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In vivo Immunological Changes Occurring at Different Time Intervals in White Spot Syndrome Virus Infected Shrimp, Treated with Anti-WSSV Drug Derived from Marine Plants

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Abstract: White spot syndrome virus (WSSV), is the most transmittable 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 drug, (MP07X -derived from the marine plant) in the host, *Litopenaeus vannamei* was evaluated. The immunological and physiological changes aggravated by WSSV in the host and the *In vivo* efficacy of the drug in the host - pathogen interaction were analyzed. The survival percentage of the treated (with MP07X) WSSV infected host was 85 %. Significant results were obtained from the cytotoxicity assays of the drug in both the brine shrimp and host. A total of 10 parameters such as, 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 (Negative - NEG), WSSV infected (Positive - POS) and test sample (TS) shrimps. Significant differences ($P<0.01$) were observed between the POS, NEG and TS in the variables at different time intervals post infection with WSSV. In the case of POS, significantly ($P<0.01$) reduced variables were observed when compared to the NEG. In contrast, significant ($P<0.01$) elevations were observed in the TS after a certain time interval due to the anti-WSSV activity of MP07X. Neither the VP 28 gene nor the immediate early genes (*ie*) were expressed in the host at the 42nd and 84th hrs. Thus, in accordance with the above results it can be concluded that acute WSSV infection triggers alterations in immunological and physiological parameters in *L. vannamei* and at the same time the drug is efficient enough to combat the deadly virus and can increase the survivability of the host.

Key words: *Litopenaeus vannamei* · Anti-WSSV Drug · Immunological Parameters · Viral Gene · Immune Gene

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 mesodermal 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

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few scientific data supporting the link between environmental stress and increased susceptibility to diseases in shrimps.

Strategies for the prophylaxis and control of WSSV theoretically include improvement of environmental conditions, stocking of specific pathogen free (SPF) shrimp post larvae and enhancement of disease resistance by using immunostimulants. Several reports have appeared in literature over a period of time stating the use of different plant extracts against enveloped, non-enveloped, DNA/RNA viruses and their mode of action against these pathogens. Numerous plants from both terrestrial and marine origin have already been tested against viral diseases to judge its immunostimulant efficacy. For several years, mangroves, seagrasses and seaweeds have been in focus, as they are a rich storehouse of phytomolecules with several biological activities. The uniqueness of these phytomolecules, that are derived from these plants have prompted us to take up this present investigation, for which we have selected 30 plants exclusively from different marine ecosystems like mangroves, seagrass, seaweed, etc. The leaves from each of these plants were studied for their anti-WSSV property in the host, *Litopenaeus vannamei*. Further, the crude drug derived from the marine plant is administered to the WSSV infected host and the host is subjected to an array of immunological and physiological analysis to judge the efficacy of the same as a potent anti-WSSV drug. The *In vivo* destruction of the host metabolism caused by the virus can be envisaged by studying the immunological parameters and molecular analysis of the host in order to fulfill the objective of the present research.

MATERIALS AND METHODS

Screening and Isolation of Anti-White Spot Syndrome Virus Drug: Thirty marine plants (mangroves, seagrasses, seaweeds, salt marshes and sand dunes) were collected from different parts of the East coast of India. Four solvents based on their polarity were used to extract phytomolecules from the dry leaves by the Soxhlet extraction method. A total of 120 crude isolates thus obtained were coded properly, viz. MP01A (Marine Plant 01 solvent A), MP01B, MP01C, likewise. These coded isolates were administered to *Litopenaeus vannamei* (white legged shrimp) weighing 5-7 g post challenge with WSSV to determine the anti-white spot syndrome virus (WSSV) efficacy in the host-pathogen interaction model. Amongst these 120 isolates, 9 showed significant anti-WSSV property. By means of several trials and chemical processes the purified anti-WSSV plant isolate, MP07X was derived and used in further bioassays.

Cytotoxicity Assay of Plant Isolate MP07X: The brine shrimps used for the cytotoxicity test was produced by hatching 5 mg of *Artemia salina* eggs in natural seawater, after incubation at a temperature around 37°C with constant oxygen supply for 48 hrs. The nauplii were maintained for another 48 hrs in seawater to ensure their survivability and maturity before use. Six doses of the MP07X (10, 20, 40, 60, 80 and 100 µg/ml) were dissolved in 5% dimethyl sulfoxide (DMSO) and/or seawater and tested. Each of the extract preparations were dispensed into clean test tubes in 10 ml volumes and tested in duplicates. For control, same procedure was followed devoid of the plant isolate. Ten living brine shrimps were added to each of the test tubes with the help of a Pasteur pipette. All the test tube containing the nauplii for the bioassay were then incubated at 29°C for 24 hrs in a water bath, after which each tube was examined and the surviving nauplii was counted. From this, the percentage of lethality of brine shrimp nauplii was calculated for each concentration of the extract [8].

Statistical analysis was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared to the control group. $P<0.05$ were considered to be statistically significant. The concentration of the plant isolate producing 50 % of the maximum response (LC_{50}) was obtained by the best visual fit from the plot of the individual experiments.

Toxicological Analysis of MP07X in Animal Model: The lyophilized plant isolate (MP07X) was used to prepare the strength solution for the toxicity studies in *L. vannamei* (6-8 g) 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 were monitored for 7 days and subjected for general health assessment following the parameters such as; characteristic coloration, 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 $p\leq 0.05$.

