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Effect of Fucoidan of Brown Seaweeds on the Immuno-haematological Change and the Disease Resistance against *Aeromonas hydrophila* in Tilapia *Oreochromis mossambicus*

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A B S T R A C T

An experiment was conducted to study the influence of dietary fucoidan from brown seaweeds viz., *Padina tetrastromatica* and *Sargassum oligocystum* on the innate immunity and disease resistance of fish *Oreochromis mossambicus*. Fucoidan was supplemented with fish feed at different concentrations such as 0.1, 0.2 and 0.3% to observe hematological changes and non-specific immunological properties for a period of 60 days. The results indicated that fucoidan supplemented diet had no significant effect on hematological changes whereas, the lysozyme activity was significantly increased in the fishes fed with fucoidan of *P. tetrastromatica* (4012 ± 2198 IU ml^{-1}) on 30^{th} day and *S. oligocystum* (2412 ± 221 IU ml^{-1}) on 15^{th} day at 0.2% and 0.3% concentration, respectively. NBT activity was found to be highest at 15^{th}, 30^{th} and 45^{th} days in fishes fed with 0.1, 0.2 and 0.3% of fucoidan derived from *P. tetrastromatica*. The results stated that the fucoidan derived from *S. oligocystum* has relatively lower lysozyme and NBT activity than from *P. tetrastromatica* but higher than the control. The fish *O.mossambicus* challenged with *A. hydrophila*, feeding offucoidan extracted from the seaweeds invariably increased the survival upto 75%, 80% at 0.2 and 0.3% and 65, 70% respectively than the control.

Introduction

The native seaweeds are dominantly abundant along the coast of Gulf of Mannar but are not effectively exploited either for commercial application or human health or agriculture and aquaculture purposes. Seaweed products like k-carrageenan and liquid fertilizer have received greater demand in the market, after the culture practice of exotic red seaweed, *Kappaphycus alverazii* has witnessed a positive sign as through implementation by self-help groups rather than corporate farms (Mantri *et al.*, 2017). Among the three major groups of seaweeds, brown seaweeds contain more biological properties compared to red and green seaweeds (Seafood Plus, 2004) and specifically used to produce polysaccharides

like alginates, laminarians and fucoidan (Lee *et al.,* 2008). The Gulf of Mannar Biosphere Reserve Trust (GoMBRT), Ramanathapuram also permits the utilization of brown seaweeds available along the coast of Gulf of mannar throughout the year. The biodiversity of brown seaweeds and their seasonal abundance along the Gulf of mannar were assessed by few authors (Rao 1972; Kannan and Krishnamurthy 1978; Oza and Zaidi 2000; Rani *et al.,* 2015).

Fucoidan is a sulfated polysaccharide found in the cell walls of brown seaweeds. In recent years, researchers have identified the biological properties of Indian seaweeds for various properties such as antibacterial and antiviral against various clinical and fish pathogens (Radhika *et al.,* 2012; Maheswaran *et al.,* 2013), anti-oxidative (Chattopathyay *et al.,* 2010) and immunomodulatory properties on shrimp (Immanuel *et al.,* 2012). There are reports utilizing the herbal immunostimulants derived from terrestrial plants for aquaculture purposes (Sakai 1999). Fucoidan from brown seaweeds as an immunostimulant has received much attention recently and several studies have reported on their immunomodulatory properties (Itoh 1993; Choi *et al.,* 2005; Yeh *et al.,* 2006; Hwang *et al.,* 2010; Yang 2014).

Aeromonas hydrophila is one of the most important bacterial pathogens in freshwater as well as brackish water aquaculture systems (Karunasagar and Rosalind 1991). It causes severe detrimental effects in carp farming, which is widely practiced in India. Tilapia has become the second most popular fish in India and its farming is flourishing nowadays. It has entered the list of best-selling fish species like shrimp and salmon. There is no literature available on the effect of fucoidan on the immuno-hematological changes in tilapia cultured in controlled condition. Hence, the present study was undertaken to analyze the effect of fucoidan as dietary supplements in the tilapia, *Oreochromis mossambicus* by observing the hematological parameters to assess their immune response by examining disease resistance against pathogenic bacteria, *A. hydrophila* in the experimental culture condition.

