CHAPTER III: IMMUNOSTIMULATORY EFFECT IN *MUGIL CEPHALUS*

7.1. INTRODUCTION

*Vibrios* are natural inhabitants of the marine environments. The genus *Vibrio* includes more than 35 species which are mainly presented in the marine environmental throughout the world. *Vibrio* sp is one of the most important pathogen in aquaculture which causes grater economic loss. Disease outbreaks occur when fish are exposed to infectious agents in the presence of stress factors (Austin and Austin, 2007). However, it has also been suggested that this species is a pathogen of several marine animals (Lee, 1995). There is controversy about the precise role of *V. alginolyticus* as a fish pathogen (Balebona et al., 1996). This species has been reported to be the causal agent of outbreaks of Vibriosis.

The wide range of pathogens present in fish farming limits the effectiveness of vaccines. Therefore, the need for alternative techniques is increasing. One of the most promising methods of controlling diseases in aquaculture is strengthening the defence mechanisms of fish through prophylactic administration of immunostimulants (Robertson, 1999). Enhancement of the immune system seems to be the most promising method of preventing fish diseases. This modulation can be achieved with vaccines, which enhance the acquired (or specific) immune response of the fish and are considered to be the most effective agents, but a single vaccine is effective against only one type of pathogens, and in the case of many pathogens no effective vaccines available due to the complex antigenic structure (Alexander et al., 2010).

Many plant-derived compounds are known to have non-specific immunestimulatory properties in animals, of which more than a dozen have been evaluated in fish and shrimp (Raa, 1996). Even though glucan and other
immunostimulants have positive effects on fish and prawn (Song and Hsieh, 1994), some disadvantages have been found with the application of these natural immunostimulants like being intolerant to heat and indigestible. Hence, it is advisable to continue searching for alternative immunostimulant products from plants. All forms of stress activate responses through diverse physiological processes, and among them, energy metabolism is of prime importance for physiological compensation by organisms (Hill and Taylor 1991). Stress can also suppress the defense system to such an extent that susceptibility to disease is increased (Anderson, 1990). Since aquatic organisms are constantly subjected to environmental fluctuations and are under challenges from potential pathogens in the aquatic environment, reciprocal changes in the physiological and immune processes are anticipated.

Immunostimulants can increase resistance to infectious diseases, not by promoting specific immune responses, but by enhancing nonspecific defense mechanisms. Use of immunostimulants is an effective means of increasing immunocompetency and disease resistance in fish. In contrast to vaccines, immunostimulants enhance the innate (or non-specific) immune response (Sheikhzadesh et al., 2012). The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, like lysozyme or complement system (Magnadottir, 2006).

Immunostimulants can be applied via injection, bathing or oral administration, the latter seems to be the most practicable (Yin et al., 2006). Many types of biological and synthetic compounds have been shown to enhance non-specific immune system of cultivated fish. The major components of the innate immune system are macrophages, monocytes, granulocytes and humoral elements, such as lysozymes or the complement system (Magnadottir 2006). Best-known immunostimulants are
components of bacterial cell wall, like lipopolysaccharide (LPS) (Goetz et al., 2004) or glucans (Engstad et al., 1992), but synthetic compounds, polysaccharides, animal and plant extracts or vitamins can enhance the non-specific immune response of fish (Thompson et al., 1995). However, the effect is dosage-dependent and there is chances to overdose (Kajita et al., 1990) neither are they always effective in early developmental stages of fish (Huttenhuis et al., 2006).

Hotwater extracts from several species of brown algae including Undaria pinnatifida and Sargassum autumnale have been reported to increase the resistance of common carp (Cyprinus carpio) against Edwardsiella tarda as well as increase the resistance of yellow tail (Seriola quinqueradiata) against Streptococcus infection (Fujiki et al., 1992). Sodium alginate extracted from brown algae U. pinnatifida and Lessonia nigrescens have been reported to increase the resistance of L. vannamei against Vibrio alginolyticus (Cheng et al., 2005).

Knowledge of the haematological characteristics is an important tool that can be used as an effective and sensitive index to monitor physiological and pathological changes in fishes. Normal ranges for various blood parameters in fish have been established by different investigators in fish physiology and pathology (Xiaoyun et al., 2009). The analysis of blood indices has proven to be a valuable approach for analysing the health status of farmed animals as these indices provide reliable information on metabolic disorders, deficiencies and chronic stress status before they are present in a clinical setting (Bahmani et al., 2001).

