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Screening, isolation and optimization of anti-white spot syndrome virus drug derived from marine plants

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors have proved the efficacy of the marine derived anti-WSSV drug. The formulation of the drug is inexpensive and at the same time cost effective for the use of the marginal farmers.
Details on Page S115

ABSTRACT

Objective: To screen, isolate and optimize anti-white spot syndrome virus (WSSV) drug derived from various marine floral ecosystems and to evaluate the efficacy of the same in host-pathogen interaction model.

Methods: Thirty species of marine plants were subjected to Soxhlet extraction using water, ethanol, methanol and hexane as solvents. The 120 plant isolates thus obtained were screened for their *in vivo* anti-WSSV property in *Litopenaeus vannamei*. By means of chemical processes, the purified anti-WSSV plant isolate, MP07X was derived. The drug was optimized at various concentrations. Viral and immune genes were analysed using reverse transcriptase PCR to confirm the potency of the drug.

Results: Nine plant isolates exhibited significant survivability in host. The drug MP07X thus formulated showing 85% survivability in host. The surviving shrimps were nested PCR negative at the end of the 15 d experimentation. The lowest concentration of MP07X required intramuscularly for virucidal property was 10 mg/mL. The oral dosage of 1 000 mg/kg body weight/day survived at the rate of 85%. Neither VP28 nor *ie 1* was expressed in the test samples at 42nd hour and 84th hour post viral infection.

Conclusions: The drug MP07X derived from *Rhizophora mucronata* is a potent anti-WSSV drug.

KEYWORDS

Shrimps, *Litopenaeus vannamei*, Anti-WSSV, Marine plants, MP07X, White spot syndrome virus

1. Introduction

The shrimp culture industry is currently going through a period of severe crisis due to the outbreak and recurrence of a viral infection. For the last two decades, the discovery of white spot disease caused by white spot syndrome virus (WSSV) is regarded as the most virulent known disease, affecting the sustainability and growth of the global penaeid shrimp farming industry. WSSV is one of the most widely distributed virus, which ranks in the top three of the harmful shrimp viruses, causing immense losses in the shrimp population[1,2]. Due to its pathogenicity and epidemic nature, WSSV has been considered notifiable by the Office Internationale de Epizooties[3]. It is an enveloped non occluded DNA virus of the family Nimaviridae under the new genus *Whispovirus* and is also the most devastating shrimp pathogen ever isolated and studied[4]; it causes total mortality in a rearing stock within 3-7 d of infection in a culture system[5]. This virus continued to cause a direct loss of approximately US $ 10 billion to the native cultured

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shrimp industry worldwide since its first appearance in 1992[6]. WSSV has a wide host range as it has been observed to infect not only shrimps but also crabs and other arthropods such as copepods, insects and pest prawns[7]. The principle clinical sign of this virus infected shrimp will be white spots in the exoskeleton and epidermis[8]. Other behavioral abnormalities include a rapid reduction in food consumption, lethargy and reddening of appendages. Sardonically, till now, no effective treatment or prophylactic measure could be developed to manage the virus.

Strategies for the prophylaxis and control of WSSV theoretically include improvement of environmental conditions, stocking of specific pathogen free shrimp post larvae and enhancement of disease resistance by using immunostimulants. Several plants from both terrestrial and marine origin have already been tested against viral diseases to judge its immunostimulant efficacy. Aqueous extracts of *Cynodon dactylon* (*C. dactylon*) (terrestrial plant) and *Ceriops tagal* (*C. tagal*) (mangrove) exhibited protective effects against WSSV in *Penaeus monodon* (*P. monodon*) [9-11]. The aqueous extract of *Sargassum weighti* (seaweed) showed significant anti-WSSV property against marine shrimp, *Penaeus indicus* and freshwater crab, *Paratelphusa hydrodomous*[12]. The extract of *Phyllanthus amarus* and *Psidium gugajava* has shown antiviral activity against yellow head baculovirus in *P. monodon*[13]. The extract of *Clinacanthus nutans* has been tested against yellow head virus in shrimp and the results indicated an effective control of yellow head virus infection in shrimp[14]. Other control measures that have been undertaken against the WSSV virus in the culture systems are oral administration of peptidoglycan, lipopolysacharides, β-1,3-Glucan, vaccination with inactivated viral preparation and viral envelope protein, VP19 and VP28, feeding with fucoidan extracted from *Sargassm polycysticus* and antiviral drug supplemented with *Spirulina platensis*[15-22]. An aqueous preparation of a composite mixture of seven Indian medicinal plants (*Aegle marmelos, Allium sativum, Curcuma longa, C. dactylon, Lantana camara, Mimosa pudica, Ocimum sanctum*) has been developed and patented in 2002, with the conclusion that the preparation was effective in controlling WSSV at the rate of 15 mg/L[23].

Marine plants are used in folk and Ayurvedic (herbal) medicines in several parts of the world. Several reports have appeared in literature over a period of time stating the use of aqueous extracts of various plant species against enveloped, non-enveloped, DNA/RNA viruses and their mode of action against these pathogens. Marine algae have exhibited their potential as important sources of antiviral and other bioactive compounds[24]. Some of the Indian marine plants have shown antiviral activity against Newcastle disease virus, anti-vaccinia virus, Semliki forest virus, human immunodeficiency virus, hepatitis B virus, encephalomyocarditis virus and herpes simplex virus I[25-31]. The antiviral activity of a polysaccharide isolated from a mangrove plant *Rhizophora apiculata* against HIV in MT-4 cells was also demonstrated previously[32]. For several years, mangroves, seagrasses and seaweeds have been in focus, as they are a rich storehouse of phytomolecules with several biological activities. The uniqueness of these phytomolecules that are derived from these plants has prompted us to take up this present investigation, for which we have selected 30 plants exclusively from different marine ecosystems like mangroves, seagrass, seaweed, *etc.* The leaves from each of these plants were studied for their anti-WSSV property in the host, *Litopenaeus vannamei* (*L. vannamei*).

