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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 Terrestrial Plants

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Abstract: White spot syndrome virus (WSSV) is one of the economically important viral pathogen 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 a drug (TP22C, derive from a terrestrial plant) was evaluated in the host, *Litopenaeus vannamei*. The biochemical changes caused 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 TP22C) WSSV infected host was 86% compared to 0% survivability in the untreated group. Significant results were obtained from the toxicological assays of the drug in the 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 (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 biochemical variables at different time intervals post infection with WSSV. In the case of POS, significantly ($p<0.01$) reduced variables were observed when compare 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 TP22C. Neither the VP 28 gene nor the immediate early gene 1 (*ie* 1) were expressed in the host at the 42nd and 84th hrs post-WSSV challenge. 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.

Key words: *Litopenaeus vannamei* · Anti–WSSV Drug · Biochemical Parameters · Viral 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]. In immune compromised host exposure to pathogen(s) often leads to a major changes in the of the organism. Stress therefore disrupts the immunity ability and metabolic performance of shrimps, increasing its susceptibility to microbial infections. WSSV 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

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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 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, plants 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 terrestrial plants. The leaf extracts from each of these plants were studied for their anti-WSSV property in the host, *Litopenaeus vannamei*. Further the leaf extracts 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 terrestrial plants were collected from different parts of the West Bengal and Tamil Nadu states in 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. TP01A (Terrestrial Plant 01 solvent A), TP01B, TP01C, 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, 7 showed significant anti-WSSV property. The best anti–WSSV plant isolate, TP22C was derived and purified and used in further bioassays.

Toxicological Analysis of TP22C in Animal Model: The lyophilized plant isolate (TP22C) 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 [8]. 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 $p \leq 0.05$.

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 10µl 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 until used.

Protocol for the *in vivo* Experimentation: For bioassay, the plant isolate (TP22C) 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 25µl per animal {5µl of viral suspension, 20 µl of plant isolate-buffer solution}.

The positive control (POS) shrimps were injected with a mixture of 20µl NTE buffer and 5µl viral suspension, while the negative control (NEG) shrimps were injected with 25µl 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 TP22C 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 measured spectrophotometrically based on the Lowry method [9], total carbohydrate (TC) by the Anthrone method [10], total glucose (TG) by the Glucose (GO) Assay Kit (Sigma), total free amino acid (TAA) using the ninhydrin method [11], total fatty acid (TFA) according to the standard method [12], Fructose 1, 6 Diphosphatase (FDPase) by slightly modifying the earlier methodology [13]. Aldolase (ALD) by the Randox AD189 assay kit, Glucose 6 Phosphatase (G6Pase) and Glucose 6 phosphate dehydrogenase (G6PDH) was estimated by the Glucose 6 Phosphate Assay Kit (Sigma) and the Glucose 6 Phosphate dehydrogenase Assay Kit (Sigma) respectively.

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) and Shrimp ß actin gene (internal control gene) were expressed at the 42nd hr and 84th hr, after the WSSV challenge using reverse transcriptase PCR, to determine whether the plant isolate (TP22C) was inhibiting the processes involved in the viral replication 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 estimated variables with respect to time. Representation of relationships was projected by using CIRCOS data visualization software.

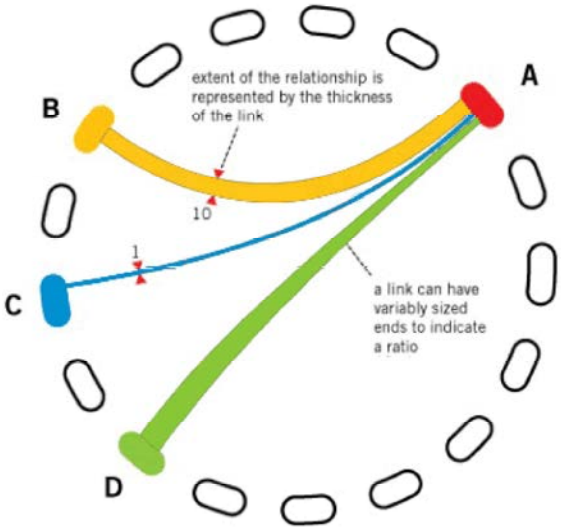

Fig. 1: Data presentation in CIRCOS

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).

