Sakthivel et al., J Mar Biol Oceanogr 2015, 4:2
http://dx.doi.org/10.4172/2324-8661.1000144

Journal of Marine Biology & Oceanography

Research Article

A SCITECHNOL JOURNAL

Immunostimulatory Effects of Polysaccharide Compound from Seaweed *Kappaphycus alvarezii* on Asian seabass (*Lates calcarifer*) and it's Resistance against *Vibrio parahaemolyticus*

Sakthivel M[1], Deivasigamani B[1]*, Rajasekar T[2], Kumaran S[3] and Alagappan KM[1]

Abstract

Kappaphycus alvarezii is a red seaweed rich in polysaccharides. In the present study an attempt was made to isolate polysaccharide from *K. alvarezii* and also tested for immunostimulatory effects on Asian seabass (*Lates calcarifer*) using *Vibrio parahaemolyticus* as a test pathogen. Two sets of experiments were designed as mixed with fed diets and intraperitoneal (IP) injection of the crude extracts. Every five days of interval, the non specific immune responses were analysed by the parameters like WBCs count, Lysozyme activity and NBT assay. After 15 days of treatment, the animals were challenged against *V. parahaemolytcius* and cumulative survival rates were recorded. The total white blood cells were decreased in intraperitoneal injection of polysaccharides extract after 5th day. But in fed diet cells count were significantly increased from 1st to 15th day. Fish fed diet with polysaccharide extract improved the lysozyme level than IP injection, which reduced simultaneously over the period compared with control. In the present study, the respiratory burst activity significantly enhanced in the fed diet polysaccharide extract in Asian seabass (*Lates calcarifer*) by oral administration and the highest activity reached in 0.5% fed diet on 15th day. After challenge with *V. parahaemolyticus,* all treated groups showed a reduced mortality compared to the control group. The best survival rate was observed in the group polysaccharide extract treated with 0.5% by oral administration. Among the other groups, fish treated by intraperitoneal injection had a lower survival rate than the fish treated by oral administration. However, the differences between the two sets of experiment (oral administration or intraperitoneal injection) were only 10%. UV, FT-IR, ^{1}H and ^{13}C NMR spectra were carried out to characterize the polysaccharide extract. The UV spectrum of the crude extract compound was showed a major peak at 282 nm. In FT-IR spectrum, major peak was observed at 1000-1100 cm^{-1} supports the presence of ring vibrations C-O, C-O-C (polysaccharides region) and minor peak of 1647.93 cm^{-1}, which supports amide group. And based on the ^{1}H and ^{13}C NMR spectra, the presence of carbonyl carbons in the purified compound was confirmed. Findings of the present study evidenced the excellent immunostimulatory activity of polysaccharide from the seaweed *Kappaphycus alvarezii.* Further complete purification and characterization of the active polysaccharide and its field evaluation is needed to bring this seaweed polysaccharide as a promising candidate for the development of good immunostimulatory agent in the field of aquaculture.

Keywords

Seaweed; Kappaphycus alvarezii; Polysaccharides; Immunostimulants; Lates calcarifer; Immune response

Introduction

Mariculture and brackish water aquaculture have potential for increasing export earnings and for creating live hoods for the coastal communities [1]. The Asian seabass (*Lates calcarifer*), also known as barramundi, is an anadromous fish which can adapt to both freshwater and seawater environments and is widely cultured and marketed especially in Southeast Asia [2]. However, the Asian seabass is susceptible to various pathogens of parasitic, bacterial and viral origin. Bacterial diseases were recognized as a significant constraint of the development of culturing Asian seabass. A wide range of bacterial pathogens has also been described in marine fish and some have gained notoriety as major limiting factors in the development of marine aquaculture in various parts of the world.

The immunostimulants, which have been tested for application to aquaculture, include peptides like FK-565, glucan, extracts from a tunicate, chitosan, vitamin C and oligonucleotides from yeast RNA [3]. Plant derived immunostimulants such as *Ocimum sanctum* [4] azadirachtin [5], *Viscum album, Urtica dioica* and *Zingiber officinale* [6], *Astragalus radix* and *Scutellari radix* [7], and *Achyranthes aspera* [8] have been reported to enhance the immunity of fish. Polysaccharide immunostimulants, extracted mainly from plants and animals, are harmless to organisms even they deposited as residues.

From the available literature, there are no encouraging reports on immunostimulatory effects of polysaccharides from the red seaweed *Kappaphycus alvarezii* on Asian seabass (*L. calcarifer*) against the infection caused by *Vibrio parahaemolyticus.* With this view, the present study was attempted to study not only the immunostimulatory effect of polysaccharides from *Kappaphycus alvarezii* on Asian seabass against *Vibrio parahaemolyticus* infection but, also focused on extraction, purification and characterization of polysaccharide.

Materials and Methods

Collection and maintenance of fish

Asian seabass *Lates calcarifer* (35 ± 2 g; n=140) were obtained from Rajiv Gandhi Centre for Aquaculture (RGCA), Sirkazhi, Tamilnadu, India. Fishes were transported alive in plastic bags containing seawater enriched with oxygen and they were acclimated for 2 weeks in 1000 litre fiber tanks filled with UV- treated estuarine water and provided with continuous aeration using air-pumping compressors. About 50% of the water was exchanged weekly twice to remove waste feed and fecal materials. During the experiments water temperature 25-28°C; pH 7.1 and salinity 26 ± 0.05 ppt were maintained. The fish were fed with pellet feed (CP aqua company, Chennai) twice a day at a rate of 3% of their body weight.

***Corresponding author:** Deivasigamani B, Department of Marine Biotechnolgy, CAS in Marine Biology, Annamalai University, Parangipettai, Chidambaram, Tamil Nadu - 608502, India, Tel: +914144237606; Fax: +914144 243641; E-mail: b.deivasigamani@gmail.com

Received: March 24, 2015 **Accepted:** July 06, 2015 **Published:** July 10, 2015

Extraction of polysaccharide from seaweed

The seaweed, *Kappaphycus alvarezii* collected from Mandapam Coastal region, Rameshwaram, Tamil Nadu. The seaweed was washed with sterile distilled water and shadow dried for one week then it was powdered. The polysaccharide extraction was done by the method described by Miguel et al. [9] with slight modifications.

Feed preparation and experimental design

To study the innate immune responses, two sets of experiments were designed. They were intraperitoneal injection (I) and fed diet (II) with each 3 group (20 fish group^{-1}) respectively. The detailed experiments were done by adopting the following steps.

