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Original Article  
Comparative study of in vivo metabolic parameters in WSSV infected Litopenaeus vannamei, treated with anti-WSSV drug derived from marine and terrestrial plants  
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Abstract  
White spot syndrome virus (WSSV), is the most contagious pathogen of cultured shrimp that causes mass mortality, leading to huge economic loss to the shrimp industry. The lack of effective therapeutic or prophylactic measures has aggravated the situation, necessitating the development of antiviral drugs. With this objective, the antiviral activity of the drugs (MP07X - derived from the marine plant) and (TP22C - derived from the terrestrial plant) in the host, Litopenaeus vannamei was evaluated. The metabolic and physiological changes aggravated by WSSV infected host were analyzed. A total of 19 parameters such as, total protein, total carbohydrate, total glucose, total free amino acid, total fatty acid, fructose 1, 6 diphosphatase, aldolase, glucose 6 phosphatase, glucose 6 phosphate dehydrogenase, total hemocyte count, clotting time, oxyhemocyanin, hemolymph pH, hemolymph ammonia, phenoloxidase activity, respiratory burst activity, superoxide dismutase, oxygen consumption and ammonia excretion were examined for healthy (NEG), WSSV infected (POS), test sample 1 (TS1) and test sample 2 (TS2) shrimps. Significant differences (p < 0.01) were observed between the POS, TS1, TS2 and NEG in the variables at different time intervals post infection with WSSV, when the treatments were subjected to two way ANOVA. Although there is not much difference in the survivability of the host when administered with the two drugs, MP07X and TP22C, but significant differences are noted in all the variables justifying that the mode of nullifying the virus in the host, is dissimilar in nature. The systematic relationships between all the variables were analyzed by CIRCOS data visualization software. The histopathology of four groups of shrimp tissues revealed the virulence of WSSV. Thus, in accordance with the above results it can be concluded that acute WSSV infection triggers alterations in metabolic and physiological parameters in L. vannamei. and the two drugs are capable enough to eradicate WSSV.  
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KEYWORDS : WSSV, Litopenaeus vannamei, anti – WSSV drug, metabolic variables, comparative analysis  
1. Introduction  
Among the lethal viruses infecting penaeid shrimp, the white spot syndrome virus (WSSV), is a fast replicating and an extremely virulent shrimp pathogen, that has emerged globally as one of the most prevalent and widespread one, resulting in a rapid decline in the global shrimp production over the last few decades [1, 2]. Disease is the result of a complex interaction between host, pathogen and the environment. Maintaining a healthy shrimp stock requires a multidisciplinary approach that mostly depends upon stress management and disease control [3]. There is considerable evidence to support links between stress caused by environmental changes and diseases mainly caused by depression of the immune system [4, 5]. Once the immune system fails, it may lead to an enormous change in the metabolism of an organism. Stress therefore disrupts the immune ability and metabolic performance of shrimps, increasing its susceptibility to microbial infections. This virus infects the vital organs of mesoderma and ectodermal origin, as evidenced by the presence of degenerated cells with hypertrophied nuclei in the infected tissues [6, 7]. Other signs of WSSV include lethargy, sudden reduction in food consumption, red discoloration of body and appendages and a loose cuticle. However, there are very few scientific data supporting the link between environmental stress and increased susceptibility to diseases in shrimps.
In the present study the comparative estimation of in vivo metabolic parameters in WSSV infected Litopenaeus vannamei, healthy and the test groups (administered with two drugs derived from marine and terrestrial plants) were carried out. Thus, the annihilation of the host metabolism caused by the virus and the protection of the same by the two drugs can be envisaged by studying the metabolic variables of the host in order to fulfill the objective of the present research.

2. Materials and Methods

2.1. Screening and isolation of anti – white spot syndrome virus drug

Sixty plants were collected from different places of India. Amongst them thirty were from marine origin and the rest were terrestrial in nature. Four solvents (water, ethanol, methanol and hexane) based on their polarity were used to extract phytomolecules from the dry leaves by the soxhlet extraction method. A total of 240 crude isolates (120 isolates from marine and 120 from terrestrial plants) thus obtained from the plants were tested for the anti-WSSV activity. These 240 isolates were administered to WSSV infected Litopenaeus vannamei (white legged shrimp) weighing 5 – 7 gms. to determine the antiviral efficacy in the host - pathogen interaction model. Amongst the 240 isolates, 9 (from marine plants) and 7 (from terrestrial plants) exhibited significant anti – WSSV property. The best anti – WSSV plant isolates, MP07X and TP22C derived from marine and terrestrial plants respectively; were purified, and used in further bioassays.

