Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.1.20132013J2

Effect of fucoidan from *Turbinaria ornata* against marine ornamental fish pathogens

Thangapandi Marudhupandi*, Thipramalai Thankappan Ajith Kumar

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, India

PEER REVIEW

Peer reviewer

Dr. N. B. Dhayanithi, Postdoctoral Fellow, Centre for Biotechnology, Anna University, Chennai-600025, Tamil Nadu, India.
Tel: +91-9965012329
Fax: +91-44-22350299
E-mail: microbiodhaya@gmail.com

Comments

The research is certainly innovative, and has the potential to be a valuable means of disease control in aquaculture. The authors have clearly demonstrated the methodology. The author's effort of confirmation of fucoidan through FTIR and NMR analysis is appreciable. However, the authors suggested that further *in vivo* study is required to get the mode of action of fucoidan against the bacterial pathogens.

Details on Page 286

ABSTRACT

Objective: To evaluate the antimicrobial capacity of fucoidan from the brown seaweed *Turbinaria ornata* against the marine ornamental fish bacterial pathogens.

Methods: Fucoidan was isolated by the ethanol extraction method and the functional groups were identified by Fourier Transform Infrared Spectroscopy analysis. Subsequently, structural characterization was done by 1H NMR analysis. *In vitro* antibacterial activity of fucoidan was performed by the agar plate diffusion method and Minimum Inhibitory Concentration.

Results: The characteristic C-O-S bending vibration of sulfate substituent the axial C-4 was observed at 839 cm^{-1}. Characterisitic signal of the fucoidan was detected in a different ppm of 1H NMR analysis. The maximum antibacterial activity (16.23±0.11) mm was obtained for *Vibrio parahaemolyticus* and the minimum activity (5.1±0.24) mm was recorded for *Yersinia enterocolitica*. The Minimum Inhibitory Concentration value was recorded between 2.5 to 10 mg/mL to the respective pathogens.

Conclusions: The present study proved that fucoidan possessed the significant antibacterial activity against the tested fish bacterial pathogens. It could be further used as a natural antibiotic in an aquaculture system to control the bacterial diseases. However, the present study suggested that the further *in vivo* study is required to get the better understanding of the mode of action of fucoidan.

KEYWORDS

Turbinaria ornata, Fucoidan, Antimicrobial activity, Fish bacterial pathogens, FTIR, NMR

1. Introduction

The marine ornamental fishes have great economic potential and the annual trade in live marine ornamentals has been estimated between USD 28 million to USD 44 million globally[1,2]. The bacterial diseases have been causing severe economic losses in the cultured marine ornamental fishes[3]. Seaweeds are considered as the important medicinal plants. It provides varieties of pharmacologically important active compounds. Nowadays, using antibiotics for controlling the propagation of pathogenic bacteria in aquaculture practice have been common. Emergence of antibiotic resistant strain is a worldwide problem in human and also in fishes. The research on the line continues due to the absence of a more effective and safer use of antibiotics. There is now a greater interest in marine seaweeds especially in polysaccharides. Utilization of seaweed polysaccharides as therapeutic agents and for manufacturing antibiotics has attracted the attention of scientists in recent years due to less toxic and effective

*Corresponding author: Thangapandi Marudhupandi, Marine ornamental fish hatchery division Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608502, India.
Tel: +91 8870366413
E-mail: tmarudhu@gmail.com

Fundation Project: Supported by the scheme of Centre with Potential for Excellence in Particular Area (CPEPA) [Grant No: F.No. 8-2/2008 (NS/PE) December 2011].

Article history:
Received 28 Aug 2013
Received in revised form 11 Sep, 2nd revised form 16 Sep, 3ed revised form 18 Sep 2013
Accepted 20 Oct 2013
Available online 28 Nov 2013

biological activities. The immunostimulant effect of fucoidan against white spot syndrome virus was in *Penaeus monodon* studied by Immanuel *et al*[4].

Fucoidan-polysaccharide contain L-fucose and sulfate is the major chemical constituent and minor amount of other sugar molecules and it exhibited various biological activities[5]. Antibacterial activity of fucoidan was poorly studied. From these accomplishments, the present study was carried out to evaluate the antibacterial effect of fucoidan against selected fish bacterial pathogens from brown seaweed *Turbinaria ornata* (*T. ornata*). The fourier transform infrared spectroscopy (FTIR) and 1H NMR study were used for confirmation of fucoidan from this alga.