Set I (Intraperitoneal injection)

Group 1: 50 µl Phosphate buffer saline (control)

Group 2: 50 mg kg^{-1} of fish

Group 3: 100 mg kg^{-1} of fish

Set II (Fed diet)

Group 4: cornmeal oil with pellet (Control)

Group 5: 0.1% of crude polysaccharide extract with cornmeal oil and pellet

Group 6: 0.5% of crude polysaccharide extract with cornmeal oil and pellet

In the set I experiment, the polysaccharide extract was injected intraperitoneally (50 µl) with 50 and 100 mg kg^{-1} body weight of fish through 1-ml tuberculin syringe with 24 gauge needle on day 1. The corresponding control fish received 50 µl of Phosphate Buffered Saline (PBS).

In the set II experiment, fish were fed with polysaccharide extract containing 0.1 and 0.5% body weight of fish with 3% of commercial pellet and fed with twice a day. The corresponding control fish received pellet feed with cornmeal oil. Cornmeal oil (10 ml kg^{-1} feed) was used to bind the powdered seaweed extract. The prepared feed was maintained at room temperature.

The immunomodulatory activity

Collection of blood and immune organs: Fish were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24-gauge needle. For serum separation, 200 µl of blood was drawn and the whole bleeding procedure was completed within 1 minute. The blood was collected in serological tubes and stored in a refrigerator overnight. The clot was then spun down at 400 g for 10 min. The serum collected was stored in sterile eppendorf tubes at -20°C until used for assays. The immune organs, spleen and gut were collected from fish and stored in 10% formalin solution for histopathological studies.

Counting of white blood cells (WBC): To determine the total white blood cell count (WBC), a 1 in100 dilution of the blood was made in phosphate saline buffer (PBS, 0.02 M, pH 7.3). Counts were carried out using a Neubauer haemocytometer (Hawksley & Son, England) and expressed as cells ml^{-1}.

Lysozyme activity: Lysozyme activity was measured by turbidimetric assay, 0.03% lyophilized *Micrococcus lysodeikticus* in 0.05 mM sodium phosphate buffer (pH 6.2) was used as substrate. Ten microlitres of fish serum was added into 250 µl of bacterial suspension in 'U' bottom microtitre plate wells in duplicate. The reduction in absorbance at 490 nm was determined after 0.5 and 4.5 minutes of incubation at 22°C using a microtiter plate reader (VERSA$_{max}$ tunable microplate reader). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per minute.

Respiratory burst activity (NBT assay): Respiratory burst activity of blood sample was quantified by the nitroblue tetrazolium (NBT) assay, which measures the quantity of intracellular oxidative free radicals. This method was slightly modified by changing the concentration of NBT solution to 0.2%.

Histopathological examination

The spleen and gut of the control and experimented fishes were fixed in Bouin's fluid for about 24 hours, processed, embedded in paraffin wax and cut in to 5 µm thick sections using were stained by Haematoxylin and Eosin (HE) method.

Experimental infection

In the present study, the bacterial pathogen *Vibrio parahaemolyticus* (Vp1) was used as a test organism. This isolate was previously isolated from shrimp ponds and maintained as slant culture in nutrient agar medium under refrigerated conditions.

The bacterial strain Vp1 subculture was centrifuged at 1000 g for 10 minutes at -4°C. The supernatant were discarded and the bacterial pellet was washed three times and resuspended in phosphate buffered saline (PBS) at pH 7.4. The OD of the solution was adjusted to 0.5 at 456 nm which corresponded to 1× 10^{7} cells ml^{-1}. After seaweed extract treatment, fish were injected (50 µl) with *V. parahaemolyticus* (Vp1) (1×10^{7} cells ml^{-1}) on day 15.

Cumulative survival rate

The control fish received 0.2 ml of saline. Experiment Fish was infected with bacterial pathogen Vp1. Survival rate of the fish were recorded from 24 hours to 144 hours. The clinical symptoms were noted including hemorrhagic septicemia, distended abdomen and lesions on the ventral surface of the body. Relative percentage survival rate (RPS) was calculated by the following formula.

RPS=1=Percent mortality in treated group/ [Percent mortality in control group] × 100

Purification and characterization of seaweed polysaccharide

Solubility of polysaccharide: The crude polysaccharide preparation was dissolved in each 2 ml of different solvents such as water, methanol, chloroform, ethyl acetate, dichloromethane, dimethyl sulfoxide (DMSO) and n-hexane and mixed well to determine the solubility.

Thin Layer Chromatography (TLC): The crude polysaccharide was dissolved completely in chloroform/DMSO. Initial separation as done by analytical thin layer chromatography using readymade silica gel coated alumina sheet. Crude polysaccharide extract was dissolved in chloroform and spotted at the bottom of the TLC sheet using glass capillary tube. TLC was run using different solvent systems (chloroform: methanol; DMSO: methanol; hexane: ethyl acetate and ethyl acetate; acetone) in different ratio. Reagents such as iodine, $KMnO_4$ were used to visualize the spots. Single white colour spot with Rf value was observed when chloroform: methanol (9:1) used as solvent system and $KMnO_4$ as spraying reagent.

Column chromatography: Large scale purification of active fraction was carried out in glass column packed with neutral alumina as stationary phase and chloroform: methanol (9:1) as mobile phase. First the alumina powder was packed in the column using chloroform. Crude extract slurry was prepared by mixing with chloroform and small amount of neutral alumina powder. Then the slurry was added in to the column started to run using chloroform as mobile phase in order to remove the impurities. Then the polarity of the solvent was increased by adding methanol. Simultaneously TLC checked the eluted solvent fraction for the presence of compounds. One major fraction was eluted and concentrated by dryness.

Physicochemical characterization and structure elucidation

The UV spectrum was measured between 200 to 600 nm ranges with a Systronics spectrophotometer. Infra Red spectra were recorded on a Perklin Elmer, No17-1159 Fourier Transform Infra Red spectrophotometer (FTIR) using KBr pellet. ^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker ADVANCE III 500 MHz (AV500) spectrometer using tetramethylsilane (TMS) as internal standard.

Results

Throughout the experimental period 5% of the fishes were died due to environmental conditions and acclimatization period. There were no infection symptoms and mortality was observed during the experimental period.

Extraction of polysaccharide from seaweed and feed preparation

Totally 2 Kg of seaweed was collected from Mandapam Coastal region. At the end of extraction procedure about 16.25 grams of crude polysaccharide was obtained. The obtained precipitate is colourless in nature and powdery in consistency. During the seaweed extract preparation for fed diet and intraperitoneal injection, no bacterial and fungal contamination were observed through plating techniques.

