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IMMUNOLOGICAL CHALLENGE STUDY IN A *VIBRIO HARVEYI* INFECTED FRESHWATER CRAB, *OZIOTELPHUSA SENEX SENEX*

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ABSTRACT

In the present study, the crab, *O. senex senex* was injected with *V. harveyi* (0.1 ml of 10^8 cfu/ml). After injection of bacteria the crabs were allowed to withstand for 96 hrs. After 96 hrs in one group of crab's haemolymph were used for haematological and immunological assays. Remaining bacterial injected crabs were treated with 0.05 ml of 80% *P. guajava* leaf ethanol extract (1000 ppm) , after 96 hours Total Haemocyte Count (THC) and Prophenol oxidase (ProPO) were significantly changed ($P<0.001$) in the experimental group. These results suggested that the *P. guajava* could combat the microbial infection by stimulating the immune response in crabs.

KEY WORDS: THC, Haemocytes, ProPO, *P. guajava*, *V. harveyi*, *O. senex senex*.

INTRODUCTION

Infectious diseases are a major problem in aquaculture causing heavy loss to the fish farmers. The recent expansion of intensive aquaculture practices has led to high interest in understanding the various fish diseases, so that they can be treated or prevented. It is widely demonstrated that the occurrence of diseases in fish farm is due to several factors concerned with the rearing methods, environmental conditions and variations. Consequently, cultivated fish can become more susceptible not only to pathogenic but also to opportunistic bacteria [1].

Apart from the viral attack, diseases caused by bacterial infection in crabs also cause a great economical loss. To maintain the animal health several antibiotics have been used to forecome the bacterial diseases which resulted in the development of resistance among pathogenic bacteria. Due to increase of antibiotic resistant among pathogenic bacteria, there has been the urgency for scientist to find new drugs against these pathogenic bacteria.

To control microbial diseases, a number of chemotherapeutic agents including antibiotics are used in shrimp farms. This has led to problems such as antibiotic resistance [2]. According to WHO fact sheet 194 (World Health Organisation Antimicrobial Resistance Fact Sheet 194, http://www.who.int/inf-fs/en/fact 194.html), the massive use of antimicrobials for disease control and growth promotion in animals increases selective pressure on the microbial world and encourages the emergence of resistant bacteria which can transfer their resistance genes to other bacteria. Another major concern associated with the use of antibiotics is the problem of residues which has already led to 'red alert' on imported shrimp in the European Union.

Therefore, the environmental consequences of antibiotic use in aquatic environment are serious. The prevalence and multiple antibiotic resistance of *A. hydrophila* in seafood samples of Chennai, Tamil Nadu, India have been reported [3].

Several antimicrobial, antistress, immunostimulant, growth-promoting plant products significantly influenced the fish/ larviculture [4-9]. Hence the present study was focused on screening of antibacterial/immunostimulant activity of *Psidium guajava* leaf extract against the *V. harveyi* infected freshwater crab, *Oziotelphusa senex senex*.

MATERIALS AND METHODS

Experimental animal and Treatment

The female crabs, *Oziotelphusa senex senex*

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collected from *Vandalur Lake* 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 in the prevailing room temperature.

The crabs were divided into three groups of thirty crabs each – Control group A, *V. harveyi* injected Group - B and *P. guajava* leaves ethanol extract treated group – C. Experimental group was injected with sub lethal dose (0.1 ml of 10^8 cfu/ml) of *V. harveyi*. After injection the crabs were allowed to withstand for 96 hrs. After 96hrs haemolymph was collected from ten crabs for haematological and Immunological assays. Remaining bacterial injected crabs were treated with 0.05 ml of 80% *P. guajava* leaves ethanol plant extract (1000 ppm) after 96 hours haematological and Immunological assays were repeated.

Collection of haemolymph

Haemolymph 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; 30mM trisodium citrate; 20mM citric acid; 100mM EDTA, pH 4.6) as anticoagulant.

Haematological analysis:

Total haemocyte count (THC)

Total Haemocyte Count was determined by the method of haemocytometer [10].

