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Immunostimulatory Effect and ProPo Gene Expression Status of Methanolic Extract of *Phyllanthus niruri* Against WSSV in Shrimp *Penaeus monodon* (Fab).

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Abstract

A methanolic extract of the medicinal herb *Phyllanthus niruri* was added to shrimp pellet diet at three concentrations (1, 2 and 3%), simultaneously a control diet was also prepared, which devoid of *P. niruri* extract. The experimental diets were individually fed to shrimp *P. monodon* for 90 days. Influence of herb *P. niruri* dietary source on prophenoloxidase gene expression in shrimp was analyzed through qRT-PCR and it indicated that the gene expression level was 16.24 to 29.63% in experimental shrimp over control shrimp. The tested shrimps were challenged with WSSV and the mortality rate was recorded everyday up to 21 days. During the challenge test, the control group had 100% mortality within 11 days, but the experimental groups showed 62 to 84 % mortality on the 21st day. During the WSSV challenge experiment, the immune functions were significantly elevated in experimental groups of shrimp over the control group. Quantification of WSSV infection through qRT-PCR analysis recorded that the control group had 429 x 10^3 WSSV DNA copies; nevertheless the WSSV DNA copy numbers in the experimental groups were considerably reduced from 4415 to 91.

Keywords: *Phyllanthus niruri*, WSSV, Immunostimulant, qRT-PCR and ProPo gene

1. Introduction

Aquatic organisms, including fish and shrimp are vulnerable to majority of disease-causing organisms such as protozoans, bacteria, fungi, viruses, etc. Among these, viruses are more threatful entities. There are 20 known shrimp viruses today, six are especially important due to their epizootic spread and economic impact, which are Monodon Baculovirus (MBV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHNNV), Taura Syndrome Virus (TSV), Yellow Head Disease Virus (YHV), Monodon Slow Growth Disease (MSGD) and White Spot Syndrome Virus (WSSV). Among these, WSSV is the most important disease-causing virus in shrimp farms (Moscardi, 1999). The major clinical sign of WSSV in diseased shrimp is the presence of white spots on the exoskeleton, along with some other signs like rapid reduction in food consumption, lethargy and reddening of appendages. It was reported that, in farmed shrimp mortality rate pertained to WSSV is more rapid and within 3 to 10 days after infection, the cumulative mortality can reach to 100% (Flegel, 1997).

Immunostimulants are one of the most important prophylactics, which are used to control diseases in aquatic organisms by improving their immune systems especially in finfish and shellfish species. Immunostimulants are considered as an alternative to chemotherapy and vaccines, because of their wide spectrum activity, cost effectiveness and eco-friendly disease preventative measure (Anderson, 1992; Nayak, 2010). A variety of immunostimulants such as fucoidan (Immanuel *et al*., 2012a; Sivagnanavelmurugan *et al*., 2014), β-1,3-glucan (Chang *et al*., 2003), *Spirulina platensis* (Rahman *et al*., 2006), sodium alginate (Cheng *et al*., 2004; Cheng *et al*., 2005), *Sargassum hemiphyllum var. chinense* powder and extract (Huynh *et al*., 2011), ferritin (Ruan *et al*., 2010), etc. have been reported for the enhancement of shrimp immune activity against pathogens.

Research on the antiviral activity by plant extract has become popular since 1950 (Balasubramanian *et al*., 2008a). Direkbusarakom *et al*. (1995) examined the ability of ethanolic extract complexes with polyvinylpyrolidone from *Clinacanthus nutans* fed to shrimp provide better resistance against YHV. Previous studies emphasized that herbal immunostimulants are one of the safest, eco-friendly alternative approach towards protecting shrimp from both bacterial and viral infections (Balasubramanian *et al*., 2007; Sivagnanavelmurugan *et al*., 2014). Balasubramanian *et al*. (2008b) reported that the oral administration of *Cynodon dactylon* plant extract could effectively enhance innate immunity and disease resistance against WSSV.

The genus *Phyllanthus* includes more than 500 species, having medicinal use in several countries. *P. niruri* L. (Euphorbiaceae) is a common kharif (rainy season) weed found in both cultivated fields and wastelands in India. It is an annual herb with height varying between 30 and 60 cm. Its roots, leaves, fruits, milky juice and whole plant are used as medicine. Fruits are useful to treat tubercular ulcers, wounds, sores, scabies and ringworm. Similarly, the roots in fresh condition are best to control urinary infection, jaundice, dropsy, etc. (Agharkar, 1991). The n-butanolic extract of *P. niruri* has been reported for the inhibition of shrimp pathogen *Vibrio parahaemolyticus* in *P.indicus* (Immanuel *et al*., 2004). Considering the

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importance of the terrestrial medicinal herb *P. niruri*, in the present study an attempt has been made to evaluate the immunostimulatory and immune gene expression effects of methanolic extract of *P. niruri* in *P. monodon* challenged with WSSV.

2. Materials and Methods

2.1. Preparation of methanolic extract of *P. niruri*

The medicinal herb *P. niruri* were collected from the barren lands of nearby villages of Kanyakumari District, Tamilnadu, India. The collected plants were washed thoroughly with sterile water to remove extraneous dirt and dried under shade. Then the whole plants were finely powdered and the extraction was done by percolation process for seven days at1:4 ratio of plant powder and methanol. Further, with the help of Whatman No.1 filter paper, the extract was filtered and it was dried in a rotary vacuum evaporator. Then the powder form of extract was stored in screw cap vial at 4°C in a refrigerator for further study.

2.2. Primary screening on antiviral activity of methanolic extract of *P. niruri* in shrimp *P. monodon* through PCR analysis.