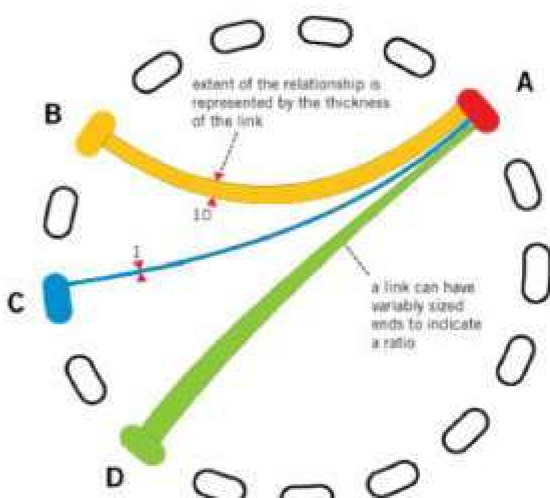

Fig. 1: Data presentation in CIRCOS

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 minutes in a refrigerated centrifuge at 4°C. The supernatant was recentrifuged at 8000 g for 30 minutes at 4°C and the final supernatant fluid was filtered through a 0.4µm 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.

Protocol for the *in vivo* Experimentation: For bioassay, the plant isolate (MP07X) was 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 (TS) (five shrimps in each tank) were injected intramuscularly with a mixture of viral suspension and the above prepared plant product 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 after post infection with WSSV.

Estimation of *in vivo* Efficacy of MP07X in Host-Pathogen Interaction Model: The survivability percentages (SURV) along with 8 immunological and 2 physiological parameters in the three groups (POS, NEG and TS) of shrimps were analyzed. The 10 parameters such as; total hemocyte count (THC) were performed using a Neubaeur's hemocytometer, Clotting time (CT) of the hemolymph was determined by the capillary method as described earlier by Sachdev [9], Oxyhemocyanin (OHC) was calculated based on the method described previously by Chen and Cheng [10], Hemolymph pH (pH) was measured by the glass electrodes of a microelectrode set [11], Hemolymph Ammonia (NH_3) was determined using the Sigma Diagnostic Kits Ultraviolet No. 170-UV [12]. The Phenoloxidase activity (PO) was determined using standard protocol [13], Respiratory burst activity (RB) was determined standard methodology [14] and Superoxide dismutase (SOD) was analysed using Ransod Kit [15, 16]. The 2 physiological parameters such as; oxygen consumption (O_2 cons.) and ammonia excretion (NH_3 exce.) of the groups (POS and NEG) of shrimps were analyzed according to the standard methods [17, 18].

To study the *In vivo* host-drug-pathogen interaction, the histopathological analysis of the gill tissues from each of the three groups were done. The gill tissue samples 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.

During this trial, shrimps {Negative (NEG), Positive (POS) and Test Sample (TS)} were subjected to comprehensive molecular analysis, post infection with WSSV. The genes namely; the VP28 (WSSV gene), *ie* 1 (immediate early 1 gene-immune related gene of shrimp) and Shrimp ß actin gene (internal control gene) were expressed on the 42nd hr and 84th hr, after the viral

challenge using reverse transcriptase PCR (RT-PCR), to find out whether the plant isolate (MP07X) was inhibiting the processes involved in the viral multiplication cycle during host pathogen interaction. The survival percentages in all the three shrimp groups were recorded. The experiments were conducted in triplicates and the results were confirmed and concluded after 100 % mortality was observed in the positive control (POS) group.

Statistical Analyses: The data obtained from the experiments were subjected to appropriate statistical analysis. Statistical a nalyses 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 immunological and physiological parameters, the results were examined using Analysis Of Variance (ANOVA) followed by a Least Significant Difference (LSD) test and correlation and regression analyses 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 10 variables with respect to time. Representation of relationships was projected by using CIRCOS data visualization software.

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).

RESULTS

Studies on the Anti-WSSV Efficacy of MP07X: The activity of the crude drug (MP07X) was examined against WSSV in *L. vannamei* to confirm its efficacy as a potent anti-WSSV drug. On completion of the experiment, after 84 hrs the shrimps were nested PCR negative and when the DNA extracted for virus from these shrimps were injected into a fresh batch of shrimps none of them showed any clinical signs of WSSV infection and remained negative to nested PCR (Fig. 2). The survivability was 85 % at the end of the 84th hr of the experimentation (Fig. 3).

Cytotoxicity Assay of Plant Isolate MP07X: Brine shrimp lethality bioassay, test sample showed the different mortality rate at different concentrations against the brine shrimp nauplii. The mortality rate of brine shrimp was found to increase with the increase in concentration of the test sample and showed significant (P<0.001) toxicity to the brine shrimp nauplii. From the plot of percentage of mortality versus log concentration on the graph paper LC_{50} and LC_{90} were deduced (LC_{50}=40 µg/ml; LC_{90}=80 µg/ml). The specific findings are illustrated in Table 1.

Determination of *in vivo* Toxicity of the Plant Isolate MP07X: *L. vannamei* (6-8 g) (n = 6) were injected with the plant isolate at different concentrations ranging from 5-50 mg/ml and monitored for 7 days (Fig. 4). The response of the animals was more or less the same without any significant mortality even up to a concentration of 35 mg/ml ($P<0.05$). However, at 50 mg/ml strength there was significant reduction (56 % average percentage survival) ($P<0.05$) in survival of shrimps during the experimental period of 7 days.