2.2. Toxicological analysis of MP07X and TP22C in animal model

The lyophilized plant isolates (MP07X and TP22C) were used to prepare the strength solution for the toxicity studies in L. vannamei (6 – 8 gms.) as the animal model. The stocks having strength of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/ml were prepared in NTE buffer. From each of the preparations, aliquots of 10 μl were administered intramuscularly into the 6th abdominal segment of apparently healthy L. vannamei. The control consisted of animals injected with 10 μl of distilled water alone. For each of the concentrations of the extract, 6 animals were used in triplicates and monitored for 7 days and subjected for general health assessment following the parameters such as; characteristic colouration, feed intake, moulting, antenal intactness and necrosis. The percentage of survivability obtained with different dilutions of the extract was statistically analyzed by a single factor ANOVA. The differences were considered significant at p ≤ 0.05.

2.3. Preparation of viral inoculum

WSSV infected L. vannamei with prominent white spots were collected from shrimp farms. Gills and soft parts of the cephalothorax region (500 mg) from these infected shrimps was macerated in 10 ml cold NTE buffer (0.2 M NaCl, 0.02 M Tris–HCl and 0.02 M EDTA, pH 7.4) with glass wool to a homogenous slurry using mortar and pestle in ice bath. The slurry was centrifuged at 3000 g for 20 mins. in a refrigerated centrifuge at 4 °C. The supernatant was recentrifuged at 8000 g for 30 mins. at 4 °C and the final supernatant fluid was filtered through a 0.4 μm filter. The preparation was streaked on ZoBell’s, Thiosulfate Citrate Bile Salts - Sucrose (TCBS) and Potato Dextrose (PDA) agar plates and incubated at 28 ± 2 °C for 72 hrs. to confirm the absence of microbial contamination. The viability of WSSV in the prepared inoculum was tested by injecting 10 μl to a batch of apparently healthy shrimps (4 nos.); whose mortality occurred over a period of 3 to 5 days, and the viral infection was confirmed by PCR results. The viral inoculum was stored at - 20 °C till used.

2.4. Protocol for the in vivo experimentation

For bioassay, the plant isolates (MP07X and TP22C) were dissolved in NTE buffer and termed as, plant isolate - buffer solution, at the concentration of 10 mg/ml (500mg/kg body weight of shrimp). During the experimental trials, shrimps (TS1 and TS2) (5 shrimps in each tank) were injected intramuscularly with a mixture of viral suspension and the above prepared plant product (MP07X and TP22C respectively) at the volume of 25 μl per animal [5 μl of viral suspension, 20 μl of plant isolate - buffer solution]. The positive control (POS) shrimps were injected with a mixture of 20 μl NTE buffer and 5 μl viral suspension, while the negative control (NEG) shrimps were injected with 25 μl NTE buffer only. All these mixtures were incubated at 29 °C for 3 hrs. before the experimentation. The experimental trial was carried until the absolute mortality of the positive control, post infection with WSSV.

2.5. Estimation of in vivo metabolic and physiological parameters

The survivability percentages (SURV) along with 19 metabolic and physiological parameters in all the four groups (POS, TS1, TS2 and NEG) of shrimps were analyzed. The 17 metabolic parameters such as; total protein (TP) was determined spectrophotometrically based on the Lowry method [10], total carbohydrate (TC) was determined by the Anthrone method [11], total glucose (TG) was determined by the Glucose (GO) Assay Kit (Sigma), total free amino acid (TAA) was determined using the ninhydrin method [12], total fatty acid (TFA) according to the standard method [13]. Fructose 1, 6 Diphosphatase (FDPase) was determined by slightly modifying the earlier methodology [12]. Aldolase (ALD) was determined by the Randox AD189 assay kit, Glucose 6 Phosphatase (G6Pase) was estimated by the Glucose 6 Phosphate Assay Kit (Sigma), Glucose 6 phosphate dehydrogenase (G6PDH) was estimated by the Glucose 6 Phosphate dehydrogenase Assay Kit (Sigma). Total hemocyte count (THC) was determined using a Neubauer's hemocytometer. Clotting time (CT) of the hemolymph was determined by the capillary method as described earlier [13], Oxyhemocyanin (OHC) was calculated based on the method described elsewhere [14], Hemolymph pH (pH) was measured by the glass electrodes of a microelectrode set [15], Hemolymph Ammonia (NH3) was determined using the Sigma Diagnostic Kits Ultraviolet No. 170-UV [16]. The Phenoloxidase activity (PO) was determined using standard protocol [17], Respiratory burst activity (RB) was determined standard methodology [18] and Superoxide dismutase (SOD) was analysed using Ransod Kit [19, 20]. The 2 physiological parameters such as; oxygen consumption (O2 cons.) and ammonia excretion (NH3) 58-72
exce.) of all the four groups (POS, TS1, TS2 and NEG) of shrimps were analyzed according to the standard methods [21, 22].