The methanolic extract of *P. niruri* was tested to screen the anti WSSV activity by bioassay method (Balasubramanian *et al.*, 2007). In the present investigation, WSSV inactivation was confirmed through bioassay and PCR method. In brief, for bioassay, 5µl of viral (WSSV) suspension, 10µl of *P. niruri* with the concentration of 200mg/kg of shrimp and 15 µl of NTE buffer were taken and mixed well. Simultaneously, a positive control was also prepared which had 25µl NTE buffer and 5µl viral suspension alone. Thereafter, both test and positive control samples were incubated for 3h at 29°C. After incubation, 30µl of mixture was injected into experimental shrimp (15±1.25g) *P. monodon* intramuscularly, further its mortality rate was recorded daily up to 15 days.

The dead shrimp were preserved in 70% ethanol and then rehydrated in distilled water for 1h before DNA extraction. The DNA was extracted from the pleopods of shrimp by following the method of Yoganandhan *et al*. (2003a) and the extracted DNA was subjected to PCR analysis. The primers used were designed as per the protocol of Van Hulten *et al*. (2001) in order to yield a 613 bp amplicon (forward primer: VP28-F 5'-ATGGATCTTT CTTTCACTCTTTC-3'and reverse primer: VP28R 5'-CTCGGTCTCAGTGCCAG-3'). The PCR product was examined through 1% agarose gel electrophoresis. The infectivity range (296 bp to 910 bp) of the WSSV in control and experimental groups of shrimps was determined. The infectivity range was considered as heavy (2000 WSSV copies), moderate (200 WSSV copies) and low (20 WSSV copies) with 910 bp, 550 bp and 296 bp, respectively. The house keeping gene (848 bp) was used as an internal control for WSSV detection (IQ 2000™ WSSV detection and prevention system, Farming Intelli Gene Tech. Corp., Taiwan).

2.3. Evaluation of immunostimulatory effect and immune gene (ProPo) expression status of *P. niruri* extract in *P. monodon* against WSSV

2.3.1. Preparation of pellet diets supplemented with *P. niruri* extract

The control and experimental diets, supplemented with various concentrations (1, 2 and 3%) of methanoloic extract of *P. niruri* extract were prepared by following the protocol described by Yeh *et al*. (2008) (Table 1).The required quantities of ingredients in fine powder form were mixed well along with the respective concentrations of *P.niruri* extract, fish oil and water and made in to dough's. Finally, the individual dough was pelletized and the pellet diets (1mm size) were oven dried (40°C) and stored in containers in refrigerated condition until further use. The proximate analysis of basal diets reveled that they contain 40.14 – 40.47% crude protein, 15.32 – 16.48% carbohydrate and 7.56 – 7.64% crude lipid.

2.3.2. Feeding experiment

The shrimp *Penaeus monodon* postlarvae (PL20) were initially stocked and acclimatized in a 1000 L capacity FRP tank having filtered seawater (32 ± 1 ppt) at room temperature (28 ± 1°C) and aerated to maintain oxygen level at > 6mg l^{-1}. Further the PL were acclimatized for 10 days on a diet of live *Artemia franciscana* nauplii. From the acclimation tank, uniform size of *P. monodon* postlarvae (0.023 ± 0.0019) at the stage of PL30 were selected and transferred in to four groups of experimental tanks (control – un supplemented pellet diet; Experimental-*P. niruri* extract with respective concentrations of 1, 2 and 3 % supplemented pellet diets) with 750 L of filtered seawater (salinity of 32 ± 1ppt) in 1000L capacity FRP tanks at an ambient temperature (28 ± 1°C). The shrimp PL at the rate of 1/15L were stocked in each experimental tank. The experimental tanks were maintained with optimal oxygen concentration (about 6ppm) by proper aeration. The shrimp PL were fed with the respective experimental or control diets 3 times (6:00, 14:00 and 18:00 h) a day at 30, 30, and 40%, respectively. 50% water exchange was maintained daily and also the unfed diets were removed daily. In each group, three experimental tanks were maintained and the feeding experiment was scheduled for 90 days. At the end of feeding experiment, the survival and growth rate of individual group of shrimps were estimated.

2.3.3. Prophenoloxidase (ProPO) immune gene expression analysis in *P. monodon* through qRT-PCR

After 90 days of feeding experiment, the prophenoloxidase (ProPO) gene expression in the haemolymph samples of the individual feeding treatments were analyzed. For this, haemolymph (0.50 ml) was individually withdrawn from the ventral sinus cavity of each group of shrimp using a 1-ml sterile syringe (with a 25-gauge needle) containing 0.5 ml of precooled (4°C) anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, at pH 7.5 and with an osmolality of 780 mOsm kg^{-1}). The diluted haemolymph was centrifuged

(500xg) at 4°C for 20 min, and the haemocyte pellet was washed once with cacodylate buffer (10mM sodium cacodylate, 0.45 M sodium chloride, 20 mM calcium chloride; pH 7.0). The resulting haemocyte pellet was then used for the total RNA isolation.

Total RNA was isolated by using TRizol reagent (Invitrogen, USA) according to manufacturer instruction. cDNA was synthesized using one step reverse transcriptase (RT) kit (Applied Biosytems). The specific primer pairs were designed for prophenoloxidase gene mRNA sequence [Forward primer: 5'-CGACTCCTGGATGCCATACAT-3';Reverseprimer:5'-CATCGCGAAGAGGAACTTTGT-3' (Accession no.: AF521948)] using Primer Express™ software (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression of prophenoloxidase gene of *P. monodon* from the respective groups was measured by qRT-PCR. Data analysis of the qRT-PCR was performed with SDS software version 2.0. Relative quantification of ProPo gene expression was performed according to the manufacturer's instructions. β – actin was used as an internal control. Expression level of the gene (up-regulation) was determined by using fluorescence tagged probes (FAM and VIC). Threshold cycle (Cq Mean) value of experimental and control groups were calculated based on the ΔCq difference between the control and treated group of shrimps.