Estimation of *in vivo* Efficacy of MP07X in Host-Pathogen Interaction Model: 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 discoloration 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 after injection. The negative control (NEG) shrimps did not exhibit any of these symptoms and did not show any mortality. The absence of WSSV infection in this group was confirmed using PCR. In the case of test sample (TS), the shrimps almost behaved like that of the negative ones.

Table 1: Brine shrimp lethality bioassay of MP07X

Extract concentration (μg/ml)	Log concentration	Number of survivals	Mortality (%)	LC_{50} (μg/ml)	LC_{90} (μg/ml)
10	1.000	9	10	40	80
20	1.315	7	30		
40	1.590	5	50		
60	1.762	2	80		
80	1.915	1	90		
100	2.000	0	100		

Notes: LC_{50} and LC_{90} were determined from 24 hrs counts using the probit analysis method described by the FINNEY computer program.

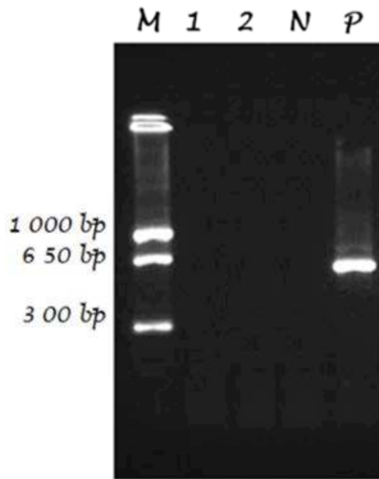

Fig. 2: PCR diagnosis of MP07X in shrimps
M = marker, 1 = WSSV negative (MP07X intramuscular injection), 2 = WSSV negative (lane 1 DNA injected to fresh shrimps), N = negative control (NEG), P = positive control (POS).

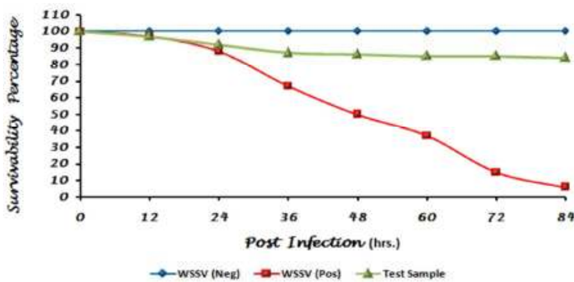

Fig. 3: Variations in survivability percentage in different experimental groups

This result was only due to the efficiency of the plant isolate (MP07X) which nullified the *In vivo* virulence of WSSV. This was also confirmed by the significant variations observed in the immune variables in the hemolymph of the animals (TS). The Total hemocyte count (Fig. 5) in the hemolymph of the animals (TS) was observed to be 61×106/ml at the zero time; however, with

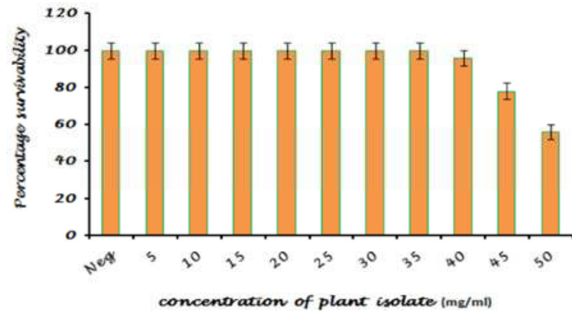

Fig. 4: Toxicity of different concentration of plant isolate (MP07X) in L. vannamei

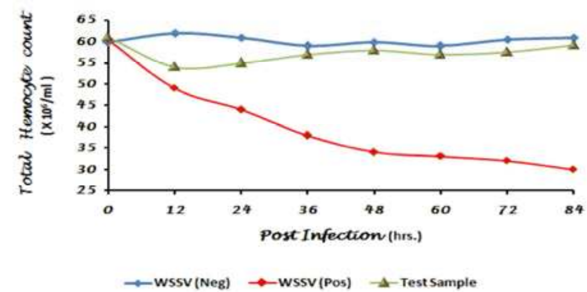

Fig. 5: Variations in total hemocyte count in different experimental groups

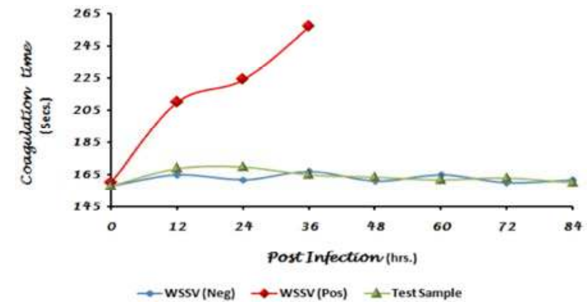

Fig. 6: Variations in coagulation time in different experimental groups

a further increase in time, stability in this count was observed. The coagulation time (Fig. 6) of the hemolymph of the animals (TS) exhibited a similar pattern to that of the healthy ones (NEG). The oxyhemocyanin (Fig. 7) content in hemolymph of the animals (TS) was at the highest level of 1.2m mol/l at the 72th hr. The pH (Fig. 8) of the hemolymph of the animals (TS) was at the highest level of 7.52 at the 36th hr. The ammonia (Fig. 9) content in hemolymph of the animals (TS) was at the highest level of 0.39 mg/ml at the 24th hr; however, with a further increase in time, stability in the ammonia level was observed. The phenoloxidase activity (Fig. 10) and respiratory burst (Fig. 11) in hemolymph of the animals (TS) was at the

