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

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PEER REVIEW

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Comments

This is a good study in which the authors have proved the efficacy of the terrestrial plants 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 S126

ABSTRACT

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

Methods: Thirty 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*. The best anti-WSSV plant isolate, TP22C was isolated and further analyzed. 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: Seven plant isolates exhibited significant survivability in host. The drug TP22C thus formulated showed 86% survivability in host. The surviving shrimps were nested PCR negative at the end of the 15 d experimentation. The lowest concentration of TP22C required intramuscularly for virucidal property was 10 mg/mL. The oral dosage of 750 mg/kg body weight/day survived at the rate of 86%. Neither VP28 nor *ie 1* was expressed in the test samples at 42nd hour and 84th hour post viral infection.

Conclusions: The drug TP22C derived from *Momordica charantia* is a potent anti-white spot syndrome virus drug.

KEYWORDS

Shrimps, *Litopenaeus vannamei*, Anti-WSSV, Terrestrial plants, TP22C, White spot syndrome virus

1. Introduction

In the aquaculture industrial sectors, shrimp farming is not an exceptional case with a steady increase of cultivated shrimp production since the early 1980s[1-3]. Shrimp products are the largest single seafood commodity by value, which accounts for about 17% of the total internationally traded fishery products. The black tiger shrimp [*Penaeus monodon* (*P. monodon*)] and the white leg shrimp [*Litopenaeus vannamei* (*L. vannamei*)] are the two species which dominate production and account for approximately 75% of global shrimp aquaculture

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production[4]. However, shrimp farms are often infected by bacterial diseases such as, vibriosis caused by *Vibrio alginolyticus* and *Vibrio harveyi*, *etc.* and viral diseases like monodon baculovirus virus, white-spot syndrome virus (WSSV), and Taura syndrome virus[5-9]. Among these viral diseases, WSSV is the most pathogenic and the longest existing virus without any preventive or control measures[10]. This virus is considered to be an extremely virulent one, and may cause 100% mortality within 3-7 d as it initiates an infective necrotising condition in *P. monodon*, *Penaeus chinensis*, *L. vannamei* and *Fenneropenaeus indicus*[11], the most important shrimp species in the aquaculture sector. WSSV is a species of the genus *Whispovirus* and belongs to the new family Nimaviridae[12]. Previous researches have focused on the pathogenicity and development of diagnostic methods to detect the virus[13-16]. WSSV has been isolated and characterized from *Penaeus indicus* and *P. monodon* and found to be similar to that described earlier[13,15]. WSSV has been found to be highly pathogenic not only to penaeid shrimp, but also to a wide host range, which includes marine crabs and copepods, freshwater crabs and prawns[16-19].

Previous researchers have tried to eradicate aquaculture viruses through various approaches, such as by treating viral infection with common chemotherapeutants that have turned out to be ineffective and at the same time the development of virus-resistant species is time-consuming[20]. The inactivation of such viruses, including shrimp viruses, by physical and chemical agents have been reported by various authors, who have determined the effects of heat, ultraviolet light, pH, desiccation and chemical disinfectants on the inactivation of baculoviral mid-gut gland necrosis virus and WSSV[21-24]. Earlier experimentation has been done with natural adjuvants, synthetic agents, that were used as immunosuppressive and immunostimulative agents. Many herbals have been used for millennia for their potent anti-viral properties. Among them, a few have been found to have anti-viral activity against fish viruses in tissue culture and some have been investigated for their ability against shrimp viruses[25,26]. These natural plant products have been reported to have various activities like antistress, growth promoters, appetiser, tonic, non-toxic, biodegradable and biocompatible immunostimulants and antimicrobials[27,28]. Several plants of both terrestrial and marine origin have already been tested against viral diseases to judge its immunostimulant efficacy. *Cynodon dactylon* (*C. dactylon*) (terrestrial plant) and *Ceriops tagal* (*C. tagal*) (mangrove) exhibited protective effect against WSSV in *P. monodon*[29-31]. The seaweed, *Sargassum weighti*, showed significant anti-WSSV property against marine shrimp, *Penaeus indicus* and freshwater crab, *Paratelphusa hydrodomous*[20]. The extract of *Phyllanthus amarus* (*P. amarus*) and *Psidium gugajava* has shown antiviral activity against yellow head baculovirus in *P. monodon*[25]. The extract of *Clinacanthus nutans* has been tested against yellow head virus (YHV) in shrimp and the results indicated an effective control of YHV infection in shrimp[32]. 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 envelop protein, VP19 and VP28, feeding with fucoidan extracted from *Sargassm polycysticus* and antiviral drug supplemented with *Spirulina platensis*[33-40]. *P. amarus* was very effective against the fish viruses such as INHV and OMV and shrimp virus YHV and its water and alcohol extracts blocked HIV-1[41,42]. The methanol extract of *Aegle marmelos* showed little antiviral activity against WSSV whereas it revealed strong antiviral activity against herpes simplex virus-1[29,43]. Glycoproteins derived from *Celosia cristata* similar to the antiviral proteins of *Bougainvillea spectabilis*, exhibited active antiviral property by inhibiting the mechanical transmission of two tobamoviruses, tobacco mosaic virus and sunnhemp rosette virus, and citrus ring spot virus into their hosts[44,45].

