Arch Virol (2012) 157:1665–1675
DOI 10.1007/s00705-012-1346-3

ORIGINAL ARTICLE

Anti–white spot syndrome virus activity of *Ceriops tagal* aqueous extract in giant tiger shrimp *Penaeus monodon*

N. S. Sudheer · Rosamma Philip · I. S. Bright Singh

Received: 27 September 2011 / Accepted: 16 April 2012 / Published online: 29 May 2012

Abstract White spot syndrome virus (WSSV), the most contagious pathogen of cultured shrimp, 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 agents. With this objective, the antiviral activity in the aqueous extract of a mangrove plant *Ceriops tagal* in *Penaeus monodon* was evaluated. The *Ceriops tagal* aqueous extract (CTAE) was non-toxic to shrimps at 50 mg/ml when injected intramuscularly at a dosage of 10 μL/animal (0.5 mg/animal) and showed a protective effect against WSSV at 30 mg/ml when mixed with WSSV suspension at a 1:1 ratio. When the extract was administered along with the diet and the animals were challenged orally, there was a dose-dependent increase in survival, culminating in 100 % survival at a concentration of 500 mg/kg body weight/day. Neither hypertrophied nuclei nor the viral envelope protein VP28 could be demonstrated in surviving shrimps using histology and indirect immunofluorescence histochemistry (IIFH), respectively. To elucidate the mode of action, the temporal expression of WSSV genes and shrimp immune genes, including antimicrobial peptides, was attempted. None of the viral genes were found to be expressed in shrimps that were fed with the extract and challenged or in those that were administered CTAE-exposed WSSV. The overall results suggest that the aqueous extract from *C. tagal* can protect *P. monodon* from white spot syndrome virus infection.

N. S. Sudheer · I. S. Bright Singh (✉)
National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin 682016, Kerala, India
e-mail: isbsingh@gmail.com; bsingh@md3.vsnl.net.in

R. Philip
Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin 682016, Kerala, India

Introduction

White spot syndrome virus (WSSV), a member of the monotypic family *Nimaviridae*, genus *Whispovirus* [23], causes 100 % mortality in shrimps within 3-10 days of infection under farming conditions [15]. The virus has double-stranded DNA with an estimated size of 300 kb, with more than 180 open reading frames encoding 59 structural proteins [33]. It has a broad host range among decapods and causes systemic infection in organs of ectodermal and mesodermal origin [9]. The virus is responsible for huge economic loss in culture systems, and until now, no therapeutic or prophylactic measure has been effective and successful to contain the disease. Suggested prophylactic measures such as water-quality management [16], administration of immunostimulants [5, 32], application of subunit [24] and inactivated whole-virus vaccines [29], herbal-extract-coated feeds [4], etc., are still in the experimental stage.

Information on the use of aqueous plant extracts to control WSSV is scanty. Nevertheless, a few reports are available in which the use of a composite mixture of aqueous extracts from seven Indian medicinal plants [1] and an aqueous extract from the medicinal herb *Cynodon dactylon* [4] were investigated.

A preliminary phytochemical study revealed that *Ceriops tagal* aqueous extract (CTAE) contains alkaloids, flavonoids, polyphenolics, cardiac glycosides, saponins and sterols, and an HPLC fingerprint was also generated [30]. *C. tagal* belongs to the family Rhizophoraceae and has been extensively studied for its bioactive potential. It has

been used to treat hemorrhages and malignant ulcers [27], sores [17], and malaria, and its root is used as a substitute for quinine [8]. The plant is a rich source of tannins and triterpenoids [11]. So far, 23 diterpenes and 29 triterpenes have been reported from the stem, twigs, roots, leaves, hypocotyls and fruits of *C. tagal* and *C. decandra* [34].

In this context, an investigation was undertaken to evaluate the efficacy of CTAE for protecting *P. monodon* from WSSV, using histopathology, immunofluorescent histochemistry, and expression of selected WSSV and shrimp immune-related genes as tools.

Materials and methods

Plant material

Specimens of *Ceriops tagal* were collected from different localities in South India (9° 58' 1.20"N, 76° 15' 0.00"E), identified according to Naskar and Mandal [25], and coded, and the voucher specimens were deposited in the herbarium collection of the National Centre for Aquatic Animal Health, Cochin University of Science and Technology.

Preparation of the extract

Leaves were shade-dried, powdered, and used for the preparation of CTAE. Accordingly, 50 g mangrove plant leaf powder was soaked in a minimum quantity of double-distilled water and frozen at −20 °C. The sample was thawed, and the freeze-thaw cycle was repeated three times. Plant material was extracted into a 500 ml final volume in double-distilled water using a Warring blender at ambient temperature (28±1 °C). The extract was sieved through a fine-meshed (100 µm) cloth and centrifuged at 10,000 g for 20 minutes, and the supernatant was lyophilized and maintained at −20 °C until used. This preparation was examined for its antiviral activity and for its efficacy for protecting shrimp from WSSV infection upon oral administration and oral challenge (Indian Patent Application no. 2607DEL/2006).

Virus inoculum

The virus inoculum was prepared according to the method of Singh et al. [29]. In brief, a composite sample of gills and soft parts of the cephalothorax (500 mg) from freshly infected *Penaeus monodon* was macerated in 10 ml cold PBS (NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.2 g; double-distilled water, 100 ml) with glass wool to a homogenous slurry using a mortar and pestle in an ice bath. The slurry was centrifuged at 8200 g in a refrigerated centrifuge at 4 °C, and the supernatant was filter-sterilized using a 0.22-µm-pore-size PVDF membrane filter. The preparation was streaked on ZoBell's marine agar plates and incubated at 28±2 °C for 72 h to confirm the absence of bacterial contamination. The viability of WSSV in suspension was checked by injecting 10 µl into each of a batch of six apparently healthy shrimps and observing them over a period of 3 to 7 days to confirm their survival. The preparation was stored at −80 °C until used.

Determination of WSSV titer

The WSSV titer was determined as described by Ha et al. [13]. The viral stock, prepared from 500 mg freshly infected first-step-PCR-positive tissue in 10 ml PBS, was serially diluted from 1×10^{-1} to 1×10^{-6}. Apparently healthy shrimps (four animals in a tank in triplicate) were injected with 10 µl suspension from each dilution. A negative control was maintained by administering the same quantity of PBS. The animals were observed for mortality for 7 days. The highest dilution at which 100 % mortality of the test animals was observed was recorded, and accordingly, the penultimate dilution was selected for application in all assays.