Materials and Methods

Brown seaweeds

Two species of brown seaweeds viz., *Sargassum oligocystum* and *Padina tetrastromatica* were collected from two locations viz., Valinokkam (09˚ 13.684'N, 078˚ 47.194'E) and Hare Island (08˚ 047.254' N, 078˚ 11.884' E) of Gulf of Mannar during the year 2014. They were used to extract the fucoidan by the standard protocol described by Yang *et al.,* 2008 with slight modification. The shade dried pulverized seaweed (20 g)was treated with 1 L of 85% ethanol with constant stirring for 12 h at room temperature in order to remove proteins and pigments. The ethanol treated seaweed was washed with acetone, centrifuged at 10000 ×g for 10 min. and then dried at room temperature. The dried biomass (5g) was extracted with 100 ml of distilled water at 65°C with continuous stirring for 1h twice, and the extracts were combined. The combined extract was centrifuged at 10000×g for 20 min. and the supernatant was treated with 1% of $CaCl_2$ and kept at 4°C for overnight to precipitate the alginic acid after centrifugation at 10000×g for 20 min. and the supernatant was collected. Ethanol was added into the supernatant to obtain a final ethanol concentration of 30%, and the solution was placed at 4°C for 4h in a chill cabinet. Again, the solution was centrifuged at 10000×g for 20 min. to remove the remaining impurities as residue. Finally, ethanol was added into the supernatant to obtain a final ethanol concentration of 70%, and then placed at 4°C overnight to precipitate out the intact fucoidan. After

centrifugation at 10000×g for 15 min, the residue fucoidan was washed with ethanol and acetone, and again dried at room temperature. The yield was calculated based on the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of the obtained fucoidan (g)} \times 100}{\text{Weight of the dried biomass (g)}}$$

Preparation of pellet feed supplemented with fucoidan

Fucoidan extracted from two brown seaweeds were individually used for the preparation of pellet feed. Three types of pellet feed containing fucoidan were prepared following the procedure described by Yeh *et al.*, 2008. The basal feed procured from commercial feed company (CP Aquaculture Feed India Ltd) without fucoidan served as the control feed.

The fucoidan was added individually to the basal feed at different concentrations viz., 0.1, 0.2 and 0.3% of the total weight to obtain test feed. All the ingredients were ground in a mixer grinder and then tapioca powder (3%) and warm water as required were added to form the dough. The dough was pelletized using a laboratory model pelletizer (RKL 120) having 1mm diameter and dried in a hot air oven set at 40^0C overnight. The dried pellet feeds were stored in plastic containers at room temperature until use.

Experimental system

Tilapia (*Oreochromis mossambicus)* collected from the natural system of Pazhayakayal estuary near Thoothukudi was first acclimated in tap water for one month before the start of the experiments. Tilapia of both sexes weighing an average size of 12.5 ± 0.5cm in length and 28.5 ± 0.5g in weight were selected from the acclimatized stock and transferred into individual experimental tanks. The experiment was carried out in circular cement tanks of 0.75m diameter and containing 500 L of freshwater to stock 30 animals per tank. There were 12 tanks for three test feed and control feed trials in triplicate. The tilapia in control tank was fed with pellet feed without fucoidan. Mild aeration was provided continuously in order to maintain the optimal oxygen level. An *ad libitum* feeding regime was followed in all tanks and 25% water was exchanged daily during the experimental period of 60 days.

Collection of blood samples

Tilapia were selected randomly from each tanks after anaesthetizing them with MS-222 added at the rate of 100 mg/L to drawn blood for hematological analysis. Approximately, 0.05 ml of blood was collected from the dorsal aorta using 20-gauge needle from the selected fish from either the right or left side on the 0^{th}, 15^{th}, 30^{th}, 45^{th} and 60^{th} day of the experimental period. The temperature was maintained at 4^0C by placing the samples in refrigerator to allow complete healing of the withdrawal site. Heparin sodium (1%) was used as an anticoagulant. The first half of the collected blood was heparinized and used for the hematological and NBT activity (Nitro Blue Tetrazolium-NBT assay) and the second half of blood was not heparinized and the serum was used to assess the lysozyme activity.

Hematological parameters

Hematological parameters viz., haemoglobin, RBC and WBC were determined in heparinized blood within 2h after sampling. The blood was diluted with appropriate diluting fluids to determine the RBC and WBC counts using improved Neubauer haemocytometer and calculation was done as per the procedure of Barcellos *et al.*, 2004.

Haemoglobin content

Haemoglobin content was estimated by haemocytometer with permanent coloured glass comparison standards (Superior Marine Field, Germany) and the value was expressed as gram per deciliter (g/dl).

Red Blood Cells (RBC) count

The heparinized blood was diluted with equal volume of RBC (Hayemis) diluting fluid solution and RBC count was determined manually with haemocytometer. The total number of RBC was counted in the five RBC squares of the central large square of the chamber in duplicate. The average values having less than 15% were taken and multiplied by 10,000 to calculate the number of RBCs per microliter.

White Blood Cells (WBC) Count

The heparinized blood was diluted 100 times with WBC diluting fluid (Hi-Media)and the count was determined in haemocytometer. The WBC count from the four large squares of the chamber was counted in duplicate. The average counts having less than 15% difference were taken and multiplied by dilution factor to calculate the number of WBC per microliter.

