1. GENERAL INTRODUCTION

Bacterial diseases are of great concern in aquaculture, mainly because they are cause of severe loss of production with high economic impact. Several species of *Vibrio* and *Aeromonas* are common bacteria in marine environments, have been reported as pathogenic for shellfish and fish. *Vibrio harveyi* has been reported as pathogenic for penaeid shrimps, and associated to mortality of abalone (*Haliotis tuberculata*). *Vibrio* sp have been associated mortality of bivalve molluscs (oysters, clams and scallops) in summer. *Listonella (Vibrio) anguillarum, Vibrio alginolyticus,* and *V. harveyi* are responsible for larval vibriosis in different mollusc species (Garnier et al., 2007; Paillard et al., 2004). *Aeromonas salmonicida* is the causative agent of furunculosis, a bacterial septicaemia of salmonid fish (Daly et al., 1996). Other species of *Aeromonas* are opportunistic pathogens or are found in commensal or symbiotic relationships with animal hosts. *Photobacterium damselae* subsp. *piscicida* has been recognized as the causative agent of fish photobacteriosis (Romalde, 2002). Emergence of multi-drug resistant pathogens now presents an increasing global challenge to veterinary medicine. Therefore, there is a continuous need to develop novel antimicrobial agents to minimize the threat of further antimicrobial resistance (Celiktas et al., 2007).

Antibiotics have revolutionised mankind’s health status, allowing treatment of life threatening infections. However with the increasing occurrence of bacterial resistance against available antibiotics, it has now become essential to look for newer antibiotics. Most of the antibiotics available today come from natural origin, especially from various microbial or marine sources (Sarker et al., 2007). Antibiotic treatment of bacterial diseases in fish culture has been applied for many years. The
occurrence of antibiotic resistant bacteria associated with fish diseases is a worldwide problem in aquaculture, which has received considerable attention in the last years and continues to increase due to the absence of a more effective and safer use of antibiotics. The prevention and treatment of these infectious diseases by applying products from marine organisms appears as a possible alternative. Hence, the interest in marine organisms as a potential and promising source of pharmaceutical agents has increased during the last years (Mayer and Hamann, 2002).

Seaweeds are suitable for animal feed applications. Seaweeds were containing carbohydrates, protein and minerals as well as bioactive compounds such as polyphenols, terpenoids, carotenoids and tocopherols. Seaweeds have been reported to produce a great variety of metabolic compounds which are not produced by terrestrial plants (Plaza et al., 2008).

Seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Compounds with cytostatic, antiviral, anthelmintic, antifungal, and antibacterial activities have been detected in green, brown and red algae (Bibiana et al., 2012). Pharmaceutical importance of seaweed is well known all over the world and extensive efforts were given to bring out substances from algae. There are number of reports regarding the medicinal importance of sea weeds belonging to Phaeophyceae, Rhodophyceae and Chlorophyceae from all over the world (Kolanjinathan et al., 2009). Many studies were reported earlier on the antimicrobial study of marine algae (Battu et al., 2011).

Number of bioactive compounds which have been isolated and identified from seaweeds include sulphated polysaccharides (laminarins and fucoidans), polyphenols
such as phlorotannins (Zou et al., 2008), carotenoid pigments such as fucoxanthin (Aranthi et al., 2011) and astaxanthin, sterols and mycosporine-like amino acids (MAAs) to name some. Presence of polyphenols such as catechin, epicatechin, epigallocatechin gallate and gallic acid are reported in the green seaweed *Halimeda* (Yoshie et al., 2002). Lopez et al. (2011) reported the presence of 14 polyphenols, namely gallic acid, catechin, epicatechin, rutin, p-coumaric acid, myricetin, quercetin and protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids in the solvent extracts of *Stypocaulon scoparium*.

Onofrejova et al., (2010) reported the extraction of bioactive phenolic acids (protocatechuic, p-hydroxybenzoic, 2, 3-dihydroxybenzoic, chlorogenic, caffeic, p-coumaric, salicylic acid), cinnamic acid and hydroxybenzaldehydes (p-hydroxybenzaldehyde, 3,4 dihydroxybenzaldehyde) from food products of *Porphyra tenera* and *Undaria pinnatifida*. Fucoxanthin and phlorotannins have been identified as active antioxidant compounds from *Hijika fusiformis* and *Sargassum kjellmanianum* (Yan et al., 1996), respectively. Aerial parts of green seaweeds have been reported to yield diterpenes, sesquiterpenes and related compounds having antibacterial and antifungal activity (Puglisi et al., 2004). In addition to those mentioned above, bromophenols are present in red algae which, have been reported to function as antimicrobial compounds (Xu et al., 2003).

Fish nutrition is a matter of great importance in aquaculture industry worldwide for better product quality. The optimum growth of this industry can be using appropriate feeds. One important ingredient used in the formulation of commercial aquaculture feed is fish meal which has high protein quality and palatability. Substituting high price fish meal in aqua feed with less expensive protein source in one way of reducing protein production cost. Trash fish, shrimp waste and acetes for
the alternative protein feed for fish. It reduced the production cost of the fish. Shrimp head waste, which represents about 33% of the shrimp weight, is almost completely discarded. The under utilization of the shrimp head waste posing a serious disposal problem, contributes to the overall cost of the production (Cavalheiro et al., 2007).

Fish have high dietary protein requirement. In aquaculture reducing the feeding costs could be key factor for successful development (Deng et al. 2006). Level of dietary protein is of fundamental importance, because it significantly influences growth, survival, and yield of fish as well as economics of a farming industry by determining the feed cost which is typically the largest operational cost. Increase in dietary protein has often been associated with higher growth rate in many species. However, there is a certain level beyond which further growth is not supported, and may even decrease (Kvale et al., 2007). Considerable research effort has been expended to determine the quantity and quality of dietary protein necessary to achieve optimum performance of fish.

Seaweeds with elevated protein content and production rates are receiving increasingly attention as novel feeds with potential nutritional benefits (Buschmann et al., 2001; Ruperez and Saura-Calixto, 2001) and as possible ingredient in fish diets. Interest in the use of edible seaweeds in the development of low-cost, highly nutritive diets for animal nutrition (Fleming et al., 1996). Rapid expansion of fish culture in recent years is demanding the development of nutritious fish feeds, as well as better feed utilization, due to the fact that feed cost may increase the cost of fish production by 50-80% (El-Sayed et al., 2003).

Seaweeds contain several immunologically active substances. The polysaccharides obtained from seaweeds can modify the activity of some components
of the immune system in the fish and increase the protection against certain diseases. Carrageenan, a polysaccharide abundant in certain red seaweeds, induced an increase in macrophage phagocytic activity and in the resistance against bacterial infections after being injected intraperitoneally in carp (Cyprinus carpio) (Fujiki et al., 1997). Sodium alginate was found to enhance migration of carp head kidney phagocytes to the peritoneal cavity, to increase phagocytic activity (Fujiki and Yano, 1997) and the survival of juvenile turbot challenged with Vibrio anguillarum (Skjermo et al., 1995).