2. Materials and methods

2.1. Isolation and purification of fucoidan

The brown seaweed *T. ornata* was collected from the Mandapam coast of Tamilnadu, India. The collected seaweed was washed well enough. Then the washed seaweeds were shadowed dried under room temperature, pulverized in a blender and sieved. The extraction of fucoidan was performed by the method of Yang *et al*[6]. Milled seaweed of about 20 g was treated with a liter of ethanol and stirred with a mechanical stirrer for about 12 h at room temperature in order to remove proteins and pigments. After washing with acetone, centrifugation was done at 5 000 r/min for 10 min. The residue obtained was allowed to dry at room temperature. After well drying, a biomass 5 g was taken and extracted in 100 mL of distilled water at 65 °C with continued stirring for 1 h. The extraction was done twice and the extracts were pooled and centrifuged at 15 000 r/min for 10 min. The collected supernatant was mixed well with 1% $CaCl_2$ and the solution was stored overnight at 4 °C to facilitate the precipitation of alginic acid. The solution was then centrifuged at 15 000 r/min for 10 min and the supernatant was collected. Ethanol (99%) was added into the supernatant in order to arrive upon the final ethanol concentration of 30% and the solution was placed at 4 °C for 4 h. The obtained solution was centrifuged at 15 000 r/min for 10 min. Ethanol (99%) was added to the collected supernatant to arrive upon the final ethanol concentration of 70% and the solution was placed on 4 °C overnight. The intact fucoidan was then obtained through filtration of the solution with a nylon membrane (0.45 μm size) and the product was washed in ethanol (99%) and acetone. A purified fucoidan was obtained after these procedures. Fucoidan yield was estimated based on the dried biomass obtained after the treatment of the milled sample with 85% ethyl alcohol as a percentage of algal dry weight, (% dry weight). Then obtained intact fucoidan was further purified by ion exchange chromatography method[7]. Briefly, 100 mg of intact fucoidan was dissolved in 10 mL of distillled water applied to a coloumn (3.5×50 cm) of diethyl-aminoethanol-cellulose. Pre equilibrated with water, flow rate at 2 mL/min (pH 7.0 adjusted with 0.1 N NaOH). It was continued till the pH at the outlet matches with the pH of equilibration of water and stepwise elution with distilled water by passing NaCl in increasing concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mol/L) solution in turn at a flow rate of 1 mL/min. Fractions were collected and the optical density was measured at 490 nm, until no more carbohydrate was detected. Each fraction was assayed for carbohydrates by phenol-sulfuric acid method[8]. Carbohydrate-positive fractions were pooled together, dialyzed for 24 h in distilled water, and lyophilized.

2.2. Hydrolysis of fucoidan

In order to convert the polysaccharide into monosaccharide, the purified fucoidan was subjected for hydrolysis. For this, 20 mg of purified fucoidan was hydrolyzed with 2 mol/L H_2SO_4 at 100 °C for 30 min. The hydrolyzed material was neutralized with 6 mol/L NaOH, then it was freeze dried and kept for further analysis[4].

2.3. Chemical analysis

Fucose was estimated by the phenol-sulphuric acid method by Dubois *et al*.[8] using L-fucose as standard. Sulfate content was determined according to Hou *et al*.[9] using sodium sulfate as standard.

2.4. FTIR analysis

Infrared analysis was performed using Shimadzu FTIR 8 300. KBr pellet was prepared by mixing 1 mg of the fucoidan with 100 mg of anhydrous potassium bromide. The spectra were recorded from 500 to 4 000 cm^{-1}.

2.5. NMR analysis

Fucoidan, sulphated-polysaccharides was dissolved in 0.5 mL Deuterium oxide and the proton number was identified and confirmed by 1H NMR experiments using a Bruker Biospin Avance 400 NMR spectrometer (1H frequency ¼ 400.13 MHz) at 298 K using 5-mm broad band inverse probe head equipped with shielded z-gradient and XWIN-NMR software version 3.5 using TMS as an internal reference. One-dimensional 1H spectra were obtained using one pulse sequence.

2.6. Antibacterial assays

2.6.1. Bacterial culture

Fish bacterial pathogens such as *Aeromonas hydrophila*, *Enterobacter* sp., *Pseudomonas aeruginosa* (*P. aeruginosa*), *Streptococcus* sp., *Escherichia coli* (*E. coli*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio alginolyticus* (*V. alginolyticus*), *Vibrio cholerae*, *Yersinia enterocolitica* (*Y. enterocolitica*) and *Proteus* sp., were obtained from the microbiology laboratory of the marine ornamental fish hatchery, CAS in Marine biology, Annamalai University. The concentration 10^7 CFU/mL was used in this study.