Non-specific immune response studies

NBT reduction assay

Nitrobluetetrazolium (NBT) assay was performed following the method described by Stasiak and Baumann 1996. The heparinized blood was placed in Eppendorf tubes and the puffy coat was separated by centrifuging at 500 xg at 4^0C for 10 min. Exactly, 50µl of the puffy coat was placed into each well of a 96 wells of 'U' bottomed microliter plates (Tarson, India) and incubated at 37^0C for 1 h to facilitate adhesion of cells. The supernatant was removed and 50 µl of 0.3% NBT solution was added. After incubation at 37^0C for 1 h, NBT solution was removed. The cells were then fixed with 100% methanol, washed thrice with 70% methanol and air-dried. Then, 60 µl of 2N potassium hydroxide and 70µl diethyl sulphoxide (DMSO) were added into each well to dissolve the formazan blue precipitate. The turquoise-blue colored solution was then read in ELISA reader (BioTek) at 655 nm.

Lysozyme activity

Lysozyme activity was measured by adapting the turbidimetric method described by Parry *et al.*, 1965 with slight modification. Serum (50µl) was placed in triplicate in a 96 well plate with 50 µl phosphate buffered saline (PBS), pH 5.8. After mixing, the serum was serially diluted from one well to another. Finally, 50 µl of mixer was discarded in the last well. To each well, 125 µl of *Micrococcus luteus* (MTCC No:106) suspension (Aliquots of 15mg culture in 20ml of PSB yields 0.075% solution of *M.luteus*) was added (100mg/mlin phosphate buffer). The reduction in the absorbance at 450 nm was measured from 0 to 15 min at room temperature in an ELISA reader. The lysozyme activity was converted to lysozyme concentration using hen egg white lysozyme (Sigma, USA) as standard.

Challenge studies with *Aeromonas hydrophila*

Preparation of culture suspension

A. hydrophila (MTCC No.1739) was grown in Mueller Hinton nutrient broth (Hi Media) by placing in shaking water bath for 12 h at 20°C and centrifuged at 9,000xg for 20 min at 4°C. The supernatant was discarded and the bacterial pellet was washed thrice with PBS

(pH 7.2) and adjusted by dilution to get a concentration of $2.5x10^6$ cfu/ml using Neubauerhemocytometer

Infectivity study

In order to determine the infectivity of *A. hydrophila*, 18 numbers of tilapia of both sexes (30 ± 1.5g) were chosen for testing six concentrations of *A. hydrophila* in triplicate was injected intramuscularly. About 100µl of *A. hydrophila* suspension of each concentration viz., 10^5 to 10^{10} cfu/ml to induce septicemia in tilapia individually. At a concentration of 10^6cfu/ml of *A. hydrophila*, the mortality recorded as 50-60%, while at 10^8cfu/ml, the mortality was 90% after an incubation period of 10 days. Hence, 10^6cfu/ml was chosen further to ensure 50% survival.

Challenge experiment set up

In the previous experimental set up, upon completion of 60 days of feeding experiment, tilapia were challenged with intramuscular injection of 100µl of 12 h grown culture of the virulent *A. hydrophila* of having concentration of $2.5 \pm 0.3x10^6$cfu/ml. Total plate count was determined by hemocytometer and the total viable bacterial count was confirmed by spread plate method. Simultaneously, a negative control group was also maintained. Mortality was observed daily up to 15 days and Relative Percentage Survival (RPS) was calculated following the method of Amend 1981 given in table.6

Results and Discussion

In the present study, the biological parameters viz., temperature, pH, DO, ammonia, nitrite, nitrate, phosphate, alkalinity and hardness were analysed by the standard APHA (1995) method and the results are indicated in Table.1. The water in the tanks had pH (7.03 to 7.25), temperature (27.5 to 28.3°C), dissolved oxygen (5.52 to 6.12ml/L), hardness (135.17 to 143.7 $CaCO_3$), alkalinity (147.5 to 155.5mg/L); with nutrients nitrite (15.25 to 18.43 µg.at.NO_2N/L), nitrate (0.75 to 0.92µg.at.NO_3.N/L), ammonia (0.058 to 0.093µg.at.NH_3.N/L)and phosphate (25.59 to 36.11µg.at.PO_4.P/L). Nutrients were high in fucoidan incorporated diet fed tanks. Alkalinity was also more, but hardness was quite low. Temperature and pH were slightly high while dissolved oxygen level was quite low and the reason was not clear.

