.3. 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; 0.1 M glucose; 30 mM trisodium citrate; 20 mM citric acid; 100 mM EDTA, pH 4.6) as anticoagulant. The collected hemolymph was stored for further analysis in aseptic conditions.

Table 1

S.NO	Groups	Male/Female Groups & Treatment
1.	Group A	Control- Male crab
2.	Group B	Control –Female crab
3.	Group C	*P. aeruginosa* infected Male crab at 96 h
4.	Group D	*P. aeruginosa* infected female crab at 96 h
5.	Group E	*Salvinia molesta* treated to Group C Male after 96 h
6.	Group F	*Salvinia molesta* treated to Group D Female after 96 h

2.4. Immunological assay

2.4.1. Total hemocyte count (THC)

Total hemocyte count was determined by using standard methods of hemocytometer (Dacie and Lewis, 1968).

2.4.2. Differential hemocytes count (DHC)

Differential counts of hemocytes were performed by using standard methods (Kondo, 2003). 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 min in methanol. The films were washed in distilled water and washed with Giemsa stain solution for 20 min and finally rinsed with distilled water. Presence of large granule cells (LGC), small granule cells (SGC) and hyaline cells (HC) were determined.

2.4.3. Prophenol oxidase (Propo) assay

Prophenol oxidase activity in hemolymph samples was determined using L- dihydroxyphenylalanine (L-DOPA) as a substrate. TBS (30 μl) was added to the experimental cuvette containing 30 μl of haemolymph 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 dilutent 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. Enzyme activity was expressed in units, defined as the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per min/mg/protein (Takahashi et al., 2000). All the obtained data were expressed as mean ± standard error of mean (S.E.M).

2.4.4. Estimation of Superoxide dismutase (SOD) activity

The enzyme was estimated by the method of Marklund and Marklund (1974).The reaction mixture for auto oxidation consisted of 2 ml of buffer containing diethylene triaminepenta acetic acid, 0.5 ml of the diluted (2mM), pyrogallol solution and 1.5 ml of double distilled water. The assay mixture contained 2 ml of buffer, enzyme and double distilled water to give a final volume of 4 ml. Diethylene tri amine penta acetic acid acts as a chelator. The enzyme activity was measured at 420 nm and was expressed as units/mg protein.

2.4.4.1. Calculation.

$$\mathrm{SOD} = \frac{\Delta \mathrm{O} \cdot \mathrm{D}\,\mathrm{Sample} \times \mathrm{O} \cdot \mathrm{D}\,\mathrm{blank} \times 100}{\Delta \mathrm{O} \cdot \mathrm{D}\,\mathrm{Sample} \times 50 \times \mathrm{Vol} \cdot \mathrm{of\ sample\ mg\ protein}}$$

2.4.5. Estimation of Catalase (CAT) activity

The catalase activity was measured by the method of Claiborne (1985). The assay mixture contained 0.5 ml H_2O_2, 1 ml buffer and 0.4 ml water, 0.1 ml of 1:10 diluted tissue extract was added to initiate the reaction. 2 ml Dichromate acetic acid reagent was added after 15, 30, 45 and 60 s to arrest the reaction to the control tube, the enzyme was added after the addition of the dichromate acetic acid reagent. The tubes were then heated for 10 min, allowed to cool and the green colour developed was read at 570 nm. The catalase activity was calculated in terms of nmol H_2O_2 consumed/minute/mg protein, with the help of the following formula:

2.4.5.1. Calculation.

$$\mathrm{CAT} = \frac{\Delta \mathrm{O} \cdot \mathrm{D/min} \times Vol \cdot \text{of assay}}{0.081 \text{ of Vol} \cdot \text{of conjugate enzyme} \times protein(mg)}$$