These previous literatures motivated us to work with the commonly available terrestrial plants, for the eradication of WSSV from the shrimp aquaculture industry and at the same time the protocol should be inexpensive for the marginal farmers. The leaves from each of these plants were studied for their anti-WSSV property in *L. vannamei*.

2. Materials and methods

2.1. In vivo anti-WSSV activity of terrestrial plants

2.1.1. Selection of terrestrial plants

The terrestrial plants selected for the present study were collected from different parts of West Bengal, namely Bongaon, Basirhat, Contai, Sundarban, Kolkata, Kharagpur and from some of the places from Tamil Nadu, namely Pichavaram, Parangipettai and Chidambaram. Thirty terrestrial plants such as, *Aloe barbadensis*, *Amaranthuus* sp., *Bougainvillea spectabilis*, *Carica papaya*, *Celosia cristata*, *Centella asiatica*, *Cinnamomum verum*, *Citrus aurantifolia*, *Citrus maxima*, *Cocos nucifera*, *Coriandrum sativum*, *Eclipta alba*, *Elettaria cardamomum*, *Ficus religiosa*, *Hibiscus rosa-sinensis*, *Impatiens balsamina*, *Justicia adhatoda*, *Lactuca sativa*, *Lantana camara*, *Manilkara zapota*, *Mentha spicata*, *Momordica charantia*, *Moringa oleifera*, *Murraya koenigii*, *Musa* sp., *Nicotiana tabacum*, *Piper betle*, *Polyalthia longifolia*, *Withania somnifera* and *Zingiber officinale* 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[46,47]. All the 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 were collected from the above enlisted locations when necessary 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 affects the nature of the crude extract[46]. The liquid extracts thus obtained were filtered through Whatman No. 1 filter paper and were 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 as shown in were coded properly, *viz*. TP01A, TP01B, TP01C, TP01D likewise; where, TP, A, B, C & D represents terrestrial 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 lts. 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 μm filter. The preparation was streaked on ZoBell's. Thiosulfate citrate bile salts -sucrose and potato dextrose agar plates 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) (5 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 μLsterile 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. 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′[48].

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, followed 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. Optimization of TP22C as an anti-WSSV drug in animal model

2.2.1. 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} in the same diluents. Apparently, healthy shrimps (4 animals in a tank in triplicate) were injected with 10 μL suspension 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 recorded, and the penultimate dilution was selected for application during all assays. The percentage mortality obtained on different dilutions of virus on different days after the intramuscular challenge was statistically analyzed by two factor ANOVA and the differences were considered significant at $P\leqslant0.05$.

2.2.2. Toxicity of the plant isolate in animal model

The lyophilized plant isolate was used to prepare strength solution for toxicity studies in *L. vannamei* (6-8 g) as the animal model. The stocks having strength 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 was administered intramuscularly at the 6th abdominal segment of apparently healthy *L. vannamei* (6-8 g). The control consisted of animals injected with 10 μL of distilled water. For every strength of the extract, 6 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 survival obtained on different dilutions of the extract was statistically analyzed by single factor ANOVA. The differences were considered significant at $P\leqslant0.05$.

