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In vivo biochemical 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 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 drug, (MP07X -derived from the marine plant) in the host, *Litopenaeus vannamei* was evaluated. The biochemical 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 9 biochemical parameters such as, total protein, total carbohydrate, total glucose, total free amino acid, total fatty acid, fructose 1, 6 diphosphatase, aldolase, glucose 6 phosphatase and glucose 6 phosphate dehydrogenase were examined for healthy (NEG), WSSV infected (POS) and test sample (TS) shrimps. Significant differences ($p < 0.01$) were observed between the POS, NEG and TS in the biochemical 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* 1) 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 biochemical 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.

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 (Primavera, 1997; Rosenberry, 2000). 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 (Sindermann and Lightner, 1988). There is considerable evidence to support links between stress caused by enviromental changes and diseases mainly caused by depression of the immune system (Dunier and Siwicki, 1993; Pipe and Coles, 1995). 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 (Chou *et al*. 1995, Chang *et al*. 1996). 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. Strategies for the prophylaxis and control of WSSV theoretically include improvement of environmental conditions, stocking of specific pathogen free (SPF) shrimp post

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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 physiobiochemical metabolic 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 biochemical 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 *Litopeneaus vannamei* (white legged shrimp) weighing 5 – 7 gms. 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. 6 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. 10 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 (Meyer *et al*. 1982).

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 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 were 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 $p \leq 0.05$.

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.

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) (5 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 9 biochemical parameters in the three groups (POS, NEG and TS) of shrimps were analyzed. The 9 biochemical parameters such as; total protein (TP) was determined spectrophotometrically based on the Lowry method (Lowry *et al.*, 1951), total carbohydrate (TC) was determined by the Anthrone method (Hedge and Hofreiter, 1996), total glucose (TG) was determined by the Glucose (GO) Assay Kit (Sigma), total free amino acid (TAA) was determined using the ninhydrin method (Yemm and Cocking, 1955), total fatty acid (TFA) according to the standard method (Cox and Pearson, 1962), Fructose 1, 6 Diphosphatase (FDPase) was determined by slightly modifying the earlier methodology (Gancedo and Gancedo, 1971). Aldolase (ALD) was determined by the Randox AD189 assay kit, Glucose 6 Phosphatase (G6Pase) was estimated by the Glucose 6 Phosphate Assay Kit (Sigma) and Glucose 6 phosphate dehydrogenase (G6PDH) was estimated by the Glucose 6 Phosphate dehydrogenase Assay Kit (Sigma).

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 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 biochemical 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 9 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).

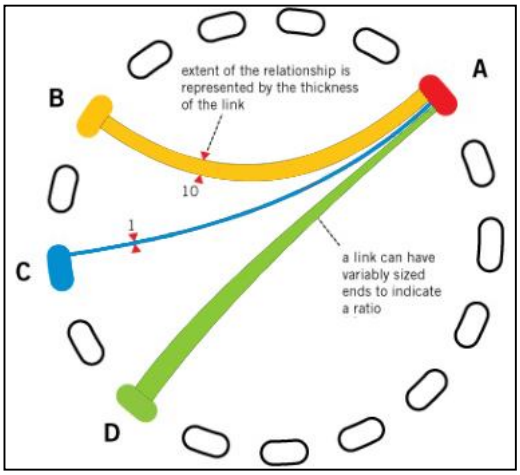

Fig. 1: Data presentation in CIRCOS.

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).

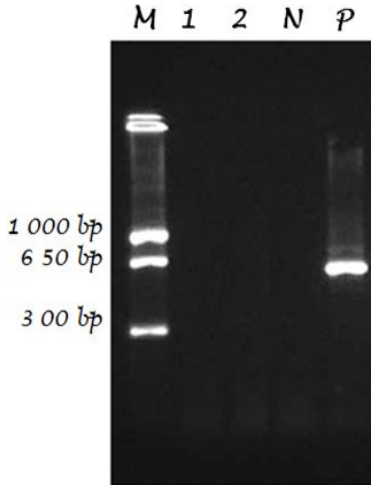

Fig. 2: PCR diagnosis of MP07X in shrimps. M = marker, 1 = WSSV negative (MP07X intramuscular injection), 2 = WSSV negative (lane 1 DNA injected to fresh shrimps), N = negative control (NEG), P = positive control (POS).