RESULTS

Studies on the anti – WSSV efficacy of TP22C: The activity of the crude drug (TP22C) was examined against WSSV in *L. vannamei* to confirm its efficacy as a potent

Fig. 2: PCR diagnosis of TP22C in shrimps
M = marker, 1 = WSSV negative (TP22C intramuscular injection), 2 = WSSV negative (lane 1 DNA injected to fresh shrimps), N = negative control (NEG), P = positive control (POS).

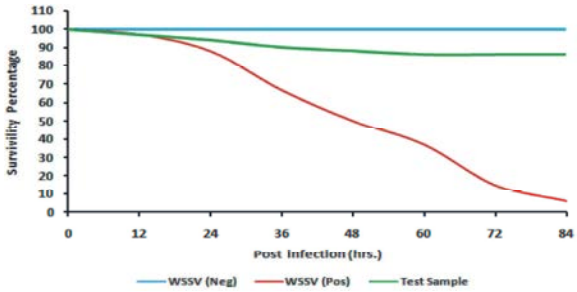

Fig. 3: Variations in survivability percentage in different experimental groups

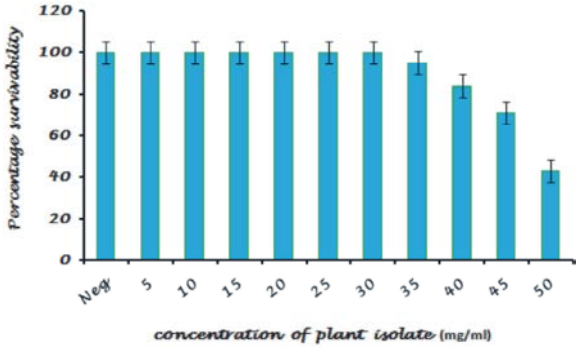

Fig. 4: Toxicity of different concentration of plant isolate (TP22C) in *L. vannamei*

anti-WSSV drug. On completion of the experiment at 84th hrs, post infection, the shrimps were found to be nested PCR negative and when the DNA extracted from these shrimps were injected into a fresh batch of shrimps, none of the injected animals displayed any clinical signs of WSSV infection and remained negative to nested PCR (Fig. 2). The survivability was 86% at the end of the 84th hr of the experimentation (Fig. 3).

Determination of *in Vivo* Toxicity of the Plant Isolate TP22C: *L. vannamei* (6-8g) (n=6) were injected with the plant isolate at different concentrations ranging from 5-50 mg/ml and monitored for 7 days (Fig. 4). Response of the animals was more or less the same even up to a concentration 30 mg/ml ($p > 0.05$). However, at 50 mg/ml strength there was significant reduction (43% average percentage survival) ($p < 0.05$) in survival of shrimps during the experimental period of 7 days.

Estimation of *in vivo* Efficacy of TP22C 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.

This result was only due to the efficiency of the plant isolate (TP22C) 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 32.3 µg/mg and 2.13 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 1.01 µg/mg and 1.06 µg/mg at the 24th hr respectively; but with a gradual increase in time, a steady decline in 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 0.456 µg/mg

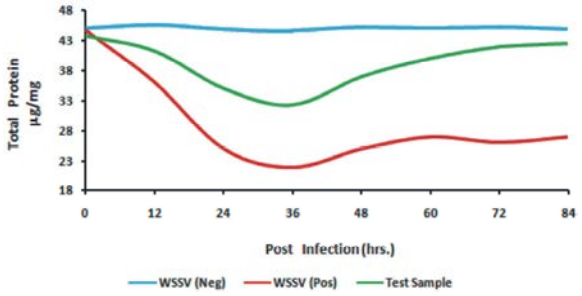

Fig. 5: Variations in total protein content in different experimental groups

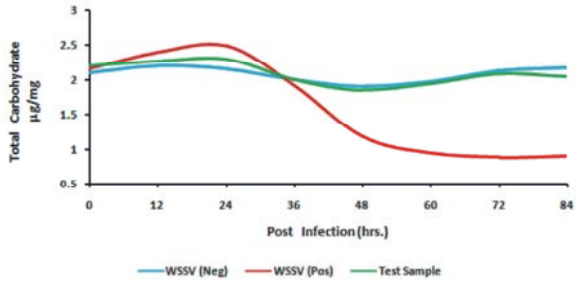

Fig. 6: Variations in total carbohydrate content in different experimental groups