2.4.6. Lipid peroxidation activity estimation (LPO)

Tissue lipid Peroxidation was measured by the method of Devasagayam and Tarachand (1987). The reaction mixture consisted of 1.0 ml of 0.15 M Tris- HCl buffer (pH 7.4), 0.3 ml of 10 mM KH_2PO_4 and 0.2 ml of tissue extract in a total volume of 2 ml. The tubes were incubated at 37 °C for 20 min with constant shaking. The reaction was stopped by the addition of 1 ml of 10% TCA. The tubes were shaken well and 1.5 ml TBA was added and were heated in a boiling water bath for 20 min. Standard tubes containing 10, 20, 30, 40 and 50 nmoles/ml were also run simultaneously. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmol MDA formed/gram tissue by using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.4.6.1. Calculation.

$$\mathrm{LPO} = \frac{\mathrm{Vol} \cdot \text{of assay} \times \mathrm{O} \cdot \mathrm{D} \times 10^9}{1.56 \times 10^5 \times 10^3 \text{ gm tissue}}$$

2.5. Statistical analysis

The SPSS software version 11.0 for Windows was used for the statistical analysis. Results are reported as mean ± SEM of three individuals per group per time point ($n = 3$). The data were processed by two-way analysis of variance (ANOVA).

3. Results

3.1. Total hemocyte count

When experimental Groups C and D infected with *P. aeruginosa* were assayed for Total hemocyte count after 96hrs, there was significant increase in hemocyte count. THC of control male and female crabs were 4232 ± 36.93 cells/cu.mm and 3982 ± 42.15 cells/cu.mm respectively, whereas it increased to 6451 ± 58.62 cells/cu.mm and 6128 ± 36.49 cells/cu.mm in infected groups C and D respectively. There was increase in hemocyte counts when compared to control groups A and B which showed the response of immune defence mechanism against the pathogen. When Groups E and F (Groups treated with ethanol extract of *Salvinia molesta*) were assayed for THC after 96hrs of incubation period, the THC levels decreased significantly to 4032 ± 21.31 and 3543 ± 28.17 in both male and female groups respectively depicting the immune protection of *Salvinia molesta.* In infected groups-C and D there was a significant increase ($P < 0.05$) in THC levels when compared to group A, whereas there was a significant decrease ($P < 0.05$) in THC levels at different time intervals and the levels of THC in treated groups retained to closer control values at 96 hrs (Fig. 1).

3.2. Differential hemocytes count

Control groups A and B when assayed for differential hemocytes count (DHC) and the following results were obtained. In that way, the large granular cells (LGC), small granular cells (SGC) and hyaline cells (HC) ranged as 24%, 54%, 26% for male control group A, whereas LGC, SGC and HC for female control group ranged as 36%, 45%, 22% respectively. There was a significant increase ($P < 0.05$) of LGC, SGC and significant decrease ($P < 0.05$) of HC in all the infected groups C and D when compared to group A and B. The values at 96 h for treated groups were closer to control group-A and B mentioning the therapeutic role of ethanolic leaf extract of *S. molesta* (Fig. 2).

3.3. Prophenol oxidase assay

In hemolymph of control crabs of Group A and B the prophenoloxidase enzyme activity level recorded was 0.798 ± 0.021 and 0.898 ± 0.016 (mg/min/protein) respectively. After 96 h of exposure to *P. aeruginosa*, the prophenoloxidase level gradually reduced in infected Group C *viz.*,0.321 ± 0.036 and Group D *viz.*,0.491 ± 0.026 (mg/min/protein). Both male and female groups showed significant decrease ($P < 0.05$) in ProPo in infected groups. Whereas, the values of treated groups-E_3 and F_3 at 96hrs were observed to be closer to the control group-A and B (Fig. 3).

