Morinda tinctoria leaf extract induced immunity in a fresh water crab

Oziotelphusa senex senex

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Abstract

The crab, Oziotelphusa senex senex was injected with V.parahaemolyticus (0.1 ml of 10⁷ cfu/ml). After injection of bacteria the crabs were allowed to withstand for 96 hrs. After 96 hrs one group of crabs haemolymph were used for haematological and immunological assays. Remaining bacterial injected crabs were treated with 0.05 ml of 80% M.tinctoria leaf methanol extract (1000 ppm), after 96 hrs. Total Haemocyte Count (THC), Differential haemocytes counts (DHC) and Prophenol Oxidase (ProPO) were significantly changed (P<0.001) in the experimental group. These results suggested that the M.tinctoria could combat the microbial infection by stimulating the immune response in crabs.

Keywords: THC, DHC, M. tinctoria, V. parahaemolyticus, O. senex senex

Introduction

Crab farming is a recent activity and is practiced in Bangladesh, China, Indonesia, Malaysia, Singapore, Taiwan, Philippines and Vietnam. In India, crab farming is being mainly carried out in West Bengal, Orissa, Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, Goa, Maharashtra and Andaman islands. The mud crabs are large portunids with high commercial value in terms of domestic markets and export by virtue of their delicacy. In spite of intensive research on several aspects of aquaculture of this species for the past two decades (Kathirvel et al., 2004) little attention has been given to the causes of disease and mortalities of cultured crab populations (Poormima et al., 2008). The diseases of mud crabs have been reviewed recently (Jithendran et al., 2010). Although as many as 30 viruses have been documented to cause infections in crabs (Bonami and Zhang 2011), most publications on viral infections in mud crab diseases pertain to white spot syndrome virus (WSSV). Natural WSSV infections have been reported in wild-caught and farmed mud crabs of various life stages in many countries of Asiatic region (Lo et al., 1996; Otta et al., 1999). Recently, an icosahedral 150 nm virus causing a disease characterised by muscle necrosis and a reovirus designated as mud crab reovirus (MCRV) with signs of ‘sleeping disease’ associated with high mortality in cultured mud crabs, S. serrata was reported from China (Weng et al., 2007). A number of bacterial diseases such as shell disease, filamentous bacterial disease, luminescent bacterial disease etc. have also been reported in mud crabs (Lavilla-Pitogo et al., 2004). It is possible that the intensification of mud crab culture is likely to result in increased occurrence of diseases, as experienced with shrimp farming, with WSSV being an important agent (Somboonna et al., 2010). The foregoing overview of diseases of mud crabs indicates that they
suffer morbidity and mortality due to viral and bacterial infections affecting productions.

Apart from the viral attack diseases caused by bacterial infection in crabs also causes a great economical loss. To maintain the animal health several antibiotics have been used to fore come 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. Several antimicrobial, anti-stress, immunostimulant, growth-promoting plant products significantly influenced the fish/shrimp larviculture (Citarasu et al., 2003; Sivaram et al., 2004; Devakumar et al., 2011 a, b; 2012) successfully controlled the Vibrio pathogen, and improved the immune system of grouper larviculture using herbal methanolic extracts. These findings suggest that phytochemicals could be an alternative to the chemotherapeutic molecules and safe to use in aquaculture. The present study focus on screening of selected antibacterial / immunostimulant (M. tinctoria) herbals against the V. parahaemolyticus bacterial infected O. senex senex crabs.

Materials and Methods

Experimental animal and treatment

The female and male crabs, Oziotelphusa senex senex collected from Vandalur Lake, Tamil Nadu were brought to the laboratory and maintained in plastic tubs. Crabs were fed with beef mutton ad libitum and the water was changed daily and was acclimatized for 15 days in the prevailing room temperature. The crabs were divided into six groups of sixty crabs each - Female Control (Group-A) V. parahaemolyticus injected female crabs (Group-B), M. tinctoria leaves methanol extract treated Female crabs (Group-C), Male Control (Group-D), V. parahaemolyticus injected Male crabs (Group-E), M. tinctoria leaves methanol extract treated Male crabs and Group-F, experimental crabs injected with sub lethal dose of V. parahaemolyticus 0.1 ml of 10^7 cfu/ml. After injection the crabs were allowed to withstand for 96 hrs. After 96 hrs haemolymph was collected from ten crabs for haematological and Immunological assays. Remaining bacterial injected crabs were treated with 0.05 ml of 80% M. tinctoria leaves methanol plant extract (1000 ppm) and after 96 hrs 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; 0.1 M glucose; 30 mM trisodium citrate; 20 mM citric acid; 100 mM EDTA, pH 4.6) as anticoagulant.

Haematological analysis: Total haemocyte count (THC)

Total haemocyte Count was determined by the method of heamocytometer (Dacie and Lewis, 1968).