Preparation of WSSV-infected tissue for oral challenge

Apparently healthy *P. monodon* shrimps (6-8 g) were challenged by injecting them intramuscularly with 10 µl of a 10^{-1} dilution of the virus suspension. The animals were monitored for development of clinical signs and mortality for 7 days. All dead or moribund animals were collected and subjected to PCR for detection of WSSV following the procedure of Lo et al. [21]. Amplification was carried out in a thermocycler (Eppendorf). The PCR products were analyzed on a 1 % w/v agarose gel using TAE buffer stained with ethidium bromide and visualized using a gel documentation system, Dolphin-Doc (Weal Tec, USA). Animals that were positive in the first step were segregated, and soft tissues from the cephalothorax were minced and stored at −80 °C in 1-g aliquots for oral challenge experiments.

Toxicity of CTAE in a shrimp animal model

The CTAE was prepared as described above. From the lyophilized material, suspensions of varying concentrations (5, 10, 20, 30, 40, 50, 60 mg/ml) were prepared in distilled water. From each of the preparations, aliquots of 10 µl were administered intramuscularly into the sixth abdominal segment of apparently healthy *P. monodon* (6-8 g). The control consisted of animals injected with 10 µl of distilled water. For each concentration of the extract, six animals were used in triplicate. They were monitored for 7 days

and subjected to a general health assessment, noting their coloration, feed intake, molting, antennal intactness and necrosis.

Strength of CTAE required for antiviral activity

Various concentrations of the extract (5, 10, 20, 30, 40, and 50 mg/ml) were prepared and mixed with a 10^{-1} dilution of the virus suspension at 1:1 ratio and incubated at 28 °C for 3 hours. As a negative control, PBS alone, and as a positive control, the virus suspension mixed with PBS at 1:1 ratio, was incubated for the same duration and at the same temperature. After incubation, 10-µl aliquots of each of these preparations were injected intramuscularly (sixth abdominal segment) into a batch of six apparently healthy *P. monodon* shrimps (6-8 gm size) in triplicate. The animals were monitored for clinical signs of white spot disease and mortality.

Quantitative determination of the amount of CTAE that protecting shrimps from WSSV infection

To determine the quantity of CTAE required to protect shrimps from WSSV infection, the animals were fed different concentrations of the extract for a period of 7 days prior to challenge. Accordingly, shrimp feed (Higashimaru, India (P) Ltd.) was coated with the extracts @ 1, 0.5, and 0.25 %, which on consumption delivered 500, 250, and 125 mg/kg body weight/day, respectively. The positive and negative controls received feed without the extract. On the eighth day, the test and positive-control animals were challenged by feeding them freshly generated WSSV-positive tissue (prepared as described above) at 10 % of the body weight/animal and subsequently maintained on their respective diets. The animals were observed for clinical signs of the disease and mortality for 7 days.

Antiviral assay

The CTAE (50 mg/ml) was mixed with an equal volume of viral suspension and incubated for 3 hours at 28 °C. The controls included mixtures of WSSV and PBS (positive control) and PBS alone (negative control). From the preparations, aliquots of 10 µl each were administered intramuscularly to the animals (n=20) in the sixth abdominal segment and monitored for 7 days for clinical signs and mortality. The animal samples for gene expression analysis were collected (three animals each) from each group on second, fourth and sixth day post-challenge and preserved in TRIzol Reagent (Sigma) for RNA extraction. For histopathology and indirect immunofluorescent histochemistry, the animals were sacrificed on completion of the experiment and preserved in Davidson's fixative and neutral buffered formalin respectively; moribund animals were also preserved in the same manner.

Oral administration of CTAE and challenge with WSSV

The CTAE was mixed with a minimum quantity of distilled water and coated onto shrimp feed at a ratio of 1 % w/w (dry weight) in order to make the extract available to the shrimp at 500 mg/kg body weight/ day. The feed pellets were further coated with 4 % aqueous gelatin as binder, dried under vacuum, and fed to apparently healthy shrimps (20 animals per tank) of 6-8 g for 7 days. On the eighth day the shrimps were challenged orally with freshly generated WSSV-infected first-step-PCR-positive shrimp tissue at 10 % of the body weight/animal. Controls included shrimps fed on placebo and challenged (positive control) or maintained unchallenged (negative control). Samples were collected from all groups of animals, three each, on the second, fourth and sixth day of challenge and preserved in TRIzol (Sigma) for RNA extraction. On completion of the experiment, surviving animals were sacrificed and fixed for histopathology and indirect immunofluorescent histochemistry (IIFH) in Davidson's fixative and neutral buffered formalin, respectively; the moribund animals were also preserved in a similar way.

RT-PCR of WSSV genes and immune-related genes in *Penaeus monodom*

A pooled sample of shrimp gill tissue (50 mg) from three animals representing each group was used for RNA extraction. In brief, gill (100 mg) tissue was macerated in 1000 µl TRIzol. The sample was kept for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. An aliquot of 0.2 ml chloroform was added to 1000 µl TRIzol, shaken vigorously for 15 seconds, allowed to stand for 15 minutes at room temperature, and centrifuged at 12,000 g for 15 minutes. From the three layers formed, the colorless aqueous phase was separated carefully transferred to a fresh tube. An aliquot of 0.5 ml isopropanol was added, and the sample was kept for 10 minutes at RT and centrifuged at 12,000 g for 10 minutes at 4 °C; RNA was found precipitated on the sides and bottom of the tube. The supernatant was discarded, and the pellet was washed twice in 75 % ethanol. The pelleted RNA was air-dried, dissolved in 20 µl DEPC-treated water by repeated pipeting, and incubated at 55 °C for 10 minutes. RNA sample was subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs). An aliquot of 0.2 units of enzyme was added to 1 µg RNA, and the sample was incubated at 37 °C for 10 minutes and inactivated at 75 °C for 10 minutes. The concentration and

quantity of RNA was measured at 260/280 nm in an UV-visible spectrophotometer. Five micrograms of RNA was used for cDNA synthesis in a 20-µl reaction mix containing M-MuLV reverse transcriptase (200 U), RNase inhibitor (8 U), oligo $(dT)_{12}$ primer (40 pmole), dNTP mix (1 mM), RTase buffer (1 X) and $MgCl_2$ (2 mM) at 42 °C for 1 hour (New England Biolabs). Subsequently, eight WSSV genes and eight immune-related genes were amplified by PCR, using 1 µl cDNA with the specific primer sets shown in Table 1. The shrimp β-actin gene was amplified as a reference. Twenty-five microliters of PCR reaction mix contained 0.5 U of Taq DNA polymerase, 200 µM dNTP mix, 10 pmol each of forward and reverse primers, and 1X PCR buffer. The hot start PCR programme used for WSSV genes was 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing for 30s, and extension at 68 °C for 30 s, followed by a final extension at 68 °C for 10 min. Annealing temperatures were 50 °C for endonuclease (*endnu*); 53 °C for latency gene 1 (*latency*) and ribonucleotide reductase (*rr*1); 54 °C for DNA polymerase