2.6. Histopathology of gill tissues of the host
The gill tissue samples from the experimental shrimps (POS, TS1, TS2 and NEG) were dissected out and immersed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS at pH 7. Histosections (30 μm) were excised using Cryostat (Leica, CM1510 S). The sections were transferred to clean microscopic slides previously coated with Chrome Alum Gelatin, dewaxed in two changes of xylene and rehydrated through 95%, 70% alcohol and finally in distilled water. The sections were stained with hematoxylin and differentiated in acid alcohol. Bluing was done in Scots tap water and counter stained with eosin. Again dehydrated in alcohol and cleared in xylene and mounted in DPX. The sections were observed under a phase-contrast microscope.

2.7. Statistical analyses
The data obtained from the experiments were subjected to appropriate statistical analysis. Statistical analyses were carried out using the software packages such as; R i386 2.15.1; SPSS ver. 19.0; Minitab Ver. 15.0; Circos v0.64; and Microsoft Office Excel 2007. To find out the relationships between survival rate and other parameters (metabolic & physiological), the results were examined using Analysis Of Variance (ANOVA) followed by a Least Significant Difference (LSD) test of the post challenge data. P - values of less than 0.05 were considered to indicate statistical significance. Along with the above statistical analysis, a new approach was introduced to present the relationship between survival rate and the 19 variables with respect to time. Representation of relationships was projected by using CIRCOS data visualization software.

Fig. 1. Data presentation in CIRCOS

NOTE:
The concept behind the CIRCOS data visualization tool is very simple. In the general case, relationships between elements in data sets are indicated by links. Links can indicate a simple relationship (A-B), a relationship that has positional information (A-C), or a unidirectional relationship (A-D). If the relationship has an associated quantity (e.g. degree of similarity, correlation, proportion ratio, traffic between elements, etc.), this quantity can be represented by the thickness of the link. By coloring the links based on one of the elements, following relationships to/from an element is made easier. For example, when the links relate a cell for a given row and column, the color of the link can be that of the row or column segment. When links are colored based on the elements that they relate, spotting patterns is easier. In particular, when relationships have a direction, links can be colored by source or target element (Fig. 1).

3. Results
3.1. Preliminary study of the WSSV challenge test
The L. vannamei with prominent white spots, collected from shrimp farms were WSSV positive (Fig. 2). The WSSV inoculum obtained from the viral infected L. vannamei was administered in the fresh shrimps, and none of the shrimps survived. The molecular diagnosis (PCR result) and survivability percentage in different experimental groups are presented (Fig. 3, 4).

Fig. 2. WSSV infected carapace of L. vannamei

Fig. 3. Detection of WSSV gene in the PCR product. M = marker, 1 = WSSV infected shrimp from the farm, 2 = freshly WSSV infected shrimp produced in laboratory, P = positive control (211 bp), N = negative control.

3.2. Determination of in vivo toxicity of the plant isolate MP07X and TP22C
L. vannamei (6-8 gms.) (n = 6) were injected with the two plant isolates at different concentrations ranging from 5 - 50 mg/ml and monitored for 7 days (Fig. 5). In case of MP07X and TP22C the response of the animals was more or less the same without any significant mortality even up to a concentration of 35 mg/ml ($p < 0.05$) and 30 mg/ml
(p<0.05) respectively. However, at 50 mg/ml strength there was significant reduction (56 % and 43 % average percentage survival respectively) (p<0.05) in survival of shrimps during the experimental period of 7 days.