The immunomodulatory activity

White blood cell (WBC) count: Feeding the fish with 0.5% of polysaccharide extract significantly increased the WBCs count than 0.1% and control throughout the experiment (Figure 1). The polysaccharide extract injected through intraperitoneal containing 100 mg kg^{-1} of fish were increased on 5th day compared with 50 mg Kg^{-1} of fish and control, but then after simultaneously decreased in count.

Lysozyme activity: The lysozyme activity was measured during the 5, 10 and 15th days of the fish fed the polysaccharide extract diets containing at 0.1 and 0.5% along with control diet fish (Figure 2). A significant increasing of plasma lysozyme activity was found in groups receiving feed containing 0.1 and 0.5% than control fish. In contrast, polysaccharide injected fish lysozyme level were decreased gradually during the experimental period as like WBC count.

Respiratory burst activity (NBT assay)

The production of intracellular oxidative radicals showed a statistically significant increase in fed diet contains 0.1 and 0.5% polysaccharide extract compared to the control (Figure 3). In the polysaccharide extract injected intraperitoneally, containing 50 and 100 mg kg^{-1} of fish were significantly increased for 10 days. However it was decreased from 11th day onwards, also in control animals.

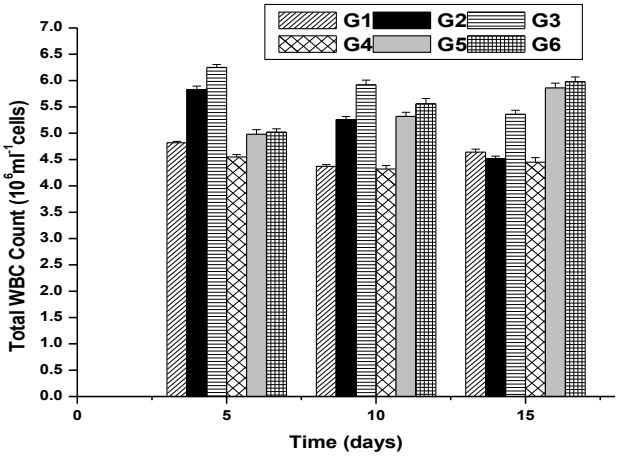


Figure 1: Total white blood cell counts of six groups with different dosage of polysaccharide extract and control diet. G1: 50 µl Phosphate buffer saline (control); G2: 50 mg kg^{-1} of fish; G3: 100 mg kg^{-1} of fish; G4: Cornmeal oil with pellet (Control); G5: 0.1% of seaweed extract with cornmeal oil and pellet and G6: 0.5% of seaweed extract with cornmeal oil and pellet on 5, 10 and 15th day of sampling.

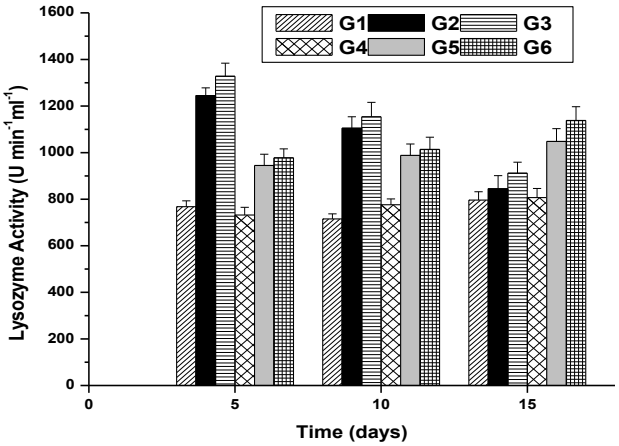


Figure 2: Lysozyme activity of six groups with different dosage of polysaccharide extract and control diet sowed the variations on 5, 10 and 15th of sampling.

Histopathology

The histopathology differences were observed in spleen and gut by fed diet. In spleen histological changes were observed by intraperitoneal injection. The spleen of seabass were reddish brown, elongated, thick and flattened structures, lying along the intestine the pulp in general consist of large venous sinuses and their branches. In this study, the 0.5% of fed diet showed more MMCs and lymphocytes than 100 mg kg^{-1} of intraperitoneal injection and control in spleen. The histopathological changes in gut showed moderate lipid content than control fishes.

Challenge test

After 15 days of seaweed polysaccharide extract treatment, fish were challenged with *V. parahaemolyticus* and cumulative survival rate was registered with 144 hours (Figure 4). The cumulative survival rate over the experimental period in the control group was 30%. All the

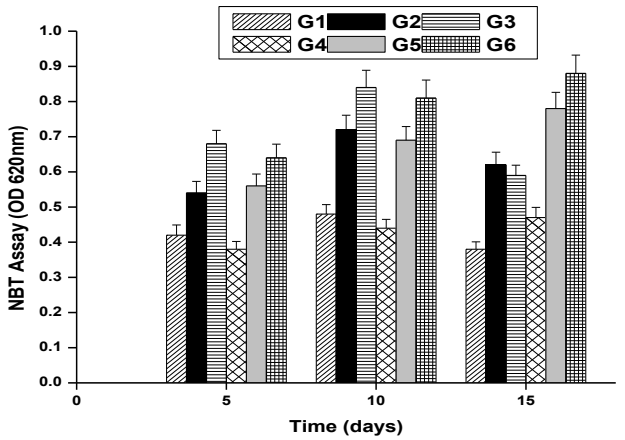


Figure 3: The respiratory burst activity of six groups with different dosage of polysaccharide extract and control diet sowed the variations on 5, 10 and 15th of sampling.

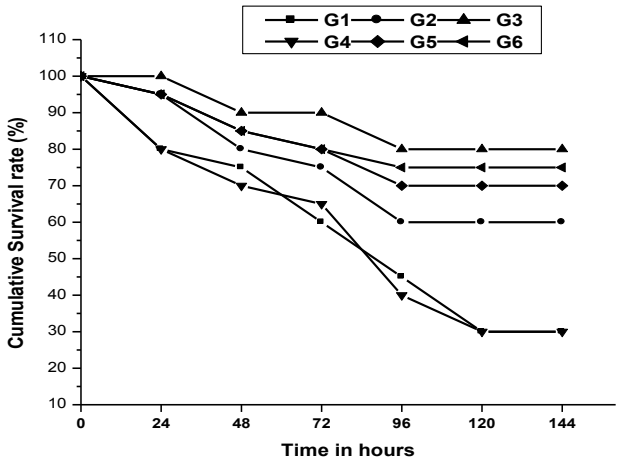


Figure 4: The Cumulative survival rate for 144 hours infected with *V. parahaemolyticus.*

other dosages of treated groups showed reduced mortality compared to the control. Cumulative survival of fish treated with 0.1 and 0.5% of polysaccharide extract reached 70 and 75% respectively. The fish injected with phosphate buffered saline in control fish showed 30% of the mortalities over the experiment. Cumulative survival rate in fish injected with polysaccharide extract at 50 and 100 mg kg^{-1} of fish reached 60 and 80% respectively.

