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Confirmation of Anti-WSSV activity from Red Algae *Hypnae spinella* in freshwater crab *Paratelphusa hydrodomous*

Dinesh S.[1], Manasi Karmarkar[1], Vinodhini S[1], Vidhya G[1], Hemalatha K[1], Sudhakaran R[1,]*

[1]Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, VIT University, Tamilnadu, India.

***Corres.author: sudhakaran.r@vit.ac.in / 2sudha@gmail.com**

Abstract: In recent years, aquaculture business in the world has been rapidly developed. Viral pathogens especially White Spot Syndrome Virus (WSSV) causes huge economic losses to aquaculture companies. Every were 100% cumulative mortality were observed in all species of cultured shrimps due to WSSV infection. To treat WSSV, several antiviral compounds obtained from both terrestrial as well as aquatic plants were tested, some of them have act as good antiviral activity and some act as immunostimuants. In the same series, we tested the antiviral potency of marine red algae *Hypnae spinella* against WSSV. The antiviral activity of crude methanolic extract of *H. spinella* were analysed *in vivo* in freshwater crabs *Paratelphusa hydrodomous*. Bio-assay showed strong activity against WSSV and results were confirmed by histopathology and DNA polymerase chain reaction.

Keywords: Marine algae, *Hypnae spinella*, White spot syndrome virus, antiviral activity, *Paratelphusa hydrodomous*, PCR.

Introduction

White spot disease is one which cause high mortality among the common shrimp diseases.[1] Globally aquaculture industries faces huge economic losses due to rapid spread of WSSV disease, this can cause 100% mortality in a short period like 3-10 days.[2] WSSV belongs to the Genus *Whispovirus* (Family Nimaviridae), which is enveloped, rod shaped, dsDNA virus of approximately 300 kbp in size.[3] The signs and symptoms include reduction in feed consumption, Lethargy, changes in the faecal matters and finally lead to the death.[4] WSSV has broad host range which includes almost all species of shrimp and other crustaceans like lobsters, crabs, krill, crayfish and branchiopods.[5]

Several protective strategies have been applied against WSSV, for example, microbial immunostimulants (*Pediococcus parvulus* and *Candida parapsilosis*) used to analysis the immune parameters, survivability in *Litopenaeus vannamei* against WSSV[6]and the WSSV infection was decreased in *Penaeus monodon* by using *Sargassum polycystum* drevied polysaccharide.[7] but still no effective remedies were found to control the spread of WSSV.

Macro algae seaweeds have been used as food and medicine in several Asian. Antiviral activity of macro algae has been confirmed by many researches. Many reports of marine macro algae derived compounds have anti-inflammatory, antibacterial, antifungal, antimitotic and antifouling activities.[8] In the present study we analysed the protective effect of crude methanol extracts of fresh marine macro algae *Hypnea spinella* against WSSV in fresh water crabs (*Paratelphusa hydrodomous*), it was highly susceptible to WSSV.[4]

Materials and methods

Animal collection

Crabs were collected from the Vellore, Tamil Nadu, India. Crabs having more than 20g weight were selected and acclimatized in 30 l aquarium tanks (5 animals per tank) at room temperature. Fresh minced fish were given twice as a feed. Sterilised ground water was used for maintaining the animals.

Collection of *H. spinella*

The marine red alga, *H. spinella* was collected from the coastal area in Rameshwaram, Tamil Nadu, India and was authenticated by CSRI institute, Mandapam camp, Rameshwaram.

Extraction procedure

Hypnae spinella were washed thrice with distilled water and dried completely under shade. Using electrical grinder the algae was powdered. 10 g of powdered algae were subjected to extraction with 100 ml absolute methanol and kept in orbital shaker for overnight and filtered using Whatman filter paper No.1. Filtrate were dried and stored at 4°C for future studies.

Viral inoculum preparation

Hemolymph obtained from WSSV-infected shrimp *P. monodon* was centrifuged at 3000 ×g for 20 min at 4°C. Supernatant was separated, centrifuged at 8000 ×g for 30 min at 4°C. After centrifuged the supernatant was passed through a sterile 0.45µm syringe filter. Then the final filtered hemolymph (Viral inoculum) was stored in -20°C for the experimental studies. The protein concentration in the viral inoculum was determined by the method of Lowry et al 1951.[9] A standard PCR was performed to confirms the presence of WSSV in the viral inoculum.[10]

Experimental design for bioassay

Healthy crabs were divided into three groups having three crabs in each group and the experimental challenge were conducted in triplicates.[11] Briefly, a mixture 100 µl contains NTE buffer and viral suspension was injected to the crabs present in the Group I named as positive control. NTE buffer alone was injected to the crabs present in the Group II named as negative control. A mixture of viral suspension, methanol extract of *H. spinella* and NTE buffer was injected to the crabs present in the Group III named as treated. In all the groups of viral suspensions were incubated for a period of 3 h at room temperature. After 3 h incubation, the viral suspensions were injected intramuscularly into the respective experimental groups. In all the experimental groups, fresh minced fish were given twice as feed. Unconsumed feed and faecal matter were cleaned before the next feeding. All the experimental groups were examined twice in a day for signs and symptoms of WSSV infection and the mortalities were recorded until end of the experiment.