**Fig. 4.** Variations in survivability percentage in different experimental groups.

**Fig 5.** Toxicity of different concentration of plant isolates (MP07X & TP22C) in L. vannamei.

### 3.3. Estimation of in vivo metabolic and physiological parameters

Administration of viral inoculum to L. vannamei resulted in development of white spot syndrome, manifesting clinical signs after 24 hrs. of injection in the positive control (POS) shrimps. The animals ceased eating, became lethargic and disoriented during swimming; showing a tendency to move towards the edges of tanks and near the surface. The morphological abnormalities included appearance of white circular inclusions or spots, developing in the cuticle, often followed by a red discolouration all over the body, especially in pleopods, periopods, telson and uropods. Mortality of shrimps started along with the appearance of clinical signs registering 100 % mortality within 80 – 84 hrs. post injection. The negative control (NEG) shrimps were devoid of any such symptoms with no mortality recorded. The absence of WSSV infection in this group was confirmed using PCR.

This result suggested the in vivo virulence of WSSV. This was also confirmed by the significant variations observed in the metabolic variables in the tissue of the four groups (POS TS1, TS2 & NEG). The total protein (Fig. 6) content in POS, TS1 and TS2 groups, exhibited the lowest level of 22 µg/mg, 33 µg/mg and 32.3 µg/mg at the 36th hr respectively. The lowest level of carbohydrate (Fig. 7) in POS group was 0.89 g/mg at the 72nd hr whereas; in case of TS1 and TS2 groups, it was 1.8 µg/mg and 2.13 µg/mg at the 36th hr respectively. The highest level of total glucose (Fig. 8) estimated in POS was 1.29 µg/mg at the 24th hr whereas; in case of TS1 and TS2 groups, it was 0.96 µg/mg and 1.06 µg/mg at the 24th hr respectively. The total fatty acid (Fig. 10) content in POS, TS1 and TS2 groups, exhibited the highest level of 0.56 µg/mg, 0.42 µg/mg and 0.456 µg/mg at the 36th hr respectively. The lowest level of fructose 1, 6 diphosphatase (Fig. 11) estimated in POS was 25 µg P/mg protein/hr at the 36th hr and in case of TS1 and TS2 groups, it was 58 µg P/mg protein/hr and 53.6 µg P/mg protein/hr at the 12th hr respectively. The aldolase (Fig. 12) content in POS, TS1 and TS2 groups showed its highest level of 1.468, 1.427 and 1.429 µ glyceraldehyde/mg protein/hr at the 36th hr respectively. The lowest level of glucose 6 phosphatase (Fig. 13) estimated in POS was 28.7 µg P/ mg protein/hr at the 36th hr; however, in case of TS1 and TS2 group, it was 33 and 33.7 µg P/ mg protein/hr at the 24th hr. The glucose 6 phosphate dehydrogenase (Fig. 14) content in POS exhibited a highest level of 1.91 units/mg protein/hr at the 72nd hr whereas; in TS1 and TS2 groups, it was 1.4259 and 1.4552 units/mg protein/hr at the 36th hr respectively.