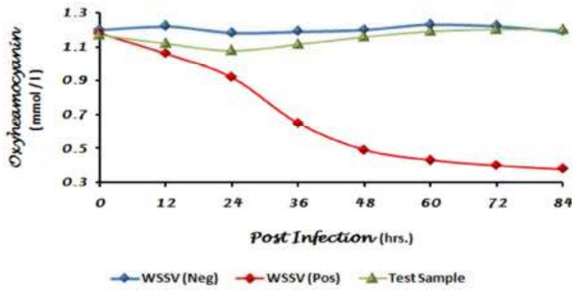

Fig. 7: Variations in oxyhemocyanin content in different experimental groups

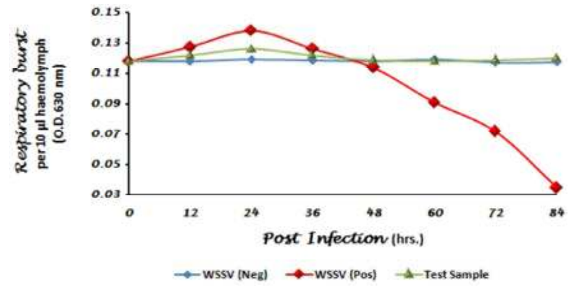

Fig. 11: Variations in respiratory burst in different experimental groups

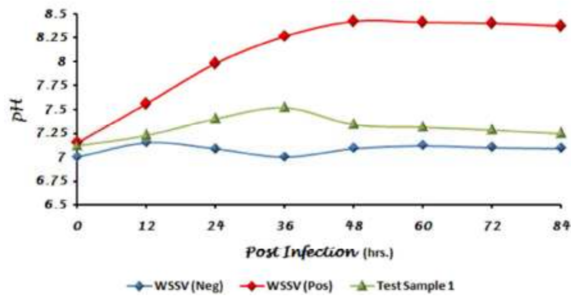

Fig. 8: Variations in hemolymph pH content in different experimental groups

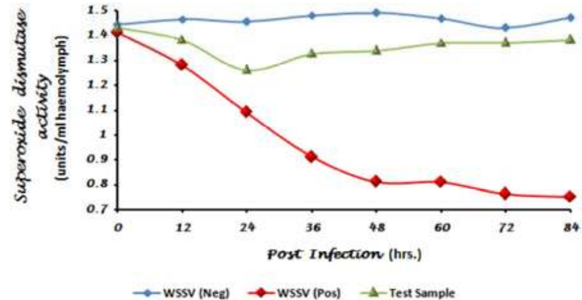

Fig. 12: Variations in superoxide dismutase in different experimental groups

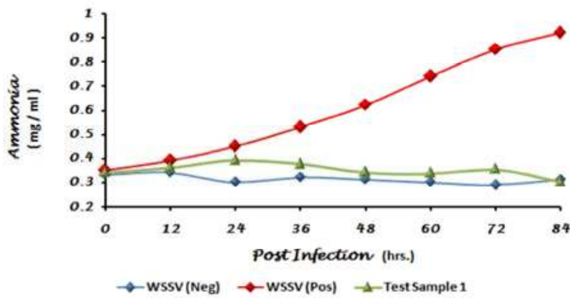

Fig. 9: Variations in hemolymph ammonia content in different experimental groups

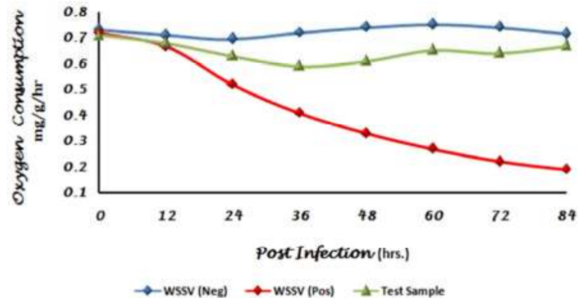

Fig. 13: Variations oxygen consumption in different experimental groups

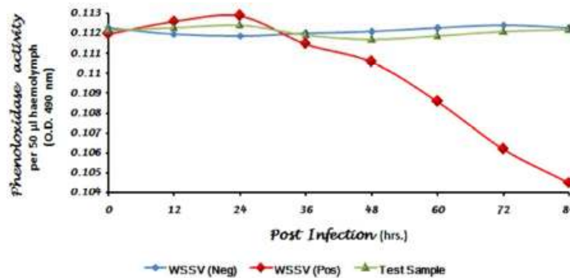

Fig. 10: Variations in phenoloxidase activity in different experimental groups

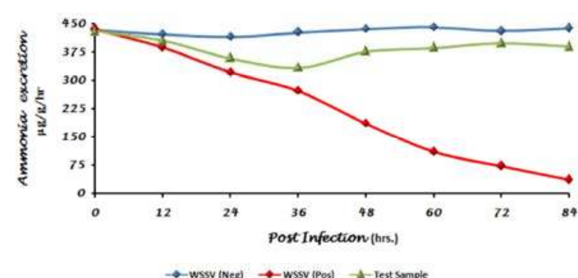

Fig. 14: Variations in ammonia excretion in different experimental groups

highest level of 0.11243/50 µl of hemolymph and 0.126/10 µl of hemolymphat the 24th hr respectively; though with a further increase in time, stability in both the variables were observed. The superoxide dismutase (Fig. 12) level in hemolymph of the animals (TS) was at the highest level of 1.43 U/ml hemolymph at the zero time and at the lowest

level of 1.26 U/ml hemolymph at the 24th hr; but with furtherance in time, stability in the level was observed. The significant variations were not only confined to immunological parameters but also reciprocating the same in the physiological ones. The oxygen consumption (Fig. 13) and ammonia excretion (Fig. 14) of the animals (TS) 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; but with furtherance of time, stability in the curve was observed.