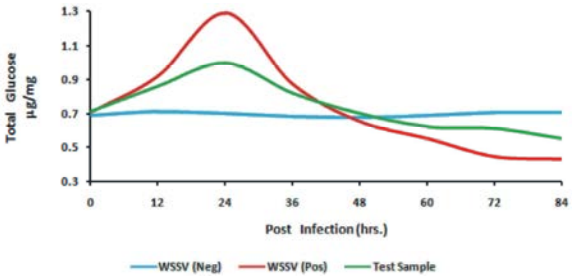

Fig. 7: Variations in total glucose content in different experimental groups

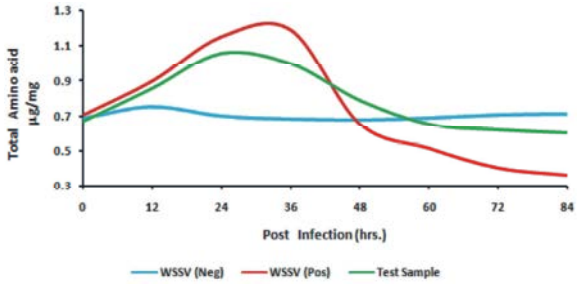

Fig. 8: Variations in total amino acid content in different experimental groups

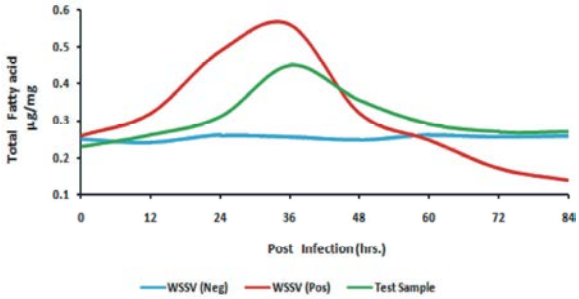

Fig. 9: Variations in total fatty acid content in different experimental groups

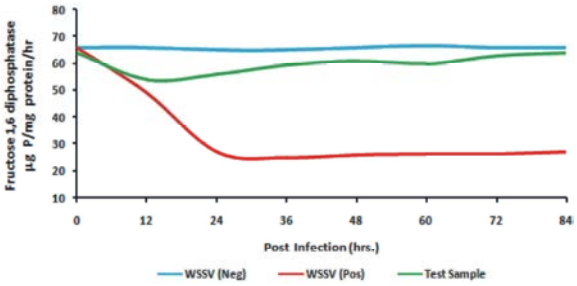

Fig. 10: Variations in fructose 1, 6 diphosphatase content in different experimental groups

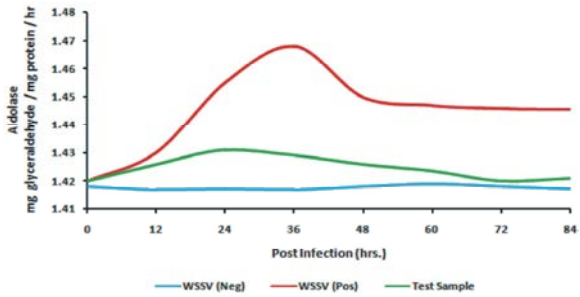

Fig. 11: Variations in aldolase content in different experimental groups

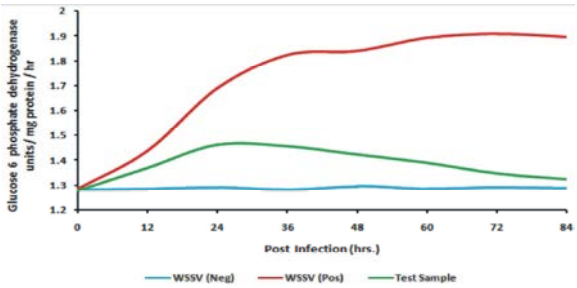

Fig. 12: Variations in glucose 6 phosphate dehydrogenase content in different experimental groups

at the 36th hr; however, with further time elapsation a steady decline in the same was observed. The lowest level of fructose 1, 6 diphosphatase (Fig. 10) estimated in the tissue of the shrimps (TS) was 53.6 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

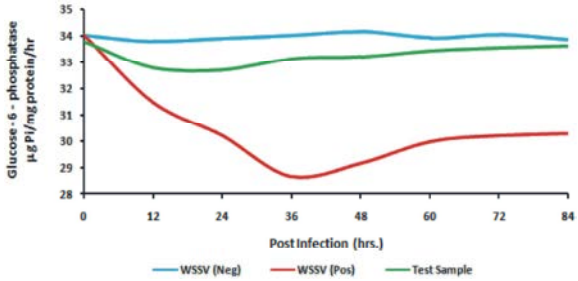

Fig. 13: Variations in glucose 6 phosphatase content in different experimental groups

time with its highest level of 1.429 µg glyceraldehyde/mg protein/hr and 1.4552 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 33.7 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.