**Fig. 6.** Variations in total protein content in different experimental groups.

**Fig. 7.** Variations in total carbohydrate content in different experimental groups.

**Fig. 8.** Variations in total glucose content in different experimental groups.
Similarly, significant variations were observed in the immune variables in the hemolymph of POS, TS1 and TS2. The Total hemocyte count (Fig. 15) in the hemolymph of POS, TS1 and TS2 groups were observed to be $6.1 \times 10^6$/ml, $6.1 \times 10^6$/ml and $6.145 \times 10^6$/ml at the 0th hr; however, with further increase in time a steady decline (in case of POS) and a stability (in case of TS1 and TS2) in its count was observed. The coagulation time (257 secs.) (Fig. 16) was observed in the hemolymph of POS till 36th hr and after that the hemolymph did not coagulate at all but in case of TS1 and TS2 groups a similar pattern to that of the healthy ones (NEG) was observed. The oxyhemocyanin (Fig. 17) content in hemolymph of the animals (TS1 and TS2) was at the highest level of 1.2 and 1.186 m mol/l at the 72nd hr respectively, whereas in case of POS a steady decline till the 84th hr was observed. The pH (Fig. 18) of the hemolymph of the TS1 and TS2 was at the highest level of 7.52 and 7.562 at the 36th hr respectively, whereas in case of POS a steady increase till the 84th hr was observed. The ammonia (Fig. 19) content in hemolymph of the TS1 and TS2 was at the highest level of 0.39 and 0.412 mg/ml at the
$24^{th}$ hr respectively, whereas in case of POS a steady increase till the $84^{th}$ hr was observed. The phenoloxidase activity (Fig. 20) observed in hemolymph of POS was at the highest level of $0.1129/50$ $\mu l$ of hemolymph at the $24^{th}$ hr respectively; though with a further increase in time, a steady decline was observed, whereas in case of TS1 and TS2 the highest level of $0.11243/50$ and $0.11266/50$ $\mu l$ of hemolymph $\mu l$ of hemolymph at the $24^{th}$ hr respectively; though with a further increase in time, a stability in both the variables were observed. The respiratory burst (Fig. 21) observed in hemolymph of POS was at the highest level of $0.138/10$ $\mu l$ of hemolymph at the $24^{th}$ hr, with further increase in time, a steady decline was observed. In case of TS1 and TS2 groups the values were $0.126/10$ $\mu l$ and $0.1288/10$ $\mu l$ of hemolymph though with a further increase in time, a stability in both the variables were observed. The superoxide dismutase (Fig. 22) level in hemolymph of the animals (TS1 & TS2) was at the highest level of 1.43 and 1.418 units/ml hemolymph at the $0^{th}$ hr and at the lowest level of 1.26 and 1.29 units/ml hemolymph at the $24^{th}$ hr, respectively but with furthermore in time a stability in the level was observed. In case of POS a steady decline till the $84^{th}$ hr. was observed.
The significant variations were not only confined to metabolic and immune parameters but also reciprocating the same in the physiological ones. The oxygen consumption (Fig. 23) and ammonia excretion (Fig. 24) of POS was steadily declining till the 84th hr. but in case of TS1, both the parameters was at the highest level of 0.71 mg/g/hr and 430 μg/g/hr initially, whereas the lowest level of 0.59 mg/g/hr and 333 μg/g/hr was observed at the 36th hr respectively, and in case of TS2, the highest level of 0.692 mg/g/hr and 424.6 μg/g/hr initially and the lowest level of 0.557 mg/g/hr and 340.8 μg/g/hr was observed at the 48th hr and 36th hr respectively. Significant differences (p < 0.01) were observed between the POS, TS1, TS2 and NEG in the metabolic, immune and physiological variables at different time intervals post infection with WSSV, when the treatments were subjected to two way ANOVA. When the data were subjected to pairwise comparison between each treatment (NEG – POS, NEG - TS1, NEG - TS2, POS - TS1, POS - TS2 and TS1 - TS2) then the p-value pointed towards the pairs which were in particular responsible for the significant differences in the ANOVA. All the treatments are significantly different among themselves in all the 20 variables (Table 1, 2, 3 & 4). It also indicates that the treatment TS1 and TS2 although yields nearly similar survival percentages but their mode of nullifying the virulence of WSSV in the host, are significantly different. Amongst these variables, only in 7 some of the treatment pairs showed non-significant differences viz., Total Carbohydrate (NEG – TS2), Total Glucose (POS – TS2), Total Fatty acid (POS – TS1, TS1 – TS2), Oxyhemocyanin (TS1 – TS2), Hemolymph pH (TS1 – TS2), Phenoloxidase activity (NEG – TS1, TS1 – TS2) and Oxygen consumption (TS1 – TS2). The CIRCOS data visualization output illustrated (A(NEG), B(POS), C(TS1) & D(TS2))
Table 1. ANOVA and LSD of the treatments in the metabolic variables (SURV, TP, TC, TG, TAA and TFA)

<table>
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<tr>
<th>No.</th>
<th>Name of Variables</th>
<th>ANOVA (p-value)</th>
<th>ANOVA (p-value)</th>
<th>Least Significant Differences (LSD)</th>
<th>Least Significant Differences (LSD)</th>
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<td>Treat p &lt; 0.001</td>
<td>Treatment pair p-value LCL UCL</td>
<td>Treatment pair p-value LCL UCL</td>
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<td>Treat p &lt; 0.001</td>
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Table 2. ANOVA and LSD of the treatments in the metabolic variables (FDPase, G6Pase, ALD and G6PDH)

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<td>NEG - POS p &lt; 0.001 0.000875738 0.00090439</td>
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### Table 3. ANOVA and LSD of the treatments in the metabolic variables (THC, CT, OHC, pH and NH₃)

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<td>5.</td>
<td>Hemolymph Ammonia (NH₃)</td>
<td>Treat</td>
<td>p &lt; 0.001</td>
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<td>Treat Time</td>
<td>p &lt; 0.001</td>
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### Table 4. ANOVA and LSD of the treatments in the metabolic variables (PO, RB, SOD, O₂ cons and NH₃ excr.)