Table 1 Primers used for the expression of WSSV-specific and shrimp immune-related genes

Sl. no.	Gene	Primer sequence (5'–3')	Size (bp) of the PCR product	Reference
WSSV-specific genes				
1.	Immediate early gene (*ie*1)	F-GACTCTACAAATCTCTTTGCCA	502 bp	[20]
		R-CTACCTTTGCACCAATTGCTAG		
2	Protein kinase (*pk*1)	F- TGGAGGGTGGGGACCAACGGACAAAAC	512 bp	
		R-CAAATTGACAGTAGAGAATTTTGCAC		
3	Thymidine kinase and thymidylate kinase (*tk-tmk*)	F-GAGCAGCCATACGGGTAAAC	412 bp	
		R-GCGAGCGTCTACCTTAATCC		
4	Ribonucleotide reductase (*rr*1)	F-ATCTGCTAGTCCCTGCACAC	408 bp	
		R-AAAGAGGTGGTGAAGGCACG		
5	DNA polymerase (*dnapol*)	F-TGGGAAGAAAGATGCGAGAG	586 bp	
		R-CCCTCCGAACAACATCTCAG		
6	Endonuclease	F-TGACGAGGAGGATTGTAAAG	408 bp	
		R-TTATGGTTCTGTATTTGAGG		
7	Vp 28	F-CTGCTGTGATTGCTGTATTT	555 bp	
		R-CAGTGCCAGAGTAGGTGAC		
8	*Latency* 1	F-CTTGTGGGAAAAGGGTCCTC	647 bp	
		R-TCGTCAAGGCTTACGTGTCC		
Shrimp immune-related genes				
1	Prophenol oxidase (pro PO)	F -TGGCACTGGCACTTGATCTA	590	[14]
		R -GCGAAAGAACACAGGGTCTCT		
2	Astakine	F -GTCGCGCATTTAACAAGGAG	455	
		R -CCCTGTGGATTGAGCTCACT		
3	Peroxinectin	F -CGAAGCTTCTTGCAACTACCA	547	
		R -GCAGGCTGATTAAACTGGCTT		
4	Alpha 2 macroglobulin	F -ATGGCCAATCCCGAGAGGTACCTACTG	345	[18]
		R -TGTTGCTGCAGAAGTTTGTTATCCTCAT		
5	Haemocyanin	F-GTCGACGAACTTCACTGGGA	598	[14]
		R-GTTCAGTGTCATCAACGGCA		
6	Transglutaminase	F-TGGGYCTTCGGGCAGTT	627	
		R-CGAAGGGCACGTCGTAC		
7	Crustin	F-GCACAGCCGAGAGAAACACTATCAAGAT	430	
		R-GGCCTATCCCTCAGAACCCAGCACG		
8	Penaeidin-3	F-AGGATATCATCCAGTTCCTG	240	
		R-ACCTACATCCTTTCCACAAG		
9	β actin	F-CTTGTGGTTGACAATGGCTCCG	520 bp	[35]
		R-TGGTGAAGGAGTAGCCACGCTC		

(*dnapol*), immediate early gene1 (*ie*1), Vp 28, thymidine kinase, and thymidylate kinase (*tk-tmk*); and 55 °C for protein kinase 1 (*pk*1) and β actin. For immune-related and AMP genes, hot start PCR was also carried out with 94 °C for 2 min, followed by cycles of 94 °C for 2 min, annealing for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The annealing temperature and the number of cycles employed for PCR of astakine and peroxinectin were 56 °C and 30 cycles; for prophenoloxidase (Pro PO) and transglutaminase, 56 °C and 35 cycles; for crustin and penaeidin, 55 °C and 35 cycles; for alpha-2-macroglobulin, 65 °C and 30 cycles; and for haemocyanin, 56 °C and 35 cycles. Aliquots of 10 µl of each of the PCR products were analyzed by 1 % agarose gel electrophoresis, stained with ethidium bromide and visualized using a gel documentation system, Dolphin-Doc (WealTec, USA).

Histopathology

Tissues were fixed overnight in Davidson's fixative, transferred to 70 % ethanol, and maintained for 24 hours. Paraffin sections were cut following standard procedures, stained with haematoxylin and eosin, and observed under a light microscope.

Indirect immunofluorescence histochemistry (IIFH)

Gill tissues fixed in neutral buffered formalin were processed according to the standard protocol. Sections were de-waxed, rehydrated and incubated for 1 hour with 3 % BSA in PBS in a humidified chamber to block free sites. They were then washed three times (3 minutes each) in a PBS–Tween-20 mixture (0.01 %) and incubated with WSSV-specific monoclonal (C 38) antibody [2] for 1 hour. After washing the slides three times (3 minutes each) in a PBS–Tween-20 mixture, anti-mouse IgG FITC conjugate (Sigma), diluted 1:40 in PBS containing BSA, was added and incubated for 1 hour in the dark in a humidified chamber. The slides were washed in PBS–Tween-20, stained with nuclear stain DAPI (10 µl, 0.02 µg/ml), and incubated for 3 minutes. They were rinsed with distilled water, air-dried, mounted with mounting medium (Vectashield, USA), and observed in a fluorescence microscope (Olympus, Germany). DAPI and FITC were viewed under filters with excitation wavelengths of 360-370 nm and 470-490 nm, respectively. The images were processed and merged using the Image-Pro Express software (Media Cybernetics Inc, MD, USA)

Statistical analysis

The results are reported as mean ± standard deviation for independent experiments. The data obtained were analyzed statistically by single-factor or two-factor ANOVA wherever applicable, and the differences were considered significant at $p \leq 0.05$.