2.2.3. Strength of the plant isolate required for virucidal activity

Various concentrations of the extract (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 1:1 and incubated at 28 °C for 3 h. As NEG NTE buffer alone and as the POS, the virus suspension mixed with NTE buffer at a ratio of 1:1 were also incubated under the same conditions given to the test. After the incubation, 25 μL aliquots each of the above preparations were injected intramuscularly on to a batch of 9 apparently healthy *L. vannamei* (6-8 g) in triplicate into their 6th abdominal segment. This on administration provided the animals with a quantity of the extract delivered at 1 500 mg, 1 250 mg, 1 000 mg, 750 mg, 500 mg, and 250 mg/kg body weight/day. The animals were monitored for clinical signs of WSSV disease and mortality. Percentage survival obtained on different dilutions of the extract was statistically analyzed by single factor ANOVA and the differences were considered significant at $P\leqslant0.05$.

2.2.4. 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 10^{-1} dilution of the virus suspension. The animals were monitored for development of clinical signs and mortality for 7 d. All dead or moribund animals were collected and subjected to PCR for detection of WSSV following the procedure described earlier. 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, IUSA). 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.

2.2.5. Quantitative determination of the plant isolate (TP22C) for protection of shrimp from WSSV

To determine the quantity of the isolate required for protection of shrimp against WSSV, the animals were fed with different concentrations of the extracts for a period of 7 d, prior to the challenge. To accomplish this objective, artificial medicated shrimp feed was produced with the extracts at 3.0%, 2.5%, 2%, 1.5%, 1% and 0.5% of the feed. This on administration provided the animals with a quantity of the extract delivered at 1 500 mg, 1 250 mg, 1 000 mg, 750 mg, 500 mg, and 250 mg/kg body weight/day. The POS and NEG animals received feed without the extract. On the 8th day, both the TS 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 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 single factor ANOVA and the differences were considered significant at $P\leqslant0.05$.

2.3. 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 virus were examined to find out whether the plant isolate (TP22C) was inhibiting the processes involved in the viral multiplication cycle during host pathogen interaction. 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 volume 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 volume 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 pmoles), 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-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. Approximately 25 μL PCR reaction mix contained 0.5 IU of Taq DNA polymerase, 200 μmol/L dNTP mix, 10 pmoles 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 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. Ten μL each of the PCR products 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 terrestrial plants

3.1.1. Viral inoculums for anti-WSSV assay

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

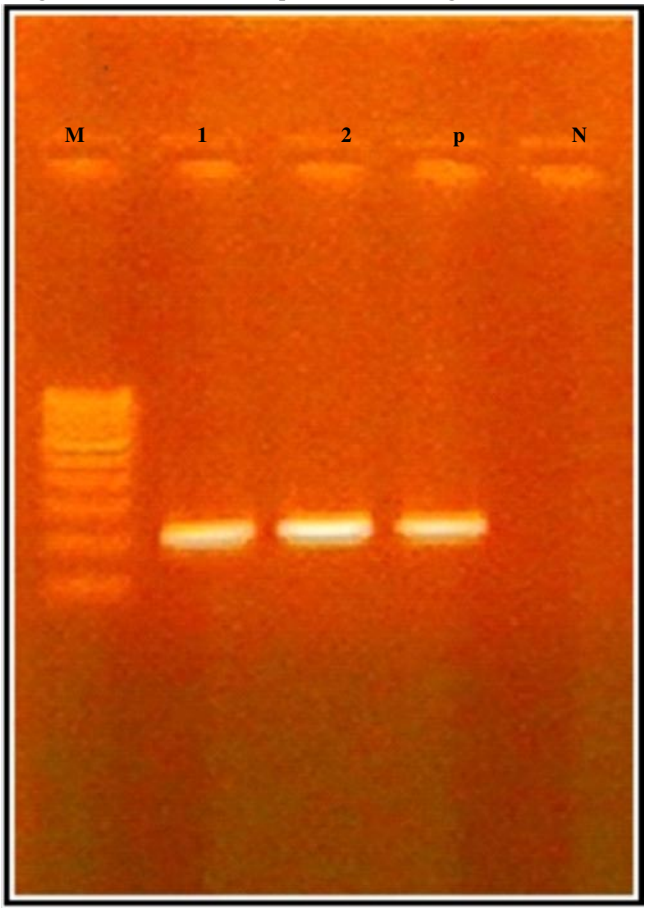

Figure 1. Detection of WSSV gene in the PCR product.
M=marker, 1=WSSV infected shrimp from the farm, 2=freshly WSSV infected shrimp produced in laboratory, p=positive control (211 bp), N=negative control.