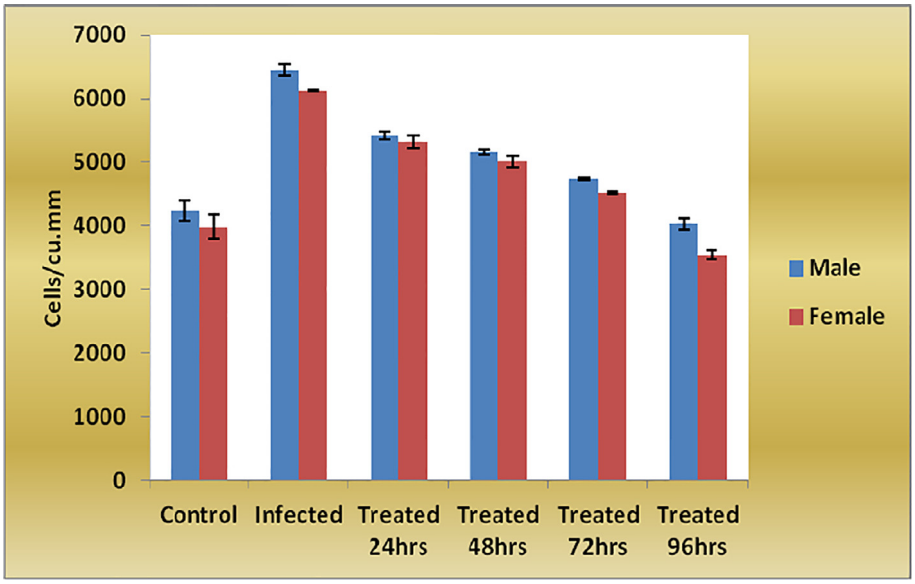

Fig. 1. Total hemocyte count (THC) in hemolymph of *O. senex senex* infected with *P. aeruginosa* and treated with ethanolic leaf extract of *S. molesta*. Each value represents mean ± SEM of 3 samples expressed as cells/cu.mm.

