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In vivo administration of fucoidan from *Turbinaria decurrens* protects shrimps from white spot syndrome virus

[*]S. Meenakshi[1], R. Saravanan[2], T. Balasubramanian[3], & A. Palavesam[1]
[1.]Department of Animal Science, Manonmaniam Sundaranar University, Tirunelveli, India
[2]Sri K.G.S Arts College, Srivaikuntam, India
[3]Faculty of Marine Sciences, Annamalai University, India
[E-mail: smeenakshi85@gmail.com]

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Fucoidan was extracted from the brown seaweed *Turbinaria decurrens* by hot water extraction and characterized with HPLC, FTIR, NMR and GPC to study the impact against the White Spot Syndrome Virus (WSSV), bacteria and fungi. Fucoidan was fed for shrimps along with the diet (before and after WSSV infection). In the end of the study, the survival percentage of shrimp was 51% (in the case of 5-8 g) and 97% (in the case of 12-15 g), respectively. Among the 10 bacterial pathogens and 7 fungal pathogens, fucoidan inhibited 2 bacterial pathogen such as *S. aureus* (0.26±0.02 mm) and *E. faecalis* (1.3±0.1 mm) and *A. Niger* (0.84 ± 0.03 mm) in fungal pathogens with clear zone of inhibition.

[Keywords: Fucoidan, Brown seaweeds, WSSV, Antimicrobial activity, Prawn feed]

Introduction

WSSV is a viral disease affecting most of the commercially culturing marine shrimp species in the world. WSSV infection is characterized by a rapid disease on set and high mortalities. The principal clinical sign of WSSV is the presence of white spot on the carapace and the body colour of diseased shrimps becomes pale or reddish in colour[1]. Out breaks of WSSV causing serious mortality to cultured shrimp have been occurring in Asia since 1992. In order to prevent WSSV infection in cultured shrimp, the present study is planned to use the purified fucoidan from the brown seaweed *T. decurrens*.

Fucoidan represent 40% of the dry weight of isolated cell walls of brown seaweeds[2,3]. It is composed of fucose, uronic acids, galactose, xylose and sulfated fucose[4,5]. They are comprised of long chains of linked sugar molecules, along with sulfate groups which make them negatively charged. They composed primarily of highly branched (1→2) or (1→3) linked α-L-fucose-4-sulfate units[6].Structures and chemical composition of polysaccharide vary among the species and sometimes among different parts of the plant[6,7]. This structural complexity, chemical composition of fucoidan of algal origin may vary depending on the method of extraction[8]. Fucoidan interfere in several cellular processes such as migration, adhesion, proliferation and apoptosis[2,9]. Thus, each new fucoidan of algal origin has unique compound with unique structural features which may display varied bioactivity and would be a potentially a new drug.

Fucoidan from algae possess important pharmacological activities such as antioxidant[10], anticoagulant and antithrombotic[11,12], antiproliferative, anti-inflammatory, antiviral[13], antitumour and immunomodulatory[14], hepatoprotective[15], neuroprotective[16]. Turbinaria species have been reported to have wide range of pharmacological activites. Only limited number of studies has been done in *Turbinaria decurrens*. So, the present study was planned to isolate fucoidan from *T. decurrens* and to study against WSSV infection in *P. monodon*.

Materials and Methods

The brown seaweed *T.decurrens* belonging to the family phaeophyceae was collected in yervadi, India. Specimens were identified and authenticated by phycologist Dr.P.Anantharaman, Faculty of Marine Sciences, Annamalai University, Parangipettai, India. The collected *T. decurrens* was initially washed with seawater to remove the macroscopic epiphytes and other extraneous matter and then rinsed in distilled water. Specimen was shade dried and coarsely powdered. 100g of dried seaweed powder was depigmented with acetone followed by hot water