The immune system is classified into innate (non-specific) and adaptive (specific) immune systems. The innate immune system of vertebrate is the first line of defense against invading pathogens (Narnaware et al., 1994). The innate system's response to infectious pathogens is determined by the evolutionary lineage and genetic makeup; it has been tailored through time by environmental factors and pathogenic associations (Alvarez-Pellitero, 2008) they maintain stable conditions (homeostasis) during development and growth and following inflammatory reaction or tissue damage (Magnadottir, 2010). The major components of the innate immune system are macrophages, monocytes, granulocytes, and humoral elements, including lysozyme or complement system (Magnadottir, 2006). In fish and shellfish the innate immune system consists of neutrophil activation, production of peroxidase and oxidative radicals, together with initiation of other inflammatory factors (Ainsworth et al., 1991).

One of the most promising methods of controlling diseases in aquaculture is by strengthening the defence mechanism of fish through prophylactic administration of immunostimulants (Robertson, 1999) which is considered as a promising alternative to chemotherapy and vaccines (Secombes, 1996) because of their broad spectrum activity, cost effectiveness, and eco-friendly disease preventative measure. A number
of immunostimulants have been developed and found to be effective in fish and shellfish including chemical agents, bacterial components, polysaccharides, and animal or plant extracts. The immunostimulants are effective means of increasing the immunocompetency and disease resistance by enhancing both specific and non-specific defence mechanisms of fish and shellfish and other animals (Zhou et al., 2003).

On the basis of this working hypothesis and the bioactive potential, the present study was initiated with the following objectives: (i) Isolate and identify bioactive compound from selected seaweeds against the fish pathogens. (ii) Study the effect of seaweeds extraction the growth and feed utilization in Mugile cephalus and Selection of best seaweed. (iii) To study the effect of potential seaweeds extract on non specific immune response against Vibrio alginolyticus.
2. REVIEW OF LITERATURE

CHAPTER I: ANTIMICROBIAL ACTIVITY OF SEAWEEDS

Freile-Pelegri and Morales (2004) studied the antibacterial activity of ethanolic and lipid-soluble extracts of 21 marine algal species against pathogenic microbes. The lipid-soluble extract of Ceramiumnitens exhibited the highest activity among the species tested.

Han et al. (2005) isolated four bromo phenols from the extract of marine red alga, Rhodomela confervoides and tested their antimicrobial activity. The compound 3-bromo-4, 5-dihydroxybenzaldehyde showed powerful antibacterial activities against S.aureus and P. aeruginosa.

Engel et al. (2006) investigated the antimicrobial activity of lipophilic and hydrophilic extracts from 49 marine algal species and 3 seagrasses against marine pathogens and saprophytes. Overall, 90% of all species surveyed yielded extracts that were active against one or more and 77% yielded extracts that were active against two or more test organisms. Broad spectrum activity against three or four assay microorganisms was observed in the extracts from 48 and 27% of all species, respectively. The extracts of green algae Halimeda copiosa and Penicillus capitatus were showed activity against all the test organisms. Results from his survey demonstrate that antimicrobial activities are prevalent among extracts from marine algae and seagrasses, suggesting that antimicrobial chemical defenses are wide spread among marine plants.

Taskin et al. (2007) investigated the antibacterial activity of the methanolic extracts of six marine algae for against pathogenic microbes. Extracts of all the tested
marine algae except *Corallina officinalis* showed inhibition against *Staphylococcus aureus*. On the other hand, highest inhibition activity among all the extracts was shown to *E. aerogenes* by *C. officinalis*. The extract from *Cystoseira barbata* has shown broader activity spectrum against all the test organisms.

Matanjun *et al.* (2008) studied about the antioxidant activity of eight edible species. Methanol and diethyl ether were used as extraction solvent. The antioxidant activities were determined by two methods, TEAC (Trolox Equivalent Antioxidant Capacity) and FRAP (Ferric Reducing Antioxidant Power) assays. The methanolic extracts of green seaweeds, *Caulerpa lentillifera* and *C. racemosa*, and the brown seaweed, *Sargassum polycystum* showed better radical scavenging and reducing power ability, and higher phenolic content than the other seaweeds. These seaweeds could be potential rich sources of natural antioxidants.

Shanmugapriya *et al.* (2008) studied the antimicrobial and antifungal property of fourteen seaweeds collected from the intertidal zone of Southwest coast of India. Methanol: toluene (3:1) was found to be the best solvent for extracting the antimicrobial principles from fresh algae. However, the ethanolic extract showed no antibacterial activity. *Acrosiphonia orientalis* showed activity against 70% of the tested organisms. *Stocheospermum marginatum* was the only seaweed that showed activity against *Klebsiella pneumoniae*. The extract from *Gracilaria corticata* was highly active against *Proteus mirabilis*. His study evidenced that seaweeds are highly active against Gram negative bacteria than Gram positive bacteria. The antimicrobial principle from seaweed was found to be a lipophilic compound.

Taemchuay *et al.* (2009) prepared the crude extract from *Centella asiatica* with either ethanol or water and tested against 30 bacterial isolates. The results showed that
ethanol extracts and water extracts had average inhibition zones ranged from 6.44-6.49 and 6.54-17.72 mm in diameter, respectively. Results showed that the ethanol extracts had an MIC50 value of 8 mg ml\textsuperscript{-1}, the water extracts of leaf powder had an MIC50 value of 32 mg ml\textsuperscript{-1}, and the water extracts of fresh leaves had an MIC value of 32-256 mg ml\textsuperscript{-1}. The ethanol extracts had an MBC value of 16 mg ml\textsuperscript{-1}. The water extracts could not kill \textit{Staphylococcus aureus}. It has been conclude, the ethanol extracts had more potential antibacterial activity than the water extracts.

Vedhagiri \textit{et al.} (2009) investigated pharmacologically active compounds that were isolated from \textit{Asparagopsis taxiformis} against the \textit{Leptospira javanica}. The GC-MS analysis of the purified compound revealed the presence of 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (56.012%), fattyacids, 14-methyl-pentadecanoic acid methyl ester (26.6%), octadecanoic acid methyl ester (8.46%), octadec-9-enoic acid 2,3-dihydroxy-propyl ester (4.11%), 9-octadecanoic acid, methyl ester (4.535%) and trace amount of chlorobenzene (0.09%). The seaweed active fraction exhibited comparable MIC and MBC values with that of the standard antibiotic doxycycline.