2. Materials and methods

2.1. In vivo anti-WSSV activity of marine plants

2.1.1. Selection of marine plants

Thirty marine plants selected for the present study were collected from different parts of the east coast of India, namely Sunderban, Digha, Konark, Vishakapatnam, Kakinada, Parangipettai, Pichavaram, Mandapam and Tuticorin. The plants such as *Avicennia marina, Avicennia officinalis, Bruguiera gymnorrhiza, Heritiera fomes, Lumnitzera racemosa, Nypa fruticans, Rhizophora mucronata* (*R. mucronata*), *Sonneratia caseolaris, Sonneratia griffithii, Citrullus colocynthis, Derris scandens, Ipomoea pes-caprae, Sesuvium portulacastrum, Suaeda monoica, Tamarix troupii, Halodule pinifolia, Porteresia coarctata, Caulerpa racemosa, Chaetomorpha crassa, Enteromorpha intestinalis, Ulva fasciata, Ulva lactuca, Padina tetrastromatica, Sargassum prismaticum, Sargassum wightii, Turbinaria conoides, Amphiroa fragilissima, Catenella repens, Gelidium pusillum* and *Gracilaria corticata* were selected for preliminary screening against WSSV based on the criteria such as, random collection of plants followed by mass screening; selection based on ethnomedical and ethnopharmacological uses in the management of diseases; follow-up of existing literature leads; chemotaxonomic approaches and easy availability of the plants for its evaluation[33,34]. The plants belong to diverse categories of marine ecosystem such as mangroves, mangrove associates, seagrass and seaweed. All the marine plants were personally identified by Dr. Kumudranjan Naskar, National Fellow, ICAR, Govt. of India. The voucher specimens were preserved in the herbarium and deposited in our laboratory.

2.1.2. Preparation of plants isolates

Fresh leaves of these above enlisted plants were collected and washed thoroughly with tap water to remove the dust particles followed by sterile distilled water. The washed plant parts were also surface sterilized with calcium hypochlorite before they were kept aside for shade-drying for not more than 3-4 d depending upon the nature of the plant. The completely dried up leaves were ground, powdered and kept in air tight polythene bags until used for the extraction process. For the extraction process, 200 g of plant powder were extracted using 800 mL of different solvents like water, ethanol, methanol and hexane subsequently (*i.e.* in the decreasing order of polarity) as the nature of the solvent used in the Soxhlet extraction method affecting the nature of the crude extract[33]. The liquid extracts thus obtained were filtered through Whatman No.1 filter paper and evaporated

to dryness in a vacuum evaporator at 40 °C and 25–30 mmHg. Thus, the respective crude plant isolates obtained from the above procedure were coded, *viz.* MP01A, MP01B, MP01C, MP01D; likewise, MP, A, B, C & D represents marine plant, water, ethanol, methanol and hexane respectively. A total of 120 plant isolates thus prepared were tested for anti-WSSV activity.

2.1.3. Collection and maintenance of experimental animals

L. vannamei (4–5 g) were collected from grow-out ponds (near Marakkanam, Pondicherry) and maintained in 1 000 litre fibreglass tanks containing natural seawater with airlift biological filters at room temperature (27–30 °C) and salinity (20 to 21 ppt). During the experimental trials, in each of the 30 liter capacity plastic tank, 5 shrimps (4–5 g) were maintained in sea water (20 ppt). Proper aeration was provided and 10% water exchange was done regularly in the tanks. The shrimps were fed with artificial pellet feed (CP feed, Thailand). The water quality parameters such as temperature (28±2) °C and pH (7.9±0.1) were recorded and the salinity was measured with a refractometer (Erma Inc, Tokyo).

2.1.4. Preparation of viral inoculum

WSSV infected *L. vannamei* with prominent white spots were collected from shrimp farms located in Cuddalore district, Tamil Nadu. Gills and soft parts of the cephalothorax region (500 mg) from these infected shrimps were macerated in 10 mL cold NTE buffer (0.2 mol/L NaCl, 0.02 mol/L Tris-HCl and 0.02 mol/L EDTA, pH 7.4) with glass wool to a homogenous slurry using mortar and pestle in ice bath. The slurry was centrifuged at 3 000 r/min for 20 min in a refrigerated centrifuge at 4 °C. The supernatant was recentrifuged at 8 000 r/min for 30 min at 4 °C and the final supernatant fluid was filtered through a 0.4 μmol/L filter. The preparation was streaked on Zobell's Thiosulfate Citrate Bile Salts – Sucrose and potato dextrose agar plates and were incubated at (28±2) °C for 72 h to confirm the absence of microbial contamination. The viability of WSSV in the prepared inoculum was tested by injecting 10 μL to a batch of apparently healthy shrimps (4 nos) whose mortality occurred over a period of 3 to 5 d and the viral infection was confirmed by polymerase chain reaction (PCR) results. The viral inoculum was stored at – 20 °C until further use.

2.1.5. Virucidal activity of different plant isolates and their molecular diagnosis in animal model

Different plant isolates prepared were tested against WSSV in *L. vannamei*. The inactivation of WSSV was confirmed by bioassay and PCR analysis. For bioassay, three tanks [positive control (POS), negative control (NEG) and test (TS)] were separately maintained for each of the 120 plant isolates. Each crude plant isolate was dissolved in NTE buffer, termed as plant isolate-buffer solution, at the concentration of 10 mg/mL (500 mg/kg body weight of shrimp). During the experimental trials, shrimps (TS) (five animals in each tank) were injected intramuscularly with a mixture of viral suspension and the above prepared plant isolate at the volume of 25 μL per animal (5 μL of viral suspension, 20 μL of plant isolate – buffer solution). The POS was injected with a mixture of 20 μL NTE buffer and 5 μL viral suspensions whereas the NEG was injected with 25 μL NTE buffer only. All these mixtures were incubated at 29 °C for 3 h before the experimentation. The experimental trial was carried up to 15 d after post infection with WSSV. During this period, mortalities were recorded for each day along with the constant monitoring of the viral infection using WSSV-Shrimple test kit.