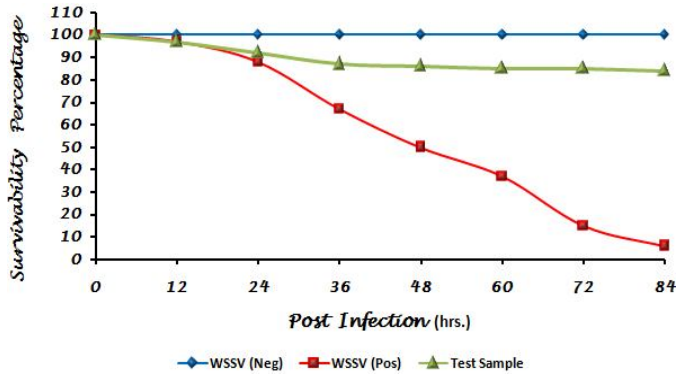

Fig. 3: Variations in survivability percentage in different experimental groups.

Table. 1: Brine shrimp lethality bioassay of MP07X.

Ectract concentration (µg/ml)	Log concentration	Number of survivals	Mortality (%)	LC_{50} (µg/ml)	LC_{90} (µg/ml)
10	1.000	9	10		
20	1.315	7	30		
400	1.590	5	50	40	80
60	1.762	2	80		
80	1.915	1	90		
100	2.000	0	100		

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

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 elaborated in Table 1.

Determination of *in vivo* toxicity of the plant isolate MP07X

L. vannamei (6-8 gms.) (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.

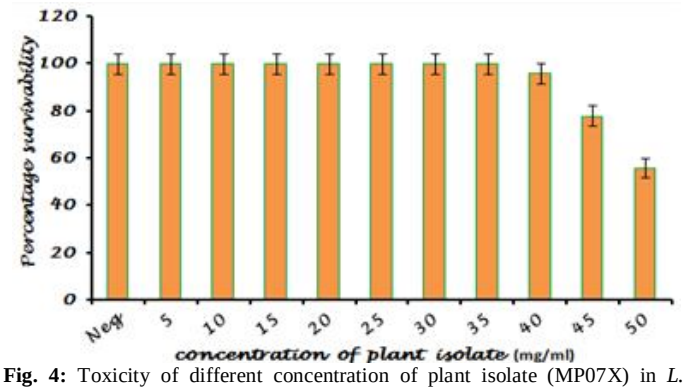

Fig. 4: Toxicity of different concentration of plant isolate (MP07X) in *L. vannamei*.

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 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. 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.

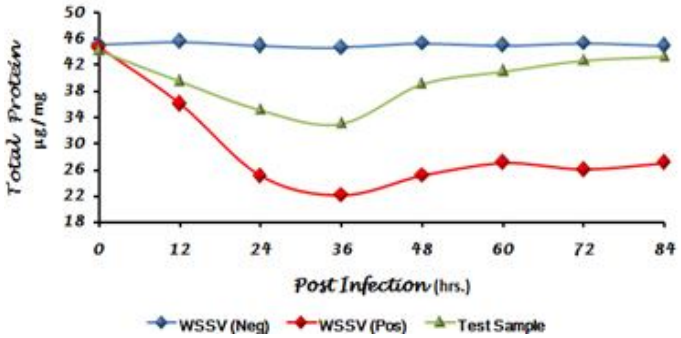

Fig. 5: Variations in total protein content in different experimental groups.