Extraction of DNA

DNA was extracted from the different organs (gills, head, muscle, intestine, Hepato pancreas) of experimental group injected with methanol extract and WSSV by Guanidine Hydrochloride.[12] Briefly, 50 mg of tissue was homogenized well with 1 mL of guanidine hydrochloride buffer (6 M guanidine hydrochloride, 10 mM Tris HCl, 0.1 M sodium acetate and 0.1 M EDTA, pH 8.0). The tissue homogenate were collected in a sterile 1.5 mL micro centrifuge tube and incubated at room temperature for 30 min and centrifuged for 5 min at 5000 × *g*. After spin the supernatant was collected into a fresh micro centrifuge tube and ice cold absolute ethanol was added in equal volume and mixed well, followed by centrifugation at 14000 × *g* for 20 min. Then the pellet was washed twice with 95% absolute ethanol. Air dried the pellet and dissolved in 50 µL sterilised TE buffer pH 7.8. DNA was quantified by taking optical absorbance at 260 nm using UV spectrophotometer (ELICO, India).

Polymerase Chain Reaction

The amplification of the WSSV specific gene VP28 was performed in Eppendorf Thermal cycler. A 20 µL standard PCR reaction contains 10 µl of 2X Mastermix (Genei, Bangalore India), 6 µl VP28 primer (VP28 Forward- ATG GAT CTT TCT TTC AC and VP28 Reverse- TTA CTC GGT CTC AGT GC), 3 µl PCR grade

water and 1 µl template DNA. The cycling conditions set for the PCR reactions are 95°C (5 min) then 35 cycles of 95°C (1 min), 50°C (1 min), 72°C (1 min) and final extension at 72°C for 10 min. The PCR products were electrophoresed on a 1.0% agarose gel, stained with ethidium bromide (Sigma Aldrich) and photographed using gel documentation system (Lark).

Histopathology

A small portion of head soft tissue and gill from all three experimental groups were dissected out and fixed in 10% formalin followed by embedded in paraffin. Thickness of 4-5 µm sections were made and stained by haematoxylin and eosin (H&E).

Results

Bioassay:

The antiviral activities of methanol extract *H. spinella* against WSSV were evaluated and we found that methanol extract of *H. spinella* have showed significant antiviral activity against WSSV. Animals in the experimental Group III (Methanol extract and WSSV) were survived without any mortality until end of the experiment. Whereas in Group I (Viral suspension and NTE buffer) treated as positive control were starts dying at 4th day or 96 h post injection (h.p.i) and 100% mortality were occurred on 8th day (168 h.p.i) with gross signs of symptoms like less active, reduced feed consumption and slow in movements. The experimental animals in Group II (NTE buffer alone) named as negative control was survived with no mortality and symptoms of WSSV infection until end of the experiment. The complete bio-assay of this experiment was plotted in a cumulative mortality graph (Fig. 1).

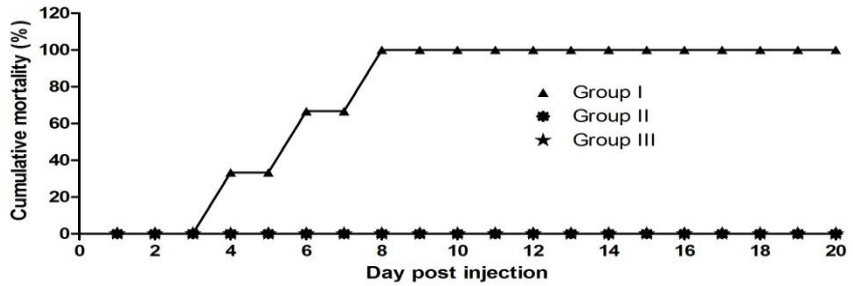

Figure 1. Cumulative mortality graph showing the survival after treatment along with controls. Group I- Positive control; Group II – Negative control; Group III – Treated group.