Immunological analysis:

Prophenol oxidase (ProPO) activity

For measurement of ProPO activity, the haemocytes collected from different groups of crabs were individually mixed with KHE anticoagulant buffer (3.2% NaCl, 0.1 M HEPES, 0.1 M EDTA, pH 7.0), resuspended in Ca-Mg HEPES buffer (5 mM CaCl2, 5 mM MgCl2, 50 mM HEPES, 3.2% NaCl, pH 7.0), disrupted by ultra sonication and filtered through a 0.22 mm membrane filter. Filtrates (0.1 ml) were mixed with 0.1 ml L-DOPA (2.9 mg ml^{-1}) and 0.8 ml Ca-Mg HEPES and incubated at 60 °C for 60 min. PO activity was measured at 490 nm by spectrophotometry [11].

The statistical analysis system (SPSS version 17.0) software was used to analyse all the data. The data were expressed as mean ± standard error of mean (S.E.M) and the data were analysed using the Student's t-test and one-way analysis of variance (ANOVA) followed by Tukeys posthoc multiple comparison test. Differences were considered statistically significant at $P < 0.05$ level.

RESULTS

Total haemocytes count (THC)

After exposure to *V. harveyi* the haemocyte counts showed significant changes in the experimental groups. (Table) In the control crabs Total haemocyte count is 4291±73.31 respectively. After 96 hours of exposure to *V. harveyi* the total haemocyte count gradually increased in the group B crabs (8366±68.85). Total haemocyte count has decreased significantly 4283±77.2 after 96 hours of treatment of *P. guajava* leaf extract in the group C crabs (Table).

Prophenol oxidase (ProPO)

In the control crabs the Pro phenoloxidase enzyme activity level in the haemolymph is 0.625±0.001. After 96 hours of exposure to *V. harveyi* pro phenoloxidase level gradually reduced in the group B (0.327±0.007) whereas *P. guajava* leaves ethanol extract treatment has induced the Pro phenoloxidase level after 96 hours (1.78±0.038) in group C. Pro phenoloxidase level has increased significantly, after 96 hours of treatment in the group C.

Table 1. Total Haemocyte count and ProPO levels in *Oziotelphusa senex senex* .

Parameters	Control (Group A)	*V.harveyi* Injected After 96hr-(Group B)	*P. guajava* Treated After 96 hr-(Group C)
Pro Po min/mg/protein	0.625±0.001	*0.327±0.007	*1.78±0.038
THC cells / cu.mm	4291±73.31	*8366±68.85	*4283±77.2

Mean ± SD of six individual observations Group-A Vs B Vs C * P< 0.01

DISCUSSION

Some chemotherapeutic agents of plant's origin have been isolated promise to deal with drug-resistant bacteria [12-14]. Compounds such as volatile oils, tannins, phenolic compounds, saponins, alkaloids polysaccharides and polypeptides were shown to be effective alternatives to antibiotics. The screening of plant extracts and natural products of antimicrobial activity from higher plants represent a potential source of new anti-infective agents as well as serve in drug discovery, antibacterial therapy is going through a crisis due to rapidly increasing developments of resistance to existing agents. Such resistance has an impact on all areas of chemotherapy. Plants-derived phytomedicines have promise in the treatments of infectious diseases.

In the present study, ethanol extracts of *P. guajava* effectively controlled the pathogen *V. harveyi*, the Total phenoloxidase activity significantly changed after 96 hr in the female crabs inoculated with *V. harveyi* and there is a significant change after 96 hr. injection of *P. guajava* ethonolic leaf extract.

The prophenoloxidase-activating system is an important defense mechanism in invertebrates against diverse pathogens [15]. Likewise ethanol extracts of *P. guajava* effectively controlled the pathogen *V. harveyi*, the Total haemocyte counts significantly changed after 96 hr. in the female crabs inoculated with *V. harveyi* and further there is a significant change after 96 hr. injection of *P. guajava* ethonolic leaf extract.

In the present investigation, the sample population of *O. senex senex*, subjected to *V.harveyi* infection survived up to ten or more days. However, the increase of dosage caused their mortality within 72 hr. The above observation also suggests that the circulatory blood cells specifically the granular haemocytes and the phenoloxidase system alongside with the haemagglutination principles may be rendering the crab's survival and the potentiation of their defense mechanism

The effectiveness of an immune system can be tested using disease resistance test which is an important tool to estimate the increased protection in the treated crabs to determine the efficacy of an immunostimulants. The present results suggested that *P. guajava* extract may provide a new therapeutic value in specific and non-specific immunity in *O. senex senex*. In addition, sanitation and good management practice will reduce *V. harveyi* outbreak in a crab production unit. Further the present study shows that the ethonolic extract of *P. guajava* has significantly enhanced the immunity in *O. senex senex* at 1000 ppm.

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