2.3.4. WSSV Challenge study

2.3.4.1. Viral inoculum preparation

The diseased shrimp *P. monodon* already infected by WSSV, having prominent white spots on the exoskeleton were collected from local shrimp farms. The soft tissues from the head region and also the gills were homogenized and centrifuged at 3000 xg for 20 min at 4°C. The supernatant was again centrifuged at 8000 xg for 30 min at 4°C and the final supernatant was filtered through a 0.4-mm membrane filter. The filtrate was then stored at -20°C for infectivity studies (Yoganandhan *et al*. 2003b; Immanuel *et al*. 2010). The occurrence of WSSV in the inoculum was checked by nested PCR and this result showed severe infection (912bp) equal to 2000 WSSV DNA copies (IQ 2000™ manual).

2.3.4.2. WSSV challenge experiment

The shrimp, which have already been reared under the experimental diets for 90 days were collected and were reared separately at the rate of 1/ 25L in 1500L capacity sterile FRP tanks with 1000L sterilized aerated seawater. Aseptic techniques were maintained throughout the experiment. The shrimp were fed on the same experimental and control diets, which were already used in the feeding experiment. The WSSV inoculum was injected intramuscularly into shrimp at a dose of 0.01 ml per shrimp, at the same time, negative control shrimp were injected with 0.01 ml saline per shrimp. During this challenge experiment, the survival of the shrimp was monitored daily up to 21 days (Chang *et al*. 2003). The dead shrimp were removed from the respective tanks during each observation and the mortality percentage was noted, from this the cumulative mortality index (CMI) was calculated (Immanuel *et al*. 2012b).

2.3.5. Immunological analysis

During challenge experiment, in the haemolymph samples of shrimp before (0 day) and after (10th& 21st days) challenged with WSSV, the immunological parameters such as total hemocyte count (THC), prophenoloxidase (ProPO), Respiratory burst (NBT assay), superoxide dismutase (SOD) and phagocytic activities were determined by following appropriate methods (Immanuel *et al*., 2012a).

2.3.6. Quantification of WSSV through qRT-PCR analysis

The WSSV infection in *P. monodon* was detected by qRT-PCR analysis after 21 days of challenge experiment. The WSSV infected control and experimental shrimps were preserved in 70% ethanol and consequently were rehydrated in distilled water for 1h before the qRT-PCR analysis. The qRT-PCR analysis for WSSV DNA quantification was determined by following Immanuel *et al*. (2012b).

2.4. Statistical analysis

The data generated were statistically expressed as Mean ± SD and were analyzed using one-way ANOVA test at 5% level of significance. Further a multiple comparison test (Tukey's test) was conducted to compare the significant differences among the parameters using computer software STATISTICA 06 (Statosoft, Bedford, UK).

3. Results

3.1. Screening of antiviral (WSSV) effect of methanolic extract of *P. niruri*

The results on primary screening of antiviral activity of methanolic extract of *P. niruri* is provided in Table 2. In control group, 100% mortality was observed within 8 days of the initial screening experiment. But in the experimental group, the mortality observed was only 58.52% within 15 days. The WSSV infectivity range of experimental and control groups was determined through PCR analysis. The result indicated that the WSSV inoculum and the positive control group showed WSSV positive with the infectivity range of severe (910bp). However, shrimp treated with methanolic extract of *P. niruri* exhibited lower WSSV infectivity range (296bp).

3.2. Determination of growth and survival of Shrimp

At the end of feeding experiment of 90 days, the growth and survival rate of control and experimental diets fed shrimps were determined. The results inferred that the survival of control group of shrimp was 78%, but it was 82 to 86% in experimental groups of shrimps fed on 1-4% of *P. niruri* methanolic extract supplemented diets. Similarly, the growth rate was observed as 7.18g in the control group, whereas, it was 7.68 to 8.32g in the experimental groups.

3.3. Relative quantification of ProPO gene expression in shrimp

After 90 days of feeding experiment, the expression rate of Pro POgene was analyzed through qRT-PCR. The mRNA expression ofProPO gene in the experimental group

of shrimp was significantly (P< 0.05) higher than the control group. The up regulation of proPo gene expression level was in the order of 16.24, 18.99 and 29.63% than the control group within Cq mean value of 21.65, 20.94 and 18.19 threshold cycles in 1, 2 and 3% of *P. niruri* extract supplemented diets fed groups, respectively (Table 3 and Fig. 1).

3.4. Cumulative mortality percentage and CMI of shrimp during WSSV challenge test

The shrimp succumbed to death started from 3rd day of challenge test. The mortality of control group was 6% on the 3rd day. On the other hand, no mortality was recorded in the experimental groups. Among the experimental groups, shrimp fed on diet containing 1 and 2% methanolic extract of *P. niruri* recorded 3 and 2% of mortality respectively on 4th day of challenge experiment; whereas shrimp fed on 3% *P. niruri* extract supplemented diet showed 3% mortality during 5th day of challenge experiment. When the duration of the challenge experiment increased, the cumulative mortality was also increased gradually. Finally, within 11 days, 100% mortality was observed in control group, however in the experimental groups, the survival of *P. monodon* was prolonged up to 21 days of challenge experiment. Within 21 days, 84, 73 and 62% mortality was recorded respectively in 1, 2 and 3 % of *P. niruri* extract supplemented diets fed experimental groups (Fig. 2).

The CMI and reduction in mortality percentage of control and experimental groups of shrimp after challenged with WSSV is given in Table 4. The CMI of control group was 21277, but it reduced considerably in experimental groups with 38.92, 49.65 and 58.14% reduction in mortality than the control group, respectively in 1, 2 and 3% methanolic extract of *P. niruri* supplemented diets fed shrimp.

3.5. Immunological analysis

In control group, the THC, ProPO activity, respiratory burst activity, SOD and phagocytic activity were observed as 66.20x10^6 cells /ml, 0.1342 OD,1.015mmol, 36.35 unit/ml and 5.69%, respectively at the beginning of the WSSV challenge experiment. At the same time, in the experimental groups, the THC recorded was 70.46 to 90.40 x10^6 cells /ml, ProPO activity recorded was 0.1593 to 0.1705OD, respiratory burst activity recorded was 1.068 to 1.110mmol, SOD activity recorded was 49.44 to 55.76 unit/ml and phagocytic activity recorded was 5.86 to 6.30% with increasing concentrations (1-3%) of methanolic extract of *P.niruri* supplemented diets fed shrimp.