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of Variables</th>
<th>ANOVA (p-value)</th>
<th>Least Significant Differences (LSD)</th>
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<tr>
<td></td>
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<td>Treatment pair</td>
<td>p-value</td>
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<td>Treat Time</td>
<td>p-value</td>
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<tr>
<td>1.</td>
<td>Phenoloxidase activity (PO)</td>
<td>Treat</td>
<td>p &lt; 0.001</td>
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<td>Treat Time</td>
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<td>2.</td>
<td>Respiratory Burst (RB)</td>
<td>Treat</td>
<td>p &lt; 0.001</td>
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<td>Treat Time</td>
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<td>3.</td>
<td>Superoxide dismutase activity (SOD)</td>
<td>Treat</td>
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<td>4.</td>
<td>Oxygen consumption (O₂ Cons.)</td>
<td>Treat</td>
<td>p &lt; 0.001</td>
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<td>Treat Time</td>
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<td>5.</td>
<td>Ammonia excretion (NH₃ excr.)</td>
<td>Treat</td>
<td>p &lt; 0.001</td>
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<td>Treat Time</td>
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...FDPase, G6Pase, ALD, G6PDH} (Fig. 25); A(NEG), B(POS), C(TS1) & D(TS2) {variables – SURV, THC, CT, OHC, pH, NH3} (Fig. 26); A(NEG), B(POS), C(TS1) & D(TS2) {variables – SURV, O2 Cons., NH3 Excr.} (Fig. 27); A (NEG), B (POS), C (TS1) & D (TS2) {variables – SURV, PO, RB, SOD} (Fig. 28)] the systematic relationship between each variable in vivo with respect to time.

3.4. Histopathology of gill tissue of the host

Amongst the prepared samples only the longitudinal sections (L.S) of gill filament (rich in viral load) of the experimental animals were examined under the phase-contrast microscope to evaluate the pathological changes. The positive (POS) control group of animals that were injected with WSSV exhibited prominent histopathological changes that included dislodgment of cuticle on the gill filaments with sub-cuticular cells having eosinophilic hypertrophied nuclei, cellular degeneration and shrinkage, multi-focal necrosis and hemocytic infiltration. The necrotic pilaster cells showed eosinophilic hypertrophied nuclei, characteristic of WSSV infection. The lacunae occluded by hemocytes showed vacuolization. On the contrary, gill tissues from NEG, TS1 and TS2 group did not show any clinical pathological changes. The gill filaments contained numerous lacunae with a sufficiently large number of hemocytes as in the case of apparently healthy shrimps (Fig 29).

Fig. 29. Histopathology of gill tissue of L. Vannamei {A(NEG), B(POS), C(TS1) & D(TS2)}.

4. DISCUSSION

Antiviral activities of aqueous extracts from plants are well established [23 – 27] that also includes reports on the anti-WSSV activity of plant extracts [28 – 34]. A combination of herbal extracts and probiotics as medicated diet could decrease the prevalence of WSSV in Litopenaeus vannamei [35]. Even though reports are available on the protective effect of plant extracts against WSSV, information on their mode of action are scanty. In this present study, an attempt has been made to look into the possibilities of using 60 plants as sources of anti-WSSV drugs. The plants were subjected to soxhlet extraction to procure a combination of phytomolecules, potent enough to be an anti-WSSV drug. In this study, 9 plant isolates (from marine plants) and 7 plant isolates (from terrestrial plants) were found to be effective against WSSV. Finally, the plant isolates MP07X and TP22C proved to be the potent anti-WSSV drug in our research.