Results

Determination of the titer of the WSSV stock

There was significant difference ($P<0.001$) in the mortality of shrimp that had received varying dilutions of the virus. Among the dilutions, 1×10^{-1} and 1×10^{-2} resulted in the death of all animals within 7 days post-injection ($P<0.001$). However, from the dilution 1×10^{-6} upwards, no mortality could be registered, and the animals behaved like the PBS-injected control group ($P<0.001$) (Fig. 1).

Determination of *in vivo* toxicity of *Ceriops tagal* aqueous extract (CTAE)

On administering CTAE up to 50 mg/ml, the response of the animals was more or less the same, without any significant mortality ($P<0.05$). However, at 60 mg/ml, only 77.7 % of the animals survived ($P<0.05$) during the experimental period of 7 days (Fig. 2).

Strength of the CTAE required for antiviral activity

Administration of WSSV (10 µl) to shrimp at a dilution of 1×10^{-1} subsequent to mixing with different concentrations of the CTAE (30, 40 and, 50 mg/ml) at equal proportions (v/v) and incubating at 28 °C for 3 hours, did not result in mortality. Meanwhile, shrimps that received the virus that was premixed with CTAE (5, 10, and 20 mg/ml) and incubated for the same period had lower survival rates: 77.8, 83.3 and 94.4 %, respectively ($P<0.001$). All positive

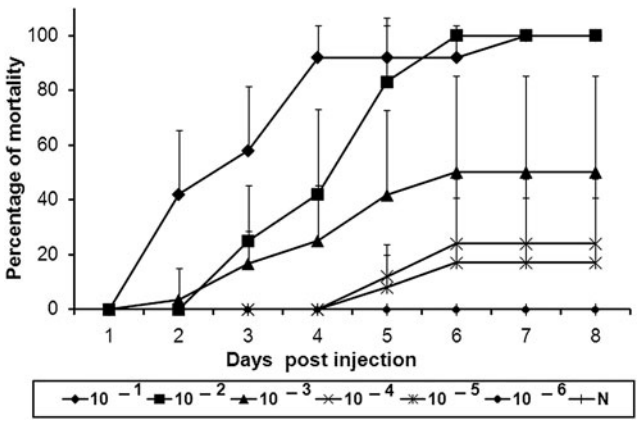

Fig. 1 Relationship between WSSV titer and mortality in WSSV-injected shrimp

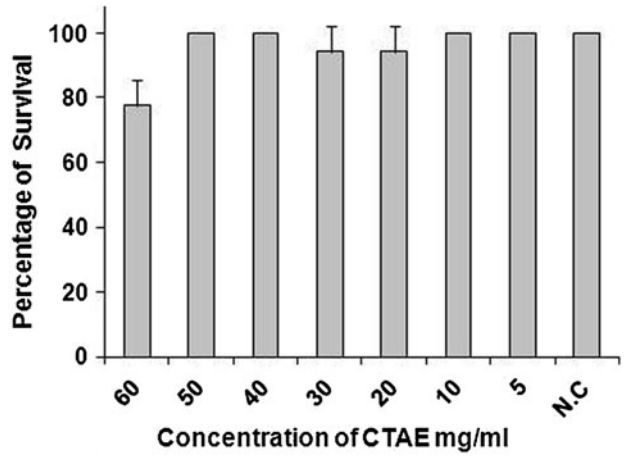

Fig. 2 Toxicity of CTAE in *P. monodon*. The bars indicate the percentage of animals that survived at the concentration tested. N.C, negative control

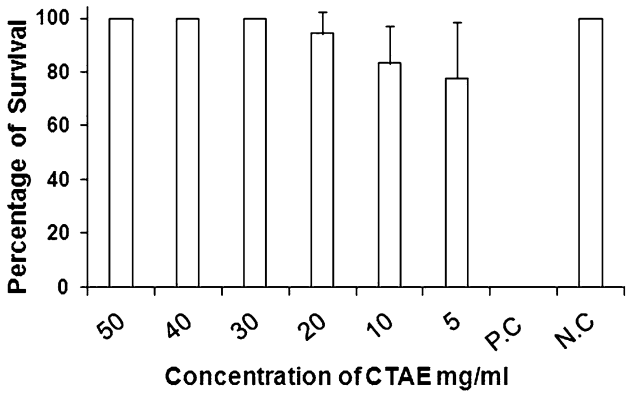

Fig. 3 Percentage of shrimp surviving within 7 days post-injection-challenge with WSSV exposed to different concentrations of CTAE in equal proportions. N.C, negative control; P.C, positive control

control animals died, and all negative control animals survived (Fig. 3).

Quantitative determination of the amount of CTAE required to protect shrimp from WSSV infection

The shrimps were fed different concentrations of the extract coated feed (500, 250 and 125 mg/kg of body weight/day) for 7 days. During this period, none the animals in any of the tanks died or showed visible abnormalities. From these data, we concluded that the aqueous extract was not toxic to the animals. The shrimps that were fed CTAE at a dosage of 500 mg/kg/body weight/day survived the oral challenge with WSSV-infected meat. Meanwhile, the survival rate of the animals that received CTAE at 250 and 125 mg/kg body weight/day was only 33.3 and 44.4 %, respectively. The difference in survival between the different batches was statistically significant ($P<0.001$). All of the positive control animals died, and all negative control animals survived (Fig. 4).

Histopathology

Neither the animals that received CTAE exposed to WSSV nor the ones that fed on CTAE and were challenged orally showed any pathological change (Fig. 5a and b). The positive control group of animals exhibited dislodgment of the cuticle on the gill filaments, with subcuticular cells having eosinophilic hypertrophied nuclei, cellular degeneration and shrinkage, multifocal necrosis, and haemocytic infiltration. The necrotic pilaster cells showed eosinophilic hypertrophied nuclei, which are characteristic of WSSV infection.

Indirect immunofluorescence histochemistry (IIFH) of gill tissue

Neither the animals that were administered CTAE-exposed WSSV nor the ones fed on CTAE and challenged orally with WSSV exhibited a positive signal with FITC-conjugated VP28 antibody (Fig. 6a and b). The positive control animal tissue sections gave strong positive signals. The infected cells showed characteristic enlarged nuclei with positive signals, and the normal nuclei were stained with DAPI.