Significant differences (P<0.01) were observed between the POS, NEG and TS in the immunological and physiological variables in various tissues and at different time intervals post infection with WSSV. In the case of POS, significantly (P<0.01) reduced variables were observed when compared to the NEG. In contrast, significant (P<0.01) elevations were observed in the TS after a certain time interval due to the anti-WSSV activity of the plant isolate, MP07X. The differences between POS and TS in all the parameters and survivability (SURV) were statistically significant. The Pearson's correlation co-efficient showed that all variables except hemolymph pH and hemolymph ammonia; exhibited positive correlation with the survival rate (Tables 2, 3). When multiple regression of survival rate on all the immunological parameters (Table 4) were considered, the amount of variability explained was 99.5 % (R Square=0.995). When significant regression co-efficient were taken into account, it was found that NH_3 (P < 0.01) alone was explaining 99 % (R^2= 0.99) of variability, indicating this one is mainly responsible for the survivability (SURV).

The data were further analyzed using factor analysis. The method of factor analysis was principal component analysis (PCA) a nd the rotation method was varimax (Fig. 15). It shows the communality of the factor analysis that expressed the percentage of parameter variability explained by the factor model and given the variance explained by each retained factor. Factor loading larger than approximately 0.5 are considered statistically significant. The factor analysis generated four significant factors, which explained 97.0 % of the data variance in data sets, among the four the first two factors itself explained 93.6 % variance (number of components of which the eigenvalues are greater than "1" was two). A scree plot explained the sorted eigenvalues from large to small as a function of the principal components' number. The first and the second factors itself have high loading and account 49% and 44.6 % response of total variance. Association of SURV, THC, CT, OHC, pH, NH_3, SOD, O_2

Table 2: Correlation matrix of survival rate (SURV) and the differences in POS & TS in parameters (THC, CT, OHC, pH, NH_3, PO, RB, SOD) of *L. vannamei*

	THC	CT	OHC	pH	NH_3	PO	RB	SOD	SURV
THC	1								
CT	-.975**	1							
OHC	.979**	-.983**	1						
pH	-.981**	.980**	-.964**	1					
NH_3	-.906**	.900**	-.939**	.882**	1				
PO	.772**	-.745**	.807**	-.738**	-.949**	1			
RB	.705**	-.676**	.745**	-.654**	-.924**	.969**	1		
SOD	.989**	-.982**	.996**	-.973**	-.924**	.790**	.721**	1	
SURV	.903**	-.895**	.939**	-.875**	-.995**	.949**	.916**	.926**	1

Table 3: Correlation matrix of survival rate and the differences in POS & TS in parameters (O_2 cons, NH_3 excr.) of *L. vannamei*

	O_2 Cons	NH_3 Excre	SURV
O_2 Cons	1		
NH_3 Excre	.981**	1	
SURV	.974**	.986**	1

Table 4: Multiple regression of survival rate (SURV) and the differences in POS & TS in parameters (THC, CT, OHC, pH, NH_3, PO, RB, SOD) of *L. vannamei*

R Square - 0.995
Adjusted R Square - 0.993
Predictors - THC, CT, OHC, pH, NH_3, PO, RB, SOD.
Dependent Variable - SURV

	THC	CT	OHC	pH	NH_3	PO	RB	SOD
Significance	0.139	0.805	0.204	0.475	0.015*	0.380	0.979	0.025*

**P < 0.01, *P < 0.05

R Square - 0.990
Adjusted R Square - 0.990
Predictors - NH_3.
Dependent Variable - SURV

	NH_3
Significance	0.000**

**P < 0.01, *P < 0.05

concentrations and NH_3 excretion in factor 1 and SURV, CT, OHC, NH_3, PO, RB, SOD, O_2 cons. and NH_3 excretion in factor 2 indicate significant effect of the parameters on the survival rate. PCA analysis results also revealed that the first component was associated with THC, OHC, SOD, O_2 cons. and NH_3 excretion The second component comprised mainly of CT and NH_3. On the other hand the CIRCOS data visualization output illustrated (Figs. 16, 17, 18) [A (NEG); B (TS); C (POS) {variables-SURV, THC, CT, OHC, pH, NH_3}; D (NEG); E (TS); F (POS) {variables-SURV, O_2 Cons. NH_3 Excr,}; G (NEG); H (TS); I (POS) {variables - SURV, PO, RB, SOD}] the systematic relationship between each variable *In vivo* with respect to time.