2.6.2. Screening of antibacterial activity

The antibacterial activity of fucoidan was performed by agar plate diffusion assay. Petri plates containing Muller-Hinton's agar medium was prepared in sterilized water. Then 0.1 mL of test organisms were taken from the stock broth and swabbed on agar medium by using sterilized buds. Then the wells 6 mm made on the agar plates by using sterilized well cutter. Fucoidan 20 mg/mL fucoidan was prepared, and 20 mg/mL of tetracycline was used as a positive control. Then it was added to the respective wells by using sterilized pipette. Then the plates were incubated at 37 °C for 24 h. The antibacterial activity of the test fucoidan was observed through zone of inhibition (in mm) on the plates. Each assay was performed in triplicate.

2.6.3. Determination of minimum inhibitory concentrations (MIC)

The MIC was tested in the listed strains (Table 1). Equal volumes of each bacterial strain culture, were applied to Muller-Hinton's broth with different concentration of fucoidan in the test tubes ranging from 10, 5, 2.5, 1.25, 0.625, 0.312 5, 0.156 2 and 0.078 13 mg/mL, respectively. Whereas, control was prepared without fucoidan. These serially diluted cultures were then incubated at 37 °C for 24 h. After the incubation period, turbidity was observed. MIC was defined as the lowest concentration of fucoidan that completely inhibited the visible growth of the test microorganisms.

Table 1
Screening of antibacterial activity of fucoidan aginst marine ornamental fish pathogens.

Bacteria	Zone of inhibition (mm)		MIC (mg/mL)
	Fucoidan (20 mg/mL)	Positive control (20 mg/mL)	
Aeromonas hydrophila	7.04±0.25	28.00±0.20	10.0
Enterobacter sp.	5.70±0.17	27.00±0.13	10.0
P. aeruginosa	7.60±0.12	28.00±0.26	5.0
Streptococcus sp.	6.20±0.27	25.00±0.22	10.0
E. coli	5.83±0.15	28.00±0.27	10.0
V. parahaemolyticus	16.23±0.11	29.00±0.13	2.5
V. alginolyticus	15.60±0.36	30.00±0.21	10.0
Vibrio cholerae	11.30±0.41	29.00±0.24	10.0
Y. enterocolitica	5.10±0.24	27.00±0.15	10.0
Proteus sp.	5.60±0.29	27.00±0.32	10.0

2.7. Statistical analysis

Unless stated otherwise, all experiments were performed in triplicate. The data were expressed as mean±SD. Statistical analysis was done by origin 6.1 version software.

3. Results

The yield of extracted fucoidan was (4.27±0.33)% and it contains the (66.86±0.23)% of fucose, (19.47±0.28)% of sulphate respectively. The IR band was obtained at 2 927 cm^{-1}. It indicated that the presence of CH stretching of pyranoid ring and C-6 group of fucose and galactose units. The characteristic IR band was observed at 839 cm^{-1}, caused mainly due to the C-O-S bending vibration of sulfate substituent the axial C-4 position. The strong IR bands were observed in the region of 1 120 and 1 045 cm^{-1}, was due to the presence of C-C and C-O stretching vibrations in the pyramid ring and C-O-C stretches of the glycosidic bonds. The IR band at 1 255 cm^{-1} was attributed to the presence of asymmetric O=S=O stretching vibration of sulfate esters with some contribution of COH, CC and CO vibrations (Figure 1).

In the present study, 1H NMR spectrum was given in Figure 2.