Gene expression

Expression of viral genes, such as immediate early gene 1 (*ie*1), DNA polymerase (*dnapol*), thymidine–thymidylate kinase (*tk-tmk*), endonuclease (*endnu*), ribonucleotide reductase (*rr*1), protein kinase (*pk*1), latency-related gene (*latency* 1), and VP28, were examined on second, fourth and sixth day of challenge with WSSV. This was conducted in both groups of animals, i.e., those that received CTAE-exposed WSSV and the others that were challenged with the virus after oral administration of CTAE. Viral

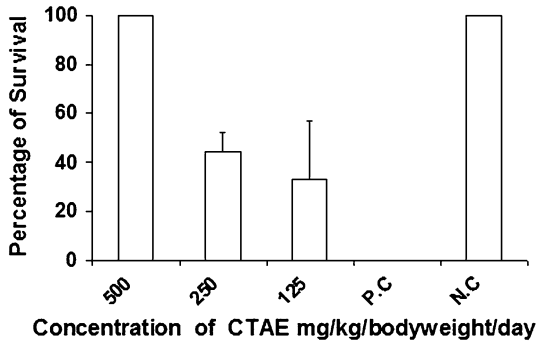

Fig. 4 Percentage of shrimps surviving for 7 days after being fed different quantities of CTAE and orally challenged with WSSV. N.C, negative control; P.C, positive control

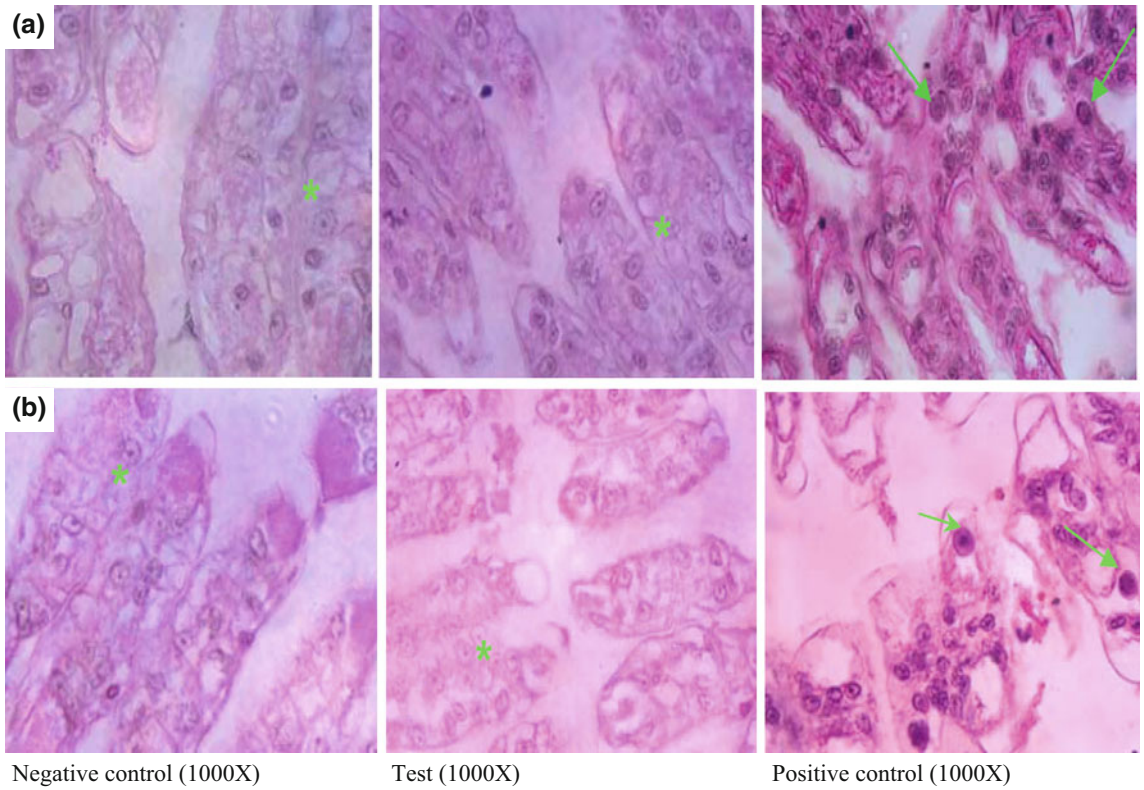

Fig. 5 Histological sections of *P.monodon* gill tissues. **(a)** Oral challenge with WSSV subsequent to oral administration of CTAE. **(b)** Intramuscular administration of WSSV exposed to CTAE. Arrow =nuclear hypertrophy, *= normal cell

transcripts could not be detected in either of the groups, whereas in the positive control, they were present, and there was a progressive increase of these transcripts after challenge, indicating viral multiplication (Fig. 7a). In general, there were variations in the expression levels of a few immune genes, including AMPs, between the groups, but these did not follow any definite pattern (Fig. 7b). Compared to the positive and negative controls, an enhancement in the transcripts of immune genes was not observed in the shrimp fed on CTAE and challenged with WSSV during the period of 6 days of the experiment

Discussion

Over the years, there have been reports on the antiviral activities of aqueous extracts from terrestrial plants, a few of which investigated their effects on WSSV [3, 4, 6, 7, 31, 33]. A combination of herbal extracts and probiotics has been found to decrease the prevalence of WSSV in *Litopenaeus vannamei* [12]. In this context, an investigation was undertaken to evaluate the efficacy of CTAE for protecting *P. monodon* from WSSV infection. In this process, as the first step, the titer of the WSSV stock used for the experiment was standardized. Accordingly, 500 mg of infected tissue (PCR first step positive) macerated in 10 ml PBS and diluted to 10^{-2} was administered at a dosage of 10 µl per animal of 6-8 g size to provide sufficient virus titres to cause mortality. However, in all experimental trials, a dilution of 10^{-1} was used to assure infection. This approach is similar to that of Ha et al., who conducted experiments to determine the neutralizing effect of a WSSV vaccine [13].