The results of the hematological parameters viz., hemoglobin, WBC and RBC examined for the tilapia fed with fucoidan incorporated diet and control are given in Table 2, 3 and 4 respectively. However, over the increase in the culture duration, the haemoglobin content in tilapia increased by approximately 0.5g/dl on the 60^{th} day irrespective of the variation in the diet. Similarly, the WBC count did not show much variation in tilapia fed with up to 0.2% fucoidan incorporated diet, but in 0.3% fucoidan incorporated diet, the WBC counts in tilapia was significantly high, irrespective of the type of seaweeds on 30^{th} day. Beyond 30 days the increase in WBC count was not substantial and had reduced in fucoidan fed tilapia. On the other hand, the RBC counts did not change significantly during the experimental period in tilapia fed with control and the test diets. Incorporation of fucoidan had not increased the RBC counts in the tilapia during the culture period.

In the present investigation, tilapia fed with fucoidan incorporated diet, the NBT reduction increased with increasing concentration of fucoidan obtained from *P. tetrastromatica* and *S. oligocystum* after 15 days. The effect continued with fucoidan of *S.oligocystum* while the effect was more pronounced with fucoidan of *P. tetrastromatica* on 30^{th} day. Beyond this duration, there was proportionate

loss in NBT reduction in tilapia fed with fucoidan incorporated diet; with the loss being more in *S. oligocystum* fucoidan. On the 60th day, the immune enhancement by fucoidan was totally lost in tilapia fed with *S.oligocystum* fucoidan; as the NBT reduction was on par with control diet. In *P. tetrastromatica* fucoidan fed tilapia, the effect was slightly high.

The lysozyme activity of control and experimental group fishes fed with fucoidan from *P. tetrastromatica* showed significant variation (Fig.3). In the present study, the maximum serum lysozyme activity was observed on the 30th day with the tilapia fed with fucoidan of 0.2% incorporated diet (4012±2198 IU ml^{-1}), followed by 0.3% fucoidan (3876±192 IU ml^{-1})on the15th day. Thereafter the activity was decreased in all the treatments as the experiment prolonged.Similar results were observed for the fishes fed with fucoidan extracted from *S. oligocystum*(Fig 4).But the maximum activity was 2412 ± 221 IU ml^{-1} in tilapia fed with fucoidan at 0.3% incorporated diet on the 15th day.

In the fish challenge experiments, the relative percentage of survival was higher in tilapia fed with fucoidan from *P. tetrastromatica* at 0.2% and 0.3% concentrations (Table 5) and 0.3% fucoidan from *S. oligocystum* (Table 6). The results revealed that fucoidan from *P.tetrastromatica* can enhance the immunity better than fucoidan of *S. oligocystum* in tilapia. Also, the immunity enhancement by fucoidan was maximum up to 60 days of culture and latter slowly dropped and diminished beyond 60 days of culture; *P.tetrastromatica* fucoidan concentration of 0.2% with was found to provide a sustainable immunity till the end of the experimental period than other concentrations.

Table.1 Biological parameters monitored in experimental tanks

S. No.	Parameters	Control	Fucoidan supplemented with 0.1%	Fucoidan supplemented with 0.2%	Fucoidan supplemented with 0.3%
1.	pH	7.03^{a}±0.07	7.19^{b}±0.03	7.23^{b}±0.04	7.25^{b}±0.04
2.	Temperature (^{0}C)	27.5±0.29	28.3±0.17	27.7±0.44	28.2±0.17
3.	DO (ml/L)	6.12^{b}±0.02	6.03^{b}±0.04	5.72^{a}±0.17	5.52^{a}±0.06
4.	Water hardness ($CaCO_3$ /L)	143.17^{c}±0.44	136.03^{a}±0.29	138.83^{b}±1.42	135.17^{a}±0.17
5.	Alkalinity (mg/L)	147.50^{a}±0.29	155.50^{c}±0.28	151.33^{c}±0.28	155.33^{a}±0.60
6.	Ammonia (µg.at.NH_3.N/L)	0.058^{a}±0.002	0.075^{b}±0.002	0.087^{c}±0.001	0.093^{d}±0.02
7.	Nitrite (µg.at.NO_2N/L)	15.25^{a}±0.03	16.21^{b}±0.05	18.43^{c}±0.03	18.36^{c}±0.19
8.	Nitrate (µg.at.NO_3.N/L)	0.85b^{c}±0.03	0.92^{c}±0.01	0.75^{a}±0.03	0.78^{ab}±0.01
9.	Phosphate (µg.at.PO_4.P/L)	25.59^{a}±0.35	27.51^{b}±0.04	29.78^{c}±0.04	36.11^{d}±0.06

Each value is the mean of three observations. Mean bearing at least one common superscript within a row do not differ significantly (P<0.05)

Table.2 Haemoglobin content (g/dl) of tilapia fed with fucoidan incorporated and control diet