Solubility of crude polysaccharide

Among the solvents tested, the crude polysaccharide was solubilized well in water, methanol and chloroform and DMSO but not in other solvents.

Purification of polysaccharide: In TLC purification, among the various solvent systems tested, only one fraction was observed when hexane: ethyl acetate, chloroform: methanol, DMSO: methanol and ethylacetate: acetone used as a solvent system. The purified spot appeared as brown color and pink color when iodine and $KMnO_4$ was used as a solvent. Rf value of the purified spot was calculated as 0.8.

The polysaccharide was further purified in column chromatography using chloroform methanol as solvent system. The purity of the column fraction was confirmed by TLC in which the column fraction showed the single spot with Rf value 0.8.

Characterization of the purified polysaccharide: The UV spectrum of the crude extract compound was showed a major peak at 282 nm (Figure 5). FT-IR spectrum of the crude extract compound was elucidated (Figure 6). The diagnostic peak present in the FT IR spectral data indicates the presence of the functional groups such as C-O, C-O-C and amide C=O (Table 1).

The ^{1}H NMR spectra of the purified compound date was given (Figures 7 and 8).

^{1}H NMR δ 7.99(t, J=4.5 Hz, 1H),7.28(d, J=9 Hz, 1H), 7.18(m, 1H), 6.84(m, 2H), 5.86(m, 1H), 4.94(m, 1H), 4.49(m, 1H)4.07(t, J=3.5 Hz, 1H), 3.83(t, J=15 Hz, 1H), 3.64(m, 1H), 3.25(t, J=14.5 Hz, 1H),3.03(m, 1H), 2.66(d, J=10 Hz, 1H), 2.38(s, 1H), 1.12(m, 1H). s-singlet; d – doublet; t-triplet; m- multiplet ^{13}CNMR δ 162.85, 153.18, 142.26, 130.30, 130.17, 125.81, 121.68, 117.12, 99.25, 92.91, 80.75, 73.32, 71.08, 68.79, 62.13, 20.42.

Results of the NMR spectra indicate the presence of carbonyl carbons in the purified compound.

Discussion

Enhancement of the immune system seems to be the most promising method of preventing fish diseases. The nonspecific immune system of fish is considered to be the first line of defense against invading pathogens, and is more important for fish than for mammals [10]. The major components of the innate immune system (non-specific) are macrophages, monocytes, granulocytes and humoral elements, like lysozyme or complement system [11]. Immunostimulants and adjuvants used in fish vaccines are of interest, as they offer an alternative to the drugs, and antibiotics currently used in fish culture to control disease. The nonspecific immune response is often reported as a function of macrophage activity such as phagocytosis and chemotaxis. Seaweed polysaccharides like sodium alginate, *k*-carrageenan and *i*-carrageenan have previously been reported to increase resistance against bacterial infections in teleost and shrimp [12].

Administration of red seaweed polysaccharides like *k*-carrageenan at a dose of 10-30 mg kg^{-1} or ι-carrageenan at 30 mg kg^{-1} via injection has been reported to increase resistance against bacterial infections in common carp *Cyprinus carpio* and grouper *Epinephelus coioides* [13]. Immunostimulants can be applied via injection, bathing or oral administration, the latter seems to be the most practicable [7].

In the present study fish were fed with two different concentrations through intraperitoneal injection and oral administration and we observed the enhanced activity in both concentrations. Some other reports also stated that intraperitoneal injection for triherbal solvent extract in *Carassius auratus* [14] and mixed with fed diet for *E. alba* extract in *Oreochromis mossambicus* [15]. Therefore the difference between the intraperitoneal injection and feed diet were experimentally evaluated for polysaccharide extract.

Table 1: Functional groups in FT-IR spectral data.

Wave number [cm^{-1}]	Band assignment
1040.70; 1117.81	C-O, C-O-C from polysaccharide region
1647.93	Amide I (C=O) different confirmation
3422.11	OH of water

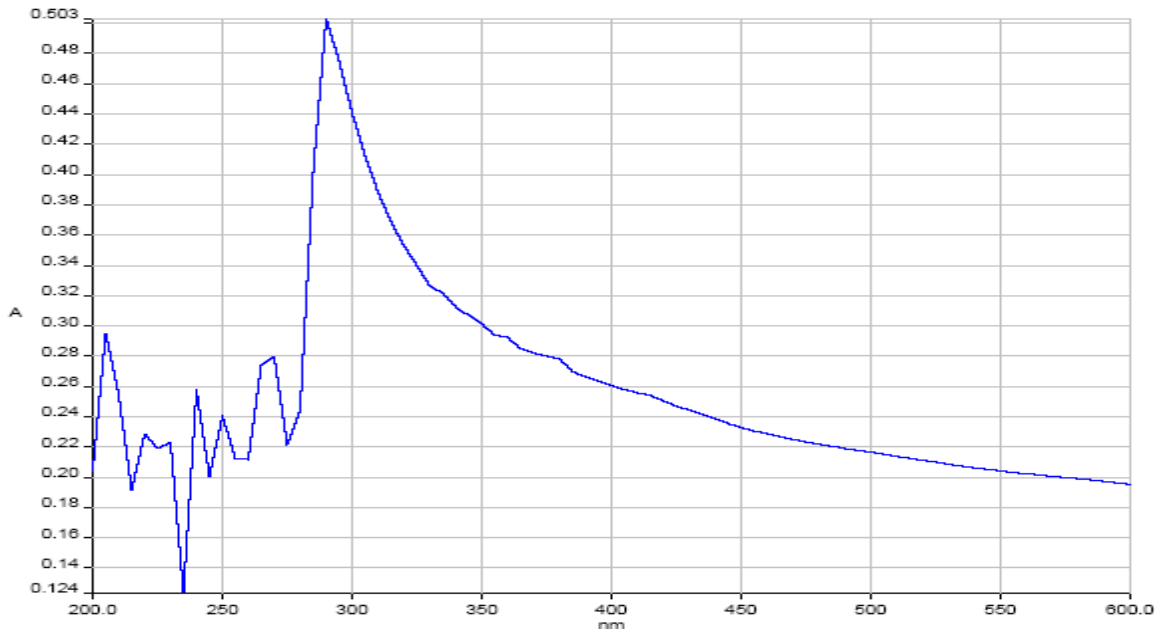


Figure 5: UV- Spectroscopy of crude polysaccharide extract.