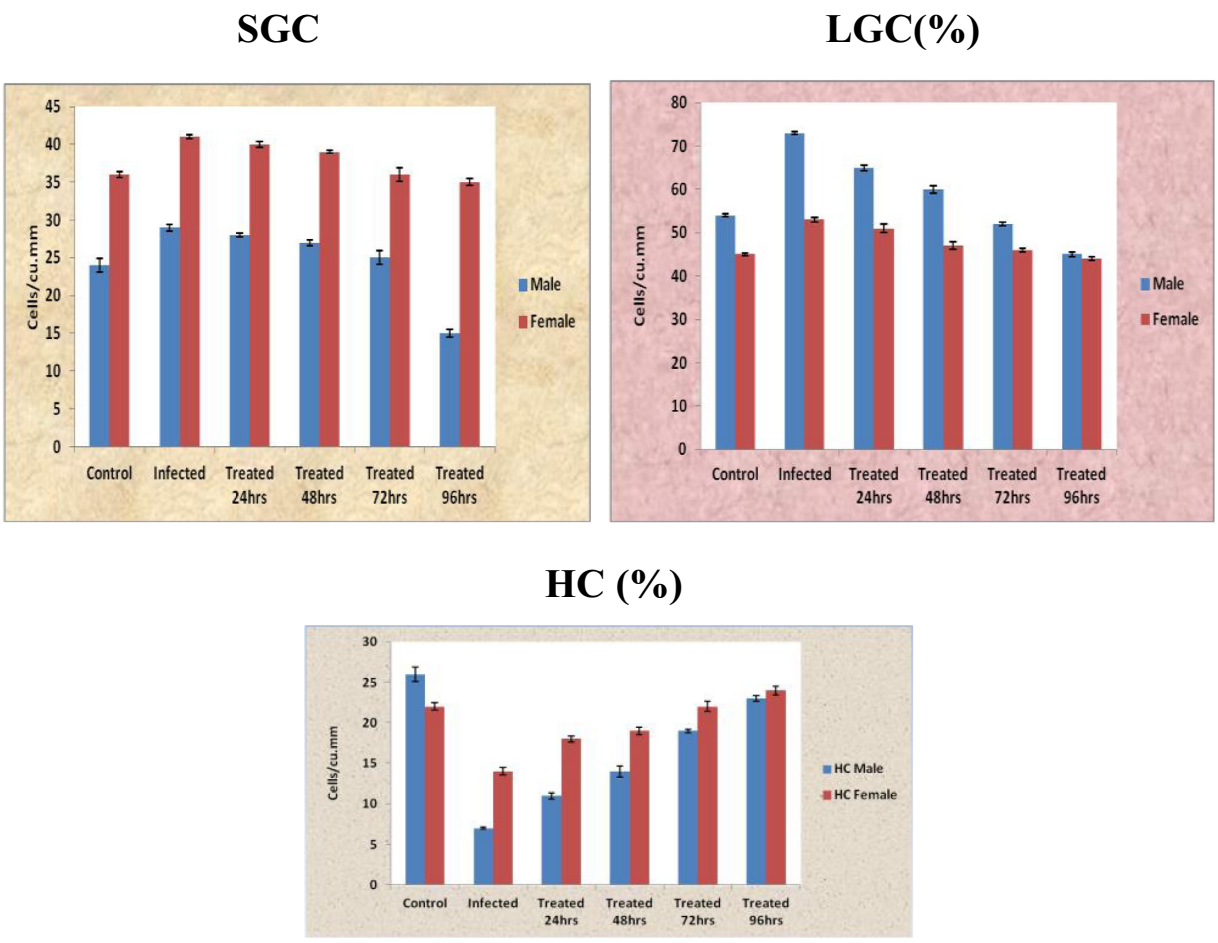

Fig. 2. Differential haemocytes count (DHC) in hemolymph of *O. senex senex* infected with *P. aeruginosa* and treated with ethanolic leaf extract of *S. molesta*. Each value represents mean ± SEM of 3 samples expressed as cells/cu.mm.

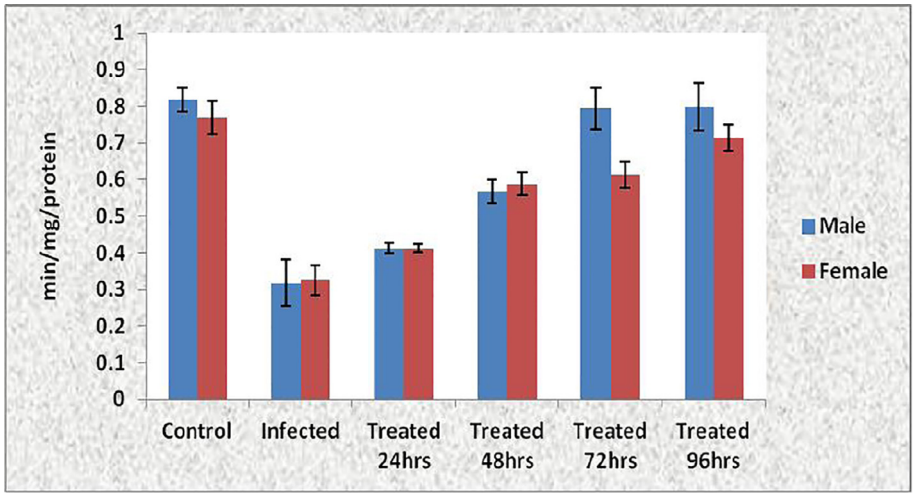

Fig. 3. Prophenol oxidase activity in hemolymph of *O. senex senex* infected with *P. aeruginosa* and treated with ethanolic leaf extract of *S. molesta*. Each value represents mean ± sem of 3 samples expressed as min/mg/protein.

3.4. SOD assay

Significant decrease ($P < 0.05$) of SOD levels were observed in infected groups C and D when compared with control groups. On comparing control and treated groups at 24 h to 96 h there was a gradual increase in SOD levels depicting the pharmacological role of ethanolic leaf extract *S. molesta* in regulating the SOD levels (Fig. 4).