Blood is known to exhibit pathological changes before the onset of any external symptom of toxicity. Fish blood is a pathophysiological indicator of the whole body function and therefore blood parameters are important in diagnosing the
structural and functional status of fish exposed to a toxicant (Sampath et al., 1998). Fish blood is being studied increasingly in toxicological research and environmental monitoring as a possible indicator of physiological and pathological changes in fishery management and disease investigations (Bansal et al., 1980). The possibility of evaluation depends on the availability of reference values as close as possible to normal values of the various blood components considered as reliable descriptors of healthy fish under natural conditions (Cataldi et al., 1998). Determination of these parameters may also be useful in assessing any changes in water quality, related soil quality and fish response as well (Darvish et al., 2010). The present study is focussed on seaweed extract and study the immunostimulatory effect in Mugil cephalus and it is resistance against Vibrio alginolyticus.
7.2. MATERIAL AND METHODS

7.2.1. Isolation of *Vibrio* sp from infected fish

Infected live fishes (*Mugil cephalus*) were dissected. The infected organ and tissues were homogenized. The homogenized samples were serially diluted by using 50% sea water and plated on Thiosulphate Citrate Bile Salts Sucrose (TCBS-Hi Media, Mumbai) prepared with 50% of seawater for specific isolation of *Vibrio* species. The TCBS plates were incubated at 28°C for 24-48h for appropriate colony formation (Figure 19) (Rajasekar, 2010).

7.2.2. Pathogenicity test

The isolated three different morphologically bacteria (BDTR01, BDTR02, and BDTR07) were injected into healthy *Mugil cephalus* (Group 1, 2 and 3) respectively, to verify the pathogenicity and prove the Koch postulate. Briefly, three isolated bacterial colonies were inoculated in to nutrient broth (with 50% seawater) and incubated at 28°C for 24-48 h. After incubation the nutrient broth were centrifuged at 6000g for 10 minutes and the pellet was suspended in to sterile PBS and injected intraperitoneally (I.P) with 0.2 ml of suspension \((6\times10^5 \text{ cfu ml}^{-1})\) per fish (Figure 20). The control group injected with 0.2 ml of sterile PBS. During the experiment, fishes behaviours and clinical signs were observed (Kumaran et al., 2010) (Figure 21).

7.2.3. Characterization of pathogen

Based on the pathogenicity assay BDTR07 strain was used further characterization. Before biochemical and molecular characterization, pure colonies were sub-cultured onto TSA plates to obtain a fresh, 24 h culture for further studies.
7.2.3.1. Biochemical identification

The colony morphology and biochemical examination were carried out. The bacterial shape and size was characterized by using Gram staining and Motility. The biochemical characters of bacterial pathogen were characterized with Biochemical kit (KB002) and Catalase, Oxidase, Glucose, Lactose, Sucrose and growth in NaCl was determined.

7.2.3.2 Molecular identification of Bacteria

7.2.3.2.1. Genomic DNA isolation

Total genomic DNA was extracted from the pure culture of the bacterial isolate by phenol-chloroform Isoamyl alcohol method, which removes the protein and other cellular components from the nucleic acid to obtain the pure DNA. Log phase culture (2 to 4 ml) was taken and centrifuged at 10,000 rpm for 10 minutes at 4°C. Centrifugation was repeated to wash the cells twice with 500µl of TE buffer. The pellet was resuspended in 500µl of TE buffer and incubated for 10 minutes in boiling water bath and centrifuged. After centrifugation, equal volume of phenol: chloroform: isoamyl alcohol was added to the supernatant and centrifuged. To the aqueous phase, 0.1 volume of 3M ammonium acetate (pH 5.2) and 2.5 volume of ice-cold ethanol were added and incubated at -20°C overnight. After incubation, sample was centrifuged at 10,000 rpm for 10 min at 4°C and 70% ethanol was added to wash the pellet and air dried. After drying, the DNA was resuspended in TE buffer (pH 8.0) and stored at 4°C. DNA sample (10µl) was mixed with 2µl of 6x loading dye and loaded in 1% agarose gel. The separated DNA was visualized by UV transilluminator (Kumaran, 2012).
7.2.3.2.2. 16S rDNA sequence amplification

The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using universal primers 27F and 1492R. The PCR mixture consisted of 5 µl of 10x buffer (Mg²⁺ free), 5 µl of 2.5 µM MgCl₂, 8 µl of dNTP mixture (2.5 µM each), 1 µl of each primer of template DNA and 0.5 µl of Taq polymerase (5U/µl) (Genei, Bangalore), making final volume of 50 µl. PCR was performed in thermal cycler (LARK) using an initial denaturation at 94°C for 5 min, followed by 94°C for 1 min and 72°C for 90 S for annealing and final extension at 72°C for 7 min (Figure 22).