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 exposed to the plant isolates such as TP05B (ethanolic extract of *Celosia cristata*), TP12C (methanolic extract of *Eclipta alba*), TP17C (methanolic extract of *Justicia adhatoda*), TP19A (water extract of

Lantana camara), TP22C (*methanolic* extract of *Momordica charantia*), TP29C (methanolic extract of *Withania somnifera*) and TP30C (methanolic extract of *Zingiber officinale*); 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 and 4th d. The TP22C isolate exhibited significant survivability and the plant is abundantly available. Hence, further experimentation was carried out with the above isolate to get a better host survival percentage.

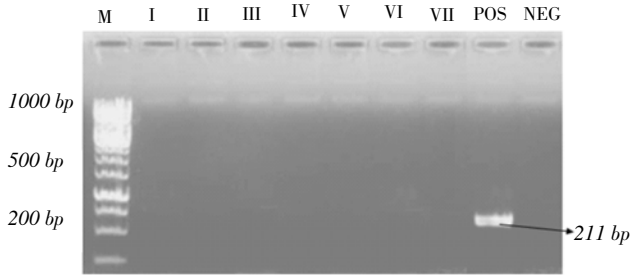

Figure 2. Detection of the virucidal property of the best 7 plant isolates. M=Marker; I=TP05B; II=TP12C; III=TP17C; IV=TP19A; V=TP22C; VI=TP29C; VII=TP30C; POS: Positive; NEG: Negative.

3.2. Optimization of TP22C as an anti-WSSV drug in animal model

3.2.1. 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 were 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 in the case of NTE buffer injected shrimps (P<0.001) (Figure 3).

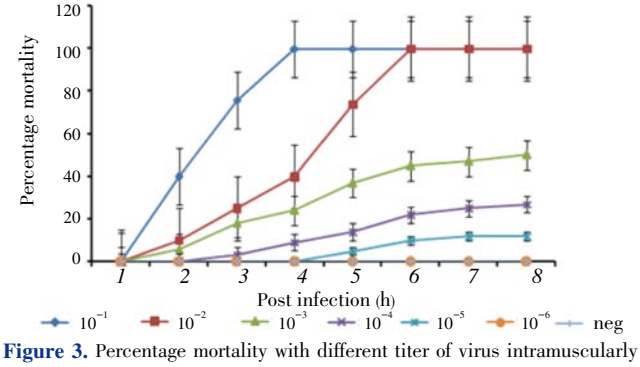

Figure 3. Percentage mortality with different titer of virus intramuscularly injected.

3.2.2. 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 4). Response of the animals was more or less the same without any significant mortality even up to a concentration 30 mg/mL (P<0.05). However, at 50 mg/mL strength, there was significant reduction (43% average percentage survival) (P<0.05) in survival of shrimps during the experimental period of 7 d.

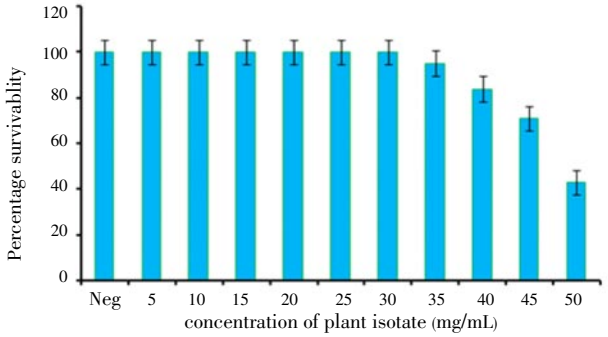

Figure 4. Toxicity of different concentration of plant isolate (TP22C) in *L. vannamei*.

3.2.3. Strength of the plant isolate required for virucidal activity

The experiments to determine the virucidal activity was carried out on *L. vannamei* (6-8 g) (n=9). On administering WSSV at a dilution of 1×10^{-1} after exposing to different concentrations of the plant isolate (TP22C) 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 TP22C such as 10-30 mg/mL. Accordingly, the shrimps did not find to get infected in these above concentrations registering overall survival of 86%. Meanwhile, the batches of shrimp administered with 5 mg/mL of TP22C resulted in a lesser survival of 58% respectively. All POS succumbed to the virus when administered with the virus exposed to NTE buffer after incubation for 3 h at 29 °C. All NEG survived the experimental period (Figure 5).