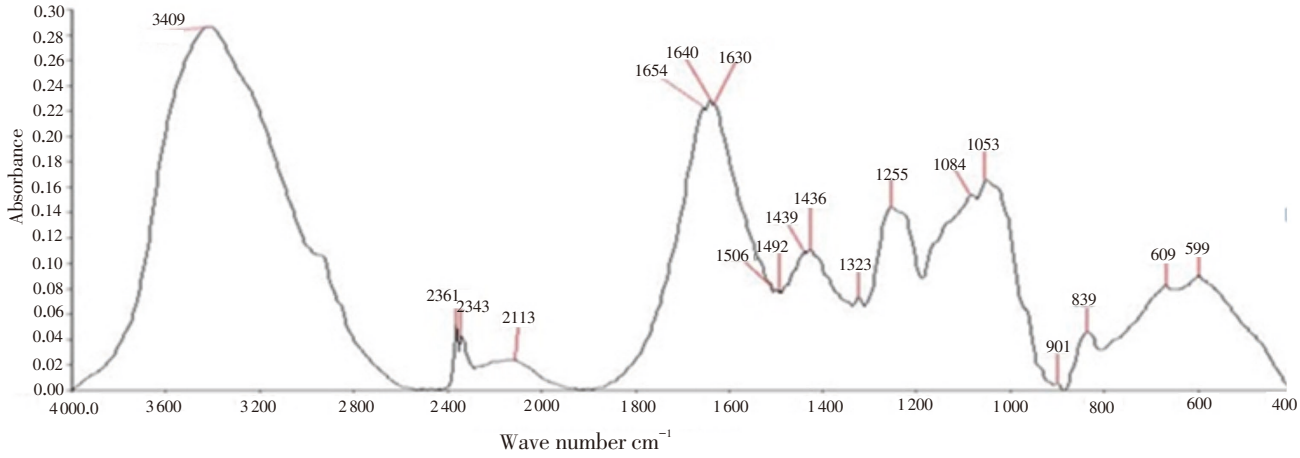

Figure 1. FTIR analysis of fucoidan from *T. ornata*.

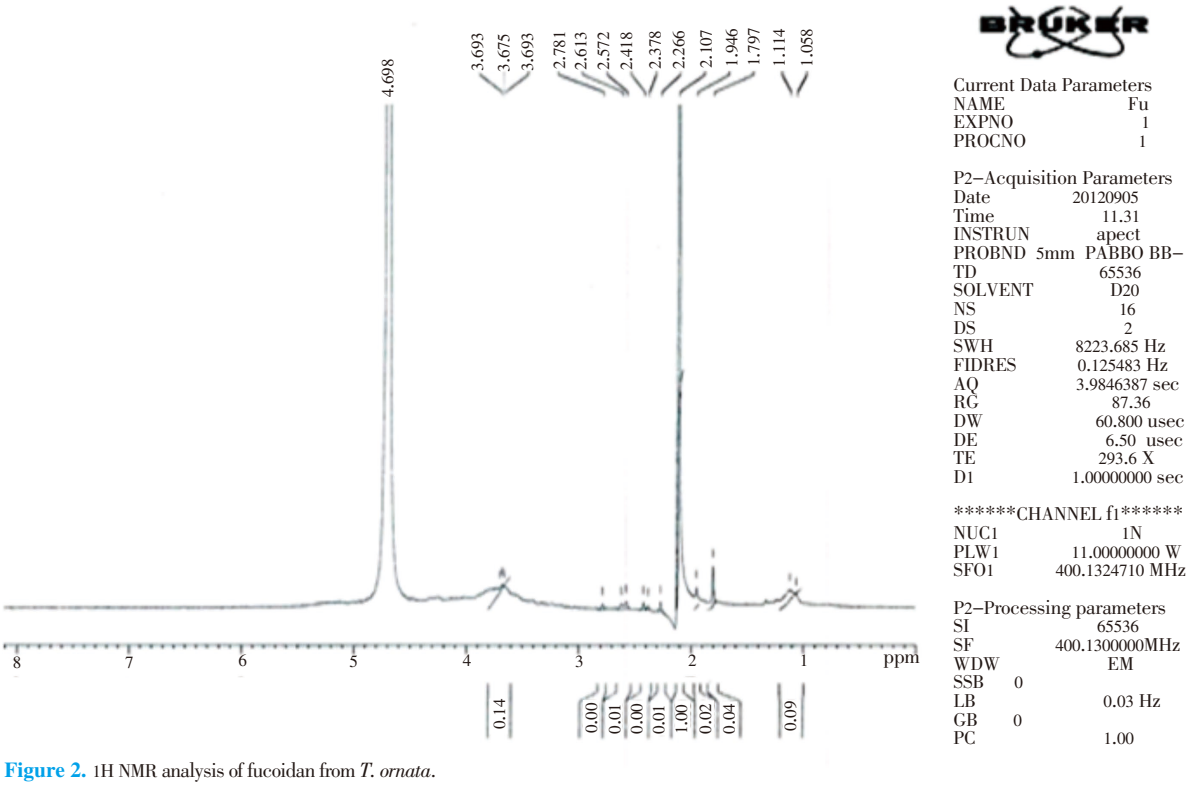

Figure 2. 1H NMR analysis of fucoidan from *T. ornata*.