Kolanjinathan \textit{et al.} (2009) prepared the ethanol extract from \textit{Gracilaria edulis}, \textit{Calorhapeltada} and \textit{Hydroclothres} sp., for screening for their antibacterial activity against six bacterial pathogens. Ethanol extract of \textit{G. edulis} inhibited growth of all the test organisms except \textit{Bacillus cereus} and \textit{Enterobacter aerogenes}. Seaweed ethanol extract of \textit{Calorpha peltada} was found effective against Gram negative and Gram positive bacteria such as \textit{Escherichia coli}, \textit{Staphylococcus aureus} and \textit{Streptococcus faecalis}. \textit{Hydroclothres} sp., ethanol extract inhibited the growth of \textit{Pseudomonas aeruginosa} only.
Manilal et al. (2009) evaluated the antibacterial property of the red algae, *Falkenbergia hillebrandii* (Born) against three multidrug resistant human pathogens. Dried samples extracted with methanol showed broadest and highest antimicrobial activity when compared to other solvent extracts. The highly active compounds from the red alga, *F. hillebrandii* were fractionated and purified using different chromatographic systems, including reverse phase HPLC and GCMS. The analysis revealed that the most abundant metabolite was oleic acid (51.33%) followed by *n*-hexadecanoic acid (42%).

Hwang et al. (2010) studied about the hot-water extract of seaweed *Sargassum hemiphyllum* for antioxidant activity by using four different methods, including DPPH free radicals scavenging activity, Fe$^{2+}$ chelating activity, superoxide anion radical scavenging activity and reducing power. It was found that the antioxidant activity was increasing in correlating with the concentration below 3.5 mg ml$^{-1}$. Hence, the hot-water extracts of seaweed *S. hemiphyllum* plays the important role as antioxidant.

Srivastava et al. (2010) made an *in vitro* antimicrobial activity study on two species of seaweed samples namely *Caulerpa racemosa* and *Grateloupia lithophila*. Both the seaweeds had shown moderate antibacterial activity with <15 mm of zone of inhibition. Out of which only butanolic extract of has shown significant activity. Phytochemical screening revealed the presence of alkaloid and phenolic compounds in both the seaweeds whereas flavonoids and steroids were found to be present in only *Caulerpa racemosa*.

Pierre et al. (2011) studied the *invitro* antimicrobial activity of the marine green algae *Chaetomorpha aerea* against bacteria and fungi. The water-soluble extract of algae was composed of a sulfate with a molecular weight of $1.160 \times 10^6$ Da. The
resuspended extracts (methanol, water) exhibited selective antibacterial activities against *Staphylococcus aureus* (ATCC 25923). MIC and MBC tests showed that the sulfated galactan could be a bactericidal agent for this strain (40 mg ml⁻¹). The results of this study confirmed the potential use of the green algae *Chaetomorpha aerea* as a source of antibacterial compounds.

Ravikumar *et al.* (2011) studied the antiplasmodial activity of 13 mangrove plants and eight seaweeds. In vivo test was carried out with rat animal model to find out the effectiveness of the polyherbal extracts in the live system, which reveals that polyherbal extracts have exhibited remarkable antiplasmodial activity against *Plasmodium berghei*. The results showed that combinations of mangrove plants and seaweeds extracts had a source of lead compounds for the development of new drugs for the treatment of malaria.

Rebecca *et al.* (2012) investigated the antibacterial potential of two species of seaweeds namely, *Sargassum ilicifolium* and *Kappaphycus alvarezii*. Crude extracts prepared from chloroform, ethanol and methanol revealed a wide range of antibacterial activity against the test pathogens. Maximum inhibition was noticed with ethanol extracts in case of *S. ilicifolium*. 
CHAPTER II: SEAWEED AS GOOD GROWTH FACTOR

Green et al. (2002) optimized the dietary essential amino acid (EAA) pattern for Oncorhynchus mykiss. Experimental diets were fed to fish in quadruplicate tanks, using the equalized satiation feeding method. In the first experiment, they used the amino acid deletion method to arrive at an estimate of optimum dietary EAA pattern for rainbow trout. Response variables included growth rate, feed efficiency ratio and N retention and excretion. The EAA pattern associated with EAA requirements as published by the National Research Council was found to result in the highest mean N retention and lowest mean N excretion, and so was considered the best estimate of optimum EAA pattern of those compared.

Telat et al. (2003) reported the effect of Ohio State University (OSU) meal, as partial or total replacement of fish meal. Thirty-two fish tanks, each containing 30 rainbow trout were fed one of 10 diets (the control had only two replicates) containing a different quantity of OSU meal for 14 weeks. The trout fed the diets containing 20% or 40% OSU meal grew similarly to the trout fed the fish meal based diet. Total replacement of the fish meal caused a significant reduction in growth ($p<0.05$) only at the 47% protein level and not at the 36%. His study suggested that diets containing up to 75% OSU meal and 25% fish meal are sufficient for good growth in rainbow trout fry.

Luo et al. (2005) determined the effect of dietary lipid levels on growth performance and body composition of grouper Epinephelus coioides. Six isonitrogenous diets (53% dietary protein) with increasing dietary lipid concentration (5.16 to 16.04 % of dry material, DM) for 56 days. Fish fed the 9L diet had the highest weight gain (WG) and specific growth rate (SGR), but they were not
significantly different from that of fish fed the 7L or 12L diet (p > 0.05). FI varied inversely with dietary lipid levels. The poorest FCR and the lowest PER were observed in fish fed the 5L diet. Nitrogen intake decreased with dietary lipid levels. Fish fed the 7L diet showed the highest N gain, which was not markedly different from that of fish fed the 9L and 12L diets (p > 0.05). Condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI) increased with increasing dietary lipid level. Based on WG against dietary lipid level, a breakpoint of 10.0% was indicated to be the optimal dietary lipid concentration for maximum growth for grouper *Epinephelus coioides* juveniles cultured in floating netcages.

Abbas *et al.* (2005) investigated the effects of dietary protein levels on growth, feed utilization, hepatosomatic index and liver lipid deposition of juvenile *Lutjanus argentinaculatus*. In this experiment, six fishmeal based diets were formulated to contain various protein levels. The fish at the end of the study had more than ten-fold (77.0 g) increase in weight compared to the initial (8.0 g). Fish fed diets of 40% and 45% protein produced significantly (p < 0.05) higher weight gain of 77.2 g and 76.5 g, and specific growth rate (SGR) of 2.65% and 2.62% than those of 67.0 g and 68.3 g, and 2.49% and 2.51% of the other diets. Feed conversion ratio decreased from 0.45 for fish fed 20% dietary protein to 0.35 for fish fed 45% dietary protein. The Hepatosomatic index of fish fed diets of 20%, 25%, 30% and 35% protein were significantly (P < 0.05) higher (2.09% - 2.57%) than those (1.44% and 1.41%) of fish fed diets containing 40% and 45% protein. Liver lipid contents decreased from 8.72% to 7.0% in fish fed dietary protein of 20% to 45% in 5% increments. His study suggest that the diet containing 40% to 42.6% protein with a P/E ratio of 17.6 mg protein kJ$^{-1}$ is required for good growth of *L. argentinaculatus* weighing between 8.0 g and 85.2 g.
Jamil et al. (2007) evaluated the nutritive value of a mixture of animal by-products (MAB) as a possible protein source in diets for juvenile mangrove red snapper. Fish were fed with one of five isonitrogenous diets (40% crude protein) by replacing 0, 25%, 50%, 75% and 100% of fish meal protein with similar percentages of MAB. After 75 d of feeding, fish fed with diets. The optimum level of MAB was estimated to be 23%. The levels of protein and ash in the whole body, carcass and viscera increased as MAB substitution in diets increased, whereas lipids and moisture remained consistent among all treatment groups. These results showed that approximately 23% of fish meal protein could be replaced by a mixture of animal by-products for juvenile snapper growing from 30 g to 167 g in 75 d without compromising growth performance and feed efficiency.