7.2.3.2.3. Sequencing and Phylogenetic analysis

The amplified PCR products were purified using a Genei PCR purification kit (Genei, Bangalore). Nearly full length sequences of the amplified 16S rRNA genes (BDTR07) were obtained by automated sequencer (Bioserve, Hyderabad). The sequences were edited by using Clustal X mega software and a BLAST search was performed in the National Center for Biotechnology Information (NCBI) database to identify the nearest neighbour of the amplified sequence. The results of the sequencing were used for homology searches. Phylogenetic trees were inferred using the neighbour-joining method. Nucleotide sequence of the partially complete 16S rDNA sequences of identified species were deposited in the Genbank database (Kumaran, 2012).

7.2.4. Preparation of bacterial suspension

Isolated Vibrio alginolyticus (BRTR07) was cultured in tryptone soy broth (Hi media) for 24-48 h at 28°C. After incubation the nutrient broth was centrifuged at 6000 for 10 mints and the pellet was suspended in to sterile PBS and injected
intraperitoneally (I.P) with 0.2 ml of suspension ($6 \times 10^5$ cfu ml$^{-1}$) per fish. This bacterial suspension was used for the challenge test (Kaleeswaran et al., 2010).

7.2.5. Feed formulation

7.2.5.1. Hot-water extract of S. wightii

Hot-water extract of S. wightii was prepared by adopting method of Fujiki et al. (1992). Briefly, the seaweed was washed with water and dried at room temperature. Then they were grounded and 10 g of the milled fronds was added to 300 ml of deionized water, and the suspension was boiled for 3 h (Figure 26). The suspension was filtered through a nylon mesh, and the filtrate was lyophilized under reduced pressure. The hot-water extract was stored at -4°C for further study (Su-Tuen et al., 2006).

7.2.5.2. Preparation of feeds

Formulated diet (Trash fish meal) was prepared at four different concentrations. Formulated diet 1, 2 and 3 were prepared with seaweed at the three different concentrations of 0.5%, 1% and 2 % respectively, where as diet 4 were prepared without seaweed. All the ingredients were mixed in a domestic mixer together with minerals and vitamins premix dissolved in a small quantity of water. The mixture was slowly mixed with hot water (80°C) in a proportion of 50:50 (v/w) to accomplish agglutination (Figure 27). The dough was passed through a meat chopper (Brand-Filizola) to obtain pellets of 2mm diameter, and dried in a forced circulation air drier at a temperature of 65 ± 2°C for 36 h. The dried pellets were stored in plastic bags at -4°C.
7.2.6. Experimental design

Healthy gray Mullet (*Mugil cephalus* 10- 25 g) was collected from Vellar estuary, Parangipettai, southeast coast of Tamil Nadu. All the fishes (*Mugil cephalus*) were transported to the wet laboratory and fishes were too stocked in circular plastic Tank (200 L and 100 L) with continuous aeration for 15 days for acclimatization. During this period, the fishes were fed with formulated diet (without seaweed) at the rate of 4% of the body weight twice a day at 6.00 and 17.00 hour. After 15 days of acclimatization the *Mugil cephalus* were stocked randomly divided into five experimental group (n=15) in triplicate groups in 200L and 100 L circular plastic tank water volume 150 L and 75 L respectively, tanks were fitted with a continuous flow-through system. Group 1, 2 and 3 fed with diet 1, 2 and diet 3 respectively, group 4 and 5 with diet 4. The respective diets were provided till the end of the experiment. On the 15th day of feeding, all the groups were injected intraperitoneally (i.p.) with 100 μl of PBS containing *Vibrio alginolyticus* at 1 × 10^6 cfu ml^-1^. Mortality and symptoms were recorded during experiments (Figure 28).