The signals at 1.797 ppm at 1[H] indicated the presence of alkyl at sulfonyl attached proton and 3.5 to 4.5 ppm assigned at methoxy attached proton at H-4 position. The signal at 4.698 ppm at 1[H] was attributed to the presence 3-linked d-galactopyranosyl.

For the present study, antibacterial effect of fucoidan was performed against the fish bacterial pathogens by agar well diffusion method. The result was displayed in Table 1. The maximum activity (16.23±0.11) mm was obtained for *V. parahaemolyticus* and the minimum activity (5.1±0.24) mm was obtained for *Y. enterocolitica*. The MIC of fucoidan was displayed in Table 1. The MIC of was recorded between 2.5 to 10.0 mg/mL to the respective pathogens.

4. Discussion

In recent years the utilization of the fucoidan as a natural medicine for treating the pathogens in aquaculture has been studied by various authors[4,10,11]. The major functional group of the fucoidan was validated by FTIR. In this study, the C-O-S bending vibration of sulfate substituent the axial C-4 was observed at 839 cm^{-1}. The IR band in 836-840 cm^{-1} which is corresponding to the characteristic of C-O-S stretching of sulfate group of fucoidan. In this study, fucoidan was validated by the 1H NMR and peaks were obtained at 1.797, 3.5 to 4.5 and 4.698 ppm respectively. It showed that the fucoidan from *T. ornata* contains the sulphate, fucose and D-galacton. In agreement with present NMR result, Ale *et al.*[12] also reported the presence of 3-linked d-galactopyranosyl unit in fucoidan.

In this study, fucoidan showed the antimicrobial effect against the bacterial pathogens. In agreement with the present study, Chotigeat *et al.*[13] proved that the crude fucoidan from *Sargassum polycystum* showed the activity against the bacterial pathogens such as *Vibrio harveyi*, *Staphylococcus aureus* (*S. aureus*) and *E. coli*. Similarly, Lee *et al.*[14] reported that the synergistic effect of fucoidan with antibiotics against oral pathogenic bacteria. In addition, Pierre *et al.*[15] also reported the antimicrobial activity of sulfated polysaccharides from *Chaetomorpha aerea* aginst the several human bacterial pathogens such as *S. aureus*, *Salmonella enteretidis*, *P. aeruginosa*, *Enterococcus faecalis*, *Bacillus subtilis*, *Micrococcus luteus* and *Candida glabnata*. Moreover, in the present study, it was showed that the fucoidan possessed significant antibacterial effect against *Vibrios* pathogens such as *V. parahaemolyticus* [(16.23± 0.11) mm] and *V. alginolyticus* [(15.6±0.36) mm]. It was suggested that the fucoidan-polysaccharide would be used as a natural antibiotic treatment to vibrios in aquaculture practice. In addition, the present study, suggested that the (19.47±0.28)% of sulphate content in the sample may be the reason for its antibacterial activity. However, further study is required to investigate the antibacterial activity of the sulfate content of fucoidan from some other seaweeds. In conclusion, the results of the present study provide the good reason for assessment of fucoidan from brown seaweed, *T. ornata* as an option to antibiotics to control bacterial diseases in aquaculture practice. The present results suggest that further *in vivo* challenging study is needed against the principal bacterial pathogens in future blooming aquaculture.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to the authorities of CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University for providing the facilities. The first author extending his sincere thanks to University Grant Commission (UGC), New Delhi, India for its financial support under the scheme of Centre with Potential for Excellence in Particular Area (CPEPA). The grant No is: F.No. 8-2/2008 (NS/PE) December 2011.

Comments

Background

The demand of marine ornamental fishes in the worldwide market has been increasing due to their attractive colors. Using antibiotics to treat diseases is not advisable because of creating resistant bacteria. Therefore this research is good trial to overcome this problem in future.

Research frontiers

The present research work depicts antibacterial potential of fucoidan against the marine ornamental fish pathogens. For that, the fucoidan-polysaccharide was isolated from the seaweed and purified. Further the fucoidan was characterized by FTIR and NMR spectroscopy and the antibacterial activity of the fucoidan was analyzed.

Related reports

Chotigeat *et al*.[13] reported that the fucoidan showed the activity against *V. harveyi*, *S. aureus* and *E. coli*. Similarly, Lee *et al*.[14] also reported that the synergistic effect of fucoidan with antibiotics against oral pathogens. Recently, Pierre *et al*.[15] reported antimicrobial activity of sulfated polysaccharides from *Chaetomorpha aerea* against the several human bacteria.