The longitudinal sections of gill filament (rich in viral load) of the experimental animals were examined under the phase-contrast microscope to

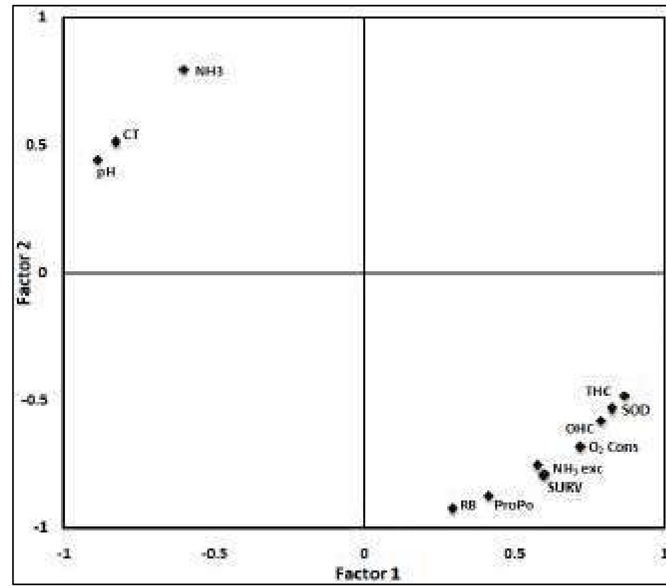

Fig. 15: Principal component analysis- The loadings after varimax rotation of the variables

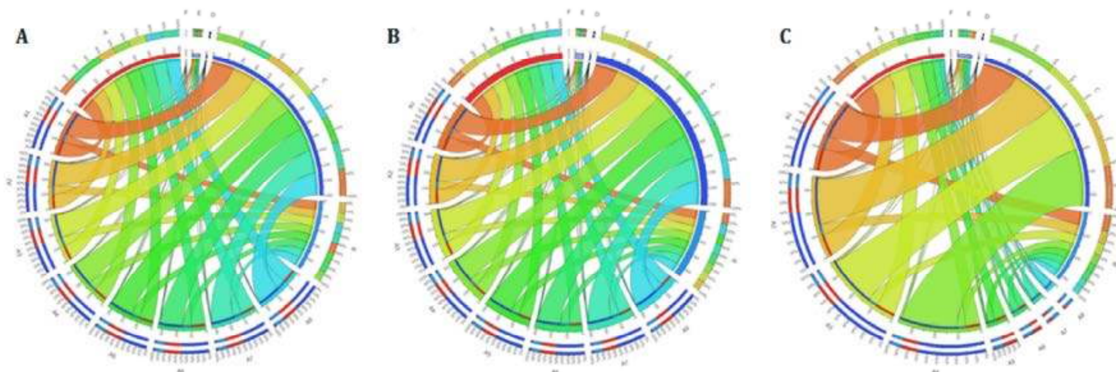

Fig. 16: CIRCOS representation of systematic relationship between survivability and other variables {SURV, THC, CT, OHC, pH, NH_3} [A _ NEG; B _ TS; C _ POS]

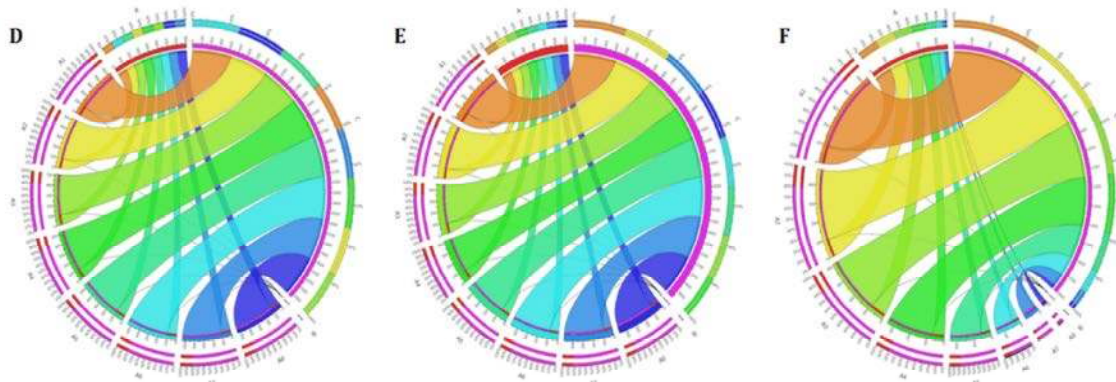

Fig. 17: CIRCOS representation of systematic relationship between survivability and other variables {SURV, O_2 Cons. NH_3 Excr,} [D _ NEG; E _ TS; F _ POS]

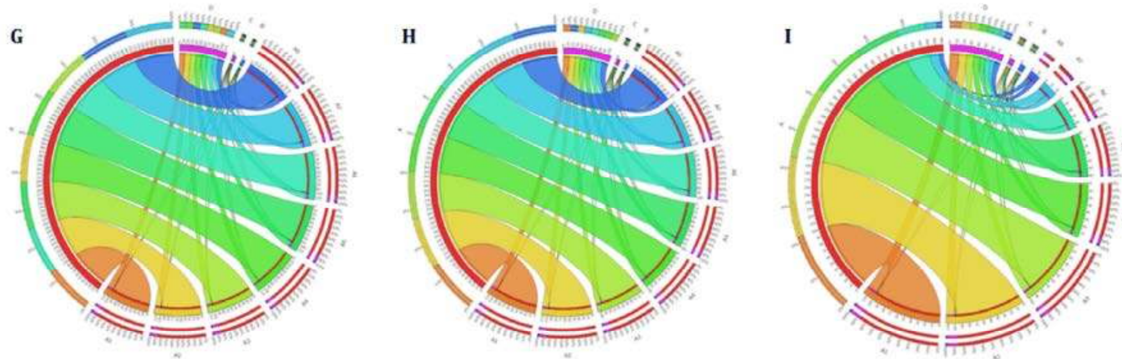

Fig. 18: CIRCOS representation of systematic relationship between survivability and other variables {SURV, PO, RB, SOD} [G _ NEG; H _ TS; I _ POS]