7.2.7. Non specific immune response in *Mugil cephalus*

7.2.7.1. Haematological parameters

RBC and WBC count were determined using a haemocytometer with Neubauer counting chamber as described by Blaxhall and Daisley (1973). The following formula was used to calculate the number of erythrocytes and leucocytes per ml of the blood sample: Number of cells = (Number of cells counted x dilution) (ml-1)/ (Area counted x depth of fluid). Thin blood smears were prepared from fresh heparinised blood on microscope slides and stained with Wright-Giemsa.
7.2.7.2. Hematocrit

Blood was drawn into heparinized hematocrit pipette up to the graduation mark. The lower opening of the pipette was closed up to 2 cm depth using sealant and heating it carefully over the spirit lamp which closes the upper opening. The pipettes were centrifuged for three minutes with a speed of 3000 rpm and placed on the reading device and read-off. The hematocrit value was expressed as % blood cells in total volume of blood (Goldenfarb et al., 1971).

7.2.7.3. Nitroblue tetrazolium (NBT) assay

The production of oxidative radicals by neutrophils in blood during the respiratory burst was measured via NBT assays, in accordance with the description of Anderson and Siwick (1994). In brief, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, and then 50 μL was extracted and dispensed into ependorff tubes. For the solubilization of the reduced formazan product, 1 mL of dimethyl formamide was added and centrifuged at 2000 for 5 min. Finally, the supernatant was acquired and the extent of reduced NBT was determined at an optical density of 540 nm with a microreader. Dimethyl formamide was used as the blank.

7.2.7.4. Lysozyme activity

Lysozyme activity was measured by adapting the turbidimetric method described. Fifty microliters of serum was placed in triplicate in a 96 well plate with 50 μl PBS, pH 5.8. After mixing, the serum was serially diluted until the last well. Finally, 50 μl of sample was discarded in the last well. To each well, 125 μl of Micrococcus lysodeikticus was added. 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 as standard (Ellis, 1990; Alcorn et al., 2002).

7.2.7.5. Disease resistance and survival experiment

The diseases resistant experiment was conducted by slightly modify the method of Harikrishnan et al. (2009). The pathogenic *Vibrio alginolyticus* were inoculated in to nutrient broth and incubated at 28°C for 24 h. The culture was centrifuged at 800 g for 15 min at 4°C. The packed cells were washed and the required dose was prepared in PBS. Groups of 15 fish each in triplicate were fed with seaweed supplemented feed at 4% of body weight on day 1. After 10 days administration, the fishes were challenged with *Vibrio alginolyticus* (6×10⁵ cfu ml⁻¹). Once the fishes were exposed with pathogen, they were observed for the clinical symptoms and mortality.

7.2.7.6 Statistical analysis

The values of the each experimental parameter were expressed as the arithmetic mean (AM) ± standard deviation (SD) and probabilities of P<0.05 were considered significant. The effects of the seaweeds diet on haematological and immunological parameter were tested using a statistical package origin 6.1 for Windows7 was used for these statistical analyses.
7.3. RESULTS

7.3.1. Pathogenicity testing

During the infection period symptoms were observed like skin haemorrhage and tail infection, control group are healthy and not infected. Infected fish were dissected and organs were used for the isolation of pathogen.

7.3.2. Isolation of bacteria from infected fish

After 24-48 h of incubation the yellow colour colonies were appeared on the selective media (TCBS). Different and individual single colonies were transferred on to tryptone soy agar (TSA) slants as pure cultures and were maintained at 4°C for further studies.

7.3.2.1. Biochemical characteristics

The isolated bacterium was Gram negative short rods, and motile. Oxidase, Indole, Methyl Red and Urease were negative. Voges Proskauer, Citrate test was positive. Glucose and Sucrose fermentation was positive and Lactose fermentation was negative, No growth in media containing 0% NaCl but well growth appeared in 6 % and 10% of NaCl concentration.