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

metabolic variables in the tissue of the test animals (TS). The total protein (Fig. 5) and carbohydrate (Fig. 6) content in the shrimps (TS) exhibited the lowest level of 33 µg/mg and 1.8 µg/mg at the 36th hr respectively; however, with the further increase in time, a steady rise in both the levels was observed. The highest level of total glucose (Fig. 7) content and total amino acid (Fig. 8) content estimated in the shrimps (TS) was 1.06 µg/mg and 0.96 µg/mg at the 24th hr respectively; but with a gradual increase in time, a steady decline in the both the levels were observed. Initially, the total fatty acid (Fig. 9) content in the shrimps (TS) was showing a steady rise with its highest level of 0.42 µg/mg at the 36th hr; however, with further time elapsation a steady decline in the same was observed.

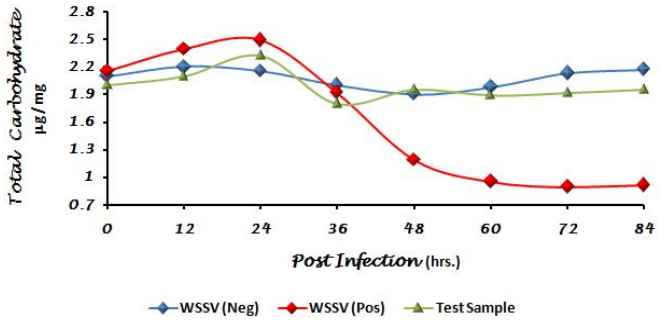

Fig.6: Variations in total carbohydrate content in different experimental groups.

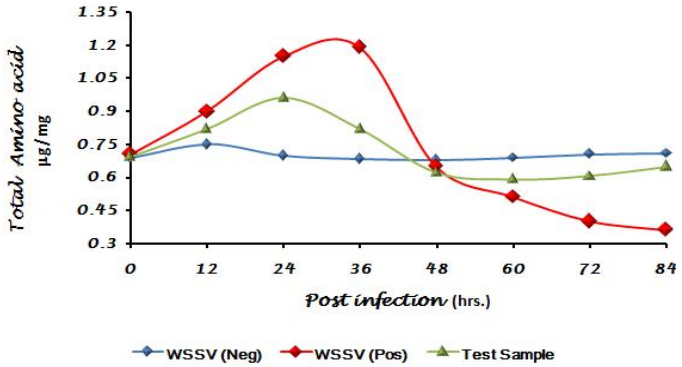

Fig. 8: Variations in total amino acid content in different experimental groups.

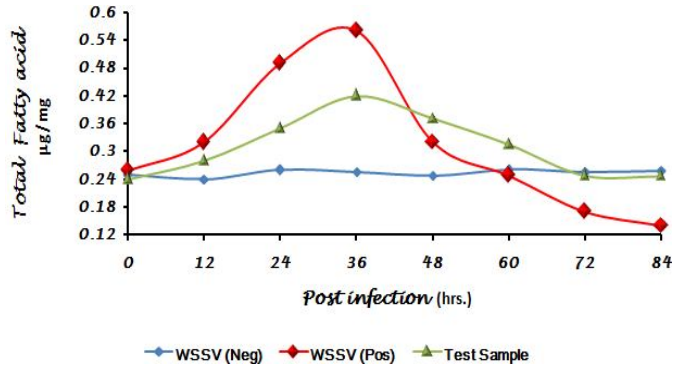

Fig. 9: Variations in total fatty acid content in different experimental groups.

The lowest level of fructose 1, 6 diphosphatase (Fig. 10) estimated in the tissue of the shrimps (TS) was 58 µg P/mg protein/hr at the 12th hr; however, with a gradual increase in time, a steady rise in this enzyme level was observed. Starting from the 0th hr. the aldolase (Fig. 11) level and glucose 6 phosphate dehydrogenase (Fig. 12) level in the tissue of the animals (TS) showed a steady rise with an increase in time with its highest level of 1.4278 µg glyceraldehyde/mg protein/hr and 1.4259 units/mg protein/hr at the 36th hr respectively; however, with a further increase in time a steady decline in this enzyme level was observed. The glucose 6 phosphatase (Fig. 13) level in the shrimps (TS) showed a steady decline with an increase in time with its lowest level of 33 µg Pi/ mg protein/hr at the 24th hr; however, with a further increase in time a constant increase in this enzyme level was observed.