Cavalheiro et al. (2007) formulated fish diet by using shrimp waste. Four feeds, in the form of pellets, were prepared by substituting shrimp head silage for fish flour at four different concentrations with other ingredients were used in the formulation. A commercial fish feed was used as the control. No significant differences in growth of juveniles fed on R1, R2, R3, or R4, or the control feed, was observed. Similarly, the proximate analysis of the flesh of juveniles did not present significant differences (p > 0.05). This result indicated that the shrimp head silage could replace fish flour as an ingredient in tilapia feed with economic advantages and without sacrificing the quality of the feed.

Tang et al. (2008) investigated the effects of fish protein hydrolysate (FPH) on growth performance and humoral immune response of the large yellow croaker. The animals were fed with 4 diets: basal diet only (control), diets supplemented with 5%, 10% and 15% (w/w) FPH. This study found that dietary FPH levels significantly influenced the growth and immunity of the large yellow croaker. In general, with the
supplementation of FPH, particularly at dose of 10%, the growth performance and immunity of the large yellow croaker can be improved effectively.

Siddiqi and Khan (2009) conducted 8-weeks growth trial to assess the effect of dietary protein on growth, feed utilization, protein retention efficiency, and body composition of young Heteropneustes fossilis (10.02 ± 0.09 g; 9.93 ± 0.07 cm). Isocaloric (4.15 kcal g⁻¹, GE) diets with varying levels of protein (25, 30, 35, 40, 45, and 50% of the diet) were fed near to satiation to triplicate groups of fish. Optimum dietary protein was determined by analyzing live weight gain (LWG %), feed conversion ratio (FCR), protein efficiency ratio (PER), specific growth rate (SGR %), and protein retention efficiency (PRE %) data. Maximum LWG% (167), best FCR (1.42), PER (1.75), SGR (1.76), and PRE (31.7%) were evident in fish fed 40% protein diet (Diet 4). Body protein data also supported the above level. However, second-degree polynomial regression analysis of the above data indicated that inclusion of dietary protein in the range of 40-43% is optimum for the growth of young H. fossilis.

Sotolu, (2010) reported the effects of dietary vegetable lipid sources on the growth performance and haematology of African catfish, Clarias gariepinus. They concluded that, vegetable oil sources including palm oil, soybean oil and groundnut oil can be utilized by catfish as the sole lipid source in fish diet with negligible impact on growth performance while the utilization of benni seed oil should be limited for efficient feed utilization by fish and ensure good food fish quality.

Akpinar et al. (2012) studied the effects of dietary lipid levels on growth and feed utilization in Shi drum (72.6 g average weight). Four isonitrogenic diets differing in dietary lipid levels between 10 and 19% were fed to triplicate groups of fish for 8
weeks. He concluded that high dietary lipid did neither had any protein-sparing effect nor other positive result, and diets for Shi drum are not recommended to contain more than 13% lipid.

Zehra et al. (2012) determined the dietary protein requirement for *Channa punctatus* fingerlings by feeding six isocaloric diets. Six types of diets were prepared with different levels of protein. Maximum absolute weight gain (AWG; 8.11 g fish\(^{-1}\)), specific growth rate (SGR; 1.82%) and best feed conversion ratio (FCR; 1.48) were recorded in fish fed diet containing 450 g kg\(^{-1}\) protein, whereas protein efficiency ratio (PER; 1.52), protein retention efficiency (PRE; 25%), energy retention efficiency (ERE; 78%) and RNA/DNA ratio (3.01) were maximum for the group fed dietary protein at 400 g kg\(^{-1}\). Considering the PER, PRE, ERE and RNA/DNA ratio as more reliable indicators, this protein requirement is recommended for developing quality protein commercial feeds for *C. punctatus* fingerlings.
Chapter III: SEAWEED ACT AS IMMUNOSTIMULANT

Yoshida et al. (1993) demonstrated that in African catfish the number of NBT-positive cells increased following oral administration of glucan or oligosaccharide over 30 days, but not over 45 days. The herbal and its products were used in fish feed ranging from 0.1% to 10% for 14 days to 70 days. Although the reasons for these increased in immune responses in fish by long-term oral administration of herbal immunostimulants is still unclear. Thus, the effective administration period should be investigated for each immunostimulant.

Robertson et al. (1994) reported that the increase in respiratory burst activity of glucan-treated macrophages was maximal at 0.1-1 mg ml⁻¹. Comparable effects were also observed in lymphocytes. High doses of FK 565 at 10 mg kg⁻¹ did not increase the number of plaque-forming cells (PFC) against Y. ruckeri, although the increase was noted at the optimum dose of 5 mg/kg. The effects of immunostimulant are therefore species specific and hence the threshold dose to elicit the response, the optimum dose to trigger maximum immune response and the efficacy of high doses which may not enhance or even inhibit the immune response has to be found out.

Yoshida et al. (1993) reported that immunostimulation is one of the useful tools in aquaculture where vaccination or treatment by injections are difficult and laborious processes, and where repeated chemotherapy poses a problem of developing drug resistant strains of pathogens. Administration through injection enabled the immunostimulant to be quickly absorbed and functional, while in the oral administration, the immunostimulant is slowly absorbed by the fish.
Figure 1: Schematic representation of fish immune response against immunostimulation.

Sarder et al. (2001) reported the importance of genetic variation in the non-specific immune responses of Nile tilapia (*Oreochromis niloticus* L.) clones. Fully inbred clones (IC) of Nile tilapia, produced using gynogenesis and sex reversal, and crosses between these lines were used in this study. Non-specific immune responses were compared between the ICs, including serum lysozyme activity and phagocytosis, and significant differences were observed between the different groups. Their natural resistance to *Aeromonas hydrophila* infection was also assessed by bacterial challenge. A positive correlation was observed between the level of infection obtained and the non-specific immune parameters measured. Cumulative mortalities of fish obtained in the study showed that when an IC susceptible to *A. hydrophila* was crossed with a resistant IC, the resulting progeny exhibited intermediate levels of resistance to that of their parents.