After 10 days of WSSV challenge, a marked reduction in THC was noticed in both control (38.70x10^6 cells /ml) and experimental (63.60 to 82.60x10^6 cells /ml) groups of shrimps. The ProPO activity of experimental groups of shrimps was increased from 0.1602 to 0.1784OD on 10th day of challenge experiment. But on the same day, in control group, the ProPO activity was decreased (0.0584OD) significantly. Similarly, the respiratory burst activity of experimental groups of shrimps was observed to be increased (1.205 to1.326 mmol) on 10th day of challenge study, however in control group it was much reduced (0.0730mmol) on the same day of challenge duration. In the case of SOD, it was decreased (11.24units/ml) in control group on 10th day of challenge study, whereas in the experimental group, the SOD activity was elevated (50.24 to 57.66 unit/ml), which depends on the concentrations (1-3%) of *P. niruri* methanolic extract supplemented diets fed shrimps on 10th day of WSSV challenge experiment. Invariably, the phagocytic activity was considerably reduced both in control (2.98%) and experimental (5.06 to 6.02%) groups of shrimps on 10th day of challenge study.

At the final day (21st day) of challenge experiment, the THC (74.80 to 95.40x10^6 cells /ml), ProPO activity (0.1652 to 0.1802OD), respiratory burst activity (1.212 to 1.338mmol), SOD activity (52.56 to 60.12 unit/ml) and phagocytic activity (5.98 to6.64%) were considerably increased in all the tested concentrations (1-3%) of *P. niruri* methanolic extract supplemented diets fed shrimp. But it was noted that all the above immunological parameters were not conducted in control group, since 100% mortality of shrimp was occurred in this group before completing the challenge period of 21 days (Fig. 3 a-e).

3.6. WSSV quantification in shrimp through qRT-PCR

On the final day of the challenge experiment, the WSSV DNA in control and experimental groups of shrimps was quantified individually by qRT-PCR (Table 5 and Fig. 4). The positive control group displayed 4.29 x 10^3DNA copies of WSSV within 22.74 threshold cycles (Ct FAM). However, the copy number of WSSV DNA in shrimps that fed on experimental diets containing *P. niruri* extracts showed a decreasing trend with respect to increasing concentrations (1 to 3%). For instance, at lower concentration (1%), 4415 WSSV DNA copies with 25.96 threshold cycles was noticed; whereas 2 and 3% concentrations displayed 717 and 91 WSSV DNA copies respectively within 28.53 and 31.46 threshold cycles. The negative control showed no WSSV DNA amplification.

Table 1. Composition of basal diet supplemented with different concentrations of methanolic extract of *P. niruri* (% by weight) for *P. monodon.*

Ingredients (% by weight)	Control diet	Experimental diets (%) 1	2	3
Fish meal	56	56	56	56
Groundnut oil cake	21	20	19	18
Soybean powder	11	11	11	11
Wheat bran	6	6	6	6
Methanolic extract of *P. niruri*	0	1	2	3
Vitamins and mineral mix	2	2	2	2
Cod liver oil	2	2	2	2
Binder	2	2	2	2

Source: Yeh *et al.* (2008) modified

Table 2. Primary screening of antiviral activity of methanolic extract of *P. niruri* (200mg/ml) in shrimp *P. monodon* against WSSV

Sample	Mortality (%)	PCR result	
		WSSV (+/-)	Infectivity range
Control	100 ± 0*	+++	Heavy (910bp)
Methanolic extract of *P. niruri*	58.52 ± 1.62**	+	Low (296bp)

*Mortality within 8 days; ** Mortality at the end of screening experiment.

Table 3. Relative quantification of prophenoloxidase gene expression in shrimp *P. monodon* fed on different concentrations of methanolic extract of *P. niruri* supplemented diets after feeding experiment for 90 days.

Plant extract	Conc. (%)	Relative quantification of prophenoloxidase gene expression	
		Cq Mean	Expressed Level (%)
Untreated (Positive control)		25.85	-
Methanolic extract of *P. niruri*	1	21.65	16.24
	2	20.94	18.99
	3	18.19	29.63

Table 4. Cumulative Mortality Index (CMI) and percentage reduction in mortality of shrimp *P. monodon* fed on different concentrations of methanolic extract of *P. niruri* supplemented diets after challenged with WSSV against control.

Plant extract	Conc. (%)	CMI	Reduction in mortality (%)
Control (C)		21277 ±1012.13^{a}	0 ± 0
Methanolic extract of *P. niruri*	1	12995 ±614.30^{b}	38.92 ± 1.320
	2	10712 ±434.62^{c}	49.65 ± 1.646
	3	8906 ±406.89^{d}	58.14 ± 1.562

Each value is a Mean ± SD of three replicate analysis; within each column, means with different superscript letters are statistically significant (one way ANOVA test, P< 0.05and subsequently *post hoc* multiple comparison with Tukey's test).

Table 5. Quantification of WSSV copies by RT-PCR analysis of shrimp *P. monodon* fed on different concentrations of methanolic extract of *P. niruri* supplemented diets after challenged with WSSV

Plant extract	Conc. (%)	Ct FAM (Cycles)	Number of DNA Copies
Negative control (NC)		0	0
Positive Control (C)		22.74	4.29 x 10^{3}
Methanolic extract of *P. niruri*	1	25.96	4415
	2	28.53	717
	3	31.46	91