Polymerase Chain Reaction

The result shows that, no bands were seen in all the organs (gills, head, muscle, intestine, Hepato pancreas) from the experimentally injected animals (Group III), it indicates that the methanol extract of *H. spinella* have strong antviral activity against WSSV. Similarly no band were came for negative control, it indicates that the primers as well as PCR machine was working properly. Whereas in the positive control, band came at 615 bp indicates positive for WSSV (Fig. 2).

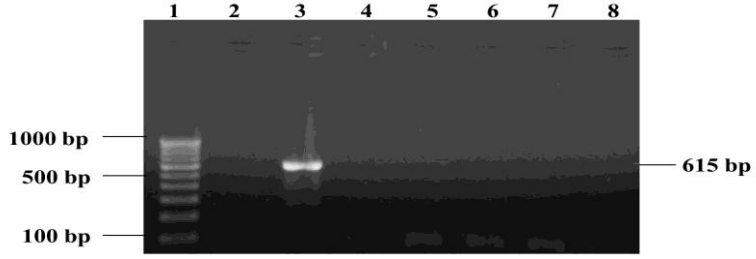

Figure 2. Polymerase chain reaction of different organs treated with extracts. Lane 1- 100 bp DNA Marker, Lane 2- negative control, Lane 3- positive control, Lane 4 - gill, Lane 5 - head soft tissue, Lane 6 – muscle, Lane 7- intestine, Lane 8 - hepatopancreas.

Histopathology

The H&E stained sections of head soft tissue and gill from the experimental animals in positive control shows histopathological changes, including cells having hypertrophied nuclei with intranuclear inclusions typical for WSSV infection (Fig. 3C & 3D). Whereas no histopathological changes were observed in negative control (Fig. 3A & B) as well as in the methanol extract injected group (Fig. 3E & F).

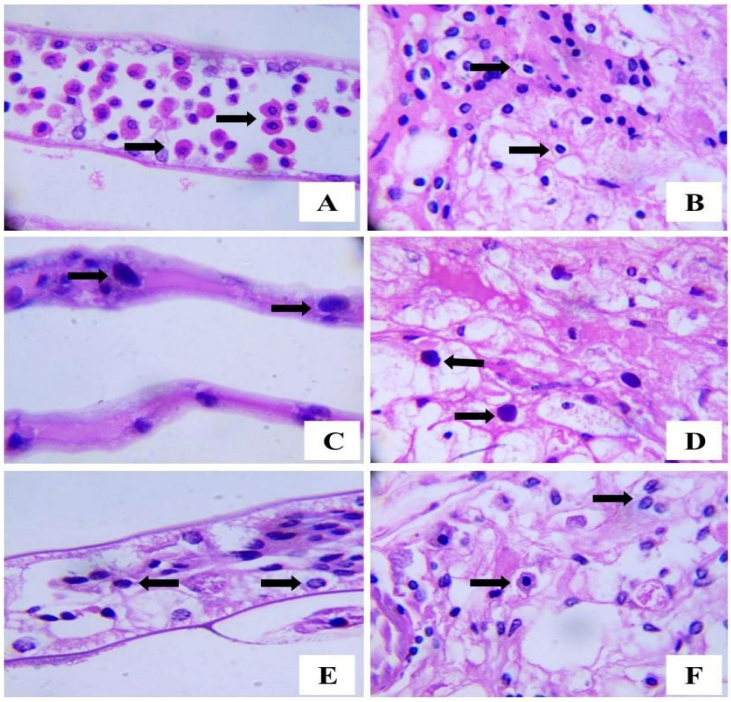

Figure 3. Photomicrographs of tissues from crabs of experimental groups: The infected tissues showing hypertrophied nuclei with intranuclear inclusions (Arrow). 3A gill and 3B head soft tissue of negative control showing normal cells, 3C gill and 3D head soft tissue of positive control, 3E gill and 3F head soft tissue of *H. spinella* treated group showing uninfected. Magnification: 1000 X.

Discussion

To prevent the WSSV disease in aquaculture farms, like shrimp, crab, crayfish etc., various treatments have been developed. Marine algal extracts possess antiviral effect as well as immunostimulatory activity.[13] Recently a study demonstrated that sulfated galactans derived from red seaweed (*Gracilaria fisheri*) have potent antiviral activity against WSSV.[14] The marine red algae *Hypnae spinella* have rich in polysaccharides and some of *Hypnae sp* were used for preparing gelling and thickening substances in food industries. A study evaluated that *Hypnae charoides* have antiviral potency against HSV type 1 and type II.[15]