The highest non-toxic concentration of CTAE was 50 mg/ml, administered as an aliquot of 10 µl, delivering 500 µg per animal of the above size range. Meanwhile, the lowest concentration of the extract required to exhibit antiviral properties against WSSV in *Penaeus monodon* was determined to be 30 mg/ml, with a selectivity index of 1.7, which is marginally safe enough to be useful. The study demonstrated that the antiviral property was concentration dependent. A dose-dependent antiviral effect against WSSV has been reported in the case of the antimicrobial peptide mytilin, and the C10 c fragment of the peptide when injected after incubating with WSSV [28]. All of the shrimps survived when injected with 50 µM or 100 µM mytilin or C10c as in the present situation. This could be compared to the findings of Balasubramanian et al., who attributed the anti-WSSV property to an aqueous extract of *Cynodon dactylon* (100 mg/kg body weight)

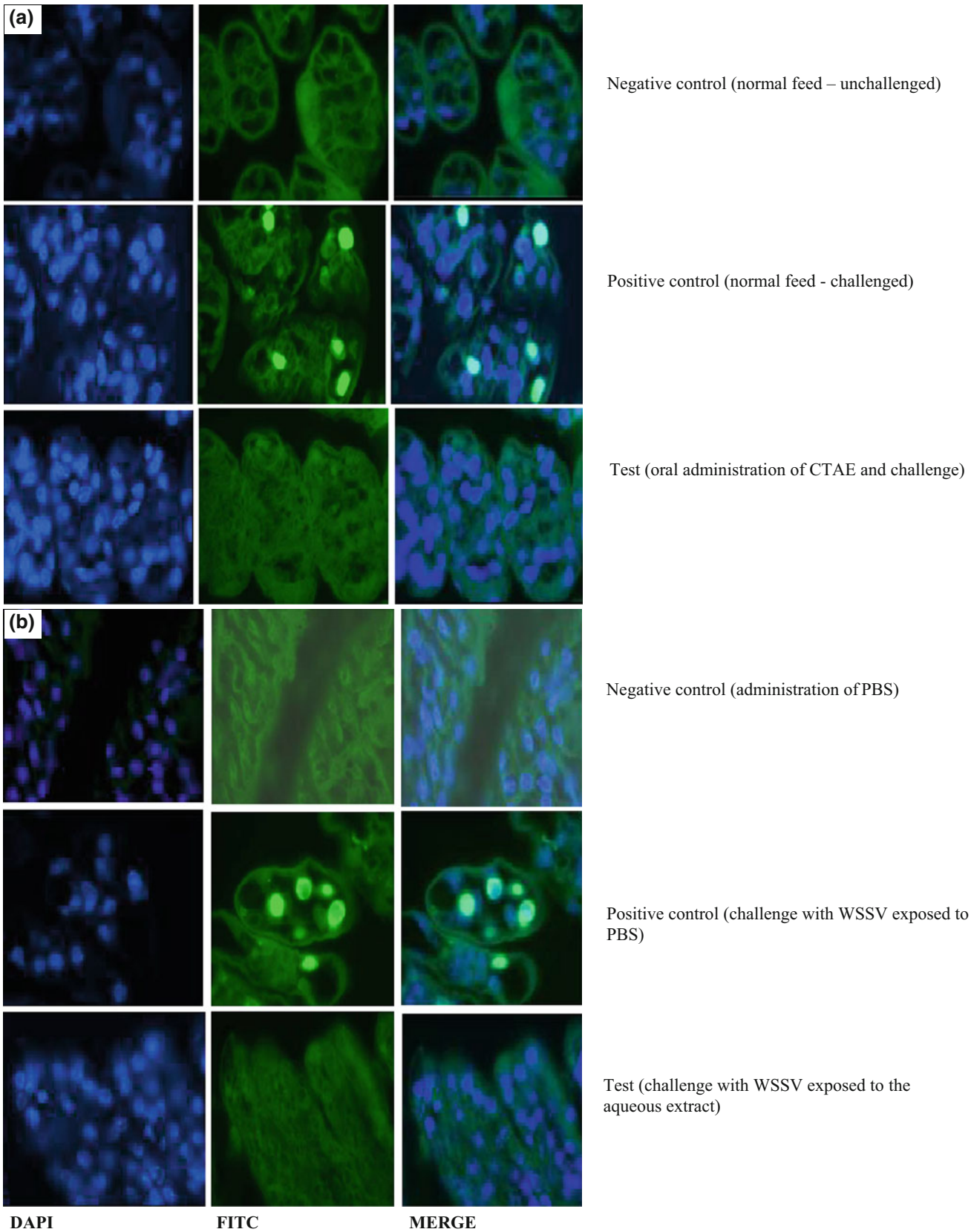

Fig. 6 Indirect immunofluorescence histochemistry images of *P. monodon* gill tissues, indicating a protective effect against WSSV, (**a**) Oral administration of CTAE and WSSV oral challenge, (**b**) Injection administration of CTAE-exposed WSSV