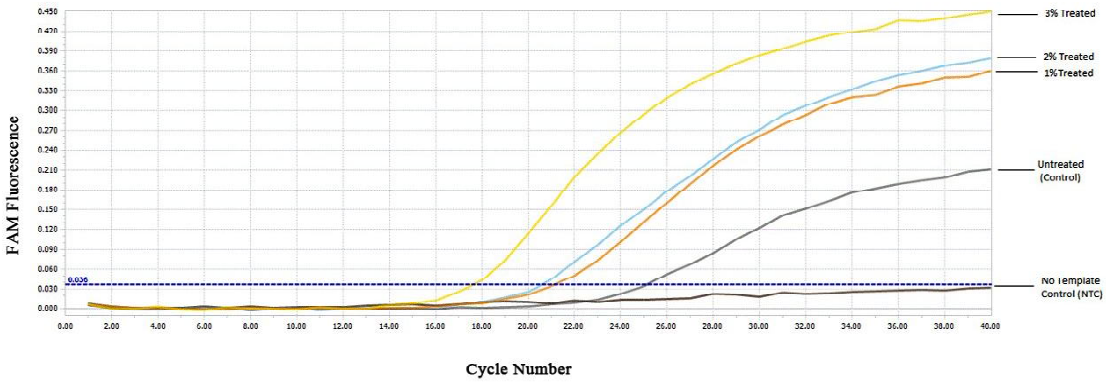

Fig. 1. Amplification curve for relative quantification of prophenoloxidase gene expression of shrimp *P. monodon* fed on different concentrations of methanolic extract of *P. niruri* supplemented diets after feeding experiment for 90 days. P1: *P. niruri* (1%); P2: *P. niruri* (2%); P3: *P. niruri* (3%); CTRL: Control; P1, P2, P3 & CTRL: respective normalizer.

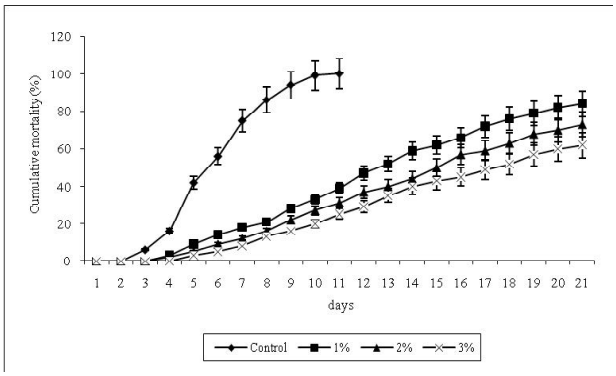

Fig. 2. Cumulative mortality (%) of shrimp *P. monodon* fed on different concentrations of methanolic extract of *P. niruri* supplemented diets after challenged with WSSV in 21 days interval. Each value is a Mean ± SD of triplicate analysis.

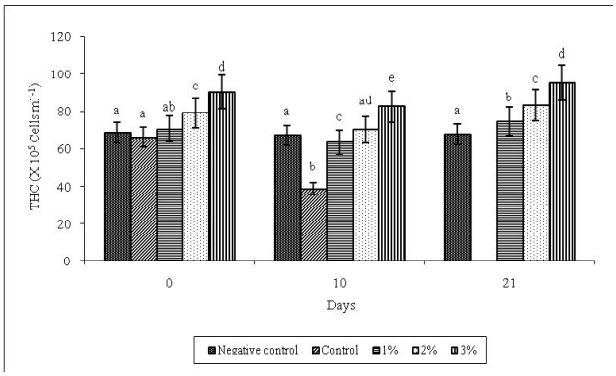

Fig. 3a. Total Haemocyte Count (THC)

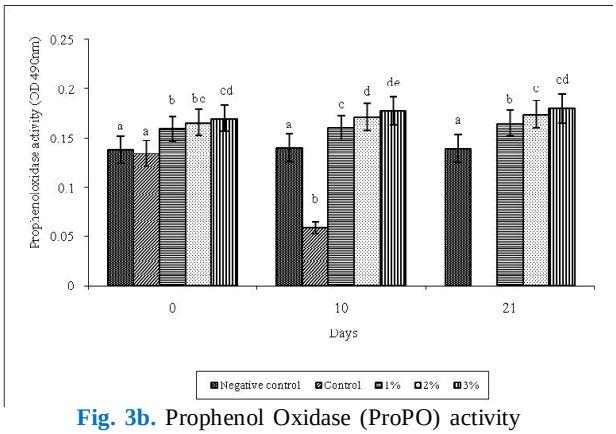

Fig. 3b. Prophenol Oxidase (ProPO) activity

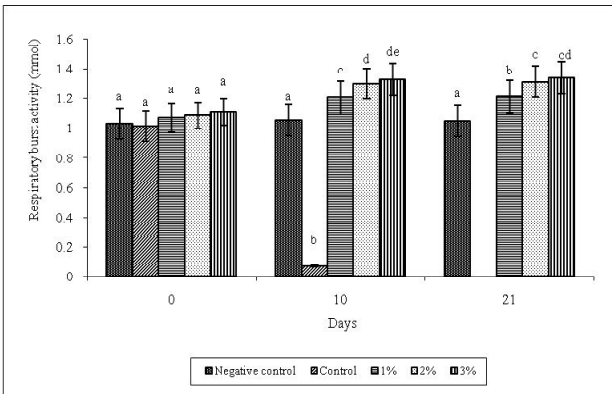

Fig. 3c. Respiratory Burst activity (NBT assay)