Innovations and breakthroughs

Based on the literature survey, this is the first kind study demonstrated the antibacterial activity of fucoidan from *T. ornata* against the marine ornamental fish pathogens. Fucoidan showed the considerable antibacterial activity against the vibriosis.

Applications

The results of the study suggested that the fucoidan from brown seaweed, *T. ornata* as an option to antibiotics to control bacterial diseases in aquaculture practice.

Peer review

The research is certainly innovative, and has the potential to be a valuable means of disease control in aquaculture. The authors have clearly demonstrated the methodology. The author's effort of confirmation of fucoidan through FTIR and NMR analysis is appreciable. However, the authors suggested that further *in vivo* study is required to get the mode of action of fucoidan against the bacterial pathogens.

References

[1] Dhayanithi NB, Ajith Kumar TT, Kathiresan K. Effect of neem extract against the bacteria isolated from marine fish. *J Environ Biol* 2010; **31**: 409-412.

[2] Pomeroy RS, Pido MD, Pontillas JFA, Francisco BS, White AT, Leon EMCP, et al. Evaluation of policy options for the live reef food fish trade in the province of Palawan, Western Philippines. *Mar Policy* 2008; **32**: 55-65.

[3] Dhaneesh KV, Ajith Kumar TT, Vinoth R, Shunmugaraj T. Influence of brooder diet and seasonal temperature on reproductive efficiency of clownfish *Amphiprion sebae* in captivity. *Recent Res Sci Technol* 2011; **3**(2): 95-99.

[4] Immanuel G, Sivagnanavelmurugan M, Marudhupandi T, Radhakrishnan S, Palavesam A. The effect of fucoidan from brown seaweed *Sargassum wightii* on WSSV resistance and immune activity in shrimp *Penaeus monodon* (Fab). *Fish Shellfish Immunol* 2012; **32**(4): 551-564.

[5] Li B, Lu F, Wei X, Zhao R. Fucoidan: structure and bioactivity. *Molecules* 2008; **13**: 1671-1695.

[6] Yang C, Chung D, You SG. Determination of physicochemical properties of sulfated fucans from sporophyll of *Undaria pinnatifida* using light scattering technique. *Food Chem* 2008; **111**: 503-507.

[7] Marudhupandi T, Ajith kumar TT, Lakshmana Senthil S, Nandini Devi K. *In vitro* antioxidant properties of fucoidan fractions from *Sargassum tenerimum*. *Pakistan J Biol Sci* 2013; doi: 10.3923/pjbs.2013.

[8] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956; **28**(3): 350-366.

[9] Hou Y, Wang J, Jin WH, Zhang H, Zhang QB. Degradation of *Laminaria japonica* fucoidan by hydrogen peroxide and antioxidant activities of the degradation products of different molecular weights. *Carbohydr Polym* 2012; **87**: 153-159.

[10] Kitikiew S, Chen JC, Putra DF, Lin YC, Yeh ST, Liou CH. Fucoidan effectively provokes the innate immunity of white shrimp *Litopenaeus vannamei* and its resistance against experimental *Vibrio alginolyticus* infection. *Fish Shellfish Immunol* 2013; **34**: 280-290.

[11] Traifalgar RF, Serrano AE, Corre V, Kira H, Tung HT, Michael FR, et al. Evaluation of dietary fucoidan supplementation effects on growth performance and vibriosis resistance of *Penaeus monodon* postlarvae. *Aquaculture Sci* 2009; **57**(2): 167-174.

[12] Ale MT, Maruyama H, Tamauchi H, Mikkelsen JD, Meyer AS. Fucose containing sulfated polysaccharides from brown seaweeds inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 *in vitro*. *Mar Drugs* 2011; **9**: 2605-2621.

[13] Chotigeat W, Tongsupa S, Supamataya K, Phongdara A. Effect of fucoidan on disease resistance of black tiger shrimp. *Aquaculture* 2004; **233**: 23-30.

[14] Lee KY, Jeong MR, Choi SM, Na SS, Cha JD. Synergistic effect of fucoidan with antibiotics against oral pathogenic bacteria. *Arch Oral Biol* 2013; **58**: 482-492.

[15] Pierre G, Sopena V, Juin C, Mastouri A, Graber M, Maugard T. Antibacterial activity of a sulfated galactan extracted from the marine alga *Chaetomorpha aerea* against *Staphylococcus aureus*. *Biotechnol Bioprocess Eng* 2011; **16**: 937-945.