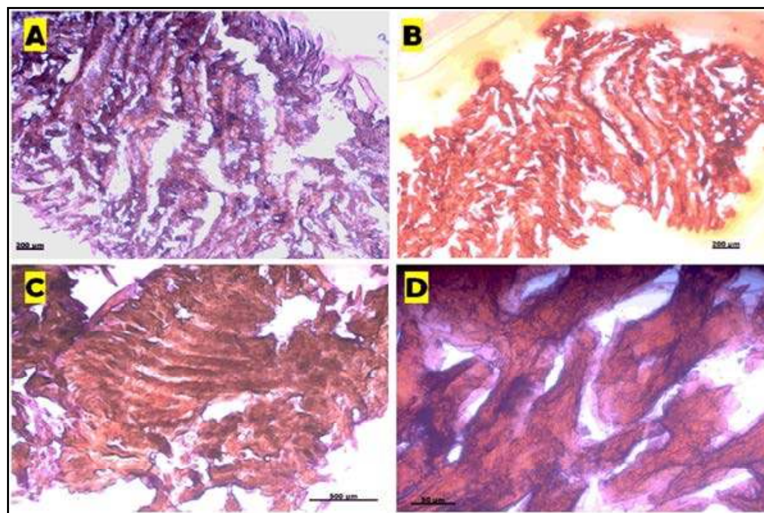

Fig. 19: Histopathology of gill tissue of *L. Vannamei*. A} positive, B} Negative, C, D} test sample

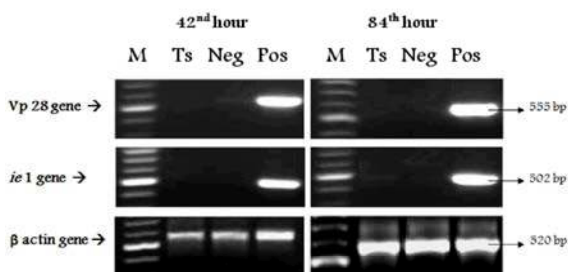

Fig. 20: Reverse transcription PCR analysis of VP28, immediate early (*ie* 1) and β actin genes in host

evaluate the histopathological 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 negative (NEG) control and the ones (TS) administered with the intramuscular injection of plant isolate (MP07X) along with WSSV did not show any clinical pathological changes and the two were almost identical. The gill filaments contained numerous lacunae with a sufficiently large number of hemocytes as in the case of apparently healthy shrimps (Fig.19).

The expressions of the genes on the 42nd hr and 84th hr after the challenge with the virus were examined to find out whether the plant isolate (MP07X) was inhibiting the processes involved in the viral multiplication cycle during host - pathogen interaction. The gene expression study

was conducted in three groups (POS, NEG and TS) of animals. Viral genes were not amplified in the test group (TS) of animals and appeared exactly like the negative controls (NEG). In the case of positive control (POS), the viral genes such as immediate early gene (*ie*1) and VP28 were found to be expressed at both 42nd hr and 84th hr after challenge with WSSV. It was observed that, as the time passed by, there was an increase in the intensity of bands of these genes suggesting more multiplication of the virus in the positive control shrimps (Fig. 20). In the case of positive control animals which received the virus intramuscularly, total mortality was observed at the 84th hr itself. Hence, animals were not available to assay beyond that timeline.

DISCUSSION

Most of the products derived from marine organisms, show many interesting activities. Their constituents are more novel than those of the terrestrial plants. Antiviral activities of aqueous extracts from plants are well established [19-23] that also includes reports on the anti-WSSV activity of plant extracts [24-30]. A combination of herbal extracts and probiotics as medicated diet could decrease the prevalence of WSSV in *Litopenaeus vannamei* [31]. 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 marine plants as sources of anti - WSSV drugs. With this objective, 30 marine plants abundantly found in different marine ecosystems of the East coast of India, were subjected to soxhlet extraction to procure a combination of phytomolecules, potent enough to be an anti-WSSV drug and at the same time applied along with diet as a prophylactic measure. In this study, nine plant isolates were found to be effective against WSSV. Finally, the plant isolate MP07X proved to be the potent anti-WSSV drug in our research. As MP07X alone could give protection to all animals tested against WSSV, under the experimental conditions, these marine plant species were identified for further studies. The viral DNA was not detected in the tissue which suggested that the virus was either had not invaded the host tissue and multiplied or it was getting eliminated subsequent to the infection.

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 [32]. Brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor etc. [33]. A study that aimed at screening the lethality of crude plant extracts commonly used as antimalarial phytomedicines in Msambweni district, Kenya against brine shrimp, *Artemia salina* larvae was conducted. In this bioassay, an LC_{50} value lower than 1000 µg/ml is considered cytotoxic. 97.6% of all the screened organic extracts and 85% of the investigated aqueous extracts demonstrated the LC_{50} values less than 1000 µg/ml, indicating the presence of cytotoxic compounds responsible for the observed toxicological activity [8]. These observations indicate that some of the antimalarial plants could not make safe herbal remedies. This calls for dose adjustment amongst the community using the plant extracts for the treatment of malaria. In the same way, in this study the cytotoxicity of MP07X was found to be (LC_{50}= 40 µg/ml; LC_{90}= 80 µg/ml) when tested with *A. salina* and the degree of lethality was found to be directly proportional to the concentration of the extract where maximum mortalities took place at a concentration of 100 µg/ml whereas least mortalities were observed at 10 µg/ml, as documented by the above mentioned study where the maximum mortalities took place at a concentration of 1000 µg/ml whereas least mortalities were at 10 µg/ml. Therefore, the positive response obtained in this assay suggests that MP07X may contain antimicrobial compounds.