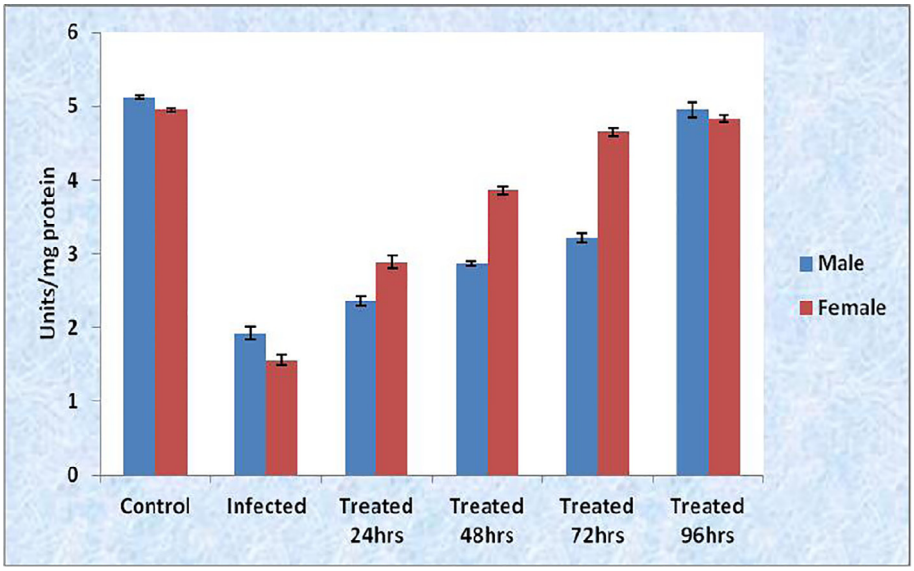

Fig. 4. Superoxide dismutase activity in hemolymph of *O. senex senex* infected with *P. aeruginosa* and treated with ethanolic leaf extract of *S. molesta*. Each value represents mean ± sem of 3 samples expressed as units/mg protein.

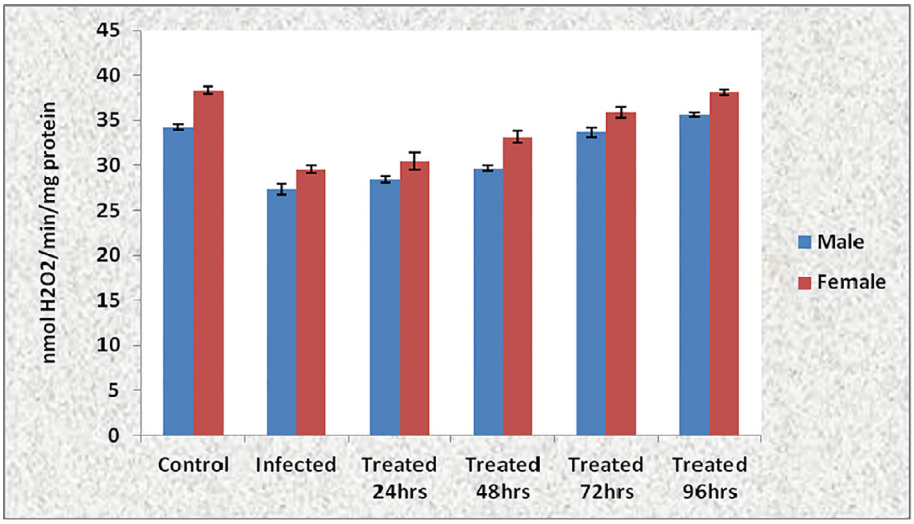

Fig. 5. Total levels of catalase in hemolymph of *O. senex senex* infected with *P. aeruginosa* and treated with ethanolic leaf extract of *S. molesta*. Each value represents mean ± sem of 3 samples expressed as nmol h_2o_2 consumed/min/mg.

3.5. CAT assay

In both male and female crabs there was a significant decrease ($P < 0.05$) of CAT levels in infected groups-C and D were observed when compared with control groups-A and B. Whereas the levels of CAT gradually increased in treated groups and the values were retained to control group-A and B at 96hrs of treatment (Fig. 5).

3.6. LPO activity estimation assay

Significant increase ($P < 0.05$) of LPO levels in infected groups-C and D were observed. On treatment with ethanolic leaf extract of *S. molesta*, gradual decrease in the LPO levels were observed. Values of group-E to E_3 and group-F to F_3 illustrate the retaining of normal physiological values of LPO on par to control group-A and B (Fig. 6).