Gill tissues from the experimental shrimps (POS, NEG, TS) were dissected out and homogenized with 500 μL of guanidine hydrochloride buffer (10 mmol/L Tris-HCl, pH 8.0, 0.1 mol/L EDTA, pH 8.0, 6 mol/L guanidine hydrochloride and 0.1 mol/L sodium acetate) in a glass homogenizer. The homogenate was allowed to react for 30 min after thorough mixing with buffer. The mixture was centrifuged at 5 000 r/min for 5 min and the supernatant fluid was collected in a fresh microcentrifuge tube. To this supernatant fluid, an equal volume of ice-cold ethanol was added and mixed thoroughly. The mixture was subjected to centrifugation at 14 000 r/min for 20 min. The pellet obtained was washed once with 200 μL of 95% ethanol followed by one more wash with 200 μL of 70% ethanol. The DNA pellet was dried in a vacuum drier and dissolved in 50 μL sterile distilled water. This DNA was used as the template for the PCR. The presence of WSSV was confirmed by PCR using the primer designed by earlier researchers to amplify the 211 bp sequence of WSSV-DNA[35]. The sequences of the primer are Forward – 5′ GAA ACT ATT GAA AAG GCT TTC CCT 3′ and Reverse – 5′ GTT CCT TAT TTA CTA CTA CGG CAA 3′.

The PCR kit contents were 2 μL of template DNA, 1 μmol/L of each primer, 200 μmol/L of deoxynucleotide triphosphate and 1.25 IU of Taq DNA polymerase in PCR buffers supplied with a commercially available kit. The mixture was incubated for 35 cycles in an automatic thermal cycler programmed for 0.5 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, following by extension for 5 min at 72 °C after the last cycle. After the PCR run, the amplified products were analyzed by electrophoresis in 1.2% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.

2.2. Confirmation of anti-WSSV activity of the final plant isolate in animal model

Fresh leaves of *R. mucronata* were collected, shade dried and coarsely powdered. About 1 kg of leaf powder was extracted by water and ethanol in succession using a Soxhlet apparatus[36]. The yield of water extract (MP07A-brownish red in colour) and the ethanolic extract (MP07B-deep green in colour) was 24.8% and 19.2% respectively, which were further condensed in a rotary evaporator. Both the syrupy extracts were mixed together, diluted in 200 mL 100% ethanol in a glass bottle and shaken vigorously. A copious quantity of gel like precipitate was formed immediately after the addition of ethanol. The supernatant was recovered by filtration and the gel pellet was discarded. The extracts thus obtained (MP07X-deep greenish red in colour) were filtered through Whatman (No. 1 filter paper) and were evaporated to dryness in a vacuum evaporator at 40 °C and 25–30 mmHg. The yield

of MP07X was 38.2% from powdered leaves. The dried plant isolate/crude drug (MP07X) was stored in the refrigerator and was used in all the analysis and experiments hereafter.

The crude drug (MP07X) was tested by injecting the host intramuscularly against WSSV to confirm its efficacy as a potent anti-WSSV drug. The same methodology was followed as described in section 2.1.5. above, and the nested PCR results are illustrated. The experimentation was carried on for 15 d and the survival percentage was also recorded. After 15 d, DNA was extracted from the surviving shrimp to check its viral load and the same was injected to fresh sets of shrimps. All the samples were subjected to nested PCR, using WSSV nested PCR kit (Bangalore Genei, India).

2.3. Optimization of MP07X as an anti-WSSV drug in animal model

2.3.1. Determination of the viral titer of WSSV using L. vannamei as the animal model

The viral stock prepared from 500 mg freshly infected first step PCR positive tissue in 10 mL NTE buffer was diluted from 1×10^{-1} to 1×10^{-6}. Apparently, healthy shrimps (four animals in a tank in triplicate) were injected with 10 μL suspensions from all the dilutions having the NEG maintained by administering with the same quantity of NTE buffer. The animals were observed for mortality every day for 7 d. The highest dilution at which 100% mortality of the test animals was obtained and selected for all the further bioassays. The percentage of mortality obtained with different dilutions of virus on different days after the intramuscular challenge was statistically analyzed by ANOVA, and the differences were considered significant at $P\leqslant0.05$.

2.3.2. Toxicity of the plant isolate in animal model

The lyophilized plant isolate (MP07X) was used to prepare the strength solution for toxicity studies in *L. vannamei* (6-8 g) as the animal model. The stocks having strength of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/mL were prepared in NTE buffer. From each of the preparations, aliquots of 10 μL were administered intramuscularly at the 6th abdominal segment of apparently healthy *L. vannamei*. The control consisted of animals injected with 10 μL of distilled water. For every concentration of the extract, six animals each were used in triplicates and they were monitored for 7 d and subjected for general health assessment such as characteristic colouration, feed intake, moulting, antenal intactness and necrosis. The percentage of survivability obtained with different dilutions of the extract was statistically analyzed by a single factor ANOVA. The differences were considered significant at $P\leqslant0.05$.

2.3.3. Strength of the plant isolate (MP07X) required for virucidal activity

Various concentrations of the plant isolate (MP07X) (35, 30, 25, 20, 15, 10 and 5 mg/mL) were prepared and mixed with 1×10^{-1} dilution of the viral suspension at a ratio of 1:1 and incubated at 28 °C for 3 h. As a NEG was only NTE buffer and the POS was the viral suspension mixed with NTE buffer at a ratio of 1:1, they were incubated under the same conditions as given to the test. After the incubation, 25 μL aliquots (each of the above preparations) were injected intramuscularly to a batch of 9 apparently healthy *L. vannamei* (6-8 g) in triplicates into their 6th abdominal segment *i.e.* the animals were injected with a quantity of 1 750 mg, 1 500 mg, 1 250 mg, 1 000 mg, 750 mg, 500 mg, and 250 mg of MP07X/kg body weight/day respectively. The animals were monitored for clinical signs of white spot disease and mortality. The percentage of survivability obtained in the administration of different concentrations of the extract was statistically analyzed by a single factor ANOVA and the differences were considered significant at $P\leqslant0.05$.