extraction at 90-95°C for 3-4 h. Brown colored syrup was then filtered through whatmann No.3 filter paper, concentrated to ¼ of the original volume, cooled and precipitate was centrifuged at 5000 rpm, dehydrated with diethyl ether and further purified in HPLC (data not shown) to get a sulphated polysaccharide fucoidan[17]. FT-IR spectroscopy of sulphated polysaccharide revealed the presence of hydrogen bonded compound, possible acid or amide salt, ionized compound and aliphatic tertiary amine salt. 1H- NMRanalysis shows the presences of signals corresponding to fucose. 3 OH groups appeared at 3.95 ppm, C= C-H (1H) (acetylenic group) signals at 3.45 ppm, C≡C-H (1H) (methylenic proton) signals at 2.35 ppm and 2.30 ppm C-H2 protons appeared at 1.34 ppm (2H). Presence of these signals clearly indicates that the isolated polysaccharide from *T. decurrens* is fucoidan. Biochemical estimation of the isolated fucoidan is carbohydrate 59.62%, sulfate 26.52%, and Uronic acid 6.3%. Molecular weight of the compound was determined in gel permeation chromatography as 2,34,000 Da[16]. All the chemicals were purchased from sigma chemical company (St. Louis, Mo) and Himedia (Mumbai, India).

Healthy shrimps weighing 5 – 8 and 12 – 15 g were used in this study. Both groups were performed separately in duplicate with 15 shrimps each treatment. Fucoidan was fed by mixing it with shrimp diet in 100, 200 and 400 mg/ kg (5-8 g) and 100 and 200 mg/ kg (12-15) of shrimp respectively. Fucoidan was fed for 4 days before WSSV infection. The shrimp were challenged by incubation in the virus solution for 2 .5 h. This virus concentration caused the controlshrimp to die in 3–5 days. The fucoidan was fed continuously for 15 days. The same diet without fucoidan was fed to the control groups, which were also challenged with WSSV. Theshrimp were monitored for 10 days after infection and all mortality were recorded.

The WSSV virus solution was prepared by using gills, heart and lymphoid organ of WSSV-infected shrimp, crushed in K-199 (1% w/v M199,1.88M Nacl,0.06 M $CaCl_2 \cdot 2H_2O$, 0.1M L-glutamine, 9.14 mM Herpes and 10% (v/v) salt mixture which consists of 0.05 M KCl, 0.12 M $MgSO_4 \cdot 7H_2O$, 0.16M $MgCl_2 \cdot 6H_2O$ and 3.2 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.3-7.6) at1:2 (W/V). Suspension was precipitated by centrifugation at 3000×g for 10min; the supernatant fluid was then centrifuged at 8000×g, 4°C for 39 min, filtered through a 0.45µm membrane and stored in15%glycerol at -70°Cuntill used[18].

Test group of the shrimp weight was 12 – 15 g fed with fucoidan 200 mg/ kg of body weight and WSSV infected (group 3). Phagocytic activity was compared with that of the normal diet control (group 1) and WSSV infected shrimp (group2). Haemolymph (0.5 ml) was collected on day 7 of the culture and mixed with 0.5 ml of KC-199 (Itami et al., 1994). Haemocytes were separated by centrifugation at 6500 rpm at 4 °C, then washed with KC-199. Haemocytes were then suspended ~10^7 cells in 0.2ml and mixed with 0.2 ml of latex beads (~10^8/ml, particle diameter 1.094µm) on a clean glass side. Mixture was incubated in a moisture chamber at room temperature for 30 min. The cells were fixed by 2.5% glutaraldehyde for 5 min, after which the nonadherent cells were removed with 0.85% NaCl, then followed by air-drying, and staining with Wright stain. Numbers of ingested cells and ingesting cells were counted from 200 cells and calculated for percentage of phagocytosis, phagocytic index (PI) and average number of the beads ingested per cell (ABPC) as follows[19]

$$\begin{pmatrix}\text{Percentage}\\ \text{phagocytosis}\end{pmatrix} = \left(\frac{\text{number of cells ingesting bead}}{\text{number of cell observed}}\right) \times 100$$

$$\begin{bmatrix}\text{Phagocytic}\\ \text{index (PI)}\end{bmatrix} = \left(\frac{\text{number of cells ingesting bead}}{\text{number of cell observed}}\right) \times \left(\frac{\text{number of bead ingested}}{\text{number of cells observed}}\right) \times 100$$

$$\begin{bmatrix}\text{Average number}\\ \text{of the bead}\\ \text{ingested per}\\ \text{cell (ABPC)}\end{bmatrix} = \left(\frac{\text{number of bead ingested}}{\text{number of cellingesting bead}}\right)$$