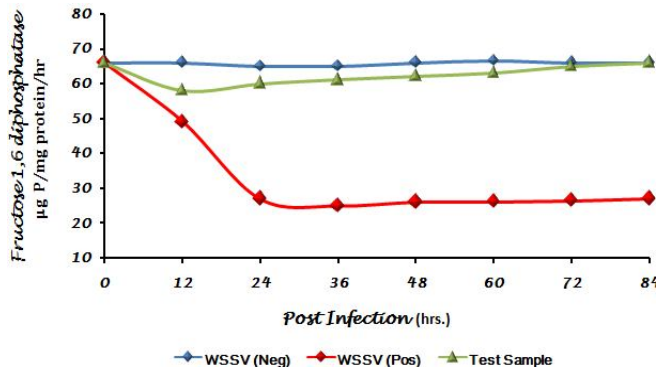

Fig. 10. Variations in fructose 1, 6 diphosphatase content in different experimental groups.

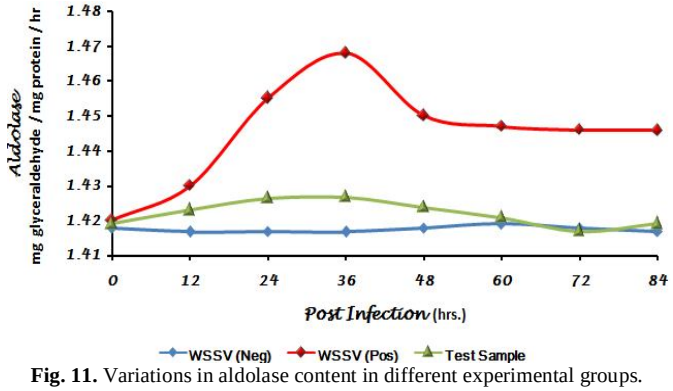

Fig. 11. Variations in aldolase content in different experimental groups.

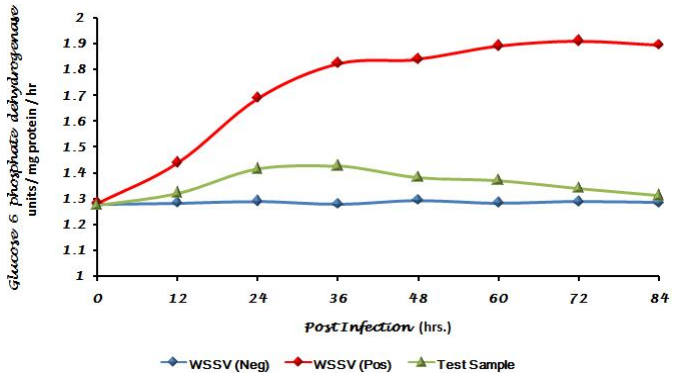

Fig. 12. Variations in glucose 6 phosphate dehydrogenase content in different experimental groups.

Significant differences ($p < 0.01$) were observed between the POS, TS and NEG in the biochemical variables at different time intervals post infection with WSSV (0th, 12th, 24th, 36th,

48th, 60th, 72nd, 84th hrs), when the treatments were subjected to two way ANOVA. When the data were subjected to pair wise comparison between each treatment (NEG – POS, NEG – TS and POS - TS) 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 10 variables. Amongst these variables, only in total fatty acid, a single treatment pairs (POS–TS) showed non-significant differences. These results were achieved 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 aldolase and glucose 6 phosphate dehydrogenase; exhibited positive correlation with the survival rate (Table 2). When multiple regression of survival rate on all the biochemical parameters (Table 3) were considered, the amount of variability explained was 99.8 % (R Square = 0.998). When significant regression co-efficient were taken into account in the case of metabolic parameters, it was found that TC ($p < 0.01$), TG ($p < 0.01$), TAA ($p < 0.01$) and G6PDH ($p < 0.01$) together is explaining 99.7 % (R Square = 0.997) of variability, indicating that these four are mainly responsible for the survivability (SURV).