2.3.4. Preparation of WSSV infected tissue for oral challenge

Apparently, healthy *L. vannamei* (6-8 g) were challenged by injecting them intramuscularly with 10 μL of a 1×10^{-1} dilution of the viral suspension. The animals were monitored for the development of clinical signs and mortality for 7 d. All dead or moribund animals were collected and subjected to PCR for the detection of WSSV following the procedure described earlier. 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 tested positively were segregated, and its soft tissues from the cephalothorax were minced and stored at − 80 °C in 1 g aliquots for oral challenge experiments.

2.3.5. Quantitative determination of the plant isolate (MP07X) for protection of shrimp from WSSV

To determine the quantity of the plant isolate (MP07X) required for protecting the shrimps against WSSV, the animals (TS) were fed with different concentrations of the same for a period of 7 d prior to the challenge. To accomplish this objective, artificial medicated shrimp feed was produced with MP07X at 3.0%, 2.5%, 2%, 1.5%, 1% and 0.5% of the feed *i.e.*, the shrimps were fed with a quantity of 1 500 mg, 1 250 mg, 1 000 mg, 750 mg, 500 mg, and 250 mg of MP07X/kg body weight/day respectively. The POS and NEG shrimps consumed feed without MP07X. On the 8th day, both the TS samples and POS shrimps were challenged by feeding them with freshly generated WSSV positive tissue at 0.25 mg/shrimp, and continued with the feed (in case of TS, medicated feed), and were observed for clinical signs of the disease and mortality for 7 d. The percentage of survivability obtained with different concentrations of the extract was statistically analyzed by a single factor ANOVA and the differences were considered significant at $P\leqslant0.05$.

2.4. Reverse transcription PCR analysis of various genes expressed during the host pathogen interaction

The expressions of the genes on the 42nd hour and 84th hour after the challenge with the virus were examined to find out whether the plant isolate (MP07X) was inhibiting the processes involved in the viral multiplication cycle during host pathogen interaction. Gill tissue from shrimps

representing each group (POS, NEG & TS) was taken for their RNA extraction. About 100 mg of gill tissue was macerated in 1 000 μL TRIzol Reagent. The sample was kept for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. A total of 0.2 mL of chloroform was added to 1 000 μL TRIzol Reagent and shaken vigorously for 15 seconds and then allowed to stand for 15 min at room temperature and centrifuged at 12 000 r/min for 15 min. Colorless aqueous phase was separated carefully from the three layers formed to a fresh tube. About 0.5 mL isopropanol was added, kept for 10 min at room temperature and centrifuged at 12 000 r/min for 10 min at 4 °C. RNA was found to precipitate on the sides and bottom of the tube after centrifugation. The supernatant was discarded, and the pellet was washed twice in 75% ethanol. The RNA pellet was air dried and dissolved in 20 μL diethyl pyrocarbonate treated water by repeated pipetting at 55 °C for a while. The RNA was then subjected to DNase treatment with RNase-free DNase 1. About 0.2 units of enzyme were added to 1 μg of RNA and incubated at 37 °C for 10 min. The enzyme was inactivated at 75 °C for 10 min. The concentration and quantity of RNA were measured at 260/280 nm using UV-Visible spectrophotometer. A total of 5 μg RNA was subjected to cDNA synthesis with 20 μL reaction mix containing M-MULV reverse transcriptase (200 IU), RNase inhibitor (8 IU), Oligo (dT) 12 primer (40 pmol), dNTP mix (1 mmol/L), RTase buffer (1×) and $MgCl_2$ (2 mmol/L) at 42 °C for 1 h. Then 2 WSSV genes immediate early gene1 (*ie 1*) and VP28 were amplified by PCR. A total of 1 μL cDNA reaction products were subjected to PCR amplification with the primer set VP28-F and VP28-R (VP28-F; 5′ - CTG CTG TGA TTG CTG TAT TT - 3′ and VP28-R; 5′ - CAG TGC CAG AGT AGG TGA C - 3′) for the VP28 gene and *ie 1*-F and *ie 1*-R (*ie 1* 1-F; 5′ - GAC TCT ACA AAT CTC TTT GCC A - 3′ and *ie 1*-R; 5′ - CTA CCT TTG CAC CAA TTG CTA G - 3′) for the immediate early gene1 (*ie 1*). Shrimp β actin gene was also amplified (Forward - 5′ - CTT GTG GTT GAC AAT GGC TCC G - 3′ and Reverse 5′ - TGG TGA AGG AGT AGC CAC GCT C - 3′) for RNA quality and amplification efficiency as a reference. About 25 μL PCR reaction mix contained 0.5 IU of Taq DNA polymerase, 200 μmol/L dNTP mix, 10 pmol each of forward and reverse primers and 1× 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 seconds, annealing for 30 seconds (54 °C for immediate early gene1 (*ie 1*), VP28, and 55 °C β actin), and extension at 68 °C for 30 seconds followed by final extension at 68 °C for 10 min. Each of the PCR products (10 μL) was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented.

3. Results

3.1. In vivo anti-WSSV activity of marine plants

3.1.1. Viral inoculums for anti-WSSV assay

The *L. vannamei* with prominent white spots collected from shrimp farms was WSSV positive. The WSSV inoculum obtained from these viral infected shrimps were injected to the fresh shrimps and the molecular diagnosis (PCR results) is presented in Figure 1.