Annick Robert-Pillot et al. (2002) compared the efficiencies of biochemical methods and polymerase chain reaction (PCR) for the identification of *Vibrio parahaemolyticus* strains. His suggest that biochemical tests are not accurate enough for the identification of *V. parahaemolyticus* isolates. They demonstrate that
amplification of the R72H fragment, whether the amplicon is 320 bp or 387 bp long, is a powerful tool for the reliable identification of *V. parahaemolyticus*.

Smitha *et al.* (2003) reviewed the growing need to control, prevent or minimize the devastating effects of disease in crustacean culture, without recourse to toxic chemicals or antibiotics. The agents currently under scrutiny for crustaceans include glucans, lipopolysaccharide and killed bacterial cells. They are thought to act as ‘immunostimulants’ because of their known effects on the crustacean immune system in vitro. Analysis of the validity of the results of many of the published studies raises questions about the value of these compounds for cost-effective control of infection in aquaculture, especially for long lasting protection in both adults and juveniles. This review further discusses the potential risks to the wellbeing of the stock animals from repeated use of these agents and makes the case for rigorous testing of putative stimulants, at the gene, protein and functional levels, as well as for the need to consider alternative strategies and approaches to disease control.

Smith *et al.*, (2003) reported immunostimulation unlike vaccination, which is applied for the purpose of conferring long-lasting protection through immunological memory, targets complement activation, phagocytosis, and cytokine secretion without necessarily or purposefully requiring a specific response to a defined antigen. Substances used for immunostimulation therefore increases the resistance of the host, not by enhancing specific memory responses, but by enhancing nonspecific defense mechanisms.

Zorrilla *et al.* (2003) isolated the bacteria from an outbreak with moderate mortalities in farmed sole, were identified as *Vibrio harveyi* and *Vibrio parahaemolyticus*. Only bacterial strains showing swarming were virulent in sole and
caused mortalities in experimentally inoculated fish. However, the signs of the disease were only reproduced with *V. harveyi*. The intramuscular inoculation of the extracellular products (ECPs) of both species produced mortalities in inoculated fish and the appearance of surface ulcers in the case of *V. harveyi*. However, the inoculation of sublethal doses of ECPs to fish showed a protective effect against *V. harveyi*.

Bobadilla *et al.* (2005) reported that partial or total replacement of fish meal by a mixture of plant protein (PP) sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) balanced with indispensable amino acids was examined in juvenile gilthead sea bream. His study conclude that, substitution of FM by a mixture of PP sources exerted an anti-oxidative effect, compromised growth performance only at the 100% level, and decreased one of the immune defense mechanisms at above 75% level.

Selvaraj *et al.* (2005) reported decades many substances have been shown to enhance the immunity of fish and the route of administration of them has differential effects on the immune system. However, the drawback of injection is that it is labour intensive and also stressful to the fish. Thus, oral administration is a useful alternative method for mass administration to fish of all sizes.

Kennedy *et al.* (2006) reported that the immunomodulation can be achieved with vaccines, which enhance the acquired or specific immune response of the fish and shellfish but a single vaccine is effective against only one type of pathogens. However at present no effective vaccines available for many fish and shellfish diseases because of the complex antigenic structure of the pathogens. Furthermore,
the development of vaccines against intracellular pathogens has not so far been successful.

Kumari and Sahoo (2006) studied the efficacy and immune reversal effects of four well-known dietary immunomodulators viz., lactoferrin, β-1,3glucan, levamisole and vitamin C on the innate immune response of healthy and cyclophosphamide (CYP) induced immunocompromised Asian catfish, *Clarias batrachus*. His results showed that all the substances delivered as feed supplements significantly (p<0.05) enhanced most of the non-specific immune parameters in both healthy and immunosuppressed subgroups compared to their respective controls. Levamisole induced significantly increase in blood phagocytic activities in both healthy and immunocompromised fish as observed from raised respiratory burst and myeloperoxidase activity as compared to control. Feeding of vitamin C significantly enhanced phagocytic activity and myeloperoxidase content. On the other hand, all parameters were positively influenced by lactoferrin and glucan feeding in both groups compared to their respective controls.

Su-Tuen *et al.* (2006) reported the total haemocyte count (THC), phenoloxidase activity, and respiratory burst were examined when the white shrimp *Litopenaeus vannamei* (10.42 ±1.39 g) were immersed in seawater (34%) containing hot-water extract of brown alga *Sargassum duplicatum* at 100, 300 and 500 mg μ l⁻¹, or injected with hot-water extract of *S. duplicatum* at 2, 6, 10 or 20 mg g⁻¹. His concluded that *L. vannamei* that were immersed in hot-water extract of *S. duplicatum* at 300 mg l⁻¹, or the shrimp that were injected with hot-water extract at 10 mg g⁻¹ or less had increased immune ability as well as resistance to *Vibrio alginolyticus* infection.
Christybabita *et al.* (2007) studied the immunostimulatory effects of the oral administration of the medicinal plant, *Eclipta alba* leaf extracts was studied in tilapia, *Oreochromis mossambicus*. For this purpose, fish were fed for 1, 2 or 3 weeks with diets containing *E. alba* leaf aqueous extract at 0, 0.01, 0.1 or 1% levels. The results indicated that *E. alba* aqueous extract administered as feed supplement significantly enhanced most of the non-specific immune parameters tested. His study concluded that dietary intake of *E. alba* aqueous leaf extract enhances the non-specific immune responses and disease resistance of *O. mossambicus* against *A. hydrophila*.

Ann-Chang *et al.* (2007) reported the lysozyme activity, alternative complement activity (ACH50), respiratory burst, SOD (superoxide dismutase) activity and phagocytic activity of orange-spotted grouper *Epinephelus coioides* were examined when the fish were injected intraperitoneally with sodium alginate at 10, 20, 30 mg kg⁻¹ and i-carrageenan at 10, 20, 30 mg kg⁻¹, respectively after 24, 72 and 120 h. His present study concluded that *E. coioides* which received sodium alginate at 20 mg kg⁻¹ or i-carrageenan at 30 mg kg⁻¹ increased the nonspecific immune response and resistance from *Vibrio alginolyticus* infection.

Laszlo *et al.* (2008) reported the effect of two Chinese medicinal herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron on non-specific immune response of Nile tilapia (*Oreochromis niloticus*) was investigated. Five diet variations in addition to a control diet (without herbs or boron) were used. These contained 0.1% *Astragalus* (with and without 0.05% boron), 0.1% *Lonicera* (with or without 0.05% boron) and a mixture of the two herbs with 0.05% boron. Results of his study showed that feeding tilapia with two herbs alone or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocytic cells. They had a moderate effect on the plasma lysozyme level. Both herbs reduced
the mortality following *Aeromonas hydrophila* infection. It can be concluded that *Astragalus* and *Lonicera* extracts and boron supplementation added to fish feed can act as immunostimulants and enhance the immune response and disease resistance of cultured fish.