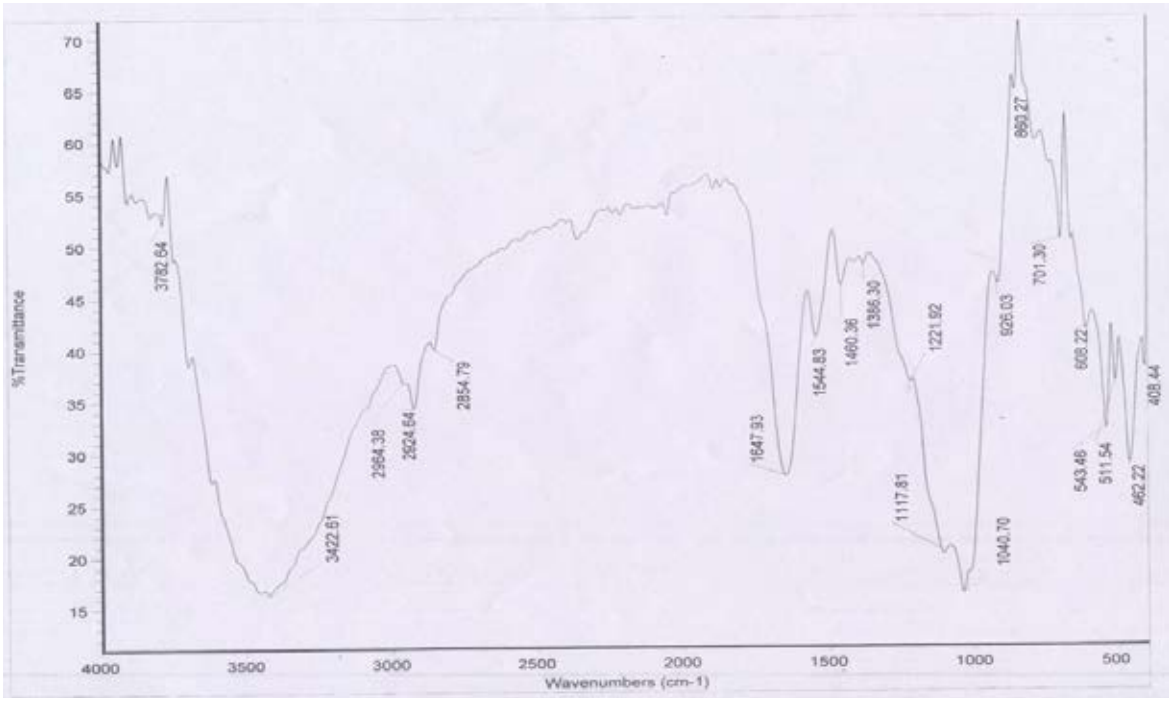


Figure 6: FT-IR analysis of crude polysaccharide extract.

White blood cell (WBC) plays an important role in the immune response in fish, particularly in inflammation [16]. In the present study, the total white blood cells were significantly increased in both fed diet and intraperitoneal injection of polysaccharide extract on Asian seabass (*L. calcarifer*). The activity of cellular immune defence system was enhanced by use of beta-glucane in the feed of salmonids and turbot. The use of high doses of vitamin C results in proliferation of rainbow trout lymphocytes. Furthermore, the white blood cells were increased in the grouper *Epinephelus malabaricus* fish fed with lipid containing diet than control fish [17]. Several studies showed that the white blood cells can be increased in infected or damaged animals. WBC and neutrophil quantities in infected samples were accepted as a response of cellular immune system to fungal infection. Palikova and Navratil [18] concluded that immune system of fish

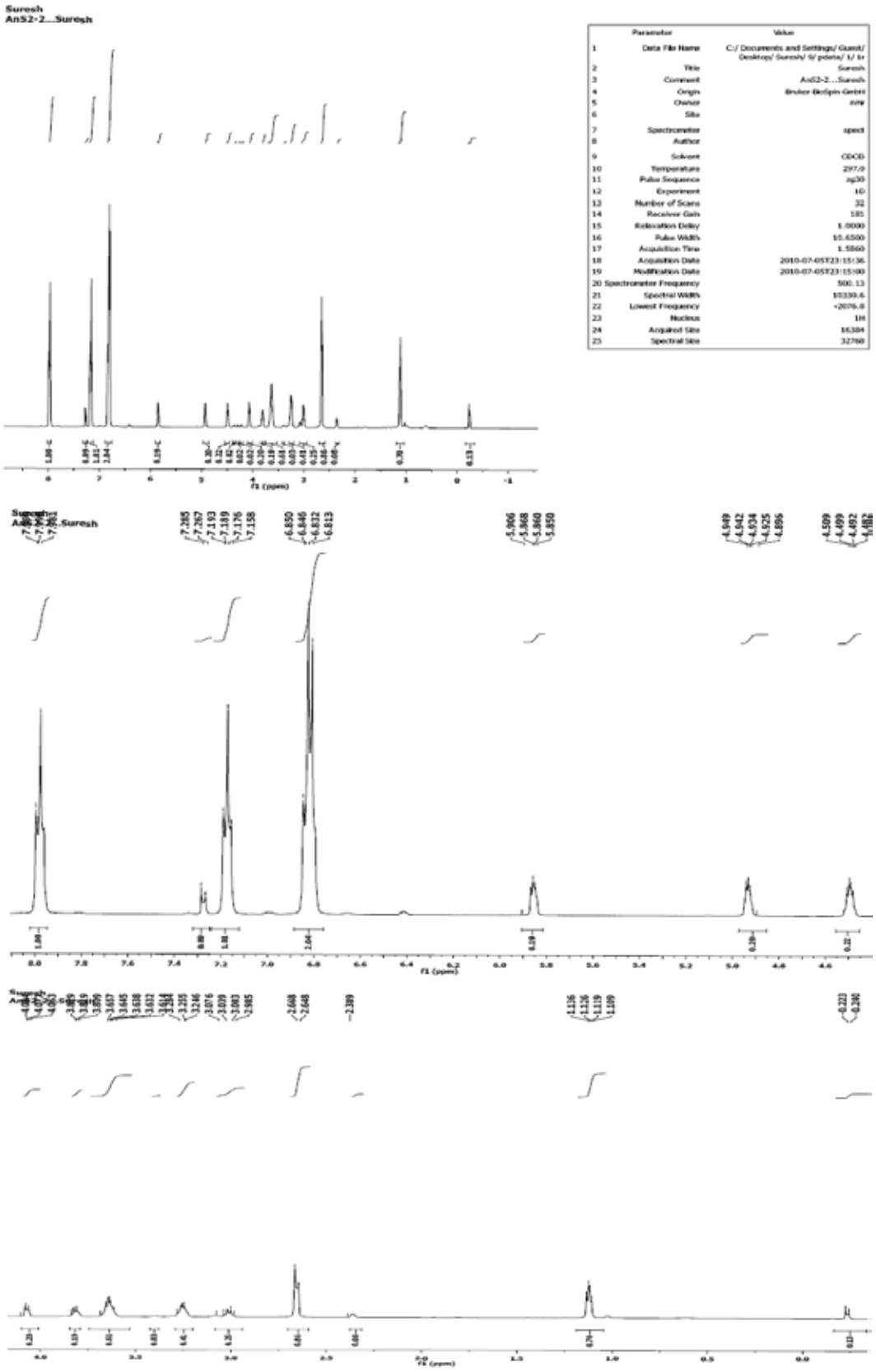
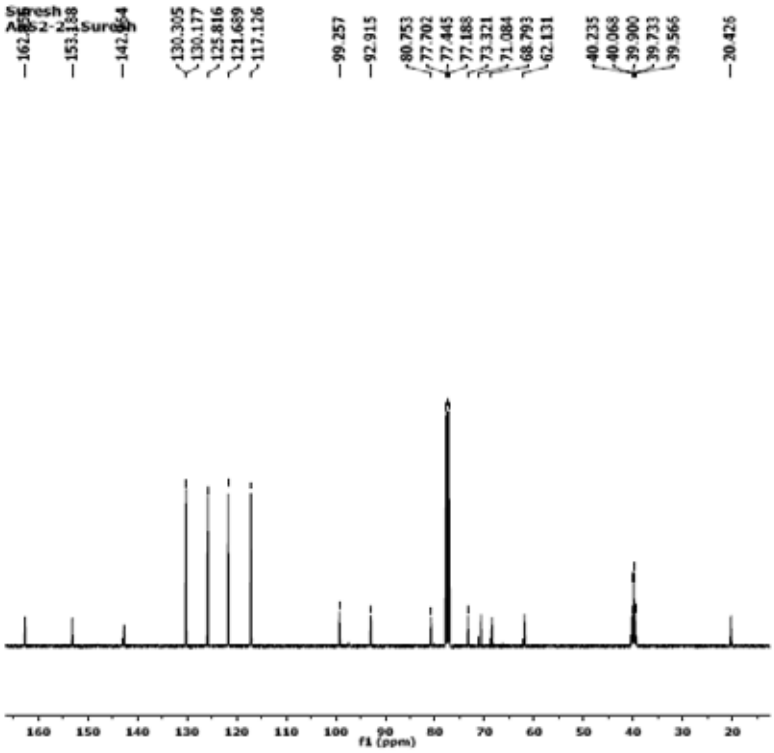