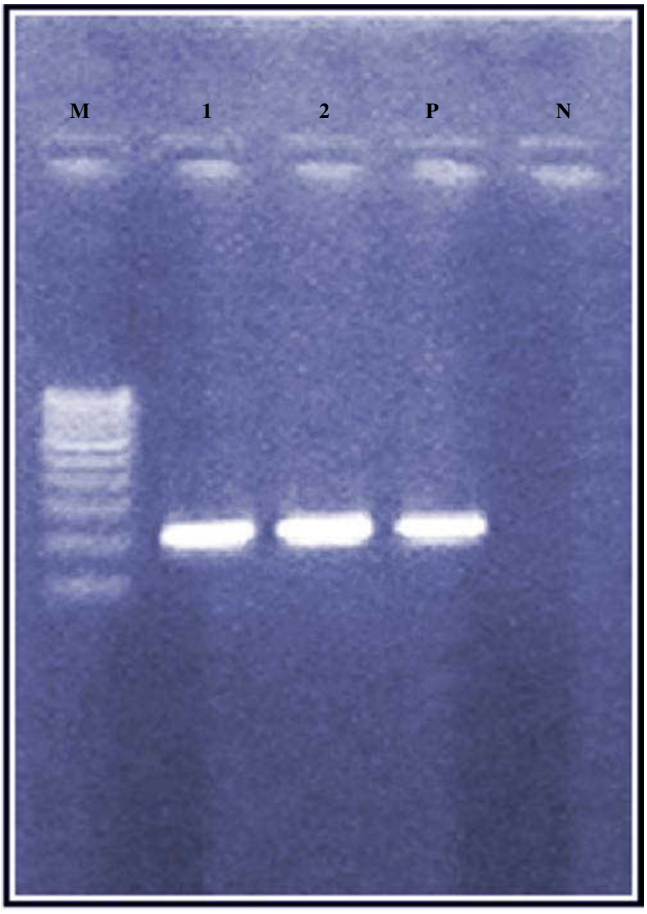

Figure 1. Detection of WSSV gene in the PCR product.
M=marker, 1=WSSV infected shrimp from the farm, 2=fresh WSSV infected shrimp produced in the laboratory, P=positive control (211 bp), N=negative control.

3.1.2. Virucidal activity of different plant isolates and their molecular diagnosis in the animal model

To check the virucidal activity of 120 plant isolates, the isolates were mixed with WSSV inoculum and challenged after incubation for 3 h at 29 °C. When shrimps were challenged with WSSV mixed with the plant isolates such as MP02C (methanolic extract of *Avicennia officinalis*), MP07A (water extract of *R. mucronata*), MP07B (ethanolic extract of *R. mucronata*), MP08A (water extract of *Sonneratia caseolaris*), MP09B (ethanolic extract of *Sonneratia griffithii*), MP11B (ethanolic extract of *Derris scandens*), MP25B (ethanolic extract of *Sargassum wightii*), MP28C (methanolic extract of *Catenella repens*) and MP30A (water extract of *Gracilaria corticata*); then a moderately higher survival percentage was obtained (Figure 2). Meanwhile, the remaining plant isolates did not produce significant survival percentage. The NEG survived till the end of the experimentation while 100% mortality was observed in the POS within the 3rd to 4th day. This abundantly available mangrove plant *R. mucronata*

has derived both MP07A and MP07B isolates that have shown significant survivability in the shrimps. Hence, further experimentation was carried out to get a better survival percentage in the shrimps by obtaining a potent anti-WSSV drug from the above two isolates.

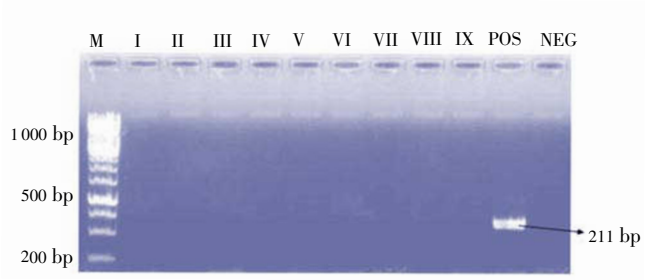

Figure 2. Detection of the virucidal property of the best nine plant isolates.
M: Marker; I: MP02C; II: MP07A; III: MP07B; IV: MP08A; V: MP09B; VI: MP11B; VII: MP25B; VIII: MP28C; IX: MP30A; POS: Positive; NEG: Negative.

3.2. Confirmation of anti-WSSV activity of the final plant isolate (MP07X) in an animal model

The activity of the plant isolate/crude drug (MP07X) was examined against WSSV in *L. vannamei* to confirm its efficacy as a potent anti-WSSV drug. On completion of the experiment, after 15 d the shrimps were nested PCR negative, and when the DNA extracted for virus from these shrimps were injected into a fresh batch of shrimps none of them showed any clinical signs of WSSV infection and remained negative to nested PCR (Figure 3). The survivability was 85% at the end of the 15th day of the experimentation (Figure 4).

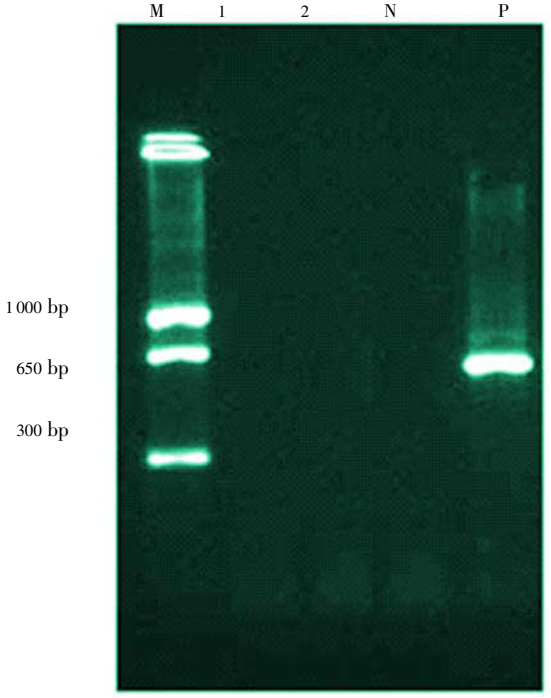

Figure 3. PCR diagnosis of MP07X in shrimps.
M=marker, 1=WSSV negative (MP07X intramuscular injection), 2=WSSV negative (lane 1 DNA injected to fresh shrimps), N=negative control (NEG), P=positive control (POS).

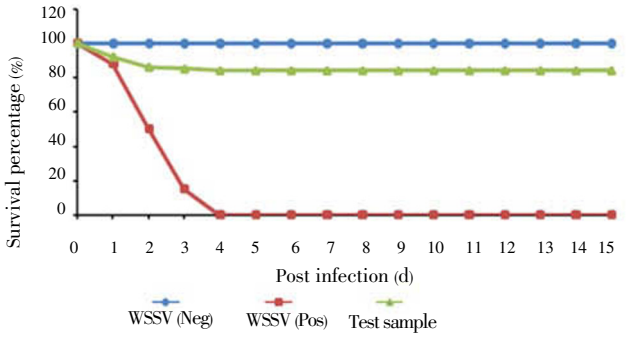

Figure 4. Survival percentage of *L. vannamei* when subjected to MP07X.