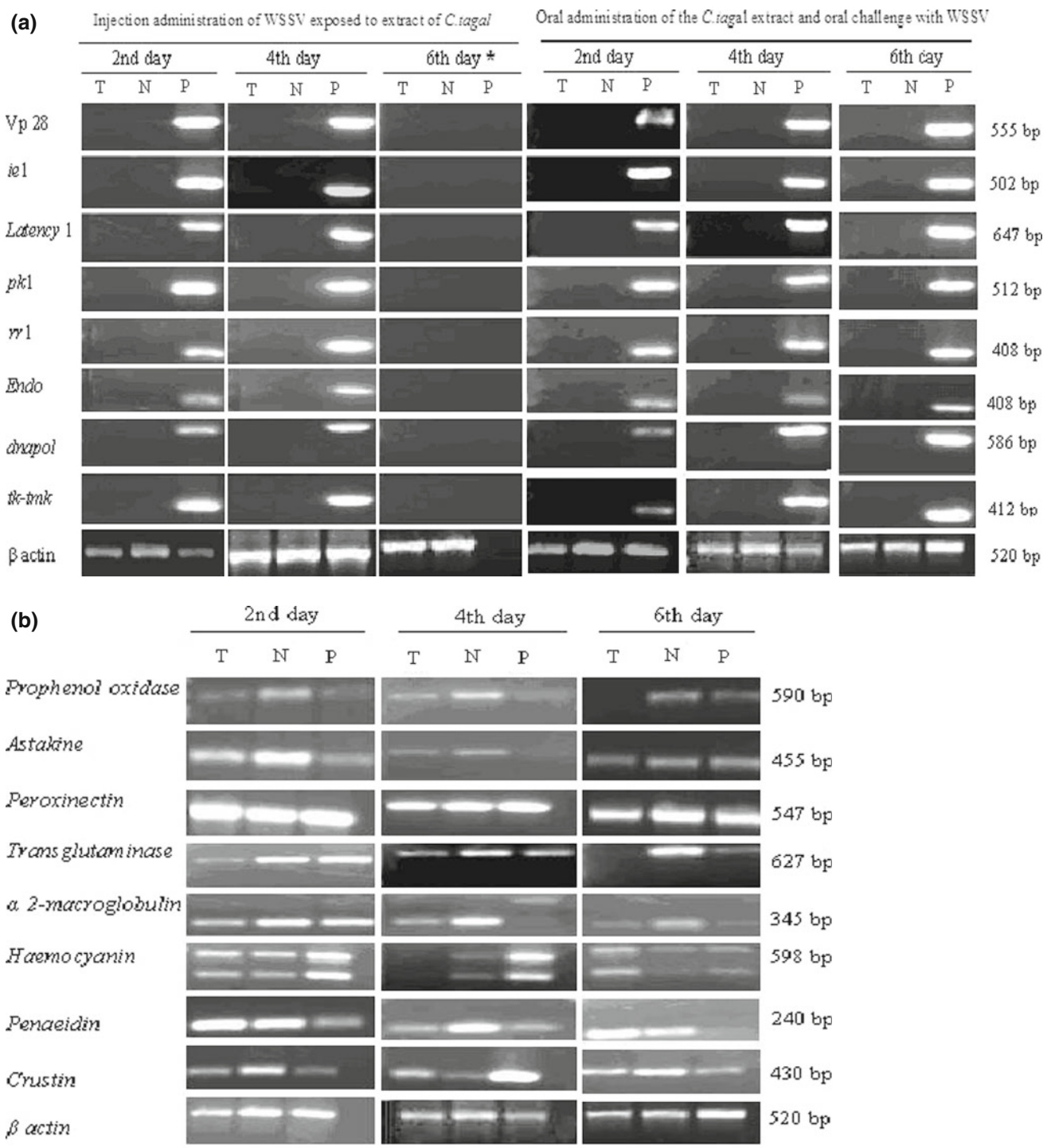

Fig. 7 Temporal expression of WSSV and immune genes. **(a)** WSSV gene expression in *Penaeus monodon* subsequent to injection administration of WSSV exposed to CTAE, and oral administration of CTAE and oral challenge with WSSV, (**b**) Immune genes in *P. monodon* in response to oral administration of CTAE and oral challenge with WSSV; T, test; N, negative control; P, positive control. * In the injection experiment, all animals in the positive control group died on the fourth day

and a methanolic extract of *Momordica charantia* (150 mg/kg body weight). However, the animals that survived the challenge were PCR positive for WSSV [3].

Evaluation of the effectiveness of the orally administered CTAE revealed that the test animals showed no sign of white spot virus disease on challenging with WSSV-infected tissue. This could be compared to the administration of peptidoglycan from *Cladosiphon okamuranus* [33], fucoidan [6], and *Dunaliella* extract [31]. However, their percent survival was comparatively lower. In a similar

study, Balasubramanian et al. reported 100 % survival of *P. monodon* when fed *Cynodon daclyone* at a concentration of 2 % of the feed [4].

In the present study, neither hypertrophied nuclei nor the envelope protein VP28 could be detected by histopathology and IIFH, respectively, in the surviving animals despite the fact that they were challenged. Meanwhile, the tissues from the positive control animals exhibited hypertrophied nuclei, cellular degeneration, shrinkage of the tissue, multi-focal necrosis, and haemocytic infiltration, as described earlier [22]. All positive animal tissues displayed positive signals for WSSV during IIFH. This indicated that the shrimps fed with the aqueous extract were protected from infection. Rahman et al. carried out IIFH to assess the clinical effects of cidofovir and diet supplemented with *Spirulina platensis* against WSSV. In their study, the surviving shrimps as well as the dead ones were positive for WSSV, and they reported that cidofovir and *Spirulina platensis*–supplemented diet could only delay death to 120 hours postinfection [26]. On this basis, it could be concluded that CTAE was much more effective in protecting shrimps from WSSV than any other natural preparation hitherto reported.

To evaluate the efficacy of CTAE for protecting *P. monodon* from WSSV infection, expression of viral genes in addition to a few well-documented immune genes and antimicrobial peptides was investigated. This study indicated that the viral transcripts involved in viral replication were not expressed in the test animals that were administered the extract, and they were also not expressed in the negative controls. This was alike in both of the experimental designs. The striking observation was that immediate early gene 1 (*ie*1) failed to be expressed in this group of animals. The expression of viral immediate early gene occurs independently of any viral *de novo* protein synthesis as the primary response to viral invasion [10]. Once expressed, the *ie* gene products may then function as regulatory trans-acting factors and may serve to initiate viral replication events during infection. Recently, it was found that white spot syndrome virus (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 [19]. In the cascade of viral regulatory events, successive stages of virus replication are dependent on the proper expression of the genes in the preceding stage. In the present study, none of these genes, starting from immediate early genes to VP 28 (late gene) were found to be expressed. This might be due to inactivation of the virus by the virucidal fractions in the extract or prevention of attachment, binding or entry of the virus. The results of different types of assays, including as survival, viral and immune gene expression, histopathology and immunohistochemistry all indicate that shrimps were protected from disease, either because they were protected from infection or because they were protected from early dissemination of the infection in the presence of the extract.

Conclusion

The antiviral activity of the aqueous extract of *Ceriops tagal* could be confirmed, and the dosage for conferring protection was standardized. We found that the extract was not toxic to shrimps at the concentrations that were effective for antiviral activity. The efficacy of the extract for protecting shrimps from WSSV infection could be evaluated through temporal gene expression pertaining to WSSV-specific genes and immune genes, including AMPs. Neither hypertrophied nuclei nor the envelope protein VP 28 could be demonstrated by histopathology and IIFH.

Acknowledgments This work was carried out with financial assistance from the Department of Biotechnology under the projects BT/PR3509/AAQ/03/173/2000 and BT/PR7419/AAQ/03/274/2006. We acknowledge the Department of Science and Technology, Lakshadweep, for the partial supply of mangrove plants, and Mr. Krishna Iyer, Scientist, CIFT, Cochin (Rtd) for statistical analysis. We thankfully acknowledge Dr. A. Mohandas, Emeritus Professor, NCAAH, for editing the manuscript. The first author thanks DBT for a fellowship.