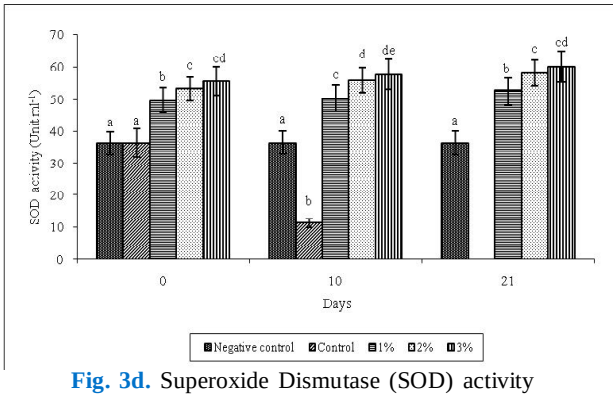

Fig. 3d. Superoxide Dismutase (SOD) activity

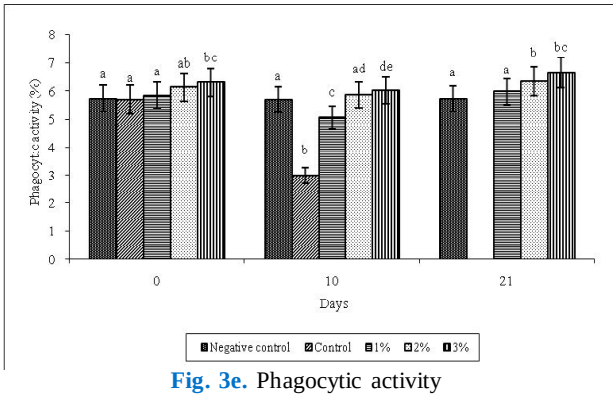

Fig. 3e. Phagocytic activity

Fig. 3 (a-e). Immunological analysis (a.THC; b. ProPO activity; c. Respiratory Burst activity; d.SOD activity & e. Phagocytic activity)of shrimp *P. monodon* fed on different concentrations of methanolic extract of *P. niruri* supplemented diets after challenged with WSSV in 21 days interval. Each value is a Mean ± SD of three replicate analysis; bars with different superscript letters are statistically significant (One-way ANOVA test, P< 0.05and subsequently *post hoc* multiple comparison with Tukey's test).

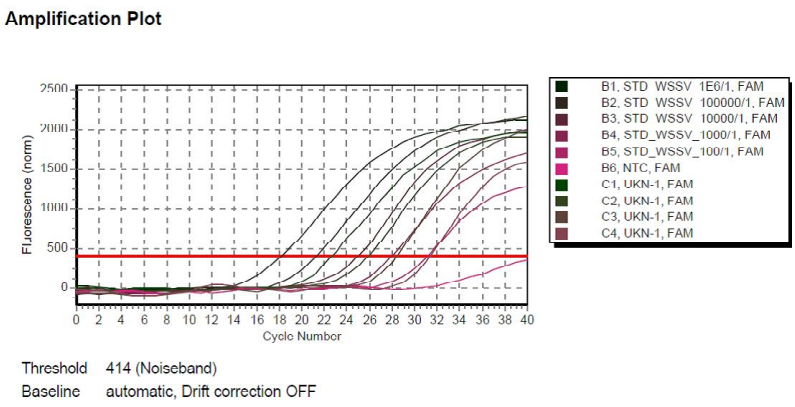

Fig. 4. Amplification curve showing 10-fold serial dilutions of standards, control and different concentrations (1 – 3%) of methanolic extract of *P. niruri* supplemented diets fed experimental shrimp samples.C1: Control; C2: *P. niruri* (1%); C3: *P. niruri* (2%); C4: *P. niruri* (3%)

4. Discussion

Viral and bacterial infections are considered as the most devastating diseases affecting the shrimp industry around the world, even though recombinant DNA, protein vaccines and RNAi are available to control these (Rout *et al*., 2007; Sanchez-Paz, 2010). Indeed, to overcome this problem, research on antiviral activity using plant extract is being made worldwide to treat such diseases with terrestrial herbal extracts, which are having immunostimulant effect on shrimp. Plants are generally considered as the storehouses and rich sources of safe and economical natural compounds. In agreement to this, several findings have evidenced that these natural products are having various activities like appetite enhancement, tonic, growth promotion, antistress, immunostimulant, and antimicrobial activities (Citarasu *et al*., 2006; Al Dabbagh *et al*., 2018; Mohamed A. Lebda *et al*., 2019). In the present study, the immunostimulatory effect of methanolic extract of *P. niruri* on shrimp *P. monodon* against WSSV was investigated.

An initial screening experiment was performed to determine the antiviral effect of methanolic extract of *P. niruri* against WSSV by antiviral bioassay method. In this study, the plant extract showed 58.52 % reduction in mortality and the PCR result displayed WSSV positive with low infection (296bp). In accordance with these, Balasubramanian *et al*. (2007) have screened 20Indian medicinal plant extracts to inactivate WSSV in shrimp. Among the tested plant extracts, *Lantana camara*, *Momordica charantia*, *Aegle marmelos*, *Cynodon dactylon* and *Phyllanthus amarus* displayed profound antiviral activity against WSSV. After initial screening of antiviral activity, different concentrations (1 – 3%) of plant extract supplemented diets were fed to shrimp *P. monodon* for 90 days and the expression of ProPO gene was determined. It was observed that the relative quantification of ProPO gene of experimental groups over control group was ranged from 16.24 to 29.63% increase; respectively in 1-4% extract supplemented diets fed shrimps. Similarly, Bae *et al*. (2012) have reported the ProPO gene expression in β1,3-glucan (BG) and rutin (RT) fed fleshy shrimp *Fennerropenaeus chinensis*. They observed the expression of proPO mRNA was 73 to 90% and 69 to 132%, respectively in0.5to 1g Kg^{-1}BG and RT diets after feeding experiment for 10 days. Cerenius *et al*. (2003) reported the injection of laminarin influenced to increasing the level of proPO mRNA in haemocytes of *Astacus astacus*. After 90 days of feeding experiment, the shrimp PL were challenged with WSSV. During challenge experiment, the percentage reduction in mortality of *P. monodon* observed was 38.92 – 58.14% in 1 – 3 % of *P. niruri* extract supplemented diets fed groups over control group. The reduction in mortality of all the tested groups increased with increasing concentrations of extract. Similarly, Balasubramanian *et al*. (2008b) conducted a study on anti WSSV activity of the medicinal plant *C. dactylon* extract in shrimp *P. monodon*. In this study, the positive control group showed 100% mortality on 4th day of post infection, whereas the shrimp fed on feed incorporated with 1% plant extract and challenged with WSSV showed 100% mortality on 9th day of post infection, however, the shrimp fed on 2% plant extract dietary source displayed no mortality and clinical sign of WSSV till the end of experimental period of 30 days. Citarasu *et al*. (2006) have reported the effect of Indian medicinal herbal (*C. dactylon*, *A. marmelos*, *Tinospora cordifolia*, *Picroorjiza kurooa* and *Eclipta alba*) extracts on WSSV resistance in shrimp *P. monodon* and observed that the control group of shrimps fed on diet devoid of immunostimulant succumbed to 100% death within 7 days. But the survival of shrimp was increased significantly ($P < 0.05$), when they fed on increasing concentrations of immunostimulants.