3.3. Optimization of MP07X as an anti-WSSV drug in animal model

3.3.1. Virulence of WSSV in animal model

To determine the virus titer required to cause mortality of shrimp within a period of 7 d on intramuscular administration, different dilutions of WSSV in NTE buffer were injected and observed for mortality (n=4). The difference in mortality occurred in shrimp which received varying dilutions of the virus and was highly significant (P<0.001). Among the dilutions, 1×10^{-1} and 1×10^{-2} resulted in mortality of all animals within 6 d (P<0.001), and from the dilution 1×10^{-6} onwards no mortality could be registered, and the animals behaved as NTE buffer injected shrimps (P<0.001) (Figure 5).

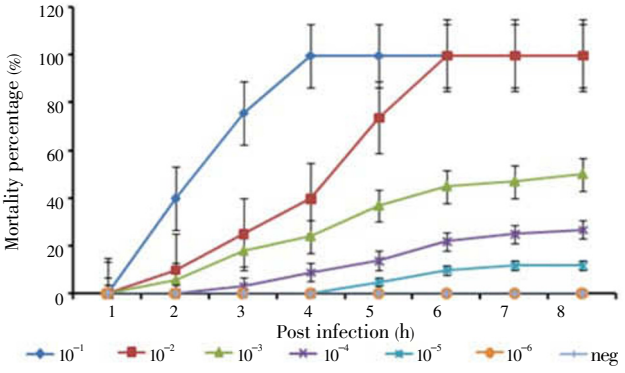

Figure 5. Percentage of mortality with different titers of virus intramuscularly injected.

3.3.2. Determination of in vivo toxicity of the plant isolate

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

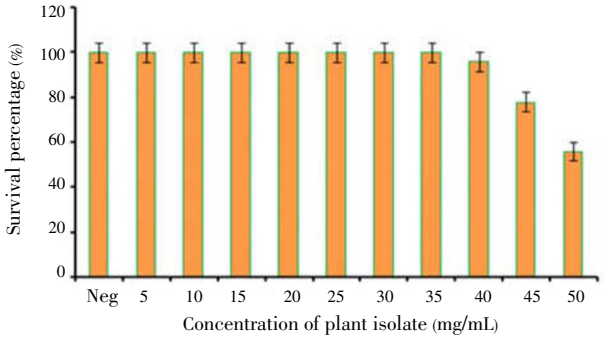

Figure 6. Toxicity of different concentration of plant isolate (MP07X) in *L. vannamei*.

3.3.3. Strength of the plant isolate required for virucidal activity

The experiments to determine the virucidal activity were carried out on *L. vannamei* (6–8 g) (n=9), by administering WSSV at a dilution of 1×10^{-1} after exposing the shrimps to different concentrations of MP07X at equal proportions for 3 h at 29 °C. There were significantly higher (P<0.001) survival rate of shrimps when administered with WSSV suspension exposed to higher concentrations of MP07X such as 10–35 mg/mL. Accordingly, the shrimps did not get infected with these above concentrations registering an overall survival rate of 85%. Meanwhile, the batches of shrimp administered with 5 mg/mL of MP07X resulted in a lesser survival rate of 64% respectively. All POS succumbed to the virus when administering with the virus exposed to NTE buffer after incubation for 3 h at 29 °C. All NEG survived the entire experimental period (Figure 7).

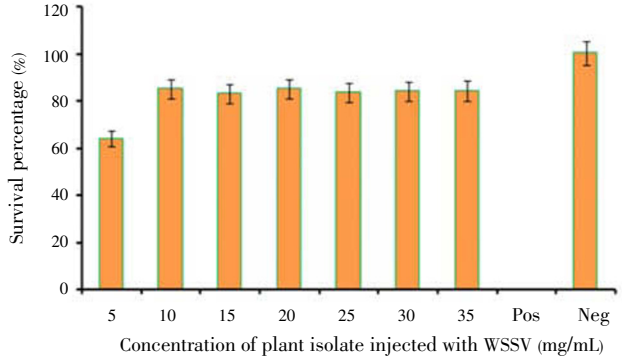

Figure 7. Virucidal property at different concentration of plant isolate MP07X.

3.3.4. Quantitative determination of plant isolate (MP07X) for protection of shrimp against WSSV

To examine the *in vivo* antiviral activity of the plant isolate, *L. vannamei* (6–8 g) (n=6) were fed with pellet feed at 1 500 mg/kg, 1 250 mg/kg, 1 000 mg/kg, 750 mg/kg, 500 mg/kg, and 250 mg/kg body weight/day for a period of 7 d. On challenging all the batches of shrimp by oral administration of freshly infected tissue, all animals that received MP07X at a dosage of 1 000 mg/kg body weight/day survived at 85%, and the differences in survivability between different batches were significant (P<0.001). Significantly, lower average survival rates of shrimps were observed in the batches that received 750, 500 and 250 mg/kg body weight/day as the average survival rate was only 65%, 57%, and 33%. The POS (shrimps supplemented with normal diet and challenged with WSSV) succumbed to WSSV registering total mortality. All NEG animals survived (Figure 8).

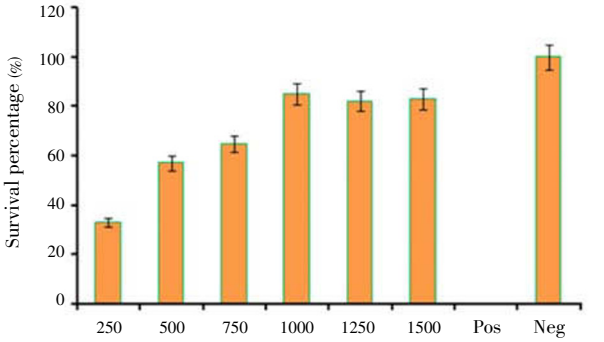

Figure 8. Efficacy of orally administered extract against WSSV.