In the present study, we tested the antiviral activity of methanol extract of *H. spinella* against WSSV. The experimental animal used in this experiment is freshwater crabs (*P. hydrodomous*) to evaluate the antiviral activity. The *in vivo* bioassay determination of antiviral activity of *H. spinella* exhibits strong antiviral potency against WSSV. The experimentally injected animals in the positive control (Group I) reached 100% mortality in 8th day of post injection. In negative control (Group II) were survived with no mortality and symptoms of WSSV infection. Similarly methanol extract (Group III) along with WSSV treated animals were survived until the experiment with no any mortality and symptoms of WSSV infection. PCR result also confirms that all the organs from the experimental animals (Group III) were found to be negative for WSSV infection. The histopathological analysis of the experimentally infected animals in the positive control have gross signs of WSSV infection like hypertrophied nuclei with intranuclear inclusions and less in number. Whereas in the negative control and *H. spinella* methanol extract and WSSV injected animals have normal in appearance, there was no significance a sign of WSSV infection were found. Overall results determines that methanol extract of *H. spinella* have potent antiviral activity against WSSV.

Conclusion

The present study shows that the methanolic extract of marine macro algae *Hypnae spinella* have strong antiviral activity against WSSV. These finding may help to increase a safe antiviral drug against WSSV and it may also save severe damage and economic losses to the aquaculture industry.

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References

1. Tendencia, E.A., Bosma, R.H., Verreth, J.A., White spot syndrome virus (WSSV) risk factors associated with shrimp farming practices in polyculture and monoculture farms in the Philippines, Aquaculture., 2011, 311, 87-93.
2. Flegel, T., Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand, World. J. Microb. Biot., 1997, 13, 433-442.
3. Leu, J.-H., Yang, F., Zhang, X., Xu, X., Kou, G.-H., Lo, C.-F., *Whispovirus*, Curr. Top. Microbiol. Immunol., 2009, 328, 197-227
4. Sahul Hameed, A., Yoganandhan, K., Sathish, S., Rasheed, M., Murugan, V., Jayaraman, K., White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphusa hydrodomous* and *P. pulvinata*), Aquaculture., 2001, 201, 179-186.
5. Lo, C.-F., Ho, C., Peng, S., Chen, C.H., Hsu, H., Chiu, Y., Chang, C., Liu, K., Su, M., Wang, C., White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods, Dis. Aquat. Org., 1996, 27, 215-225.
6. Peraza-Gómez, V., Luna-González, A., González-Prieto, J.M., Fierro-Coronado, A., González-Ocampo, H.A., Protective effect of microbial immunostimulants and antiviral plants against WSSV in *Litopenaeus vannamei* cultured under laboratory conditions, Aquaculture., 2014, 420-421, 160-164.
7. Chotigeat, W., Tongsupa, S., Supamataya, K., Phongdara, A., Effect of fucoidan on disease resistance of black tiger shrimp, Aquaculture., 2004, 233, 23-30.
8. Díaz, R.T.A., Chabrillón, M., Cabello-Pasini, A., Gómez-Pinchetti, J.L., Figueroa, F.L., Characterization of polysaccharides from *Hypnea spinella* (Gigartinales) and *Halopithys incurva* (Ceramiales) and their effect on RAW 264.7 macrophage activity, J. Appl. Phycol., 2011, 23, 523-528.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., Protein measurement with the Folin phenol reagent, J. Biol. Chem., 1951, 193, 265-275.
10. Yoganandhan, K., Sathish, S., Murugan, V., Narayanan, R., Sahul Hameed, A., Screening the organs for early detection of white spot syndrome virus in *Penaeus indicus* by histopathology and PCR techniques, Aquaculture., 2003, 215, 21-29.
11. Balasubramanian, G., Sarathi, M., Kumar, S.R., Hameed, A., Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp, Aquaculture., 2007, 263, 15-19.
12. Manjanaik, B., Umesha, K., Karunasagar, I., Karunasagar, I., Detection of hepatopancreatic parvovirus (HPV) in wild shrimp from India by nested polymerase chain reaction (PCR), Dis. Aquat. Org., 2005, 63, 255-259.
13. Rajasekar, T., Usharani, J., Sakthivel, M., Deivasigamani, B., Immunostimulatory effects of *Cardiospermum halicacubum* against *Vibrio parahaemolyticus* on tiger shrimp *Penaeus monodon*, J. Chem. Pharm. Res., 2011, 3(5), 501-513.
14. Wongprasert, K., Rudtanatip, T., Praiboon, J., Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp, Fish Shellfish Immunol., 2014, 36, 52-60.
15. Smit, A.J., Medicinal and pharmaceutical uses of seaweed natural products: a review, J. Appl. Phycol., 2004, 16, 245-262.

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