References

1. Achuthankutty CT, Desai UM (2004) Treatment of white spot syndrome virus (WSSV) in penaeid shrimp aquaculture using plant extract. In: Proceedings of MBR 2004 national seminar on New Frontiers in marine bioscience research, India, pp 63–67
2. Anil TM, Shankar KM, Mohan CV (2002) Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. Dis Aquat Org 51:67–75
3. Balasubramanian G, Sarathi M, Kumar S, Hameed A (2007) Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. Aquaculture 263:15–19
4. Balasubramanian G, Sarathi M, Venkatesan C, Thomas J, Hameed ASS (2008) Oral administration of antiviral plant extract of *Cynodon dactylon* on a large scale production against white spot syndrome virus (WSSV) in *Penaeus monodon*. Aquaculture 279:2–5
5. Chang C, Su M, Chen H, Liao I (2003) Dietary β-1, 3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish Shellfish Immunol 15:297–310
6. Chotigeat W, Tongsupa S, Supamataya K, Phongdara A (2004) Effect of fucoidan on disease resistance of black tiger shrimp. Aquaculture 233:23–30
7. Citarasu T, Sivaram V, Immanuel G, Rout N, Murugan V (2006) Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* with reference to haematological, biochemical and immunological changes. Fish Shellfish Immunol 21:372–384

8. Duke J, Wain K (1981) Medicinal plants of the world. Computer index with more than 85,000 entries. 3 vols Medicinal plants of the world. - cited in Duke J. A (1983): Handbook of Energy Crops. Unpublished. [http://www.hort.purdue.edu/newcrop/Indices/index_ab.html]
9. Escobedo-Bonilla CM, Alday-Sanz V, Wille M, Sorgeloos P, Pensaert MB, Nauwynck HJ (2008) A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J Fish Dis 31:1–18
10. Friesen P (1997) Regulation of Baculovirus early gene expression. In: Miller LK (ed) The Baculoviruses. Plenum Press, New York, pp 141–170
11. Ghosh A, Misra S, Dutta A, Choudhury A (1985) Pentacyclic triterpenoids and sterols from seven species of Mangrove. Phytochem 24:1725–1727
12. Gomez VP, GonzaŁ lez AL, Co¤rdova AC, Meyer ML, Coronado JAF, Ruiz AŁP (2009) Probiotic microorganisms and antiviral plants reduce mortality and prevalence of WSSV in shrimp (*Litopenaeus vannamei*) cultured under laboratory conditions. Aquac Res 40:1481–1489
13. Ha YM, Soo JG, Thi-Hoai N, Chae HR, Ki HK, Yoon-Kwon N, Sung-Koo K (2008) Vaccination of shrimp (*Penaeus chinensis*) against white spot syndromevirus (WSSV). J Microbiol Biotechnol 18:964–967
14. Jiravanichpaisal P, Puanglarp N, Petkon S, Donnuea S, Söderhäll I, Söderhäll K (2007) Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol 23:815–824
15. Lightner D (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge, p 304
16. Lightner D (2005) Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. J World Aquac Soc 36:229–247
17. Lin P, Fu Q (1995) Environmental ecology and economic utilization of mangroves in China. Higher Education Press, Beijing, pp 1–95
18. Lin Y, Vaseeharan B, Chen J (2008) Molecular cloning and phylogenetic analysis on alpha2-macroglobulin (alpha2-M) of white shrimp *Litopenaeus vannamei*. Dev Comp Immunol 32: 317–329
19. Liu H, Soderhall K, Jiravanichpaisal P (2009) Antiviral immunity in crustaceans. Fish Shellfish Immunol 27:79–88
20. Liu WJ, Chang YS, Wang CH, Kou G, Lo CF (2005) Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cycloheximide-treated shrimp. Virol 334:327–341
21. Lo C, Leu J, Ho C, Chen C, Peng S, Chen Y, Chou C, Yeh P, Huang C, Chou H, Wang C, Kou G (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25:133–141
22. Manjusha M, Varghese R, Philip R, Mohandas A, Bright Singh IS (2009) Pathological changes in *Fenneropenaeus indicus* experimentally infected with white spot virus and virus morphogenesis. J Invertebr Pathol 103:225–232
23. Mayo M (2002) A summary of taxonomic changes recently approved by ICTV. Arch Virol 147:1655–1656
24. Namikoshi A, Wu JL, Yamashita T, Nishizawa T, Nishioka T, Arimoto M, Muroga K (2004) Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquaculture 229:25–35
25. Naskar K, Mandal R (1999) Ecology and bio diversity of Indian mangroves. Daya Books 2:397–400
26. Rahman MM, Escobedo-Bonilla CM, Wille M, Alday Sanz V, Audoorn L, Neyts J, Pensaert MB, Sorgeloos P, Nauwynck HJ (2006) Clinical effect of cidofovir and a diet supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles. Aquaculture 255:600–605
27. Rastogi RP, Mehrotra BN (1991) Compendium of Indian medicinal plants. Central drug research institute and publications and information directorate, New Delhi, p 515
28. Roch P, Yang Y, Toubiana M, Aumelas A (2008) NMR structure of musselmytilin, and antiviral-antibacterial activities of derived synthetic peptides. Dev Comp Immunol 32:227–238
29. Singh ISB, Manjusha M, Pai SS, Philip R (2005) *Fenneropenaeus indicus* is protected from white spot disease by oral administration of inactivated white spot syndrome virus. Dis Aquat Org 66:265–270
30. Sudheer NS, Philip Rosamma, Bright Singh IS (2011) *In vivo* screening of mangrove plants for anti WSSV activity in *Penaeus monodon*, and evaluation of *Ceriops tagal* as a potential source of antiviral molecules. Aquaculture 311:36–41
31. Supamattaya K, Kiriratnikom S, Boonyaratpalin M, Borowitzka L (2005) Effect of a Dunaliella extract on growth performance, health condition, immune response and disease resistance in black tiger shrimp (*Penaeus monodon*). Aquaculture 248:207–216
32. Takahasi Y, Uchara K, Watanabe R, Okumura T, Yamashita T, Omura H, Yomo T, Kawano T, Kanemitsu A et al (1998) Efficacy of oral administration of fucoidan, a sulphated polysaccharide in controlling white spot syndrome in kuruma shrimp in Japan. In: Flegel TW (ed) Advances in shrimp biotechnology. National center for genetic engineering and biotechnology, Bangkok, pp 171–173
33. Tan Y, Shi Z (2008) Proteomic analyses of the shrimp white spot syndrome virus. Virol Sin 23:157–166
34. Wu JY, Xiao Q, Xu J, Li MY, Pan JY, Yang M (2008) Natural products from true mangrove flora: source, chemistry and bioactivities. Nat Prod Rep 25:955–981
35. Zhang J, Li F, Wang Z, Xiang J (2007) Cloning and recombinant expression of a crustin-like gene from Chinese shrimp, *Fenneropenaeus chinensis*. J Biotechnol 127:605–614