The immunological parameters were analyzed in the present study during WSSV challenge experiment. It is well known that, hemocytes play a vital role in mediating cellular immune response in shrimps through prevention of blood loss, recognition of non-self, phagocytosis and encapsulation (Soderhall, 1999). Here, at the beginning of the challenge experiment, the THC level was significantly ($P < 0.05$) raised with respect to increase in concentration of *P. niruri* extract in the experimental groups over the control group. Further increase in experimental days interval, i.e. on 10th day, a considerable decrease in THC was noticed; however, at the end of 21st day, the THC was recovered and it raised with increasing concentration of *P. niruri* extract supplemented diets fed shrimp. In accordance to this, Balasubramanian *et al*. (2008a) evaluated the effect of *C. dactylon* extract on THC of *P. monodon* challenged with WSSV. They inferred that, shrimp were treated via *in vitro* (intramuscular injection) and *in vivo* (orally with feed)methods at a concentration of 2 mg per animal and 2% of the C. *dactylon* extract incorporated in commercially available artificial pellet feed, respectively showed a significant reduction in THC at the early stage of WSSV challenge and then recovered to normal level in later period of challenge in both methods. Nevertheless, in control shrimp, the THC was significantly ($P < 0.05$) decreased by WSSV infection in both methods. Similarly, Pholdaeng and Pongsamart (2010) extracted polysaccharide gel (PG) from plant *Durio zibenthinus* and studied its effect on THC in shrimp *P. monodon* against WSSV infection. They prepared a basal diet with three different concentrations (1-3%) of PG and a control diet without PG and fed to shrimp for 12 weeks. Further, they observed that shrimp fed on 1-3% PG diets displayed higher THC level at the end of feeding trial experiment. Further these shrimps were challenged with WSSV for 14 days displayed higher relative percentage of survival than the control group. Likewise, Citarasu *et al*. (2006) reported the effect of equal proportion of methanolic extracts of Indian medicinal herbs (*T. cordifolia*, *E. alba*, *C. dactylon*, *A. marmelos*, and *P. kurooa*) incorporated diet fed *P. monodon* and challenged with WSSV. They observed a good result on increase in level of THC in the experimental group than the control group at the end of WSSV challenge.

The ProPO system is considered to be one of the main innate immune system in shrimp (Soderhall *et al*., 1994). It is widely reported that administration of plant extracts and polysaccharides along with basal diet would effectively

boost up the ProPO system in shrimp and confers protection against bacterial and viral infection in shrimp *Penaues* sp. (Rameshthangam and Ramasamy, 2007; Immanuel *et al*.,2012b; Harikrishnan, 2012). Often ProPO system gets activated by binding to various biomolecules such as zymosan, microbial cell wall components, trypsin, etc. and thereby induces cellular immune responses *viz*. non-self-recognition, melanisation, nodule formation, adhesion and cell-cell communication (Soderhall *et al*., 1994; Liu *et al*., 2005). In the present investigation, during WSSV challenge test, it was found that ProPO activity increased significantly ($P < 0.05$) higher in experimental shrimp than the control shrimp. For instance, during initial day (0 day) of the experiment, the PO activity in experimental groups was 0.1593 to 0.1702 OD; whereas it was 0.1342 OD in control group. Further increase in challenge experiment (10^{th} day), the PO activity reduced; however, at the end of the experiment, the shrimp retrieved with normal PO activity in experimental diet fed shrimp. In accordance to the result of the present study, Balasubramanian *et al.* (2008a) evaluated the efficiency of *C. dactylon* extract on ProPO activity of *P. monodon* challenged with WSSV and depicted that, the PO activity was much higher in experimental groups than the control group. Likewise, Pholdaeng and Pongsamart (2010) affirmed that, the shrimp *P. monodon* fed on diets supplemented with polysaccharide gel (PG) extracted from plant *D. zibenthinus* for 12 weeks had pronounced ProPO activity than the control group without PG. Further, the immunostimulated shrimp challenged with WSSV displayed higher relative percentage survival against WSSV than the control group at the end of 14 days of challenge experiment.

Reactive oxygen intermediates (ROIs) including superoxide anion are released during respiratory bursts of phagocytosis, which represent a defense mechanism against microbial infection (Bell and Smith, 1993). However, excessive accretion of such ROIs is reported to be highly toxic to the host cells. The harmful action of such ROIs generated in the host organisms are in turn counter balanced by powerful antioxidant defense strategy (Holmbland and Soderhall, 1999). In the present study, shrimp that received experimental diets containing *P. niruri* extract had significantly ($P < 0.05$) higher respiratory burst activity (superoxide anion activity) compared to control shrimp after WSSV challenge experiment. At the beginning of experiment, the respiratory burst (RB) noticed in the control group was 1.015 mmol; whereas it ranged between 1.068 and 1.110 mmol in shrimp fed on experimental diets. Further increase in experimental duration showed a remarkable increase in RB activity in experimental groups; whereas control group showed a decreasing trend. Supporting the results of the present study, Balasubramanian *et al*. (2008a) investigated the effect of *C. dactylon* extract on RB activity of *P. monodon* challenged with WSSV and inferred that, the shrimp infected with WSSV through intramuscular injection (*in vitro*) and oral administration (*in vivo*) showed a marked increase in RB activity up to 5 days of post injection and following recovery, it reached a normal level. According to the findings of Huynh *et al*. (2011) white shrimp, *L. vannamei* that had been immersed in 10 L of seawater containing varying concentrations (0, 100, 300 and 500 mg L^{-1}) of *S. hemiphyllum* var *chinese* powder and its extract separately for different duration (0, 1, 3 and 5 h) showed an increase in RB activity with respect to progressive increase in concentrations and thereby shrimp acquired enhanced immunity and resistance against WSSV.