The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose [36]. We found that the crude drug MP07X and TP22C was less toxic to the shrimps at the concentrations required for the antiviral activity. Similarly, the highest non-toxic level of Ceriops tagal in P. monodon is 50 mg/ml [34]. The average percentage of survivability of shrimps injected with MP07X and TP22C was 85 % and 86 % respectively, at a concentration of 10 mg/ml. Marginal mortality was due to cannibalism subsequent to moulting may be considered. The result indicated that the minimum concentration of the extract required for extending the virucidal activity was less than its in vivo toxic level with high selectivity index, which is the ratio of toxic concentration to the effective concentration, and shows higher antiviral activity at a concentration below the toxic value. The results generated unambiguously suggest that the virucidal property of the two plant isolates were concentration dependent. Different concentrations of Cidofovir (an antiviral drug) were injected and observed that it was non-toxic to shrimps up to a concentration of 200 mg/kg of body weight and they could successfully use the same for further assays [37]. In a similar pattern on screening 20 Indian medicinal plants, anti-WSSV activity was exhibited by the aqueous extract
of Cynodon dactylon on administering 100 mg/kg of body weight when injected intramuscularly. Dosage dependent antiviral effects against WSSV have been reported in the case of antimicrobial peptide mytilin when injected after incubating with WSSV. It was proposed that the antiviral activity of mytilin was mediated by its binding onto the viral envelope [38, 39]. The results showed significant differences in the metabolic parameters of POS, TS1, TS2 and NEG. There was a sharp decrease in total protein and amino acid levels in the muscles of WSSV infected shrimp. The possibility for the decrease of protein in muscle of infected shrimp is that baculoviruses encode a variety of proteases and other enzymes that ‘melt’ the tissues [40], and that the proteins of the ‘Melted’ cells (muscle and hepatopancreas) would be incorporated into the shrimp hemolymph. The total carbohydrate and glucose levels decreased in muscle of WSSV infected shrimp in comparison with healthy shrimp. Generally, the decrease in the glucose level of infected or stressed animals might be due to the transport of glucose and carbohydrate from hepatopancreas and muscle to hemolymph. During stress, shrimp use carbohydrate as a source of energy [41]. In contrast, researchers [42] have observed disappearance of glucose and lactic acid from the hemolymph of the lobster infected with Gaffky’s homari. The fatty acid level decreased in muscle. This is a usual phenomenon in the infected shrimp [43, 44]. The mechanism responsible for excessive accumulation of fatty acid in infected shrimp is not known. It has also been reported that stress affected qualitative and quantitative nature of circulating carbohydrates [45, 46]. Glycolysis is reported to be a major pathway for the generation of energy (ATP) in all living organisms. Glycolytic intermediates were also reported to serve as a precursor for the biosynthesis of other cellular constituents [47]. To study the changes in glycolysis during white spot virus infection in L. vannamei, the activity of aldolase was measured in muscle. Aldolase is an ubiquitous glycolytic enzyme that catalyzes the reversible change of fructose 1, 6 diphosphate to glyeraldehyde 3-phosphate and dihydroxy aceton phosphate. This enzyme has a central position in the glycolytic pathway. The maintenance of aldolase activity indicated that the glycolysis continued and production of energy from glucose by catabolism also proceeded in the infected animal. It is interesting to note that even at moribund stage, the glycolytic pathway was not affected, as evident from the normal activity of aldolase observed in the present study. The data from this investigation clearly show that the enzymes in the anabolic pathway, i.e. production of glucose from pyruvate, the fructose 1, 6 diphosphatase and glucose 6 phosphatase were adversely affected during viral infection. Viral infection resulted in significant lessening in feed intake. This, coupled with normal rate of glycolysis, as evidenced by the aldolase activity and near total inhibition of gluconeogenesis, because of loss of activity of fructose 1, 6 diphosphatase must have contributed to the severe energy crisis in the infected animal. The activity of glucose-6-phosphate dehydrogenase in muscle of the shrimp infected with the white spot virus was different from the activity in uninfected animals. This enzyme is involved in the metabolism of glucose through the pentose phosphate pathway (PPP) to generate NADPH [47]. The increase in activity of this enzyme might therefore result in the production of more NADPH. The significance of this is the NADPH required for adequate levels of reduced GSH in turn would be helping to overcome oxidative stress. PPP in fishes is considered as a minor pathway, but in decapods it is a major one during their intermoult period [48]. It has been reported that the PPP with its major enzyme glucose 6 phosphate dehydrogenase provides the tissues with specific molecule, the reduced NADPH [49]. The significant increase in the activity of this enzyme in the WSSV infected shrimp may be part of the overall defense mechanism against the excessive oxidative stress during the infection. In our