Significant differences ($p < 0.01$) were observed between the POS, NEG and TS in the biochemical variables from various tissues and at different time intervals post infection with WSSV (0, 12, 24, 36, 48, 60, 72, 84 hrs). 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, TP22C.

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 1). When multiple regression of survival rate on all the biochemical parameters (Table 2) were considered, the amount of variability explained was 98.3% (R Square=0.983). When significant regression co-efficient were taken into account in the case of metabolic parameters, it was found that G6PDH ($p<0.01$) itself was explaining 88.5% (R Square=0.885) 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

Table 1: 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	.774**	1								
TG	.414*	.846**	1							
TAA	.599**	.893**	.759**	1						
TFA	.477*	.882**	.935**	.889**	1					
FDPase	.939**	.657**	.295	.407*	.306	1				
G6Pase	.700**	.386	.143	.128	.107	.818**	1			
ALD	-.696**	-.399	-.131	-.119	-.111	-.795**	-.739**	1		
G6PDH	-.946**	-.893**	-.618**	-.672**	-.637**	-.916**	-.664**	.662**	1	
SURV	.850**	.961**	.772**	.820**	.827**	.747**	.463*	-.501*	-.941**	1

Table 2: 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 – 0.983
Adjusted R Square – 0.972
Predictors - TP, TC, TG, TAA, TFA, FDPase, G6Pase, ALD, G6PDH.
Dependent Variable – SURV

	TP	TC	TG	TAA	TFA	FDPase	G6Pase	ALD	G6PDH
Significance	0.88	0.256	0.616	0.336	0.266	0.095	0.682	0.228	0.015*

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

R Square – 0.885
Adjusted R Square – 0.880
Predictors - G6PDH.
Dependent Variable – SURV

	G6PDH
Significance	0.000**

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

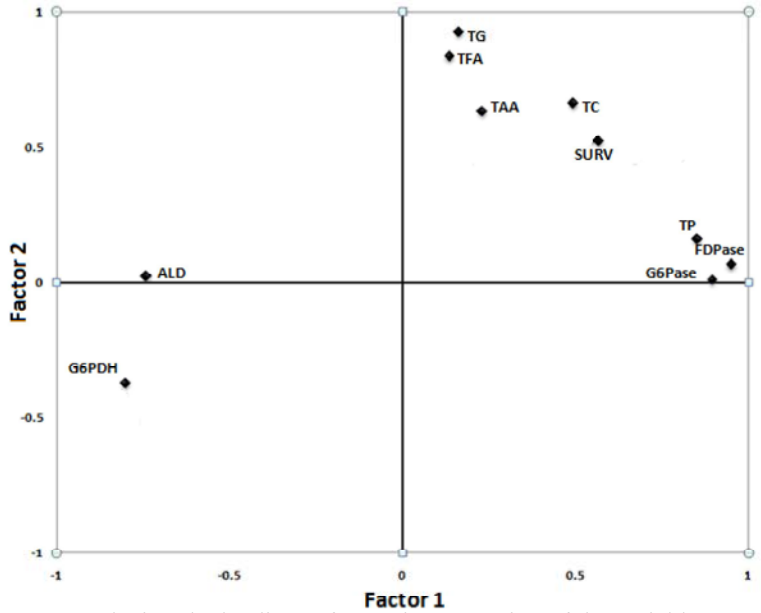

Fig. 14: Principal component analysis- The loadings after varimax rotation of the variables

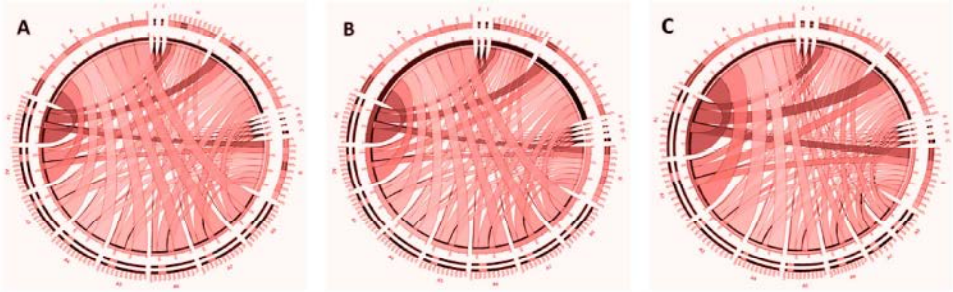

Fig. 15: CIRCOS representation of systematic relationship between survivability and biochemical variables [A _ NEG; B _ TS; C _ POS]