4. Discussions

The pathogens causing infections are usually indigenous to the aquatic environment and been associated with disease outbreaks in aquatic animals and carries a higher risk of transmitting to human beings also (Paulo Martins da Costa et al., 2013). Hence the growth of intensive aquaculture production has parallel serious challenges in preventing and treating aquatic diseases accordingly. Effective disease control is highly required within aquatic farming systems to stop the spread of infectious pathogens which may cause huge economic loss. Implementation of an effective health management system with well organized process, ideas, practices, hygienic measures and improved tools to resist disease onset can widely help in reducing and controlling the diseases at farm sites (Alexandra Adams and Kim Thompson, 2011).

Boopathi Mahalaxmi et al. (2013) have studied the distribution of microbial population associated with crabs from Ennore

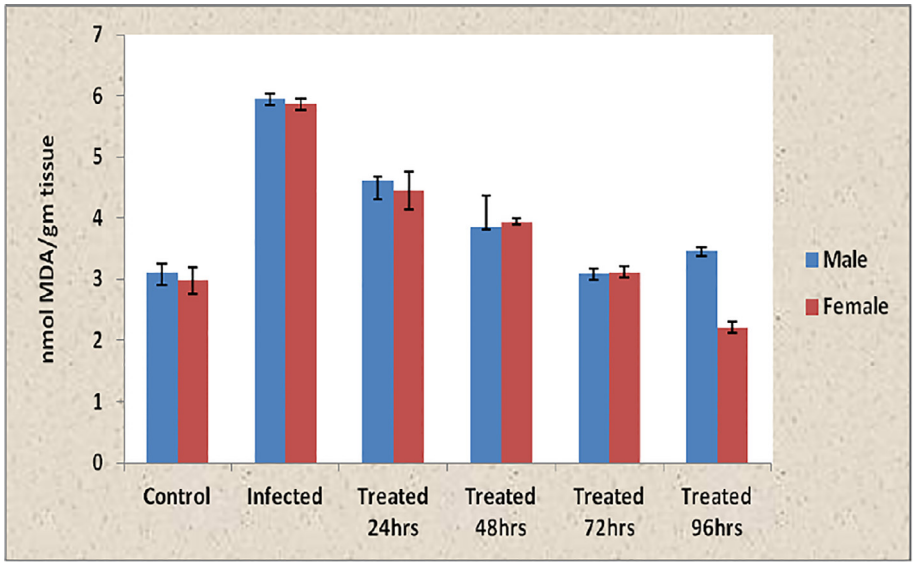

Fig. 6. Lipid peroxidase activity in hemolymph of *O. senex senex* infected with *P. aeruginosa* and treated with ethanolic leaf extract of *S. molesta*. Each value represents mean ± sem of 3 samples expressed as nmol mda/gm tissue.

seacoast, India and have reported that the sewage effluent is highly contaminated with *Pseudomonas spp*. Sharmila Joseph et al. (2014) have reported that increasing incidence of shell diseases in the freshwater crab, *Barytelphusa cunicularis* is caused by various range of bacterial infections predominantly by *Pseudomonas spp* and *Aeromonas* spp.

One of the most promising methods of controlling diseases in aquaculture is strengthening the defence mechanisms of fishes/crabs/shrimps and other aquatic animals by prophylactic administration of synthetic or natural immunostimulants.

In the present study, fresh ethanolic extract of *S. molesta* leaf extracts were analysed for its pharmacognostic properties and used as an antagonist agent in treating bacterially challenged crabs.

Choudhary et al. (2008) has reported the phenolic constituents of freshwater fern *S. molesta* D.S. Mitchell collected from Haliji Lake (Sindh, Pakistan), by a non-physiological assay and showed that compounds of *S. molesta* showed potent antioxidant radical scavenging activity. Hence in the present investigation the weed was taken as a study material to assay its antioxidant and immunomodulatory role in animal models.

Total and differential haemocyte counts are the important immunological parameters required mandatorily to assess the primary physiological state of the animal.