7.3.2.2. PCR amplification.

The genomic DNA of the bacterial strain BDTR07 was isolated and confirmed by gel electrophoresis. Isolated DNA was used for PCR amplification of 16s rRNA genes followed by sequencing. The 16s rRNA gene was amplified from isolated DNA of the strain BDTR07 under optimal condition. The amplified product was analysed by resolving it in 1% of agarose gel. The gel shows a clear band of 1.5 kb (Figure 23) and hence confirms the presence of amplified DNA.
7.3.2.3 16s rDNA sequencing analysis

Molecular identification of the 16s rRNA genes shows that the identified strain belongs to the genera Vibrio. The sequenced 16s rRNA genes of strain BDTR07 (Genebank accession no KF758571) that was identified as Vibrio alginolyticus is 630bp in the length and exhibited high similarity 99% (Figure 24) with the 16s rRNA genes of Vibrio alginolyticus from Genbank database. This sequences showed 99% similarity with the sequences of Gene bank accession number JX976307 (V. alginolyticus), JQ780446 (V. alginolyticus). Results from colony morphology, biochemical tests and 16S rDNA indicated that the isolates were Vibrio alginolyticus (BDTR07).

7.3.2.4 Phylogenetic analysis

The partial sequence of 16s rRNA genes were used to construct phylogenetic tree was generated with sequences from same group of Vibrio strains (Vibrio sp (KC777294), V. alginolyticus (AB680916), V. alginolyticus (JQ780446), V. parahaemolyticus (EU814515), V. alginolyticus (JX976307), V. natriegens (EU636230) and Streptomyces sp (AB845420) (out group) get from NCBI database (Figure 25).

7.3.3 Non specific immune response

7.3.3.1 Haematological Parameters

RBC count of group 1, 2 and 3 were significantly higher than control. In the infected fishes (diet 5), RBC count were decreased in the fish. In the 4th week of the experiment diet 2 showed a highest RBC count than other diets. Exact variations observed in the treatments, infected and control group (Figure 29).

During the study the control group of WBC count was normal. In the diet 5 infected group WBC counts was increased in the 1st two week of the experiments after
the WBC count was decreased after 3 and 4th weeks. In the experimental group, highest blood count was found in diet 2. In the diet 2, showed an increased blood count throughout the experiments when compared with control and other group (group 1 and 3), but diet 5 (infection fish) showed RBC decreased week by weeks (Figure 30).

7.3.3.2. Hematocrit

In control group of Hematocrit (HT %) was in the normal condition. Due to infection the hematocrit percentage was decreased in infected groups, when compare with control (Figure 31). In this experimental group hematocrit percentage was increased in the diet 1, diet 2 and diet 3 when compared with fed diet 5 and control. Diet 2 showed an increased hematocrit (HT %).

7.3.3.3. NBT and Lysozyme

Studies on neutrophil activity showed the enhancing effect of dietary supplements on neutrophil respiratory burst as evidences from the increased NBT reduction (Figure 32). The respiratory burst activity in infected fish fed with normal diet significantly increased when compared with the control. The seaweeds supplement diet 2 (1%) to enhanced the respiratory burst activity in infected *Mugil cephalus*. The lysozyme activity was progressively increased in experiment and in group 2 lysozyme activity increased in the first week onwards. In the infected fish lysozyme activity was very low when compared with seaweeds feed diet. After 3rd week the infected group lysozyme was decreased (Figure 33).
7.3.4. Disease resistant

The cumulative survival was shown in figure 34. In this experiments were checked the resistance against *V. alginolyticus* in fish (*Mugil cephalus*). In the 2\textsuperscript{nd} day onwards mortality were started in the entire group. In the 18\textsuperscript{th} day in the group 5 showed 20\% survival rates, group 1, 2 and 3 showed a 68\%, 78\% and 66\% survival. End of the experiment in the group 5 showed 0\% survival rate, and group 4 there is no mortality. Group 2 showed highest survival rate 73\%, followed by group 1 (63\%) and group 3 (55 \%).
7.4. DISCUSSION

Most of the pathogenic bacteria are gram negative, aerobic and facultative anaerobic bacteria and belong to the genus *Vibrio*, *Yersinia*, *Pasteurella*, and *Edwardsiella*. Members of the genus *Vibrio* are opportunistic pathogens that have been associated to infections of marine animals (Austin, 2010).