3.4. Reverse transcription PCR analysis of various genes expressed during the host pathogen interaction

The expressions of the genes on the 42nd hour and 84th hour after the challenge with the virus were examined to find out whether the plant isolate (MP07X) was inhibiting the processes involved in the viral multiplication cycle during host pathogen interaction. The gene expression study was conducted in three groups (POS, NEG and TS) of animals. Viral genes were not amplified in the TS group of animals and appeared exactly like the NEG. In the case of POS, the viral genes such as immediate early gene (*ie 1*) and VP28 were found to be expressed at both 42nd hour and 84th hour after challenge with WSSV. It was observed that, as the time passed by, there was an increase in the intensity of bands of these genes suggesting more multiplication of the virus in the POS shrimps (Figure 9).

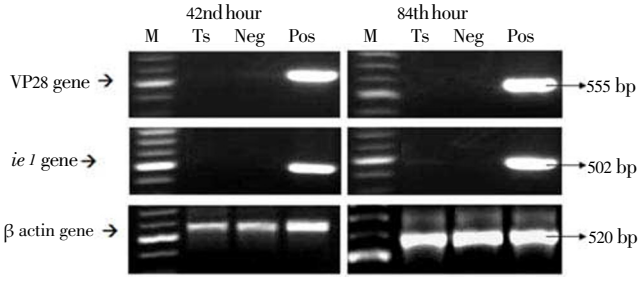

Figure 9. Reverse transcription PCR analysis of VP28, immediate early (*ie 1*) and β actin genes in host.

In the case of POS animals which received the virus intramuscularly, total mortality was observed at the 84th hour itself. Hence, animals were not available to assay beyond that timeline.

4. Discussion

Most of the products derived from marine organisms show many interesting activities. Their constituents are more novel than those of the terrestrial plants. Seaweeds have

long been recognized as rich and valuable natural resources of bioactive compounds because of their various biological properties[37]. The water soluble extracts of seaweeds have been shown to exhibit antiviral activity against a wide spectrum of viruses[38]. Extracts from mangrove plants and its associates have been used worldwide for medicinal purposes and also as a producer of around 349 metabolites, which turns out to be a rich source of steroids, diterpenes, triterpenes, saponins, flavanoids, alkaloids and tannins[39-42]. Marine algae have shown their potential as important sources of antiviral as well as other bioactive compounds[24].

In this present study, an attempt has been made to look into the possibilities of using marine plants as sources of anti-WSSV drugs. With this objective, 30 marine plants abundantly found in different marine ecosystems of east coast of India were subjected to Soxhlet extraction to procure a combination of phytomolecules, potent enough to be an anti-WSSV drug and at the same time applied along with diet as a prophylactic measure. In this study, MP02C, MP07A, MP07B, MP08A, MP09B, MP11B, MP25B, MP28C and MP30A were found to be effective against WSSV. Both the isolates MP07A and MP07B derived from the same plant that showed significant survivability, and the plant was abundantly available. Hence, further experimentation was carried out to get a better survival percentage in the shrimps by obtaining a potent anti-WSSV drug from the above two isolates. The final plant isolate MP07X proved to be the potent anti-WSSV drug in our research. As MP07X alone could give protection to all animals tested against WSSV, under the experimental conditions, this plant species (*R. mucronata*) was identified for further studies. Under the conditions of feeding, when the medicated feed administered with subsequent challenge, the viral DNA was not detected in the tissue which suggested that the virus had either not invaded the host tissue and multiplied or gotten eliminated subsequent to the infection.

Several attempts have been made earlier by several workers to detect anti-WSSV property in plants. Previously, the methanolic extracts of five medicinal plants such as *C. dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa* and *Eclipta alba* were mixed together in equal proportions, and the combined extracts were supplemented through the shrimp diet at different concentrations[43]. In administering the extract at 800 mg/kg body weight/day, 74% of *P. monodon* was obtained. In a similar study, the percentage survivability of shrimp fed on the ethanolic leaf extract of the terrestrial plant (*Pongamia pinnata*) was 40% on administering at 200 mg/kg of body weight and 80% on administering at 300 mg/kg body weight/day[44]. On feeding *P. monodon* with 2% aqueous extract of *C. dactylon*, coated feed could obtain 100% survival rate and at the same time the live animals were PCR negative[10]. There were reports of feeding *P. monodon* with diet containing extracts of herbs which have improved their immune system and also accorded protection against WSSV[43]. Approximately 100% survivability of *P. monodon* was obtained on feeding with 1% aqueous extract of *C. tagal* at 500 mg/kg of body weight/day[11]. Virucidal property of the aqueous extracts of *R. mucronata*, *Sonneratia* sp. and *C. tagal* when administered along with WSSV suspension in 1:1 ratio after incubation for 3 h at 25 °C suggested the presence of molecules in the preparation which could inactivate the virus. In a similar pattern, preincubation of WSSV with a synthetic antibacterial peptide from *Mytillus galloprovincialis* reduced mortality due to WSSV in Palaemonid shrimp *Palaemon* sp[45]. They suggested that this might be due to contact of virus with mytilin before injection into shrimp. PCR analysis showed that the surviving animals were not accommodating the viral DNA.