Tingting *et al.* (2009) reported the immunostimulatory effect of oral administration of different preparations (conventional fine powder (CP) and superfine powder (SP)) of *Astragalus membranaceus* root or its polysaccharides (APS) in sea cucumber (*Apostichopus japonicus*) was investigated. Sea cucumbers with an average initial weight of 49.3-5.65 g were fed with a diet containing 3% CP or SP or 0.3% APS over a period of 60 days. The non-specific humoral and cellular responses were determined and compared with controls (no supplement) after 20, 40 and 60 days of feeding. His study indicated that dietary intake containing *A. membranaceus* root or its polysaccharides could enhance the immune responses of *A. japonicus* and improve its resistance to infection by *Vibrio splendidus*.

Harikrishnan *et al.*, (2009) studied the effect of traditional *Prunella vulgaris* extracts enriched diets were investigated the non-specific immune response and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Uronema marinum*. The effect of *P. vulgaris* extracts enriched diets on respiratory burst activities, plasma lysozyme, and complement activity. Feeding with 0.1% and 1.0% diets was the most effective and reduced the mortality then 0.01% diet. These results indicate that 0.1% and 1.0% doses of *P. vulgaris* extracts enriched diets potentially enhanced the non-specific immune response and disease resistance of *P. olivaceus* against *U. marinum*. 
Alexander et al. (2010) described the effect of water-soluble fraction of the leaves of the Indian medicinal plant, *Tinospora cordifolia* (Miers) on the non-specific immunity and disease resistance in *Oreochromis mossambicus* (Peters). Fish were intraperitoneally injected the water soluble fraction. The non-specific humoral and cellular responses and disease resistance against *Aeromonas hydrophila* were tested. All the doses of water-soluble fraction tested, significantly enhanced the serum lysozyme, antiprotease and natural haemolytic complement activities on most of the days tested. All the doses of water-soluble fraction when administered as a single or double dose gave protection in terms of reduced percent mortality which is reflected in the increased relative percent survival (RPS) values. The results clearly indicate the immunostimulatory and disease resistance properties of *T. cordifolia* leaf fraction and so its potential to be used as an immunoprophylactic in finfish aquaculture.

Caruso et al. (2011) studied the growth, hematological (hematocrit), biochemical (serum cortisol and glucose), and non-specific immune (lysozyme, serum hemolytic and haemagglutinating activities, extracellular respiratory burst activity) parameters, were monitored in European sea bass *Dicentrarchus labrax* and black spot sea bream *Pagellus bogaraveo* subjected to a 31 days starvation compared to fed fish, to assess the responses to feed deprivation of these health status indicators. Haemagglutinating activity was significantly lower in starved sea bass than in fed fish after 31 days. In blackspot sea bream, a slight, not significant, reduction in haemagglutinating activity occurred 11 days after starvation. Respiratory burst activity decreased significantly in the starved fish. In spite of the limited number of examined parameters, the opportunity to use a panel of several indicators to obtain a more complete picture of health status in fish was underlined.
Truong-Giang et al. (2011) examined the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus* and WSSV when shrimp received the *Sargassum hemiphyllum* and Chinense powder and its hot-water extract. Both powder and extract showed activation of immune parameters. Shrimp was immersed and challenge with WSSV and both *V. alginolyticus*. Survival rate of shrimp immersed in 300 mg l⁻¹ extract was significantly higher than that of control shrimp after 72-120 h in both *V. alginolyticus* and WSSV challenge tests. It was concluded that the shrimp immersed in seawater containing the powder at 500 mg l⁻¹, and the extract at 300 mg l⁻¹ had increased immunity and resistance against *V. alginolyticus* infection, and the shrimp that received extract at 300 mg l⁻¹ showed resistance against WSSV infection.

Noorlis et al. (2011) investigate the prevalence and concentration of *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish using the Most Probable Number-Polymerase Chain Reaction (MPN-PCR) method. The study was conducted on 150 samples from two types of freshwater fish commonly sold at hypermarkets, i.e. *Pangasius hypophthalmus* (catfish) and *Oreochromis* sp. (red tilapia). Sampling was done on the flesh, intestinal tract and gills of each fish. The prevalence of *Vibrio* spp. and *V. parahaemolyticus* was found to be 98.67% and 24% respectively with higher percentages detected in samples from the gills followed by the intestinal tract and flesh. *Vibrio* spp. was detected in almost all red tilapia and catfish samples. *V. parahaemolyticus* was detected in 25% of the catfish samples compared to 22.6% of red tilapia fish. The density of *Vibrio* spp. and *Vibrio parahaemolyticus* in the samples ranged from 0 to 1.1x10⁷ MPN g⁻¹. Although the maximum value was 1.1x10⁷ MPN g⁻¹ most samples had microbial loads ranging from 0 to >10⁴ MPN g⁻¹. The outcome on the biosafety assessment of *Vibrio* spp. and *V.
*parahaemolyticus* in freshwater fish indicates another potential source of food safety issues to consumers.

Immanuel *et al.*, (2012) extracted polysaccharide-fucoidan from *Sargassum wightii*. The extracted fucoidan was supplemented with pellet diets at three different concentrations (0.1, 0.2 and 0.3%). During the challenge test, the control group showed 100% mortality within 10 days, but in the experimental groups reduction in mortality percentage over control group was ranged from 50.81 to 68.06%. During challenge experiment, the immunological parameters were measured before injection of WSSV (0 day) and after the injection of WSSV. All the immunological parameters of experimental groups were significantly (*p* < 0.05) increased than control group. It was concluded that *P. monodon* fed with fucoidan of *Sargassum wightii* supplemented diet had enhanced the innate immunity and increased resistance against WSSV infection.

Talpur *et al.*, (2013) determining the effects of ginger (*Zingiber officinale* Roscoe) as feed additive on Asian sea bass, *Lates calcariferculture*. Experimental diets containing ginger 1, 2, 3, 5 and 10 g kg⁻¹ of feed were fed to *L. calcarifer* and control was fed with no ginger. After the feeding trial for 15 days, fish were challenged with *Vibrio harveyi* and mortality was recorded for two weeks. Ginger diet led to control of experimental infection in *L. calcarifer*. Highest survival (86.6%) was achieved in groups fed with ginger at 5 and 10 g/kg feed respectively, compared to the control (26.7%). In addition, there was a significant increase in weight gain, growth and feed conversion in those fish fed ginger diet. Ginger diet influenced the haematological parameters, biochemical indices and immunological activities.
Gomathi et al. (2013) reported incidence of *V. alginolyticus* in sea foods (Finishes and crustaceans) as a human pathogen. Antibiotic resistant and Hemolytic activity test used for selection of *V. alginolyticus*. The entire biochemical test was carried out. Among 2 categories of samples, crustaceans have high number (80%) and finishes (73%) of *V. alginolyticus*. A total of 15 strains were tested for hemolytic reaction 12 strains were positive for hemolytic production in blood agar plates and the remaining 3 strains were failed to lyses blood erythrocytes.