Differential haemocytes counts (DHC)

Differential counts of haemocytes were performed (Kondo, 2003). The smears were prepared by carefully spreading a drop of the fixed and thoroughly mixed hemocyte suspension on glass slides. These films were then air dried, incubated for 5 min in methanol. Washed in distilled water and flooded with Giemsa stain solution for 20 min and rinsed with distilled water, finally.

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 ultrasonication 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 spectrophotometer (Takahashi et al., 2000).

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 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.

Results

Total haemocytes count (THC)

After exposure to V. parahaemolyticus the haemocytes counts showed significant changes in the experimental groups (Table 1). In the control female crabs Total haemocyte count is 5720 ± 28.674, control male crabs Total haemocyte count is 3360 ± 30.970 respectively. After 96 hrs of exposure to V.parahaemolyticus Total haemocyte count gradually increased in the Group B, E female crabs 8598 ± 24.495, male crabs 6168 ± 34.66 respectively in the groups B, E haemolymph. Total haemocyte count has decreased significantly, after 96 hrs of treatment of M.tinctoria in male and female crabs of in the group C and F (Table 1).

Differential haemocytes counts (DHC)

In the control, female crabs (Group-A) and male crabs (Group-D) differential haemocytes count (DHC) large granule cells (LGC), small granule cells (SGC) and hyaline cells (HC) are 17.5 ± 0.278, 55.3 ± 0.720 and 26.5 ± 0.623, 24 ± 0.471, 43.1 ± 0.5741 and 32.1 ± 0.490 respectively (Table 1). After 96 hrs exposure of crabs to V. parahaemolyticus. Differential haemocyte large granule cells (LGC) and small granule cells (SGC) gradually increased but hyaline cells (HC) gradually decreased in the group B and E Differential haemocytes counts level of group B and E large granule cells (LGC), small granule cells (SGC) and hyaline cells (HC) are 23.8 ± 0.574, 67.6 ± 0.544 and 8.1 ± 0.490, 13.5 ± 0.623, 71.8 ± 0.443 and 13.1 ± 0.574 respectively. M.tinctoria leaves methanol plant extract treatment has increased the Differential haemocytes counts level after 96 hrs in the group C and F large granule cells (LGC), small granule cells (SGC) and hyaline cells (HC) are 15.5 ± 0.623, 61.1 ± 0.490 and 24.8 ± 0.490, 20.6 ± 0.720, 58.5 ± 0.623, 22 ± 0.471 respectively.

Prophenol oxidase (ProPO)

In the control female crabs male crabs the Pro phenoloxidase enzyme activity level in the haemolymph are 0.828 ± 0.038, 0.790 ± 0.030 respectively. After 96 hrs of exposure to V. parahaemolyticus pro phenoloxidase level gradually reduced in the group B and E, Pro phenoloxidase level in haemolymph is 0.449 ± 0.025, 0.314 ± 0.039 respectively in the group B and E haemolymph (Table 1). M. tinctoria leaves methanol extract treatment has effected in increased the pro phenoloxidase level after 96 hrs are 0.754 ± 0.028, 0.622 ± 0.021 in group C and F Pro phenoloxidase level has increased significantly, after 96 hrs of treatment.

Discussion

In aquaculture, chemotherapeutic agents such as commercial antibiotics and disinfectants are commonly
employed for disease management, although this is not advisable due to high cost, environmental hazards, and high cost, environmental hazards, and

Table 1. Haematological and immunological levels in *V. parahaemolyticus* infected *Oziotelphusa senex senex* treated with *Morinda tinctoria* leaf extract

<table>
<thead>
<tr>
<th>Groups</th>
<th>THC cells / cu.mm</th>
<th>Differential haemocytes counts (DHC)</th>
<th>Pro Po min/mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5720 ± 28.67</td>
<td>LGC % 17.5 ± 0.28 SGC % 55.3 ± 0.72 HC % 26.5 ± 0.62</td>
<td>0.828 ± 0.038</td>
</tr>
<tr>
<td>B      <em>8598 ± 24.50</em></td>
<td><em>23.8± 0.57</em></td>
<td><em>67.6 ± 0.54</em></td>
<td><em>8.1 ± 0.49</em></td>
</tr>
<tr>
<td>C      <em>6391 ± 30.97</em></td>
<td><em>15.5 ± 0.62</em></td>
<td><em>61.1 ± 0.49</em></td>
<td><em>24.8 ± 0.49</em></td>
</tr>
<tr>
<td>D      3360 ± 30.97</td>
<td>24 ± 0.47</td>
<td>43.1 ± 0.574</td>
<td>32.1 ± 0.49</td>
</tr>
<tr>
<td>E      <em>6168 ± 34.66</em></td>
<td><em>13.5 ± 0.62</em></td>
<td><em>71.8 ± 0.44</em></td>
<td><em>13.1 ± 0.57</em></td>
</tr>
<tr>
<td>F      <em>4516 ± 27.21</em></td>
<td><em>20.6 ± 0.72</em></td>
<td><em>58.5 ± 0.62</em></td>
<td><em>22 ± 0.47</em></td>
</tr>
</tbody>
</table>