Duration (Days)	Control (g/dl)	Fucoidan supplemented with 0.1% (g/dl)	Fucoidan supplemented with 0.2% (g/dl)	Fucoidan supplemented with 0.3% (g/dl)
P. tetrastromatica				
Initial	5.07±0.03	5.07±0.03	5.06±0.02	5.06±0.01
15	5.09±0.01	5.12±0.02	5.07±0.03	5.20±0.05
30	5.20±0.05	5.31±0.06	5.13±0.15	5.80±0.19
45	5.38±0.05	5.72±0.06	5.77±0.08	5.74±0.07
60	5.59±0.13	5.61±0.03	5.69±0.13	5.67±0.02
S. oligocystum				
15	5.13±0.04	5.14±0.03	5.09±0.03	5.18±0.07
30	5.16±0.02	5.22±0.04	5.19±0.01	5.43±0.05
45	5.41±0.11	5.39±0.08	5.32±0.06	5.54±0.04
60	5.54±0.06	5.62±0.11	5.64±0.01	5.69±0.12

Table.3 WBC count of tilapia fed with fucoidan incorporated and control diet

Duration (Days)	Control (μl^{-1})	0.1% fucoidan (μl^{-1})	0.2% fucoidan (μl^{-1})	0.3 % fucoidan (μl^{-1})
P. tetrastromatica				
Initial	2988±21	2986±14	2992±21	2995±16
15	3146±41	3145±24	3255±27	3331±18
30	3250±29	3353±20	3405±10	3881±17
45	3301±13	3493±23	3508±17	3790±15
60	3308±22	3573±23	3580±15	3817±33
S. oligocystum				
15	2833±14	2830±15	3100±29	3217±90
30	3043±23	3173±15	3433±20	3803±23
45	3116±18	3273±21	3557±23	3543±24
60	3313±90	3443±18	3383±90	3453±36

Each value is the mean of three observations. Mean bearing at least one common superscript within a row do not differ significantly ($P<0.05$)

Table.4 RBC count of tilapia fed with fucoidan incorporated and control diet

Duration (Days)	Control ($x10^6 \mu l^{-1}$)	0.1% fucoidan ($x10^6 \mu l^{-1}$)	0.2% fucoidan ($x10^6 \mu l^{-1}$)	0.3 % fucoidan ($x10^6 \mu l^{-1}$)
P. tetrastromatica				
Initial	2.66±0.03	2.66±0.04	2.67±0.04	2.64±0.03
15	2.63±0.04	2.75±0.13	2.73±0.09	2.87±0.03
30	2.70±0.07	2.84±0.11	2.87±0.03	2.71±0.08
45`	2.46±0.23	2.78±0.04	2.66±0.08	2.67±0.07
60	2.76±0.12	2.75±0.08	2.78±0.06	2.86±0.02
S.oligocystum				
15	2.74±0.07	2.66±0.13	2.72±0.12	2.68±0.11
30	2.54±0.063	2.58±0.10	2.69±0.07	2.74±0.12
45	2.71±0.05	2.61±0.15	2.64±0.04	2.69±0.09
60	2.70±0.15	2.54±0.13	2.59±0.08	2.71±0.08

Table.5 Mortality and Relative Percentage Survival (RPS)of fish *Oreochromis mossambicus* fed on different concentrations of fucoidan (*P. tetrastromatica*)supplemented diets after challenged with *Aeromonas hydrophila*in 60 days

Concentration	No. of challenged Fish	No. of Mortalities	Survival (%)	Relative Percentage Survival
0.1% fucoida	20	7 (35)	65	47
0.2% fucoidan	20	5 (25)	75	62
0.3%fucoidan	20	4 (20)	80	70
Control	20	13 (65)	35	-

Table.6 Mortality and Relative Percentage Survival (RPS)of fish *Oreochromis mossambicus* fed on different concentrations of fucoidan (*S. oligocystum*)supplemented diets after challenged with *Aeromonas hydrophila*in 60 days

Concentration	No. of challenged Fish	No. of Mortalities	Survival (%)	Relative Percentage Survival
0.1% fucoidan	20	10 (50)	50	24
0.2% fucoidan	20	7 (35)	65	47
0.3%fucoidan	20	6 (30)	70	54
Control	20	13 (65)	35	-

Fish were challenged by intramuscular injection with *A. hydrophila* strain. Relative percent survival=1-[% mortality in the vaccinated group/ % mortality in the control group] x 100. RPS values over 50 indicate positive effect of the vaccine (Amend, 1981)

Fig.1 NBT reduction in tilapia fed with *P. tetrastromatica* fucoidan incorporated diet and control