The data were further analyzed using factor analysis. The method of factor analysis was principal component analysis (PCA), and the rotation method was varimax (Fig. 14). 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, TP, TC, FDPase, G6Pase, ALD and G6PDH in factor 1 and SURV, TC, TG, TAA and TFA in factor 2 indicates significant effect of the parameters on the survival rate. On the other hand the CIRCOS data visualization output illustrated (Fig. 15) [A (NEG); B (TS); C (POS) {variables – SURV, TP, TC, TG, TAA, TFA, FDPase, G6Pase, ALD, G6PDH}] the systematic relationship between each variable *in vivo* with respect to time.

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. 16). 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.

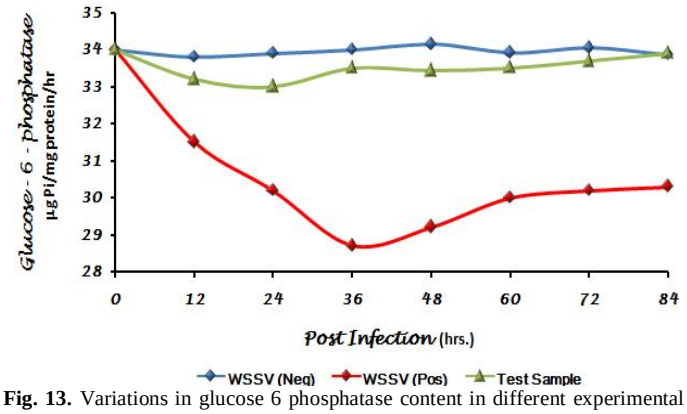

Fig. 13. Variations in glucose 6 phosphatase content in different experimental groups.

Table. 2: Correlation matrix of survival rate (SURV) and the differences in POS & TS in parameters (TP, TC, TG, TAA, TFA, FDPase, G6Pase, ALD, G6PDH) of *L. vannamei*.

	TP	TC	TG	TAA	TFA	FDPase	G6Pase	ALD	G6PDH	SURV
TP	1									
TC	.814**	1								
TG	.485*	.825**	1							
TAA	0.394	.776**	.880**	1						
TFA	.438	.845**	.896**	.905**	1					
FDPase	.965**	.666**	0.281	0.166	0.214	1				
G6Pase	.699**	0.362	0.012	-0.193	-0.018	.786**	1			
ALD	-.748**	-0.352	-0.049	0.155	.061	-.841**	-.828**	1		
G6PDH	-.984**	-.862**	-.591**	-.452*	-.521**	-.934**	-.679**	.713**	1	
SURV	.846**	.938**	.871**	.753**	.770**	.708**	0.384	-.445*	-.903	1

**$P < 0.01$, *$P < 0.05$.

Table 3. Multiple regression of survival rate (SURV) and the differences in POS & TS in parameters (TP, TC, TG, TAA, TFA, FDPase, G6Pase, ALD, G6PDH) of *L. vannamei*.