Figure 7: 1^{H} NMR Spectrum of purified polysaccharide.



	Parameter	Value
1	Data File Name	C:/ Documents and Settings/ Guest/ Desktop/ Suresh/ 10/ pdata/ 1/ 1r
2	Title	Suresh
3	Comment	An52-2...Suresh
4	Origin	Bruker BioSpin GmbH
5	Owner	nmr
6	Site	
7	Spectrometer	spect
8	Author	
9	Solvent	CDCl3
10	Temperature	298.9
11	Pulse Sequence	zgpg30
12	Experiment	1D
13	Number of Scans	1024
14	Receiver Gain	203
15	Relaxation Delay	2.0000
16	Pulse Width	7.8000
17	Acquisition Time	0.5505
18	Acquisition Date	2010-07-06T00:00:43
19	Modification Date	2010-07-06T00:00:00
20	Spectrometer Frequency	125.76
21	Spectral Width	29761.9
22	Lowest Frequency	-2305.6
23	Nucleus	13C
24	Acquired Size	16384
25	Spectral Size	32768

Figure 8: 13^{C} NMR Spectrum of purified polysaccharide.

displays similar responses to unfavorable conditions. Sahan et al. [19] reported an increase in leukocyte cells of fish infected with the parasite in a European eel, *Anguilla anguila*.

Lysozyme is a fish defense element, which causes lysis of bacteria and activation of the complement system and phagocytes by acting as opsonin [11]. Elevated lysozyme activity was noted on 20, 25 and 30 days after feeding Jian carp [20] and large yellow croaker, *Pseudosciaena crocea* [10] with traditional Chinese medicine (TCM) formulated from Astragalus root (*Radix astragalin seu heydsari*) and Chinese Angelica root (*R. angelicae sinensis*) at a ratio of 5:1 (w/w). In *Oreochromis niloticus* fed with 0.1 and 0.5% Astragalus radix root for 1 week, lysozyme activity was enhanced [7]. Dietary L-ascorbic administration at 288 mg kg^{-1} and dietary vitamin E administration at 100 mg kg^{-1} for 8 weeks have been reported to increase alternative complement and lysozyme activity of juvenile grouper *Epinephelus malabaricus* [21].

Seabass *Dicentrarchus labrax* which had been fed a diet containing sodium alginate from brown algae *Laminaria digitata* and *Ascofillum nodosum* after 15 days showed increased alternative complement and lysozyme activity [22]. In the present study, all the doses of polysaccharide extract-incorporated in the diet and injection in Asian seabass *Lates calcarifer* significantly enhanced the lysozyme activity on the 5^{th} day. Fish fed diet with polysaccharide extract improved the lysozyme level than injection which reduced simultaneously over the period compared with control. The lysozyme activity of grouper that received sodium alginate or *i*-carrageenan at 20 and 30 mg kg^{-1}, respectively, increased significantly after 24 and 72 h, but thereafter slightly decreased or returned to the original level after 120 h [23].

Phagocytes produce toxic oxygen forms during a process called respiratory burst [24]. Since superoxide anion is the first product to be released from the respiratory burst, the measurement of O^{-}_{2} has been accepted as a precise way of measuring respiratory burst [25]. In the present study, the respiratory burst activity significantly enhanced in the fed diet polysaccharide extract in Asian seabass (*Lates calcarifer*) by oral administration and the highest activity reached in 0.5% fed diet on 15^{th} day. Sajid et al. [26] reported that 250 mg kg^{-1} of dry diet levamisole showed the immunomodulatory activity in *Cyprinus carpio* on 57^{th} day than 100 and 500 mg kg^{-1} of dry diet levamisole. The respiratory burst activity of *Penaeus mondon* fed with ß-glucan gave the highest OD of 0.08 at 630 nm at the concentration of 0.2% glucan [27].