Bacteria from the genus *Vibrio* are frequently isolated from outbreaks in marine fish such as *V. alginolyticus* in the gilthead seabream *Sparus aurata* (Akaayli et al., 2008), *V. harveyi* in seabass *Lates calcarifer* (Tendencia, 2002) and in the summer flounder *Paralichthys dentatus* (Gauger et al., 2006), *V. pelagius* in turbot *Scophthalmus maximus* (Villamil et al., 2003), *V. splendidus* and *V. scophthalmi* in common dentex *Dentex dentex* (Sitja-Bobadilla et al., 2007). In the present study *V. alginolyticus* was isolated from infected *Mugil cephalus*. *Vibrio* could be isolated from gills, mucus of fish and their intestinal contents (Hoi et al., 1998). In our present study also *V. alginolyticus* was isolated from infected organs. Some *Vibrio* strains are pathogenic and can cause Vibriosis, its serious infectious disease in both wild and cultured finfish, shellfish and shrimp (Selvin and Lipton, 2003).

*Vibrio* sp described in Bergey’s manual of Systematic Bacteriology, *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus* and *V. harveyi* have been described as pathogenic for fish (Lightner 1983, Takahashi et al. 1985). In the present study also isolated identified *Vibrio* sp was identified using Bergey’s Manual of Systematic Bacteriology. Changes in environmental factors such as salinity, pH and ammonia are capable of altering the virulence of *V. harveyi* and *V. alginolyticus* (Liu and Chen 2004).
The present work confirms the pathogenic effects of *Vibrio* sp that were the causative agents of mortality in *Mugil cephalus*. In the present study, to prove the Koch’s postulate the same pathogen must present in every case of the disease. The pathogen must be isolated from the diseased host and grown in pure culture. The pathogen from the pure culture must cause the disease when it is inoculated into a healthy, susceptible laboratory animal. The pathogen must be isolated from the inoculated animal and must be shown to be the original organism (Koch 1884). The present result showed that, this bacterial species has been associated with mortality of *Mugil cephalus*. Liu et al., (2004) isolated *V. alginolyticus* from infected shrimp and upon reinjection to healthy shrimp, pathologiical symptoms were observed in its as well. In this present study also proves the the pathogenicity of *V. alginolyticus* which was isolated from Mullet (*Mugil Cephalus*) fish.

The sequence of 1500bp 16S r DNA PCR amplicon from isolates BDTR07 was determined and deposited at Gene bank under the accession number KF758571. This sequences showed 99% similarity with the sequences of Gene bank accession number JX976307 (*V. alginolyticus*), JQ780446 (*V. alginolyticus*). Results from morphology, biochemical tests and 16S rDNA indicated that the isolate was *V. alginolyticus*.

In the phylogenetic tree analysis *V. alginolyticus* and *V. parahaemolyticus* are closely related. Similarly Blake et al. (1980) reported that *V. alginolyticus* and *V. parahaemolyticus*, which are closely related, have similar flagellar systems. The occurrence of *V. alginolyticus* in the marine water samples, as well as in the fish samples was strongly determined by sampling season, i.e. with water temperature.

Enhancement of the immune system by using natural immunostimulants seems to be the most promising method of preventing fish diseases (Anderson et al., 1992).
The nonspecific immune system of fish is considered to be the first line of defence against invading pathogens, and is more important for fish than for mammals (Narnaware et al., 1994). Natural immunostimulants are biocompatible, biodegradable and safe for the environment and human health (Ortuno et al., 2002), offering an alternative to the drugs, chemicals and antibiotics currently used in fish culture to control disease. The major components of the innate immune system (non specific) are macrophages, monocytes, granulocytes and humoral elements, like lysozyme or complement system (Secombes and Fletcher, 1992; Magnadottir, 2006).

Immunostimulants and adjuvant used in fish vaccines are of interest, as they offer an alternative to the drugs, chemicals 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 (Fujiki et al., 1997). Immunostimulants can be applied via injection, bathing or oral administration, the latter seems to be the most practicable (Yin et al., 2006). Therefore in the present study we applied the immunostimulant through oral administration.

The study of blood parameters is one of the most valuable diagnostic tools because it has been shown that the physiological values of these parameters are species-specific and age-dependent (Anver, 2004; Darvishbastami et al., 2009). In medicine, haematological techniques are used for the following three objectives (listed in order of frequency as reported by Blaxhall, 1972). The variation degree on the haematological response is an important tool to fish health diagnosis and may vary
according to stressor stimulus, treatment, parasitic or infectious diseases (Chen et al., 2004; Rehulka, 2002). In present study infected fish RBC count were decreased in the fish. Decreased RBC counts and hematocrit concentration indicate that RBCs are being destroyed by the leucocytosis activity in an erythrocytic anemia with subsequent erythroblastosis (Haney et al., 1992).