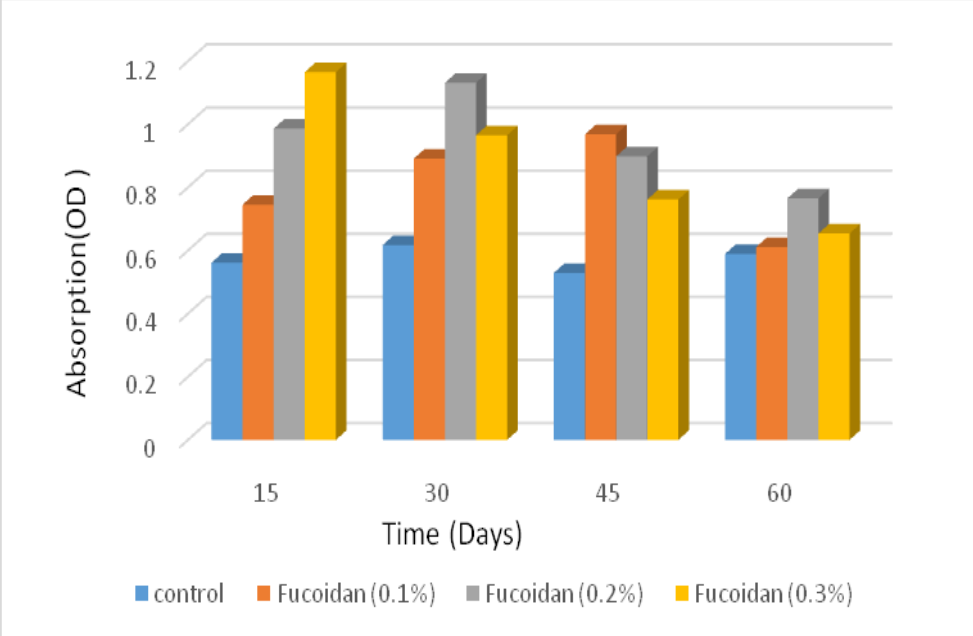


Fig.2 NBT reduction in tilapia fed with *S. oligocystum* fucoidan incorporated diet and control

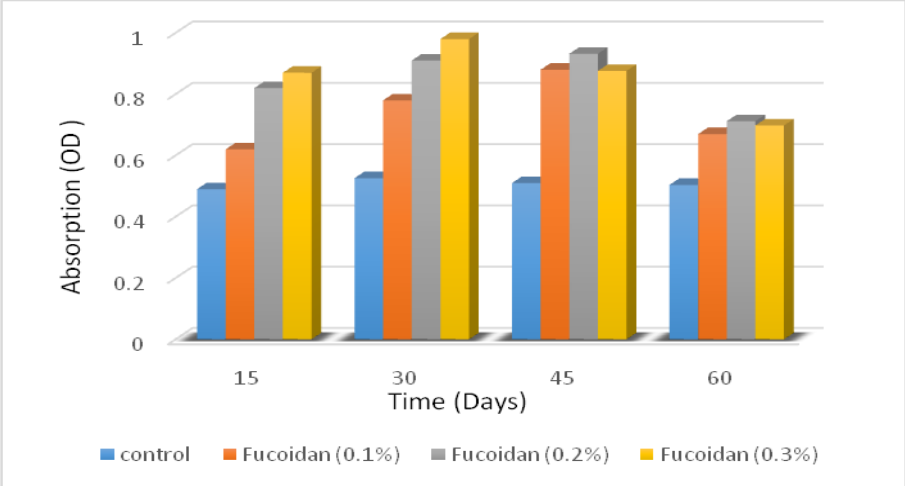


Fig.3 Lysozyme activity of tilapia supplemented with fucoidan of *P. tetrastromatica*