Superoxide dismutase (SOD) is one of the main antioxidant defense enzymes generated in response to oxidative stress (Liu*et al*., 2006). In the present investigation, SOD activity of the shrimp that had experimental diets was significantly ($P< 0.05$) higher than the shrimp fed on control diet after WSSV challenge experiment. Here, the SOD activity of control group was found to be 36.35units/ml at the initial stage of the challenge experiment, but at the same time, experimental groups displayed the SOD activity of 49.44-55.76 unit/ml with increasing concentrations. When the duration of the challenge experiment prolonged to 10 days, control group showed decrease (11.24 unit/ml) in SOD activity as compared to that of experimental group which had enhanced SOD activity of 50.24 to 57.66 unit/ml, respectively. Finally, at the end of the challenge study, it was observed that the SOD activity was further increased. The above results are in consistence with the findings of Balasubramanian *et al*. (2008b), who reported that administration of diet with *C. dactylon* extract could significantly improve SOD activity in *P. mondon* after challenged with WSSV. They observed that, the control shrimp infected with WSSV had a gradual reduction of SOD activity till the end of the challenge experiment; nevertheless, shrimp fed on diets incorporated with extract showed decrease of SOD activity at the beginning of challenge, followed by recovery, and reached a normal level at the end of challenge. Similarly, Chang *et al*. (2003) have also observed a similar increase in SOD activity in shrimp *P. monodon* that fed on diet containing various concentrations (0.2, 1 and 2%) of β-1,3 glucan (BG) than that of control shrimp after challenged with WSSV.

Phagocytosis is a common cellular defense reaction, which is generally recognized as the primitive way to eliminate microorganisms and other foreign particles. In fact, phagocytosis was performed by hemocytes, lymphoid organs and hepatopancreas (Ratcliffe *et al*., 1985; Van de Brak *et al*., 2002). Phagocytosis is widely used by researchers to investigate the health status of decapod crustaceans upon treatment with probiotics and immunostimulants. Previous studies have underlined that, animals with good phagocytic activity have better disease resistance (Rengpipat *et al*., 2000; Chiu *et al*., 2007). In this study, the phagocytic activity of shrimp fed on the diets incorporated with *P. niruri* extract was significantly ($P < 0.05$) higher when compared to shrimp received control diet after WSSV challenge experiment. During the initial stage of the experiment, the phagocytic activity of experimental groups was found to be 5.86 to 6.32%, but it was only 5.69% in control group. After 10 days of the challenge experiment, the phagocytic activity decreased in both control and experimental groups. At the end of 21^{st} day of challenge study, the experimental

groups showed a significant and rapid increase in phagocytic activity from 3.98 to 6.64% as against to that of low phagocytic activity registered by the control group. This result concurs with the findings of Chang *et al*. (2003) when they studied the dietary effect of β-1,3-gucan (BG) on phagocytic activity of *P. monodn* challenged with WSSV. They inferred that, the percentage of cells that were phagocytic as described by phagocytic index (PI) was much higher in shrimp fed on BG incorporated diets than BG free diet fed group. Coincidentally, Chotegeat *et al*. (2004) have also reported that, fucoidan of seaweed *S. polycystum* enhanced the phagocytic activity of *P. monodon* challenged with WSSV. They observed a significant raise (9.1%) in phagocytic activity in experimental groups than that of control group (3.72%). This study was also extended to quantify WSSV infection in both control and experimental groups at the end of challenge experiment by analyzing WSSV DNA copies through qRT-PCR. Results implied that, the WSSV infection in positive control group has 4.29 x 10^3 WSSV DNA copies within 22.74 threshold cycles (Ct FAM). On the other hand, the WSSV DNA copy number in experimental groups decreased (4415 to 91 numbers within 25.96 to 31.46 threshold cycles) with respect to increase in concentrations. The observed results were statistically proved and accordingly a strong linear correlation ($r^2 = 0.999$) was observed between WSSV DNA copies and threshold cycles (Ct FAM). Our results were quite consistent with the reported previous investigation, wherein dietary administration of polysachharide fucoidan derived from brown seaweed *S. wightii* to shrimp *P. monodon* at different concentrations (0.1-0.3%) has shown lesser WSSV DNA copy numbers (756-11 copy numbers within 27.23-36.26 threshold cycles) as compared to that of positive control group, which displayed 1.42 x 106 WSSV DNA copies within 16.96 threshold cycles (Ct FAM) (Immanuel *et al*., 2012b). Likewise, Zhu and Zhang (2011) have quantified the WSSV DNA in shrimp *Marsupenaeus japonicus* after treated with VP28-siRNA expressed in bacteria and challenged with WSSV. Results of their study indicated a significant ($P < 0.01$) reduction in WSSV DNA copies in shrimp treated with VP28-siRNA than that of positive control (WSSV only). A study by Balasubramanian *et al*. (2008a) depicted that administration of diet containing *C. dactylon* plant extract to shrimp *P. monodon* significantly enhanced disease resistance against WSSV. They performed qRT-PCR and western blot analysis of normal shrimp, positive control shrimp and shrimp that received diet containing plant extract (1 or 2%) after challenged with WSSV and WSSV VP28-specific transcript. Results of their study propagated that shrimp received diets containing 2% plant extract did not show occurrence of VP28-specific transcript in hemolymph as observed in normal shrimp (negative control). On the other hand, existence of VP28-specific transcript was noticed in almost all the tissues of shrimp that received diet with 1% plant extract as that of positive control group.

5. Conclusions

In conclusion, the present study suggested that dietary administration of methanolic extract of *P. niruri* could effectively stimulated nonspecific immune mechanisms and disease resistance of *P. monodon* against WSSV. However, further studies on purification and structural elucidation of active compound from methanolic extract of *P. niruri* may provide a new therapeutic avenue to protect shrimp from WSSV and for the effective management of aquaculture industry.

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