Bravo et al. (2013) study the infections with nodavirus affect a wild and farmed fish species throughout the world, mostly from the marine environment. The aim of this work was to determine the immune status of gilthead sea bream that comes as a result of a nodavirus infection, induced by activation of the interferon response pathway by lipopolysaccharides from *Vibrio alginolyticus* and the expression of interferon induced Mx protein in liver samples. The enhancement of Mx protein gene expression was detected in liver samples of experimentally nodavirus infected fish and, furthermore, the immunostimulant LPS of *V. alginolyticus* decreased almost three times the virus titration with respect to no-immunized or infected with nodavirus group of fish.
3. OBJECTIVES

Infectious diseases are the major cause of economic loss in commercial aquaculture. Application of antibiotics caused many other problems such as the spread of drug resistant pathogens, environmental hazards and food safety problems. This situation resulted in increasing interests in the alternatives for the antibiotics. Prevention and control through using the natural immunostimulatory compounds is one of the safer and eco-friendly approaches. In this present investigation, Feed development and Immunostimulatory effect of seaweeds to enhance the immune response and disease resistance of Gray Mullet (Mugil cephalus) against Vibrio alginolyticus, Specific objectives of this study are as follows:

Chapter I
- Collection of seaweeds from Rameshwaram, Tamil Nadu.
- Isolation and screening of bioactive compounds from marine seaweeds against fish and shell fish pathogens.
- Purification and characterization of bioactive compound from potential seaweeds.
- Analysis of bioactive compounds by GCMS.

Chapter II
- Collection of Trash fish, shrimp, acetes.
- Feed formulation by using trash fish with seaweeds.
- Collection and acclimation of Mugil cephalus
- To study the growth rate and feed utilization of Mugil Cephalus.

Chapter III
- Isolation and characterization of fish pathogen.
- Extraction and feed formulation with Sargassum wightii
- Analysis of nonspecific immune response in Mugil cephalus against Vibrio alginolyticus
- Cumulative survival rate was calculated.
4. DESCRIPTION OF STUDY AREA

*Mugil cephalus* were collected from Parangipettai (Lat 11° 29’; Long 79° 46’ E) landing centre, situated at South East coast of India, Tamil Nadu.

![Map of Parangipettai](image)

**Figure 2**: Sample collection site
CHAPTER I: ISOLATION OF BIOACTIVE COMPOUND FROM SEAWEEDS

5.1. INTRODUCTION

Aquaculture fish production has increased significantly over the past few decades which has leads to intensive fish culture practices where stressors like overcrowding, transport, handling, grading and poor water quality are common (Li et al., 2004). It is widely demonstrated that farmed fish are more susceptible to disease agents due to the stressors posed by intensive rearing. Bacterial infection causes a high rate of mortality in aquaculture organisms (Kandhasamy and Arunachalam, 2008). Bacteria are the primary pathogens of fishes, which causes systemic infection in fishes leading to life threatening. The development of aquaculture has seen considerable economic losses due to pathogens (Austin and Austin, 1999).

Nowadays, the use of antibiotics increased significantly due to the emergence of drug resistance among the microorganisms due to indiscriminate use of antibiotics. It becomes a greater problem of giving treatment against drug resistant pathogenic bacteria (Sieradzki et al., 1999). Decreased efficiency of drugs and resistance of pathogens to antibiotics has necessitated the development of new alternatives (Smith et al., 1994). Moreover, the cost of drugs is high and also they cause adverse effect on the host, which include hypersensitivity and depletion of beneficial microbes in the gut (Idose et al., 1968). Due to the outbreak of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and the researchers are now searching for new antibacterial agents (Morones et al., 2005).
Bioactive natural products are widely distributed in the plants, marine microorganisms (Soobrattee et al., 2005). Among the various resources of natural product marine algae represent an inexhaustible reservoir of raw materials used in medicine, food industries and cosmetics (Badea et al., 2009). The antimicrobial compounds derived from the marine algae consist of a diverse group of chemical compounds (Nor Afifah et al., 2010).

Marine macroalgae are the most interesting group of algae because of their broad spectrum of biological activities such as antimicrobial (Bouhlal et al., 2010), antiviral (Bouhlal et al., 2011), antifungal (Felicio et al., 2010), anti-allergic (Na et al., 2005), anticoagulant (Dayong et al., 2008), anticancer (Kim et al., 2011), antifouling (Bhadury and Wright, 2004) and antioxidant activities (Devi et al., 2011). They produce a wide variety of chemically active metabolites in their surroundings as an aid to protect themselves against other settling organisms (Bhadury and Wright, 2004).

The antimicrobial activity in seaweed extracts has been reported since 1917. Biological compounds extracted from some seaweed species, namely, Phaeophyceae, Rhodophyceae and Chlorophyceae, were proven to have potentials medicinal activities such as, antibacterial, antiviral, antitumour, antifungal, antiprotozoa, and mosquito and larva control (Bansemir et al., 2006). To date, only certain antibacterial activities of brown seaweed species have been studied in details [evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)]. Brown seaweeds like Dictyota (Jebakumar and Satheeea, 2008) and Sargassum (Kim and Lee, 2008) have been studied and they showed promising antibacterial activity. Phenolic compounds which play a major role in antibacterial
and antifungal activities are found abundantly in brown seaweeds when compared with the green and red seaweeds (Chkhikvishvili and Ramazanov, 2000).

Algae are organisms that are able to generate a wide range of secondary metabolites that are not found in other organisms. These compounds are produced in response to situations of oxidation and extreme environmental conditions in which they exist. Algae, besides being a healthy food due to its low calorie content and its high fiber and mineral content, can be a potential natural source of functional ingredients (Kumar et al., 2008).

The aquaculture industry facing a major problem due to infectious diseases caused by pathogenic bacteria especially Vibrio sp. The present investigation was carry out for the isolation of bioactive compound from the marine seaweeds against the fish pathogenic bacteria and partial purification and identification of compound by using GC- MS.
5.2. MATERIAL AND METHODS

5.2.1. Sample collection

Fresh marine seaweeds such as *Gracilaria edulis*, *Kappaphycus alvarezii* and *Sargassum wightii* were collected from Mandapam (*Lat 8° 35’ - 9° 25’ N; Long 78° 08’ - 79° 30’ E*), Rameshwaram, South East coast of Tamil Nadu. Collected samples were washed with tap water in order to remove epiphytes and other marine organisms and then washed with distilled water and dried at 45°C and ground (Figure 3).

5.2.2 Preparation of seaweeds extract

Extract of *Sargassum wightii*, *Gracilaria edulis* and *Kappaphycus alvarezii* were prepared based on the method of Fujiki *et al.* (1992). Seaweed material was mixed with organic solvents such as methanol, ethylacetate, acetone, chloroform and diethyl ether (1:50 w/v ratio) (Figure 4). Each extraction was carried out in a soxhlet apparatus for 24h and after evaporation in vacuum and extracts was stored at -20°C until use (Kanjana *et al.*, 2011). Seaweeds were also extraced with hot-water for the preparation of aqueous extract.