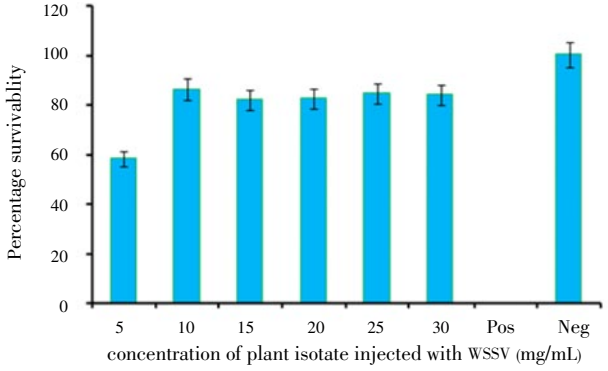

Figure 5. Virucidal property at different concentration of plant isolate TP22C.

3.2.4. Quantitative determination of plant isolate (TP22C) 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, 1 250 mg, 1 000 mg, 750 mg, 500 mg, and 250 mg/kg/body weight/day for a period of 7 d. On challenging all batches

of shrimp by oral administration of freshly infected tissue, all animals which received the TP22C at a dosage of 750 mg/kg body weight/day survived at 86%, and the differences in survival between different batches were significant (P<0.001). Significantly, lower average survival of shrimp observed in the batches which received 500 and 250 mg/kg body weight/day is noteworthy as the average survival was only 63% and 18%. The POS (shrimps administered with normal diet and challenged with WSSV) succumbed to WSSV registering total mortality. All NEG survived (Figure 6).

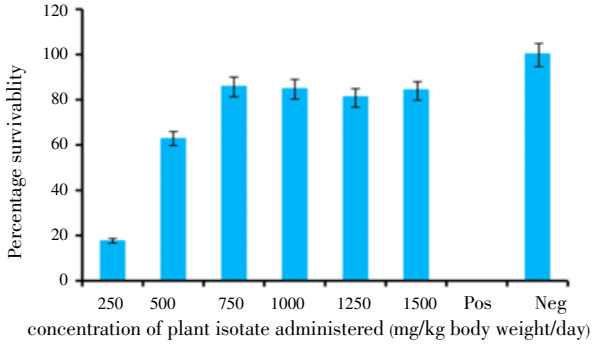

Figure 6. Efficacy of orally administered extract against WSSV.

3.3. 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 virus were examined to find out whether the plant isolate (TP22C) was inhibiting the processes involved in the viral multiplication cycle during host pathogen interaction. The gene expression study was conducted in three groups (POS, NEG and TS) of animals. Viral genes were not amplified in TS 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 expressed on both 42nd hour and 84th hour after challenge with WSSV. It was observed that, as the time went by there was increase in the intensity of bands of these genes suggesting more multiplication of the virus in the POS shrimps (Figure 7). 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.

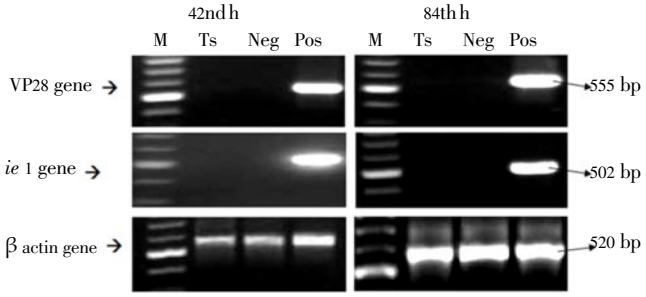

Figure 7. Reverse transcription PCR analysis of VP28, immediate early *(ie 1)* and β actin genes in host.

4. Discussion

The medicinal plants with a history of traditional use, are a potential source of substances with significant pharmacological and biological activities providing an alternative approach for the discovery of antiviral agents[49]. In this present study, an attempt has been made to look into the possibilities of using terrestrial plants as source of anti-WSSV drugs. With this objective, 30 terrestrial plant species abundantly found in different places 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, TP05B, TP12C, TP17C, TP19A, TP22C, TP29C and TP30C were found to be effective against WSSV. TP22C isolate showed significant survivability and the plant is 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 isolate. As TP22C could give protection to all animals tested against WSSV, further studies were carried on with *Momordica charantia*. When TP22C isolate was administered intramuscularly with subsequent challenge, the viral DNA was not detected in the tissue which suggested that the virus was either had not invaded the host tissue and multiplied, or it was getting eliminated subsequent to infection.