In animal model the highest non-toxic concentration went upto 35 mg/ml, from which 10 µl extract was injected to shrimps (6-8 g). We found that the crude drug MP07X 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 [30]. The average percentage of survivability of shrimps injected with MP07X was 85%, 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 MP07Xis concentration dependent. Different concentrations of Cidofovir (an antiviral drug) were injected and observed that it was non-toxic to shrimps upto a concentration of 200 mg/kg of body weight and they could successfully use the same for further assays

[34]. 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 [35, 36].

The results showed significant differences in the metabolic parameters of POS and NEG. 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 [11]. The hemocyte count varies among crustaceans and is known to be affected by a variety of factors such as infection and environmental stress [37-39]. It has been demonstrated a decrease in the hemocyte numbers of crayfish harboring a parasitic fungus (*Aphanomyces astaci*) [38]. A decline in total hemocyte count in shrimp infected with penaeid rod-shaped DNA virus has been observed [40]. 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 [41 - 43]. 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 [44]. 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 [45]. An increase in hemolymph clotting time has been observed in our study so as in *G. homari* infected lobsters as observed earlier [11]. 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 [46]. Hemocyanin represents 80 - 95% of total protein in the hemolymph of crustacean [47, 48]. The hemocyanin has been affected by moulting cycle, nutritional conditions and stress [49-51]. 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 [11]. 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 [52]. Our PO activity results were in comparison with those in closely related species, *Fenneropenaeus chinensis* [53]. 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 [14]. The enhanced production of reactive oxygen (O2-) in shrimp treated by immersion with the *Vibrio* bacterin and glucan noted earlier [54]. Variations in respiratory burst activity could be attributed to the disparity in NADPH oxidase activity, phagocytic rate and/or the number of hyaline cells [55, 56]. 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 l owered in WSSV infected *F. indicus* [57, 58]. 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* [59].

The relationship between the survival rate and the 10 variables with respect to time are represented by CIRCOS data visualization software. CIRCOS has an edge over several statistical tools. The raw data obtained from the experiment can be directly computed using this software. The relationship between the survival rate and the other parameters with respect to the time interval in the case of POS, NEG and TS are presented. The uniqueness of this software is that by having a look at the figures one can easily differentiate the TS from POS and NEG. Thus the significance of MP07X can be well demonstrated using this particular software.

Histopathological analysis of gill tissues of the experimental animals (NEG and TS) revealed the absence of hypertrophied nuclei. Gill tissues were chosen as it was reported that WSSV infects tissues of ectodermal and mesodermal origin [60]. The absence of histopathological evidence (in the case of TS) for WSSV might be due to the lack of translation product of the viral gene. Meanwhile in the experimental animals (POS) subjected to WSSV challenge, the gill tissues revealed the presence of hypertrophied nuclei. It is hypothesized that, in animals (TS) administered with MP07X; the viral genes did not integrate into the host genome. To evaluate the efficacy of MP07X for protecting *L. vannamei* from WSSV infection, expression of immediate early gene (*ie*1) and VP28 and ß actin genes were investigated. This study indicated that the viral transcripts involved in viral replication were not expressed in the animals (TS) that were administered with the crude drug. This was alike for both the 42nd hr and 84th hr, post challenge with WSSV. The striking observation was that immediate early gene (*ie* 1) failed to be expressed in this group of animals. The expression of viral *ie* gene occurs independently of any viral *de novo* protein synthesis as the primary response to the viral invasion [61]. Once expressed, the *ie* gene products may then function as regulatory transacting factors and serve to initiate viral replication events during infection. Recently, it was found that WSSV used a shrimp STAT as a transcription factor to enhance viral gene expression in the host cells. STAT directly transactivates WSSV *ie*1 gene expression and contributes to its strong promoter activity [62]. In the cascade of viral regulatory events, successive stages of viral replication are dependent on the proper expression of the genes in the preceding stage. In the present study none of these genes, {immediate early gene (*ie* 1) and VP28} was found to be expressed, that might be due to inactivation of the virus by the virucidal activity of MP07X. The results of different types of assays, viral and immune gene expression indicates that shrimps were protected from disease, either by getting protection from infection, or by getting the same from early dissemination of the infection in the presence of the crude drug.

CONCLUSION

WSSV is the deadliest of all viruses among the crustaceans ever discovered with immense pathogenicity. Serious variations in the immunological and physiological parameters of the WSSV infected *L. vannamei* was observed due to the *In vivo* host-pathogen interaction. Similarly, the parameters of the test sample (TS) group revealed significant differences at each time intervals with the POS group indicating that the drug (MP07X) is efficient enough to inactivate or nullify the virulence of WSSV. The histopathological findings revealed that the drug (MP07X) is potent enough to restrict the multiplication of the virus. The same results were demonstrated in the RT-PCR assays, once again stating the efficacy of MP07X as a potent anti-WSSV drug. Based on the results further in-depth molecular analysis can be focused to identify the mode of action of the particular drug against WSSV. The present work can thus be considered as a foundation of further anti-WSSV studies.

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