A decline in RBC and HCT combined with signs of anaemia was also described by Hoffmann and Lommel (1984) in cases of proliferative kidney disease (PKD). In the Diet 1, Diet 2 and Diet 3, showed an increased blood count when compare with control diet. WBC, RBC, HB and HT was decreased in the infected fish when compared with control group. Waagbo et al. (1988) reported that in Atlantic salmon, Salmo salar with the ‘Hitra disease’ decrease in the values of RBC, HCT and HB, associated with symptoms of severe anaemia. Cardwell and Smith (1971) did find a progressive effect on HCT and HB in juvenile chinook salmon with Vibriosis. Harbell et al. (1979) recorded the same in coho salmon, Oncorhynchus kisutch experimentally infected with a highly virulent Vibrio anguillarum.

In our present study, the values of WBC, RBC, hemoglobin, and hematocrit were lower in the normal condition. Similarly Hrubec et al. (2000) reported that the values of hematocrit, RBC and thrombocytes were lower in the tilapia in normal conditions (Tavares-Dias et al., 2000; Barros et al., 2002 and Ghiraldelli et al., 2006). Rafiq et al. (2001) did not observe any alteration in the differential counts of white blood cells in tilapia challenged with A. hydrophila. In carp experimentally infected with A. hydrophila. Harikrishnan et al. (2003) have related increased WBC counts and also reported that decreased RBC counts and hematocrit indicate that erythrocytes are being affected or destroyed with the infection.
In our present study, experimental group RBC’s was increased during experiments. RBC and HB concentration tend to increase with length and age of the fishes (Das 1965). Blaxhall and Daisley (1973) have reported the possibility of using HCT as a tool in the aquaculture and fishery management for checking the anaemic condition. HCT value in the present study was within the range 20- 33%, fish HCT values were usually between 20% and 35% and rarely attain greater than 50% (Clark et al., 1976).

In fish, the activation of non-specific defense mechanisms is evident by increased production of oxygen radicals by monocytes/macrophages and neutrophils (Miyazaki, 1998). Phagocytes produce a toxic oxygen forms during the process called as respiratory burst (Neumann et al., 2001). Destruction of ingested micro-organisms may be due to degranulation or metabolic activation, when toxic intermediates of oxygen reduction are produced. Since superoxide anion is the first product to be released from the respiratory burst, the measurement of O₂⁻ has been accepted as a precise way of measuring respiratory burst (Secombes and Olivier, 1997). This activity can be measured photometrically by detecting the amount of superoxide (O₂⁻)
anion. In the present study, the nitroblue tetrazolium (NBT) assay, this measures the amount of intracellular superoxide anions. The significant increase in NBT activity and MPO content by lactoferrin, glucan, levamisole and vitamin C feeding in healthy subgroup were also reported earlier in other fish species (Kumari et al., 2003). In the present study NBT was significantly increased in the experimental diet 2 when compared with other groups.

Fish fed with 0.1% and 1.0% Prunella vulgaris extract enriched diet showed significantly enhanced phagocytic activity from weeks 1 to 4 compared to the control
(Harikrishnan et al., 2011). In this study S. wightii (1%) with fed diet 2 increased the
NBT activity from 1\textsuperscript{st} week to 4\textsuperscript{th} week when compared with control. Shieh-tsung et
al. (2008) reported that, phagocytic activities and respiratory bursts of fish fed with
2\% of E. fuscoguttatus (sodium alginate) diets were significantly higher than those of
fish fed the control diet after 6 days of feeding. Our present study also reported that
NBT was increased after 6 days. But in this result, 1\% of Sargassum wightii showed
respiratory bursts activity significantly higher than control in Mugil cephalus.