The plant that has been identified earlier as a potential source of antiviral drug belongs to the family Rhizophoraceae[32]. The present study also points out that the mangrove plant *R. mucronata* belonging to family Rhizophoraceae is a potential source of virus inactivating agents. Antiviral activities of aqueous extracts from plants are well established and this includes reports on the antiviral activity of plant extracts against WSSV, too[9-11,21,43,46-53]. A combination of herbal extracts and probiotics as medicated diet could decrease prevalence of WSSV in *L. vannamei*[54]. Even though reports are available on the protective effect of plant extracts against WSSV, information on their mode of action are scanty. In this study, antiviral property of MP07X was investigated employing molecular tools, evaluating their role in protecting shrimps from WSSV. The titer of WSSV stock used for the experiment was determined *in vivo* animal model so as to have adequate virus load to initiate an infection. Accordingly, the viral suspension was prepared by macerating 500 mg of infected tissue in 10 mL buffer diluting to 1×10^{-1} to 1×10^{-6}. In all the further experiments, a titer of 1×10^{-1} was used. Previous researchers also used 1×10^{-1} viral titer in their experiments[11]. Similar kind of experiments were conducted to determine the dilution of WSSV required to be used for neutralization by WSSV vaccine[55]. According to them, a dilution of 1×10^{-3} could cause 100% mortality within 7 d. In animal model, the highest non-toxic concentration went up to 35 mg/mL, from which 10 μL extract was injected to shrimps (6-8 g). Similarly, the highest non-toxic level of *C. tagal* in *P. monodon* is 50 mg/mL[11]. The average percentage of survivability of shrimps injected with the MP07X was 85% at a concentration of 10 mg/mL. Marginal mortality due to cannibalism subsequent to moulting may be considered. Different concentrations of cidofovir (an antiviral drug) were injected and observed that it was non-toxic to shrimps up to a concentration of 200 mg/kg of body weight and they could successfully use the same for further assays[22].

Accordingly, an experiment was conducted in the present study to determine the lowest concentration of the extract required to exhibit virucidal property against WSSV in *L. vannamei*. In this experiment, a range of concentrations of the extract between 5 mg/mL and 35 mg/mL were chosen, and the concentration of above 10 mg/mL was found to be effective in exhibiting its virucidal property. In these processes, the lowest concentration required for virucidal property was 10 mg/mL. 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 was the ratio of toxic concentration to the effective concentration, and showed

higher antiviral activity at a concentration below the toxic value. The results generated unambiguously suggest that the virucidal property of MP07X is concentration dependent. In a similar pattern on screening 20 Indian medicinal plants, anti-WSSV activity was exhibited by the aqueous extract of *C. dactylon* on administering 100 mg/kg of body weight when injected intramuscularly. Dosage dependent antiviral effects against WSSV have been reported in the case of antimicrobial peptide mytilin when injected after incubating with WSSV[45,56]. It was proposed that the antiviral activity of mytilin was mediated by its binding onto the viral envelope[45,56].

On evaluating effectiveness of orally administered MP07X through medicated feed in protecting shrimps against WSSV, none of the test animals exhibited signs of distress during the period of drug administration. Shrimps orally administered with MP07X at a dosage of 1 000 mg/kg of body weight exhibited 85% survival rate on challenging with WSSV infected tissue. In a comparable way, administration of peptidoglycan from *Cladosiphon okamuranus* at the rate of 100 mg/kg of shrimp body weight resulted in 76.2% survival rate and administration of fucoidan from *Sargassum polycystum* at 400 mg/kg of body weight resulted in 93% survival rate[21,51]. The ethyl acetate extract of *Calotropis procera* enhanced the survival rate of shrimps to 80% against WSSV challenge groups due to the antimicrobial factors in the extracts. Also it was backed by the evidence that no signal was obtained from molecular detections[57]. Shrimps fed with 300 mg of *Dunaliella* extract per kg of feed showed higher resistance to WSSV infection, besides becoming tolerant to stress[52].

To evaluate the efficacy of MP07X for protecting *L. vannamei* from WSSV infection, expression of immediate early gene (*ie 1*) and VP28 and β actin genes were investigated. This study indicated that the viral transcripts involved in viral replication were not expressed in the animals TS and were administered with the crude drug. This was alike for both the 42nd hour and 84th hour after 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 immediate early gene occurs independently of any viral *de novo* protein synthesis as the primary response to the viral invasion[58]. Once expressed, the *ie* gene products may then function as regulatory transacting factors and may 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[59]. 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, [immediate early gene (*ie 1*) and VP28] was found to be expressed. This might be due to inactivation of the virus by the virucidal activity of MP07X. The results of different types of assays, viral and immune gene expression and histopathology all indicated 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 crude drug.

The overall results suggest that the mode of action of MP07X against WSSV is virucidal. Consequently, the inactivated virus fails to multiply in the host and subsequently gets eliminated. Three possible modes of action of plant extracts against WSSV were proposed earlier[9]; (i) Viral inactivation due to interaction between the extract and the envelope protein, (ii) Influence of the plant extract on the replication of the virus, which prevents virus multiplication in a host cell, and (iii) Immunostimulatory activity of the plant extract.

On analyzing the results generated in this study, in the light of the postulates described above, it could be inferred that the virus was getting inactivated by the plant isolate and thus getting prevented from establishing an infection. In conclusion, through this study, we could confirm the antiviral activity of MP07X and could standardize the quantity of the plant isolate required to protect shrimps from WSSV infection against a defined titer of virus. We found that the crude drug was less toxic to the shrimps at the concentrations required for the antiviral activity. However, more studies are required to evaluate the possible mode of action of MP07X using different tools of biochemistry and molecular biology.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

WSSV is a main threat to the shrimp farming industry as the virus can cause 100% mortality of the infected shrimps within few days. An effective inexpensive anti-WSSV drug is the need of the hour, in order to help the shrimp farmers.

Research frontiers

The present research work depicts the screening of the marine plants for the isolation of the anti-WSSV drug and optimization of its dosage by administering the same via intramuscular and oral routes into WSSV infected *L. vannamei*, as a host.

Related reports

Marine plants are of potent medicinal values that can be used to cure many diseases and disorders in human as well as other life forms. The plants have also derived effective antiviral drugs for some of the viruses of both plant and

animal origin.

Innovations and breakthroughs

The effective dosage of the drug is non-toxic in nature and can be easily administered to the host. This study reveals the *in vivo* efficacy of the drug to combat WSSV in the host.

Applications

The drug can be modified and further research is required in this aspect in order to make the administration of this drug possible into different hosts, harbouring WSSV.

Peer review

This is a good study in which the authors have proved the efficacy of the marine derived anti-WSSV drug. The formulation of the drug is inexpensive and at the same time cost effective for the use of the marginal farmers.

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