study, the initial hemocyte counts were reduced by more than 40 % in WSSV infected shrimp at moribund stage as observed in G. homari infected lobster [15]. The hemocyte count varies among crustaceans and is known to be affected by a variety of factors such as infection and environmental stress [51 - 52]. It has been demonstrated a decrease in the hemocyte numbers of crayfish harboring a parasitic fungus (Aphanomyces astaci) [51]. A decline in total hemocyte count in shrimp infected with penaeid rod-shaped DNA virus has been observed [53]. The decrease in THC in infected animals was probably caused by hemocytic accumulation at the site of injection for wound healing and phagocytosis of foreign bodies [44, 54, 55]. Another possibility is that the THC decline would be due to cell burst resulting from budding of the virus, or by virus induced apoptosis, since this type of cell ‘‘suicide’’ may be induced or repressed during some viral infections [56]. The hemolymph of WSSV infected shrimp failed to clot. A reduction in the hemocyte number and prolongation of coagulation in Vibrio infected shrimp has been observed [57]. An increase in hemolymph clotting time has been observed in our study so as in G. homari infected lobsters as observed earlier [15]. The WSSV might be responsible for the failure in coagulation of hemolymph in infected shrimp. Our results are consistent with the conclusion of others regarding the damage of clotting mechanism in G. homari infected lobsters [58]. Hemocyanin represents 80 – 95% of total protein in the hemolymph of crustacean [59, 60]. The hemocyanin has been affected by moulting cycle, nutritional conditions and stress [61 - 63]. Our results revealed a significant reduction in oxyhemocyanin content as well as in oxygen consumption in WSSV infected shrimp. This indicates the dysfunction of respiratory system and causing hypoxia in tissue; reduction in oxygen affinity of hemocyanin and infected animals may die of anoxia. It has been found to have no difference in hemolymph pH between normal and infected lobsters [15]. But in our studies, hemolymph pH increased from 7.15 to 8.384 in WSSV infected shrimp. The reason for this is unknown. The ammonia excretion decreased significantly in WSSV infected shrimp in comparison to normal shrimp and ammonia level in hemolymph of infected shrimp increased. This might be due to failure of excretory organs in infected shrimp. The results indicate the failure of vital functions such as clotting of hemolymph, defense mechanism, exchange of respiratory gas and excretion in WSSV.
infected shrimp. The ProPO activating system is a vital part of shrimp immune response, which includes recognition of foreign invaders and non-living entities, activation of a wide range of defense reactions, such as phagocytosis and antibacterial activity, encapsulation and nodule formation. Increase in total hemocytes count is generally accompanied by changes in phenoloxidase activity because these cells are the major store for the ProPO system. Activated phenoloxidases generate high cytotoxic quinines that can inactivate viral pathogens [64]. Our PO activity results were in comparison with those in closely related species, Fenneropenaeus chinensis [65]. Generation of reactive oxygen species as indicated by NBT assay has been used by a few investigators to measure immunostimulation in shrimp. To study the effect of treating shrimp hemocytes with glucan in vitro NBT assay was used [18]. The enhanced production of reactive oxygen (O$_2^-$) in shrimp treated by immersion with the Vibrio bacterin and glucan noted earlier [66]. Variations in respiratory burst activity could be attributed to the disparity in NADPH oxidase activity, phagocytic rate and/or the number of hyaline cells [67, 68]. Super oxide dismutase (SOD) is one of the main antioxidant defense enzymes generated in response to oxidative stress. It has been observed that the activity of SOD was significantly lowered in WSSV infected F. indicus [69, 70]. In the present study, the activity of SOD was also significantly lowered in the WSSV infected hemolymph of L. vannamei. These results coincide with that obtained earlier documenting the reduction of SOD in WSSV infected P. monodon [71]. Hence, it could be proposed that the above estimated variables are the most potential biomarkers that may be used in periodic assessment of the health status of shrimps. In accordance with the above results it can be concluded that acute WSSV infection triggers alterations in metabolic and physiological parameters in L. vannamei. Significant differences ($p<0.01$) were observed between the POS, TS1, TS2 and NEG in the metabolic, immune and physiological variables at different time intervals post infection with WSSV, when the treatments were subjected to two way ANOVA. Although there is not much difference in the survivability of the host when administered with the two drugs, MP07X and TP22C, but significant differences are noted in all the variables justifying that the mode to nullifying the virus in the host, is dissimilar in nature. The overall investigation on the comparative estimation of in vivo metabolic parameters in the four treatments indicates that the virulence of WSSV can be nullified by these two drugs derived from marine and terrestrial plants.

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supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles”, *Aquaculture*, vol. 255, pp. 600-605, 2006.


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