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

the antibiotic resistance developed by many pathogens (Kruse and Soram 1994). To overcome this present situation, herbal medicine can be used as an effective antimicrobial drug. These are the ancient Indian systems of health care and longevity (Citarasu et al., 2002), but are still rejected by the scientific community due to a lack of standardization (Ponni, 2002). In the present study, methanol extracts of *M. tinctoria* effectively controlled the pathogen *V. parahaemolyticus* the total and differential haemocyte counts significantly changed after 96 hrs in the female and male crabs inoculated with *V. parahaemolyticus* and there is a significant change after 96 hrs injection of *M. tinctoria* methonolic leaf extract. The hemocyte in *O. senex senex* comprise three major groups, including hyaline cells (HC), small granule cells (SGC) and large granule cells (LGC) depend on cell and granules size and the nucleocytoplasmic ratio. Semi-granulocytes are the immature LGC. Granulocytes involved the immune system as well as hyaline cells. However, it seems hyaline cells start some reaction such as clot formation. Lavine and Strand (2002), Cerenius and Söderhäll (2004) proved that these reactions are often observed to become melanized, through the action of phenoloxidase. In the present study, the phenoloxidase activity significantly decreased after 96 hrs in the haemolymph of female and male *O. senex senex* inoculated with *V. parahaemolyticus*, there was a significant increase after 96 hrs injection of *M. tinctoria* methonolic leaf extract. In crustaceans and insects the phenoloxidase is not merely considered as an enzyme of cuticle tanning process but also considered to play an important role in the immune functions (Brethlin et al., 1989; Lanz et al., 1993). Studies have demonstrated that various elicitors of phenoloxidase or prophenoloxidase include (Nelliappan and Ramalingam, 1980), i) B-1,3 glucan from fungal cell wall or lipopolysaccharides; ii) Bacterial peptidoglycans; iii) Temperature; iv) Calcium concentrations; v) *Vibrio* cells; vi) Divalent cations Ca2+ and Mg2+; vii) Trypsin activates. Sung et al., (1998) have reported that both calcium and magnesium are required to enhance the phenoloxidase activity, both in tiger prawns and giant freshwater prawns. Cheng et al. (2000) have opined that the quality of water is critical in maintaining proper
concentrations of calcium or magnesium in order to enhance the pro-phenoloxidase activity to strengthen the defense mechanism of the shrimps.

Sung et al. (1998) have also observed that phenoloxidase activity in the giant freshwater prawn is significantly greater than that of tiger prawn and attributed it to higher levels or large number of granular haemocytes. Soderhall and Smith (1983) have revealed that semi-granular and granular haemocytes participate in the pro-phenoloxidase system and are involved in the antibacterial activity. With the above inference from the reports, the decreased phenoloxidase activity in the present study may be attributed to the inhibitors of the above enzyme system in vivo as well as to the circulatory haemocytes, their nature or types and the specific disease of those cells involved in immune function. The fact that Macrobrachium rosenbergii survives in freshwater for longer periods with greater hardiness (Ra’anana et al., 1983) suggests that in the experimentally inoculated prawns the microbial metabolism may be interfering with its hardiness by affecting the phenoloxidase activity which has been said to play a role in the phagocytic and encapsulation processes of the non-self or foreign antigen (Hose et al., 1990). The conspicuous increase in the phenoloxidase activity in the haemolymph at 96 hrs in the bacterial inoculated prawns is of interest to suggest that the suppression or inhibition of phenoloxidase activity is not of a permanent nature and that it could also be revived. However, the intricate mechanisms in enhancing the phenoloxidase activity remain to be explained in the in vivo condition. Towards the above, the reports of a few investigators with regard to the function of phenoloxidase are of interest to mention. Chisholm and Smith (1992) observed no correlation between antibacterial activities and phenoloxidase in the shore crab carcinoma maenus. However, Du et al. (1997) revealed that phenoloxidase activity increased with the bacteriolytic activity in Penaeus chinensis against Vibrio alginolyticus after immunopotentiation with the incorporation of garlic oil in the feed.

In the present investigation, the sample population of O. senex senex, subjected to V. parahaemolyticus infection survived up to ten or more days. However, the increase of dosage caused their mortality within 72 hrs. 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 crabs 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 immune-stimulants. The present results suggested that M. tinctoria 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.parahaemolyticus outbreak in a crab production unit. Further the present study shows that the methonolic extract of M. tinctoria has significantly enhance the immunity in O. senex senex at 1000 pmn concentration level study provides scientific evidence that the methonolic extract of M. tinctoria activate the specific immune mechanisms and against V.parahaemolyticus.

References


Kumaran et al., 2013