Lysozyme is also one of the defensive factors against invasive microorganisms
in vertebrates (Janbaz et al., 2002). It lyses Gram-positive bacteria by splitting the β-
1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine in cell walls,
and as well as kill some Gram negative bacteria after complement and other enzymes
have disrupted the outer cell walls (Hjelmeland et al., 1983). A total lysozyme level
is a measurable humoral component of the non-specific defence mechanism.
Lysozyme is a fish defence element, which causes lysis of bacteria, parasite and viral
infection, fish blood is considered as a good indicator of its immune function
(Puangkaew et al., 2004) and activation of the complement system and phagocytes by
acting as opsonin (Magnadottir, 2006)

Lysozyme is a cationic enzyme that breaks β-1, 4 glycosidic bonds between N-
acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell
walls and is known to attack mainly Gram positive bacteria as well as some Gram
negative bacteria in conjunction with complement. Since O\textsubscript{2} is the first product
released during the respiratory burst, O\textsubscript{2} concentration has been accepted as an
accurate parameter to quantify the intensity of a respiratory burst (Secombes, 1990).
For example, stimulation of lysozyme activity has been recognized after three days
and four weeks of injection with levamisole in rainbow trout. An increased protection against fish bacterial infection correlated to an increment in serum lysozyme levels, phagocytic activity and bactericidal activity of head kidney leukocytes (Robertson et al., 1994).

Hot water extract of S. wightii seaweeds enhanced the lysozyme activity in M. cephalus. In Oreochromis niloticus fed with 0.1 and 0.5% Astragalus radix root for 1 week, lysozyme activity was enhanced (Yin et al., 2006). In the present study, all the doses of hot water extract of Sargassum wightii incorporated in the fed diet significantly enhanced the lysozyme activity in the first week of the experiments. Similarly Sea bass Dicentrarchus labrax which had been fed a diet containing sodium alginate from brown algae Laminaria digitata and Ascophyllum nodosum after 15 days showed increased alternative complement and lysozyme activity (Bagni et al., 2005).

Christybakita et al. (2007) reported that aqueous extract-incorporated in the diet significantly enhanced the lysozyme activity after 1, 2 or 3 weeks. In the present study aqueous extract of Sargassum wightii. Elevated lysozyme activity was noted on 20, 25 and 30 days after feeding in Jian carp (Jian and Wu, 2004) and large yellow croaker, Pseudosciaena crocea (Jian and Wu 2003) with traditional Chinese medicine (TCM) formulated from Astragalus root (Radix astragalin seuheydsari) and Chinese Angelica root (R. angelicae sinensis) at a ratio of 5:1 (w/w).

Angka et al. (1995) reported that intramuscular injection of A. hydrophila into cat fish, Clarias gariepinus, caused severe skin and muscle lesion at the injection sites of fingerlings, and fish began to die 18h after injection. Sharifpour (1997) observed that intramuscular injection of A. hydrophila (T4) (at a concentration of 5.3×10⁶ cells ml⁻¹) caused infection in common carp, Cyprinus carpio, and fish started to die within
12 h with one quarter of the injected fish dying between 12 and 24h post injection. During bacterial challenge, fish injected i.p. with *A. hydrophila* (T4 strain) suspension 5×10⁶ cells ml⁻¹ started to die within 12h. High levels of extracellular proteolytic activity are associated with *A. hydrophila* (Thapanee *et al.*, 2000). In our present study challenge with *Vibrio alginolyticus*, all the test diet showed lowest mortality compared to the without treatment.

All treated groups showed a reduced mortality compared to the control group. The best survival rate was observed in the diet 2 treated with 1% of seaweeds extract by oral administration. Survival rates of infected fish are usually increased after treatment with various immunostimulants (Anderson, 1992; Sakai, 1999), vaccines (Bakopoulos *et al.*, 2003) and probiotics (Brunt *et al.*, 2007). Present study diet 2 (1%) feed diet showed best survival rates when compare with group 5 (infection). Feeding carp with chitosan and levamisole reduced mortality of common carp after challenge with *A. hydrophila* (Gopalakkanan and Arul, 2006). A similar result was reported after feeding large yellow croaker with glucan and challenging with *Vibrio harveyi* (Ai *et al.*, 2007).

The present study concluded that, *V. alginolyticus* was one of the causative agents of tail and skin haemorrhagic infection in the fish *Mugil cephalus*. This attempt confirms the pathogenicity by using Koch’s postulates. The pathogenic *V. alginolyticus* was identified by biochemical and molecular identification. Aqueous extract of *Sargassum wightii* added to a formulated fish diet could activate the non-specific immune mechanisms and disease resistance against *V. alginolyticus* in *Mugil cephalus*. However, the most effective doses, application methods and administration
regimes for different age groups of fish have to be investigated and confirmed before application of this product in culture situations.