The antimicrobial activity of the fucoidan from *T. decurrens* was assayed using the paper disc diffusion method. Human pathogens were obtained from the Rajamuthaihah medical college, Annamalai University. Bacterial pathogens namely *Escherichia coli,Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa,Enterococcus faecalis, Enterobacter aerogenes, Micrococcus luteus, Salmonella typhi, Bacillus subtilis* and *B. megaterium* were used to study the antibacterial activity using muller hinton agar medium. Fungal pathogens namely

Aspergillus niger, Aspergillus flavus, Penicillium sp, *Trichoderma* sp, *Trichothecium* sp, *Hormodendrum* spand *Rhizophus* sp were used to study the antifungal activity using potato dextrose agar medium. 1, 1.5 and 2 mg/ml of fucoidan from *T. decurrens* were dissolved in sterile distilled water. 50µl from each concentration was poured into sterile filter paper discs (5 mm diameter) and dried for 30 min, prior to the inoculation. The disc was placed onto the surface of bacterial and fungal pathogens swabbed petriplates and incubated for 24-72 hours. After incubation, the clearance zones around the discs were measured in millimeters and the assays were carried out in triplicate. Erythromycin was used as control for antibacterial and nystatin for antifungal activity.

Results

Polysaccharide was extracted using hot water and yield of crude polysaccharide was 10.43 g/kg. The crude polysaccharide was precipitated with calcium chloride to separate fucoidan from alginates. The precipitated polysaccharide was partially purified in DEAE cellulose52 using 2M sodium chloride and the yield was 6.46 g/kg. The active fraction was identified by carbohydrate estimation using phenol sulfuric acid method and it was used for the further study. Partially purified sample was further purified in HPLC using C18 column and the yield was 3.38 g/kg.

100, 200, 400 mg/kg of body weight of fucoidan was fed to 5-8 g and 12-15 g of shrimps before and after immersing in WSSV solution. Survival rate of 5-8 g of shrimp was 5.8%, 19.3% and 61% respectively. In case of 12-15 g of shrimp was 54% and 97% survived respectively. For shrimps, which did not get fucoidan, 50% were dead 3 – 5 days after WSSV infection and 100% after 15 days. Phagocytic activity (Table 1) for the fucoidan fed shrimps with WSSV infection was found to have a higher phagocytic index, a higher percentage of phagocytosis and a higher ABPC than both of the control groups.

Antimicrobial activity was determined in fucoidan from *T. decurrens* at 1, 1.5 and 2 mg/ml (Table 2). Antibacterial activity was observed at 2 mg/ml against *S. aureus* and *E. faecalis* with a clear zone of 0.26±0.02 mm and 1.3±0.1 mm respectively. In antifungal activity clear zone of inhibition was observed only against *A. niger* at 2mg/ml with 0.84 ± 0.03 mm.

Table 1 — Phagocytic activity of shrimp

Immunity indexes	Group1 (control shrimps)	Group 2 (WSSV infected)	Group 3 (fed with fucoidan and WSSV infected)
Phagocytic index (n=10)	1.2 ±0.4	0.41±0.31	3.41±1.23
%phagocytosis (n=10)	6.27±2.43	3.63±1.32	10.4±1.98
ABPC (n=10)	2.0±0.62	1.5±1.2	3.1±0.75

Discussion

Viral and bacterial infections together with poor water quality are the main reason for shrimp mortality. Reinforcing resistance against the invading pathogens of shrimp by improving shrimp immunity is an acceptable strategy for defending diseases. Unlike the mammals and fishes, crustaceans over lacks a specific immune system[20]. Usage of natural immunostimulant as a better remedy than administrating vaccines and antibiotics is accepted for the control of pathogen in aquaculture.