Several attempts have been made earlier by several researchers to derive anti-WSSV compounds from plants. Equal proportion of methanolic extracts of 5 medicinal plants such as *C. dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa and Eclipta alba* were mixed together and the combined extracts were supplemented through shrimp diet at different concentrations[50]. There are reports of feeding shrimps with diet containing extracts of herbs which have improved immune system and also accorded protection from WSSV in *P. monodon*[51]. Virucidal property of the aqueous extracts of *Rhizophora mucronata*, *Sonneratia* sp. and *C. tagal* when administered along with WSSV suspension at 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[52]. They suggested that this might be due to the contact of virus with mytilin before getting injected into the shrimp. PCR analysis showed that the surviving animals were not accommodating the viral DNA.

The present study also points out that the *Momordica charantia* belonging to family Cucurbitaceae 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[29-31,39,50,53-60]. The organic solvent extracts of *Calotropis procera* leaves showed considerable

antibacterial, antifungal and antiviral activities against *Pseudomonas aeruginosa*, *Vibrio harveyi*, *Aeromons hydrophila*, *Fusarium* sp. and WSSV[61]. A combination of herbal extracts and probiotics as medicated diet could decrease the prevalence of WSSV in *L. vannamei* [62]. Even though reports are available on the protective effect of plant extracts against WSSV, information on their mode of action is scanty. In this study, antiviral property of TP22C was investigated employing molecular tools to evaluate their role in protecting shrimps from WSSV. 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 extract and thus getting prevented from establishing an infection. In conclusion, through this study, we could confirm the antiviral activity of TP22C isolate. However, more studies are required to elucidate the further processes.

In this study, antiviral property of TP22C 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 viral 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 a viral titer of 1×10^{-1} in their experiments[31]. Similar kind of experiments was conducted to determine the dilution of WSSV required to be used for its neutralization by WSSV vaccine[63]. According to them, a dilution of 1×10^{-3} could give 100% mortality within 7 d. In animal model, the highest non-toxic concentration went up to 30 mg/mL, from which 10 μL extract was injected to shrimps (6-8 g). Similarly, the highest non-toxic level of *C. tagal* in *P. monodon* is 50 mg/mL[31]. The average percentage of survivability of shrimps injected with a concentration of 10 mg/mL of TP22C was 86%. Marginal mortality was due to cannibalism subsequent to moulting. 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 body weight and they could successfully use the same for further assays[40].

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 from 5 mg/mL to 30 mg/mL was chosen, and the concentration higher than 10 mg/mL was found to be effective in exhibiting its virucidal property. In this 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 is the ratio of toxic concentration to the effective concentration, and shows higher antiviral activity at a concentration below the toxic value. The results generated unambiguously suggest that the virucidal property of TP22C is very much dependent on concentration. Similarly, on screening 20 Indian medicinal plants, anti-WSSV activity was exhibited by the aqueous extract of *C. dactylon* on administering 100 mg/kg of animal 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 and was proposed that the antiviral activity of mytilin was mediated by its binding onto the viral envelop[52,64].

On evaluating the effectiveness of orally administered TP22C 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 TP22C at a dosage of 750 mg/kg body weight exhibited 86% survival rate on challenging with WSSV infected tissue. In a similar way, the 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 body weight resulted in 93% survival rate[39,58]. 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 is backed by the evidence that no signal was obtained from molecular detections[61]. Shrimps fed with 300 mg of *Dunaliella* extract per kg of diet demonstrated higher resistance to WSSV infection besides becoming tolerant to stress[59]. The survival rate of *P. monodon* increased to 74% on administering herbal immunostimulant incorporated diet at a concentration of 800 mg/kg feed[50]. 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[51]. On feeding 2% aqueous extract of *C. dactylon* extract coated feed to *P. monodon*, researchers could obtain 100% survival rate and the live animals were PCR negative[30]. On feeding 1% aqueous extract of *C. tagal* at 500 mg/kg of body weight/day to *P. monodon*, researchers could obtain 100% survivability[31].

To evaluate the efficacy of TP22C 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) that were administered with the crude drug. This was alike for both the 42nd h and 84th h after challenged 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 viral invasion[65]. 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[66]. 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 TP22C. The results of different types of assays, viral and immune gene expression and histopathology 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 crude drug.

The present results suggest that the mode of action of TP22C on 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[29]; (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 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 TP22C 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 shrimp at concentrations of the extract required for antiviral activity. The mode of action of the virus was found virucidal; however, more studies are required to evaluate the possible mode of action using different tools of biochemistry and molecular biology.

Conflict of interest statement

We declare that we have no conflict of interest.

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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 terrestrial 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

Terrestrial 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 terrestrial plants 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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