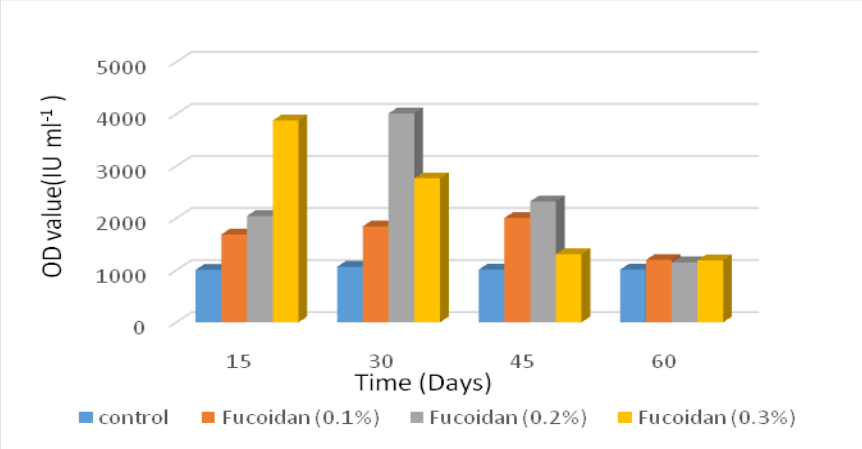
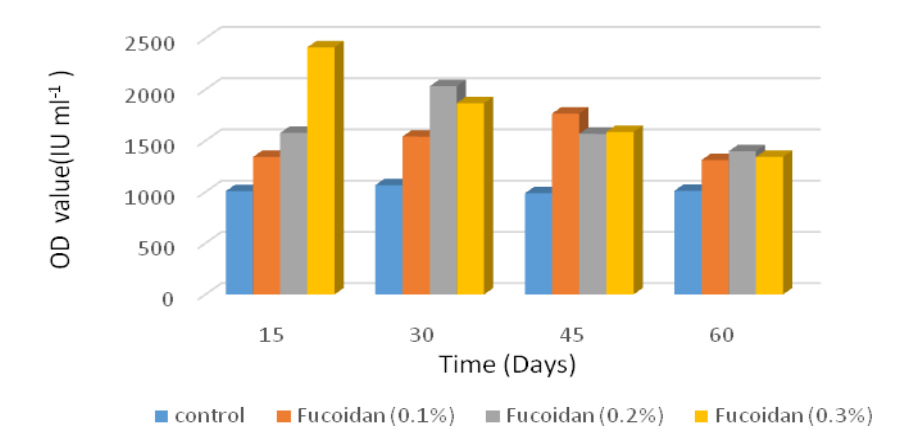


Fig.4 Lysozyme activity of Tilapia supplemented with fucoidan of *S. oligocystum*



The results of fucoidan extracts from *P. tetrastromatica* and *S. oligocystum* indicated that there was no significant variation in control and fucoidan supplemented diets at three concentrations and control group in Hb and RBC content. However, White blood cells (WBCs) of fish play a crucial role in the cellular immunity and resistance to infectious diseases (Whyte 2007). In the present study, WBC count was found to be significantly increased in tilapia fed with fucoidan from *P. tetrastromatica.* This result corroborates with Yang *et al.*, 2014 who studied the effect of dietary fucoidan on the blood constituents, anti-oxidative and innate immunity in juvenile cat fish *Pleteobagrus fulvidraco* and reported that dietary fucoidan had no significant effects on the WBC, RBC, hemoglobin of the yellow cat fish. However, oral administration of fucoidan at an optimal level decreased the serum parameters such as serum total protein, total cholesterol, glucose and triglyceride and improved the anti- oxidation and innate immunity of the treated fish. Similar result observed in fish Nile tilapia was fed with dietary supplementation of CloSTAT, black cumin, or combination of the two enhanced the overall immune response due significant increase of the WBC numbers, globulin proteins and the phagocytic activities of fish phagocytes (Elkamel and Mosaad 2012). Thus, the fucoidan extracts from *P. tetrastromatica* and *S. oligocystum* did not bring about significant variation in Hb and RBC counts of tilapia when added with the diet, but had caused some increase in WBC counts.

As fish depends mainly on innate immunity for protection against disease, attention was given in this study to assess the innate immune response with respect to fucoidan incorporated diet. Many studies have earlier demonstrated that different types of polysaccharides could improve the immunity and antioxidant capacity of cultured fish (Smit 2004; Hwang *et al.,* 2010; Yeh *et al.*, 2006; Immanuel *et al.,* 2012). In the present investigation, tilapia fed with fucoidan incorporated diet, the NBT reduction increased with increasing concentration of fucoidan obtained from *P. tetrastromatica* and *S. oligocystum* after 15 days upto 30 days. However, there was drastic reduction in NBT assays.

Phagocytosis is a primary, non-specific defense mechanism against invasion of pathogenic organisms of hosts. The NBT assay is a quick inexpensive test focusing on the ability of phagocytes to induce the dye by the production of oxygen radicals in macrophages, and a very good indicator of health status or their immunization effectiveness in fish (Anderson 1992). In mammals, the oxygen radicals are aimed at the destruction of bacterial invaders.

The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against disease among fishes. It has been reported that an increased phagocytic activity in shrimps fed with fucoidan (200mg/kg of body weight, group 12–15g) was achieved when compared to the control group due to the immunostimulatary activity of fucoidan (Immanuel *et al.,* 2012). Fucoidan would have stimulated the immuno system in several ways and most of them are related to their ability to modify cell surface properties (Usov *et al.,* 2001). Oral intake of the fucoidan derived from dietary brown seaweed had inhibited the protective effects through direct inhibition of viral replication and stimulation of the immune system (innate and adaptive) function (Hayashi *et al.,* 2008). *Sargassum thunbergii* and *S. kjellmaniaun* were also reported to possess antitumor activity, which is related to the enhancement of immune responses according to Itoh*et al.,* 1993.