Among the different extraction method for polysaccharide, hot water extraction contributed higher yield[17,21]. The present study supports the above statement by providing a good yield (10.43 g/kg) of polysaccharide using hot water extraction. The crude polysaccharide was further purified in HPLC to isolate fucoidan and the yield was 3.38 g/kg. Due to the high antioxidant potential, fucoidan from *T.decurrens* showed promising activity against the liver damage[15] and parkinsons disease[16].

Fucoidan from *Cladosiphon okamuranus*was reported to control WSSV in*P. japonicus*[22] and crude fucoidan of *Sargasum polycystum* was reported to

Table 2 — Antimicrobial activity of fucoidan from *T. decurrens*

S. No.	Pathogens	1 mg/ml	1.5 mg/ml	2 mg/ml
	Bacterial pathogens			
1.	*Escherichia coli*	-	-	-
2.	*Aeromonas hydrophila*	-	-	-
3.	*Staphylococcus aureus*	-	-	+
4.	*Pseudomonas aeruginosa*	-	-	-
5.	*Enterococcus faecalis*	-	-	+
6.	*Enterobacter aerogenes*	-	-	-
7.	*Micrococcus luteus*	-	-	-
8.	*Salmonella typhi*	-	-	-
9.	*Bacillus subtilis*	-	-	-
10.	*Bacillus megaterium*	-	-	-
	Fungal pathogens			
1.	*Aspergillus niger*	-	-	+
2.	*Aspergillus flavus*	-	-	-
3.	*Penicillium* sp	-	-	-
4.	*Trichoderma* sp	-	-	-
5.	*Trichothecium* sp	-	-	-
6.	*Hormodendrum* sp	-	-	-
7.	*Rhizophus* sp	-	-	-

control WSSV in *P. monodon*[18]. Fucoidan from *T. decurrens* fed to the WSSV infected shrimps reduced the mortality rate in both groups (5– 8 and 12 – 15 g) of the experiment. Mechanism of inhibition of WSSV is unclear. Fucoidan from *C. okamuranus* was also reported to control WSSV in *P. japonicus*[18,22] but the mechanism of the fucoidan extracted from *C. okamuranus* were not disclosed.The 26.52% sulfate of the Fucoidan from *T. decurrens* inhibited WSSV and the sulfate content effects on antiviral (WSSV) activity as previously proposed by Witvrouw & De Clercq[23] therefore, thelower dose may be manipulated by hypersulfation of the fucoidan, or the fucoidan from other sources with a higher content of sulfate are needed.

The cell extracts and active constituents of various algae have been shown to have antibacterial activity *in vitro* against Gram-positive and Gram-negative bacteria[24]. Fucoidan from *T. decurrens* showed antibacterial activity against *S. aureus* and *E. faecalis* and in antifungal activity *A.niger* with minimum zone of inhibition. The polysaccharide extracted from algal species showed a high content of sulfate, but the sulfate content did not show a clear relation to the antimicrobial activity. In contrast, Berteau and Mulloy[2] reported that the antimicrobial activity of the polysaccharides was related to their chemical structure and ester sulfate groups. On the other hand, several species of brown seaweed that have a high sulfate content have been reported to have antimicrobial activities[25,26]. Fucoidan from macro algae possess antibacterial activity[18,27]. Crude fucoidan from brown algae *Sargassum polycystum* at 12 mg/ml inhibited the growth of *V. harveyi*, *S. aureus* and *E.coli* which was the supportive evidence for the present study and the concentrations were higher than the present study.

Conclusion

In future Fucoidan will be as one of the immunostimulant which increases phagocytic activity of the shrimps. From this study it was concluded that Fucoidan from *T.decurrens* can be used as the immunostimulant and as in feed formulation to control WSSV in culture ponds.

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