Kumaran et al. (2013) reported the change of immunity parameters on treatment with plant source and have recorded the relevant changes in immune parameters such as THC, DHC and ProPo. In the present study, the total hemocyte count significantly increased in both *P. aeruginosa* infected groups and the ranges of THC were high at 96 h depicting the immune response of the hemolymph system when experienced a pathogen attack in the system. Both male and female infected groups when treated at 96hrs of incubation period, the levels returned to physiological levels mentioning the potent role of *S. molesta* extract treatment. The hemocyte counts increased significantly in all infected groups and decreased in all the treated groups and thus *S. molesta* could immune modulate the system and agent can regulate the levels of hemocyte during post infection period.

Similar changes were found in differential hemocytes cell counts, were there was significant increase of LGC, SGC and significant decrease of HC in both male and female *P. aeruginosa* infected groups, whereas in the treated groups all the obtained values were closer to the normal physiological values.

The prophenoloxidase system is a complement like enzyme cascade, responsible for the formation of melanin. Phenol oxidase enzyme is mainly present in the large granule haemocytes. Thus, they are the main performers in encapsulation, which ultimately leads to the deposition of melanin. Phenoloxidase is sometimes seen diffused in the granular and electron dense cytosol of large granule haemocytes.

In the present study, Prophenoloxidase levels decreased significantly during infection at 96 hrs of infection as a reflection of pathogenic attack. Whereas, when treated with relevant concentrations of *S. molesta* leaf extract, the ProPo levels were significantly retained back to physiological levels.

Bernard Mark Asirvatham and Sekhar (2015) reported the antibacterial and immunostimulant activity of *Psidium guajava* leaf extract against the *V. harveyi* infected freshwater crab, *O. senex senex* and had recorded the relevant positive changes in total hemocyte, differential hemocyte counts in treated groups thus elucidating the role of hemocytes in performing immune functions.

SOD is the first antioxidant enzyme which scavenges superoxide radicals and CAT is responsible for detoxification of H_2O_2 formed as a result of the reaction catalyzed by SOD. In the present study conducted, the hemolymph of both male and female crabs was assayed for major antioxidant (Pham-Huy et al., 2008). In the present study, the superoxide dismutase (SOD) levels reduced significantly in infected groups and the levels slowly increased with time and reached physiological levels in treated groups and sustained further. And hence the retained levels on treatment stayed closer to physiological levels, proving *S. molesta* extracts are capable of regulating the levels of SOD in the hemolymph of *O. senex senex*.

Catalase is a hemoprotein which catalases the reduction of hydrogen peroxides and protects tissues from highly reactive hydroxyl radicals (Indradevi et al., 2012). Lan Wang et al. (2011) have reported the changes in levels of CAT during oxidative stress conditions in the freshwater crab, *Sinopotamon henanense.* In the present study, the levels of catalase in the hemolymph of *Oziotelphusa senex senex* decreased significantly in *P. aeruginosa* infected groups and on administration of ethanolic leaf extract of *S. molesta* to the infected crabs, the levels were restored close to 96 h of incubation.

Lipid peroxidation mediated by free radicals is considered to play a major role in antioxidant mechanism in tissues. Increased LPO is the index of oxidative stress in the animals. In the present study both male and female infected crab groups showed significant increase in LPO levels whereas there was a decrease in LPO levels on treatment with *S. molesta* ethanolic extract. Natalia Kurhalyuk et al. (2010) have reported that lipid peroxidation system is the major contributor to the loss of cell function under oxidative stress conditions. The peroxidation process was preceded by a decrease in the cell antioxidant defense system followed by the production of lipid and protein oxidation products. Hence levels of LPO play a vital role in normalising the immune parameters of a crab system. In the present study, the levels of LPO increased significantly during the infection period, whereas there was an decrease in LPO levels at treated groups indicating its coordination with the pharmacological role of *S. molesta* leaf extracts.

To conclude, the present study revealed that *S. molesta* a freshwater weed is available abundant in nature but still not explored in detail for its medicinal properties. The present study of the aquatic weed in animal models revealed that *Salvinia molesta* leaf extracts possess remarkable pharmacological and therapeutic values and can act as an potent immuno-modulatory agent.

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