In another study, groupers (*Epinephelus* sp.) injected with sodium alginate at 20 mg kg^{-1} or *i*-carrageenan at 30 mg kg^{-1} showed increased respiratory burst activity. Similar results have been obtained in turbot *P. maximum*, and white shrimp *L. vannamei* [12]. It appears therefore that seaweed polysaccharides enhance the respiratory burst of teleost and shrimp. However in this study, the fed contain polysaccharide extract injected through i.p showed decreased respiratory burst activity after 10 days in both 50 and 100 mg kg^{-1} of fish. This difference is considered due to the source of polysaccharide extract, concentration of polysaccharide, administration method and exposure time. Generally the highest respiratory activity can be achieved by occurrence of highest reduction in NBT [27].

Most of the histopathological studies were done for identifications of the tissue and cell damages caused by the pathogens in immune organs [28]. Circulating antigens are rapidly intercepted in the spleen by activated macrophages [29]. The histological investigations of the

spleen in teleosts have been mainly focused on the compartments that are important for the defense systems of the fishes; the lymphocytes and the macrophages [30], and/or alterations in this organ caused by the presence of different toxicants in the environment [31]. In the present study, high number of MMCs and lymphocytes were observed in fish fed diet with 0.5% than other concentrations and control (figures not shown). Deivasigamani [32] stated that humoral and cell mediated immunity have the ability to endeavor immune response against sheep red blood corpuscles (SRBC) in cat fish and also the head kidney is the major antibody producing diseases. In the sections of gut region moderate lipid content were observed than control fishes.

To know our knowledge there were only few people were reported about *V. parahaemolyticus* in fish infection. Slavica et al. [33] reported that the major seafood samples contain *V. parahaemolyticus* (47.83%) than *V. alginolyticus* and *V. vulnificus* Adriatic Sea. The similar report was stated by Feldhusen [34]. After challenge with *V. parahaemolyticus,* all treated groups showed a reduced mortality compared to the control group. The best survival rate was observed in the group polysaccharide extract treated with 100 mg kg^{-1} by oral administration.

Among the other groups, fish treated by intraperitoneal injection had a lower survival rate than the fish treated by oral administration. However, the differences between the two sets of experiment (oral administration or intraperitoneal injection) were only 10%. Survival rates of infected fish are usually increased after treatment with various immunostimulants, vaccines and probiotics [35]. Feeding carp with chitosan and levamisole reduced mortality of common carp after challenge with *A. hydrophila* [36]. Alcaide et al. [37] stated that, the *V. parahaemolyticus* infection occurred in low and normal salinity (5-30 ppt) and not occurred at high salinities (45-60 ppt) in Iberian toothcarp *Aphanius iberus* fish.

In addition to the study of immunostimulatory effect of polysaccharide, an attempt has been made to purify and also to characterize the polysaccharide. From the seaweed *K. alvarezii*, the solubility of the polysaccharide in highly polar solvents such as water, methanol and others supports its mixing with aquaculture feed and easily digested when it administered orally. In TLC separation, the presence of single spot (Rf value 0.8) indicated the purity of the compound and it also supports the specificity of the protocol used for polysaccharide extraction.

The results of UV spectrum indicated the presence of conjugation moiety in the purified compound. FT-IR spectroscopy is mainly used for the detection of functional groups or specific groups of polysaccharide present in the extracted compound. In FT-IR, the presence of band at 1000-1100 cm^{-1} supports the presence of ring vibrations C-O, C-O-C from polysaccharides [38]. In the present study, the presence of intense absorption band in the polysaccharide region indicated that the purified compound is a polysaccharide.

NMR spectroscopy is most useful to study the number of protons and carbon molecules and its position of the given compound [39]. In the present study, the results of ^{1}H and ^{13}C NMR spectra indicated the presence of carbonyl group. Based on the available UV, FT-IR and ^{1}H and ^{13}C NMR data, it is very difficult to elucidate the structure of the purified polysaccharide. So Further 2D- NMR studies and X ray diffraction analysis is needed for the complete structure elucidation of the purified compound.

Findings of the present study evidenced the excellent immunostimulatory activity of polysaccharide from the seaweed *Kappaphycus alvarezii.* Further complete purification and characterization of the active polysaccharide and its field evaluation is needed to bring this seaweed polysaccharide as a promising candidate for the development of good immunostimulatory agent in the field of aquaculture.

Acknowledgement

The authors are grateful to Prof. S.T. Balasubramanian, Former Dean & Director, Faculty of Marine Sciences, CAS in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu, India for provided requisite facilities and University Grants Commission (UGC), New Delhi for their financial support during the study.

References

1. Gupta VM (2010) Challenges for the sustaining and increasing aquaculture production. J Fish Chimes 29: 13-16.
2. Divyagnaneswari M, Christybapita D, Michael RD (2008) Immunomodulatory activity of Solanum trilobatum leaf extracts in Oreochromis niloticus. Diseases in Asian Aquaculture VI: 221-234.
3. Sakai M, Taniguchi K, Mamoto K, Ogawa H, Tabata M (2001) Immunostimulant effect of nucleotide isolated from yeast RNA on carp, Cyprinus carpio L. J Fish Dis 24: 433-438.
4. Venkatalakshmi S, Michael RD (2001) Immunostimulation by leaf extract of Ocimum sanctum Linn. in Oreochromis mossambicus (Peters). J Aquac in the Tropics 16: 1-10.
5. Logambal SM, Michael RD (2000) Immunostimulatory effect of Azadirachtin in Oreochromis mossambicus (Peters). Indian J Exp Biol 38: 1092-1096.
6. Dugenci SK, Arda N, Candan A (2003) Some medicinal plants as immunostimulant for fish. J Ethnopharmacol 88: 99–106.
7. Yin G, Jeney G, Racz T, Xu P, Jun X, Jeney Z, et al. (2006) Effect of two Chinese herbs (Astragalus radix and Scutellaria radix) on non specific immune response of tilapia, Oreochromis niloticus. Aquac 253: 39-47.
8. Rao YV, Chakrabarti R (2005) Stimulation of immunity in Indian major carp Catla catla with herbal feed ingredients. Fish Shellfish Immunol 18: 327-334.
9. Miguel DN, Siumara T, Maria ERD (1999) Polysaccharides from the red seaweed Bostrychia montagnei: chemical characterization. J Appl Phycol 11: 35-40.
10. Jian J, Wu Z (2003) Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, Pseudosciaena crocea (Richardson). Aquac 218: 1–9.
11. Magnadottir B (2006) Innate immunity of fish (overview). Fish Shellfish Immunol 20: 137- 151.
12. Cheng W, Liu CH, Kuo CM, Chen JC (2005) Dietary administration of sodium alginate enhances the immune ability of white shrimp Litopenaeus vannamei and its resistance against Vibrio alginolyticus. Fish Shellfish Immunol 18: 1-12.
13. Cheng AC, Tu CW, Chen YY, Nan FH, Chen JC (2007) The immunostimulatory effects of sodium alginate and iota-carrageenan on orange-spotted grouper Epinephelus coioides and its resistance against Vibrio alginolyticus. Fish Shellfish Immunol 22: 197–205.
14. Harikrishnan R, Balasundaram C, Kim MC, Kim JS, Han YJ, et al. (2009) Innate immune response and disease resistance in Carassius auratus by triherbal solvent extracts. Fish Shellfish Immunol 27: 508–515.
15. Christybapita D, Divyagnaneswari M, Michael RD (2007) Oral administration of Eclipta alba leaf aqueous extract enhances the non-specific immune responses and disease resistance of Oreochromis mossambicus. Fish Shellfish Immunol 23: 840-852.
16. Secombes C.J (1996) The nonspecific immune system: cellular defenses. The Fish Immune System. Fish Physiology, Academic Press, San Diego, CA, USA 15: 63-103.
17. Hung YL, Shiau SY (2003) Dietary lipid requirement of grouper, Epinephelus malabaricus, and effects on immune responses. Aquac 225: 243-250.