significant. The factor analysis generated four significant factors, which explained 95.20% of the data variance in data sets, among the four the first, second and third factors itself have high loading and accounts 43.1%, 25.6% and 23.8% response of total variance. 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, FDPase, G6Pase, ALD and G6PDH in factor 1 and SURV, TC, TG, TAA and TFA in factor 2 and SURV and TAA in factor 3 indicates significant effect 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 (TP22C) 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 appears that as time progressed upon WSSV exposure, the expression level of the target genes increased as evidenced by the increase in the intensity of PCR amplicons representing these genes in the positive control group (Fig. 16). In the case of positive control group, there was 100% mortality at the 84th hr. The virus assay was therefore terminated at 84 hr post-challenge.

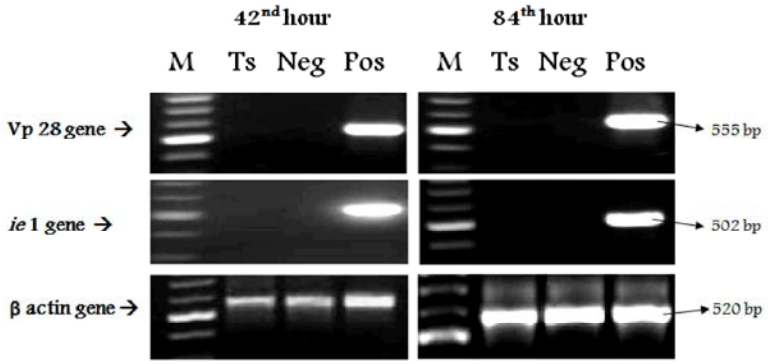

Fig. 16: Reverse transcription PCR analysis of VP28, immediate early (*ie* 1) and â actin genes in host

DISCUSSION

Most of the products derived from plants show many interesting activities. Antiviral activities of aqueous extracts from plants are well established [14-18] that also includes reports on the anti-WSSV activity of plant extracts [19-25]. A combination of herbal extracts and probiotics as medicated diet could decrease the prevalence of WSSV in *Litopenaeus vannamei* [26]. 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 assess the possibilities of using terrestrial plants as sources of anti - WSSV drugs. With this objective, 30 plants abundantly found in different parts 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, 7 plant isolates were found to be effective against WSSV. Finally, the plant isolate TP22C proved to be the potent anti-WSSV drug in the present study. As TP22C alone could give protection to all animals tested against WSSV, under the experimental conditions, this terrestrial plant 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 being eliminated subsequent to the infection.

In animal model, the highest non-toxic concentration went up to 30 mg/ml, from which 10 µl extract was injected to shrimps (6-8 g). We found that the crude drug 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 [25]. The average percentage of survivability of shrimps injected with TP22C was 86%, at a concentration of 10 mg/ml. Mortalities observed in TP22C treatment tank was due to cannibalism subsequent to moulting. 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 TP22C is concentration dependent. Different concentrations of Cidofovir (an antiviral drug) were injected and observed that it was not toxic to shrimps up to a concentration of 200 mg/kg of body weight and they could successfully use the same for further assays [27]. Similarly, 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 [24]. 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 [28, 29].

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 [30] 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 [31]. In contrast, researchers [32] 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 [33, 34]. 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 [35, 36]. 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 [37]. 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 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 [37]. 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 [38]. It has been reported that the PPP with its major enzyme glucose-6-phosphate dehydrogenase provides the tissues with specific molecule, the reduced NADPH [39]. 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 nine 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 TP22C can be well demonstrated using this particular software.

To evaluate the efficacy of TP22C for protecting *L. vannamei* from WSSV infection, expression of *ie* 1 and VP28 and ß actin genes were investigated. This study indicated that the viral encoded genes *ie* 1 and VP28 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 [40]. 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 [41]. 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 neither of the two WSSV genes examined *ie* 1 and VP28, were expressing which might be due to inactivation of the virus by the virucidal activity of TP22C. 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. Significant variations in the biochemical parameters of the WSSV infected *L. vannamei* was observed due to the *in vivo* host and pathogen interaction. The biochemical parameters examined in the present study revealed significant differences at each time intervals with the POS group indicating that the drug (TP22C) 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 TP22C 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 researches.

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