R Square – 0.998
Adjusted R Square – 0.997
Predictors - TP, TC, TG, TAA, TFA, FDPase, G6Pase, ALD, G6PDH.
Dependent variable – SURV

	TP	TC	TG	TAA	TFA	FDPase	G6Pase	ALD	G6PDH.
Significance	0.615	0.015*	0.000**	0.002**	0.806	0.263	0.482	0.972	0.014*

***P<0.01, *P<0.05*

R Square – 0.997
Adjusted R Square – 0.997
Predictors – TC, TG, TAA, G6PDH.
Dependent variable – SURV

	TC	TG	TAA	G6PDH
Significance	0.000**	0.000**	0.000**	0.000**

**P<0.01, *P<0.05

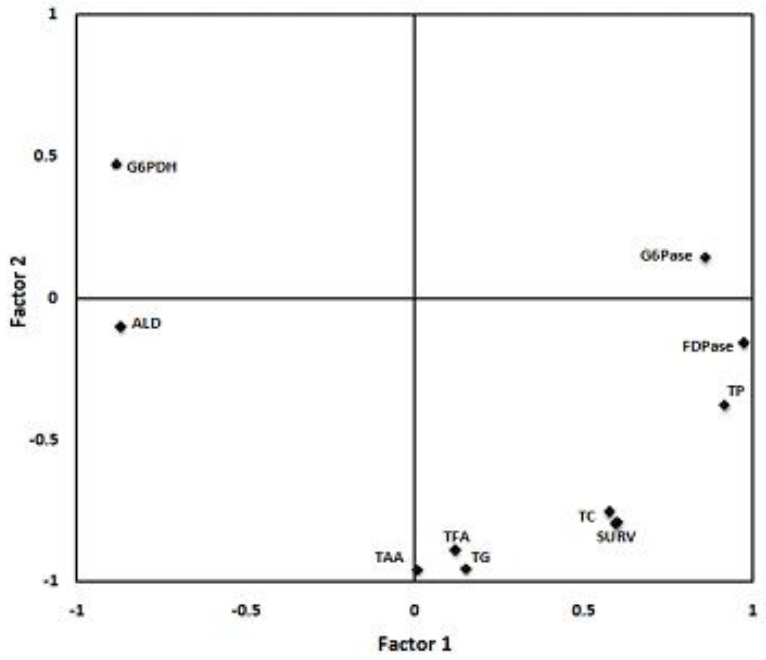

Fig. 14: Principal component analysis- The loadings after varimax rotation of the variables.

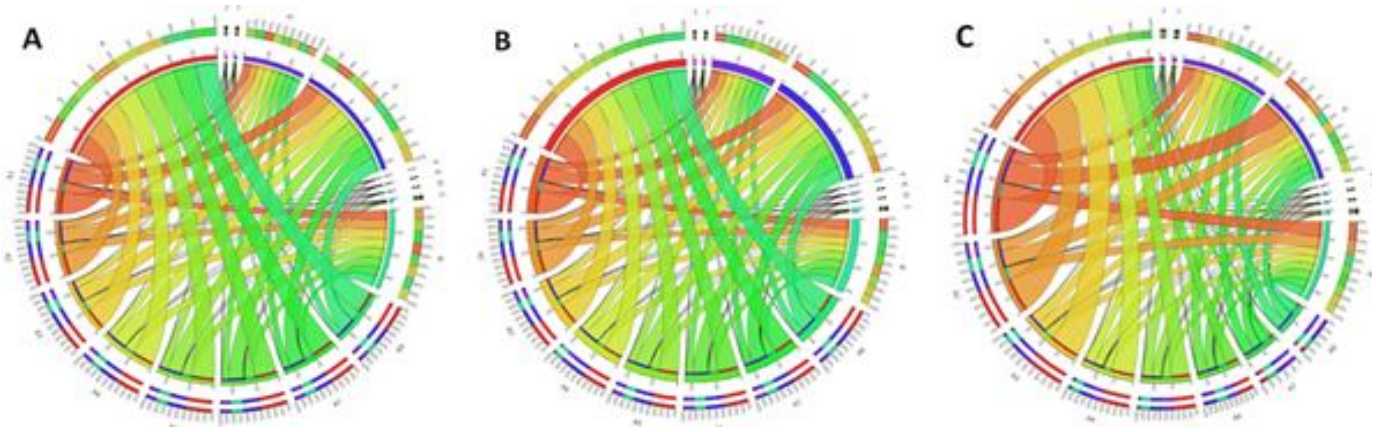

Fig. 15: CIRCOS representation of systematic relationship between survivability and biochemical variables. [A → NEG; B → TS; C → POS].