18. Palikova M, Navratil S (2001) Occurrence of Anguillicola crassus in the water reservoir Korycany (Czech Republic) and its influence on the health condition and heamatologial indices of eels. Acta Vet Brno 70: 443-449.

19. Sahan A, Altun T, Cevik F, Cengizler I, Nevsat E, et al. (2007) Comparative Study of some Haematological Parameters in European Eel (Anguilla anguilla) Caught from Different Regions of Ceyhan River (Adana, Turkey). J Fisher Aquat Sci 24: 167-171.

20. Jian J, Wu Z (2004) Influences of traditional Chinese medicine on nonspecific immunity of Jian carp (Cyprinus carpio var. Jian). Fish Shellfish Immunol 16: 185-191.

21. Lin MF, Shiau SY (2005) Dietary L-ascorbic acid affects growth, nonspecific immune response and disease resistance in juvenile grouper, Epinephelus malabaricus. Aquac 244: 215-221.

22. Bagni M, Romano N, Finoia MG, Abelli L, Scapigliati G, et al. (2005) Short- and long-term effects of a dietary yeast b-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in seabass (Dicentrarchus labrax). Fish Shellfish Immunol 18: 311-325.

23. Ann CC, Chen YY, Chen JC (2007) Dietary administration of sodium alginate and k-carrageenan enhances the innate immune response of brown-marbled grouper Epinephelus fuscoguttatus and its resistance against Vibrio alginolyticus. Vet Immunol Immunopathol 121: 206-215.

24. Neumann NF, Stafford JL, Barreda D, Ainsworth AJ, Belosevic M (2001) Antimicrobial mechanisms of fish phagocytes and their role in host defense. Dev Comp Immunol 25: 807-825.

25. Dhayanithi NB, Ajith Kumar TT, Arockiaraj J, Balasundaram C, Harikrishnan R (2015) Dietary supplementation of Avicennia marina extract on immune protection and disease resistance in Amphiprion sebae against Vibrio alginolyticus. Fish Shellfish Immunol 45: 52-58.

26. Sajid M, Samoon MH, Singh P (2009) Immunomodulatory and Growth Promoting Effect of Dietary Levamisole in Cyprinus carpio Fingerlings Against the Challenge of Aeromonas hydrophila. Turk J Fish Aquat Sci 9: 111-120.

27. Chang CF, Su MS, Chen HY, Liao IC (2003) Dietary b-1,3-glucan effectively improves immunity and survival of Panaeus monodon challenged with white spot syndrome virus. Fish Shellfish Immunol 15: 297-310.

28. Kumaran S, Deivasigamani B, Alagappan KM, Sakthivel M, Prasad SG (2010) Isolation and characterization of Pseudomonas sp. KUMS3 from Asian seabass (Lates calcarifer) with fin rot. World J Microbiol Biotechnol 26: 359-363.

29. Taussig JM (1984) Processes in Pathology and Microbiology. (2nd Edtn), Blackwell Scientific Publications, Oxford, UK.

30. Kurtovic B, Teskerezi E, Teskerezi Z (2008) Histological comparison of spleen and kidney tissue from farmed and wild European seabass (Dicentrarchus labrax L.). Acta Adriat 49: 147-154.

31. Garcia-Abiado MA, Mbahinzireki G, Rinchard J, Lee K J, Dabrowski K (2004) Effect of diets containing gossypol on blood parameters and spleen structure in tilapia, Oreochromis sp., reared in a recirculating system. J Fish Dis 27: 359-368.

32. Deivasigamani B (2007) Structure of immune organs in edible catfish, Mystus gulio. J Environ Biol 28: 757-764.

33. Jaksic S, Uhitil S, Petrak T, Bazuli D, Karolyi LG (2002) Occurrence of Vibrio spp. in sea fish, Shrimps and bivalve molluscs harvested from Adriatic Sea. Food Control 13: 491-493.

34. Feldhusen F (2000) The role of seafood in bacterial foodborne diseases. Microbes Infect 2: 1651–1660.

35. Brunt J, Newaj FA, Austin B (2007) The development of probiotics for the control of multiple bacterial diseases of rainbow trout, Oncorhynchus mykiss (Walbaum). J Fish Dis 30: 573-579.

36. Gopalakkanan A, Arul V (2006) Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of Cyprinus carpio and control of Aeromonas hydrophila infection in ponds. Aquac 225: 179-187.

37. Khouadja S, Lamari F, Bakhrouf A (2013) Characterization of Vibrio parahaemolyticus isolated from farmed seabass (Dicentrarchus labrax) during disease outbreaks. International Aquatic research 5: 1-11.

38. Schmitt J, Flemming HC (1998) FTIR spectroscopy in microbial and material analysis. Int Biodeterior Biodegradation 41: 1-11.

39. Vanessa LC, Kawano DF, Silva DB, Carvalho I (2009) Carrageenans: Biological properties, chemical modifications and structural analysis: A review. Carbohydrate Polymers 77: 167-180.

Author Affiliations

Top

[1]*Department of Marine Biotechnology , CAS in Marine Biology, Annamalai University, Parangipettai, Chidambaram, Tamil Nadu -608502, India*
[2]*Research and Development (R&D) Centre, Centre for Drug Discovery and Development,Sathyabama University, Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai - 600 119*
[3]*Sri Sankara Arts and Science College (Affiliated to Madras University), Enathur, Kanchipuram- 631561, Tamil Nadu, India*