Lysozyme is a cationic enzyme that breaks ß-1, 4 glycosidic bonds between N-acetyl glucosamine in the peptidoglycan of bacterial cell walls. This action is known to attack mainly Gram positive bacteria as well as some Gram negative bacteria in conjunction with complement (Alexander amd Ingram 1992).

A lysozyme activity unit was defined as the amount of enzyme inquired to decrease the absorbance at a rate of 0.001/min/ml. *Cyprinus carpio* fed with chitosan (1%)and levamisole (250mg kg^{-1} of diet)enhanced the serum lysozyme activity in the beginning which later declined after 60 days of culture operation in ponds (Gopalakannan and Arul 2006).

The lysozyme activity of control and experimental group fishes fed with fucoidan from *P. tetrastromatica* showed significant variation in the present study. Fucoidan activates the macrophages which in turn increases lysozyme activity (Yang *et al.,* 2008). The leaf extract of O*cimum sanctum* fed at the rate of 20mg/kg fed for a single day was found to be optimal for enhancing phagocytic and NBT reduction in tilapia (Logambal *et al.,* 2000). Although research findings on the effect of fucoidan on immunological parameters in shrimp and rats are available, similar reports in fish is very scarce.

The serum lysozyme activities of the yellow catfish fed with fucoidan from *Sargassum spp* was found to be significantly higher than the control and they have opined that fucoidan significantly influences the blood characteristics, antioxidant status and non-specific immune responses in juvenile yellow catfish (Yang *et al.,* 2014).

The observed variations in the levels of different immune related parameters assessed in this study at different concentrations of fucoidan fed incorporated diet in tilapia at various time intervals have indicated that the dosages and timing followed for administration plays an important role as reported by Sakai (1999). Immune modulating substances have the ability to increase immune function, when it is depressed, and also to reduce it, when it is over-stimulated (Kuznetsova *et al.,* 2003). Fucoidan may provide an abundant supply of fucose, one of the necessary saccharides, and smaller amounts of several other required sugars. This is likely to be one mechanism by which the fucoidan exert their immunomodulatory effect. Determination of the exact level of incorporation required for optimal immune responsiveness is very helpful for feed formulation as fucoidan is not cheap and the feed cost will increase at higher incorporation level. However, the active principle compound responsible for the immunostimulatory property observed in the present study has to be identified.

The reduction in mortality of all the test groups in challenge experiments had increased with the increasing concentrations of fucoidan, which could be due to the enhancement of the non-specific immune system of the fish. There is strong experimental evidence that oral administration of fucoidan from brown seaweeds viz., *S. wightii* and *S. polycystum* reduced the impact of the WSSV infection in *P. monodon* (Chotigeat *et al.,* 2004; Choi *et al.,* 2005; Immanuel *et al.,* 2012; Kanimozhi *et al.,* 2013).

Fucoidan is a heteropolysaccharide and is mainly composed of fucose, sulphate, uronic acid, and small quantities of monosaccharides. The composition may vary between species and the extraction techniques used to extract the fucoidan which will have a large impact on the determination of the final

structure of fucoidan (Chizhov *et al.*, 1999; Duarte *et al.*, 2001; Bilan *et al.*, 2002).

Fucoidan thus possess an immunostimulating effect in tilapia, O. *mossambicus*, under optimal concentration of fucoidan based on the species and its composition. In the present study, it is observed that fucoidan derived from *S. oligocystum* has relatively lower lysozyme and NBT activity than *P. tetrastromatica* but higher than the control. This could be due to difference of molecular structure, relative molecular mass, polysaccharide compositions in particular degree of sulphate content between the species of *P. tetrastromatica* and *S. oligocystum*.

The results specified that fishes fed with fucoidan extracted from *P. tetrastromatica* and *S. oligocystum* invariably reduce the mortality than the control and significantly enhance the relative percentage survival when challenged with *Aeromonas hydrophila*. However, the detailed mechanism of action of the fucoidan to inhibit pathogens or improve immunity need to be investigated more thoroughly.

In conclusion based on the results of the study, it is evident that 0.2%, 0.3% of fucoidan from *P. tetrastromatica* and 0.3% fucoidan from *S. oligocystum* certainly enhance the non-specific immunity of tilapia *O. mossambicus* which may be considered to improve the immune status of the fish. Thus, it can be concluded that fucoidan could be used as immunostimulant for the fish, *O. mossambicus* as it can improve its resistance to disease causing pathogen.

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