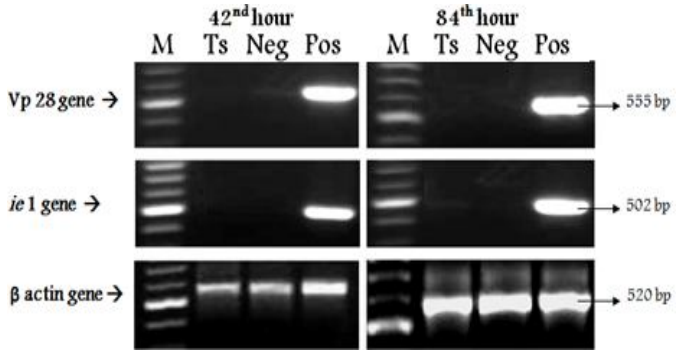

Fig. 16: Reverse transcription PCR analysis of VP28, immediate early (*ie* 1) and β actin genes in host.

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 (Summerfield *et al*. 1997; Calderone *et al*. 1998; Garcia *et al*. 2006; Roner *et al*. 2007; Reichling *et al*. 2009) that also includes reports on the anti - WSSV activity of plant extracts (Takahasi *et al*. 1998; Supamattaya *et al*. 2005; Citarasu *et al*. 2006; Citarasu 2009; Balasubramanian *et al*. 2007; Balasubramanian *et al*. 2008). A combination of herbal extracts and probiotics as medicated diet could decrease the prevalence of WSSV in *Litopenaeus vannamei* (Gomez 2009). 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, 9 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, this marine plant species was 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 (Padmaja *et al.,* 2002). Brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor etc. (Anderson *et al.,* 1988). 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 < 1000 µg/ml, indicating the presence of cytotoxic compounds responsible for the observed toxicological activity (Meyer *et al.,* 1982). 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 up to 35 mg/ml, from which 10 µl extract was injected to shrimps (6-8 gms.). 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 (Sudheer *et al*. 2011). 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 MP07X is 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 (Rahman *et al*. 2006). 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 (Dupuy *et al*. 2004; Roch *et al*. 2008). The results showed significant differences in the metabolic parameters of POS 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 (Beckage, 1996), 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 (Paterson, 1993). In contrast, researchers (Stewart and Cornick, 1972) have observed disappearance of glucose and lactic acid from the hemolymph of the lobster infected with *Gaffkya homari*. The fatty acid level decreased in muscle. This is a usual phenomenon in the infected shrimp (Bowser *et al.*, 1981; Hameed, 1989). 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 (Telford, 1968; Lynch and Webb, 1973). 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 (Wilson, 2003). To study the changes in glycolysis during white spot virus infection in *L. vanammei*, the activity of aldolase was measured in muscle. Aldolase is a ubiquitous glycolytic enzyme that catalyzes the reversible change of fructose 1, 6 diphosphate to glyeraldehyde 3-phosphate and dihydroxy acetone 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 (Wilson, 2003). 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 (McWhinnie and Kurchenberg, 1962). It has been reported that the PPP with its major enzyme glucose 6 phosphate dehydrogenase provides the tissues with specific molecule, the reduced NADPH (Cuzon, 2000). 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. The relationship between the survival rate and the 9 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. 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 42^{nd} hr and 84^{th} 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 (Friesen, 1997). 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 (Liu, 2009). 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 biochemical parameters of the WSSV infected *L. vannamei* was observed due to the *in vivo* host and pathogen interaction. Similarly the biochemical 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 same results were depicted 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 in WSSV. The present work can thus be considered as a foundation of further anti – WSSV research.

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