5.2.3. Bacterial pathogens

Bacterial strains such as *Vibrio vulnificus* (MTCC1145), *Vibrio parahaemolyticus* (MTCC 451) and *Vibrio harveyi* (MTCC 3438) used in this study were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. *Vibrio alginolyticus* (BRTR07, Genebank accession no KF758571) was isolated from fish *Mugil cephalus*. Colonies were selected from the bacterial stock and cultured on nutrient agar prepared with 50% of Sea water and incubated at 28°C for 24 to 48 h. After incubation, a pure bacterial colony was selected for each tested organism prior
to the antibacterial assays. Viability of all the strains were maintained on nutrient agar slants and stored at -20°C for further use.

5.2.4. Antibacterial activity by disc diffusion method

Antibacterial activity of the three seaweeds crude extracts were tested by disc diffusion method. Whatman No. 3 filter paper disc with 5mm diameter was prepared and sterilized by autoclaving for 15 min at 121°C. Sterile discs were impregnated with each seaweed extracts at 50 mg ml⁻¹ microgram concentration and allowed to dry. The bacterial pathogens with 1.2 X 10⁷ cfu ml⁻¹ concentrations were inoculated on Muller hinton agar (MHA) plates and spreaded using sterile cotton swab. The crude extract impregnated discs were placed over the MHA plates inoculated with test pathogens. All the plates were incubated at 28°C for 24 h. Paper discs treated with solvent alone was served as negative controls and assay was done in triplicates. Zones of inhibition were measured in millimetre in diameter (Selvin et al., 2004).

5.2.5. The minimal inhibitory concentration

The minimum inhibitory concentration (MIC) of the active extracts was determined by macro broth dilution method (NCCLS, 1993). The extracts were serially diluted two fold with nutrient broth to give concentrations of 10.00, 5.00, 2.50, 1.25, 0.625, 0.312, and 0.156 mg ml⁻¹. The 50 µl of each dilution was used to check the antibacterial activity against pathogens using the minimum inhibitory concentration method in triplicate. The tubes were inoculated with 100 µl of each bacterial suspension (5×10⁶ cfu ml⁻¹). Sterile nutrient broth was used as negative control while different concentrations of 5.2, 2.56, 1.28, 0.64, 0.32, 0.16, 0.08, 0.04, 0.02 and 0.01 µg ml⁻¹ of tetracycline prepared by serial dilution was used as positive controls. The tubes were incubated at 27°C for 24 h (Sung et al., 2006).
5.2.6. Partial purification of crude extract

5.2.5.1. TLC

Based on the minimum inhibitory concentration *Sargassum wightii* was taken for the further study. The crude extract was purified by thin layer chromatography (TLC) using Silica gel coated chromatography plates. To find out the best solvent system for good separation of crude compound, solvents such as chloroform, methanol, were used in the following proportions: 9:1, 8:2, 6:4, 4:6, 2:8, and 1:9.

Retention factor (Rf) value of the spot separated on the TLC plate was determined by adopting the following formula.

\[
R_f \text{ value} = \frac{\text{Movement of solute from the origin}}{\text{Movement of solvent from the origin}}
\]

5.2.5.2. Preparative TLC (PTLC)

Concentrated crude methanol extract of *Sargassum wightii* was purified by thin layer chromatography using chloroform: methanol as solvent systems. The crude extract was separated in a TLC plate with silica gel G 60 as stationary phase and chloroform: methanol mixture in the ratio of 8:2 as mobile phase. The eluted spots, were representing various compounds. TLC resolved spots of methanol extract at various Rf values were scrapped from the TLC plate and the scrapped spots were dissolved in methanol, mixed well and centrifuged at 12,000 x g for 5 min. The supernatant (10mg ml⁻¹) 50 µl of each fraction was used to check the antibacterial activity against pathogens using the MIC method in triplicate. Tetracycline was used as positive control. Experimental data represent mean ± SD of each sample. The partially purified compound obtained from preparative TLC was tested for antibacterial activity against fish pathogenic organisms by MIC method as described earlier (Yuvaraj et al., 2011).
5.2.6. Analysis of Compound

5.2.6.1. Fourier-transformed infrared (FT-IR)

The purified active TLC fraction was analysed by FTIR. IR spectra were recorded in the 400-4000 cm\(^{-1}\) range with a resolution of 1 cm\(^{-1}\). The room was kept at a controlled ambient temperature (25°C) and relative humidity (30%) (El-Sayed et al., 2005).

5.2.6.2. Gas Chromatography Mass Spectrometry (GC-MS)

The purified fractions were analyzed using an Agilent 6890 series high temperature gas chromatography-mass spectrometer (GCMS), fitted with an auto injector. For GCMS analysis, a high temperature column (DB-5ht; 30 m x 0.25 mm id x 0.25 μm film thickness) was used. By employing a high temperature column, we eliminated the need for derivatization of each sample. The injector and detector Temperatures were set at 350°C while the initial column temperature was set at 80°C. A 2 μl sample volume was injected into the column and ran using split less mode. After 2 min, the oven temperature was raised to 150°C at a ramp rate of 10°C min\(^{-1}\). The oven temperature was then raised to 250°C at a ramp rate of 5°C/min and finally the oven temperature was raised to 280°C at a ramp rate of 20°C min\(^{-1}\) and maintained at this temperature for 40 min. The helium carrier gas was programmed to maintain a constant flow rate of 1 ml min\(^{-1}\). Identification of organic compound was matching their recorded spectra with the data bank mass spectra of NIST library V11 provided by the instruments software (Hussain et al., 2011).
5.3. RESULT

5.3.1. Antibacterial activity of the seaweeds

In the antibacterial activity test, the methanol extract of *G. edulis* showed maximum zone of inhibition (32.66 mm) against *V. vulnificus*, followed by ethylacetate (26.66 mm), acetone (21.33 mm), diethylether (21.33 mm) and minimum zone of inhibition (14.33 mm) was produced by acetone extract (Table 1). Acetone extract of *S. wightii* showed maximum zone of inhibition (26.33 mm) against *V. vulnificus*, followed by diethylether extract (23.66 mm), methanol extract (24 mm), ethylacetate (22.66 mm), and minimum zone of inhibition was produced by chloroform (21.66 mm). *K. alvarezii* extract has not produced any zone of inhibition against *V. vulnificus* (Figures 5, 6 and 7).

The Diethylether extract of *G. edulis* showed maximum zone of inhibition (24 mm) against *V. anguillarum*, followed by diethylether extract (23.66 mm), acetone extract (22 mm), and ethylacetate extract (22.66 mm). Chloroform extract showed a minimum zone of inhibition (21.33 mm). Acetone extract of *S. wightii* produced a maximum zone of inhibition (26 mm) against *V. anguillarum*, followed by diethylether extract (25mm), methanol extract (24.66 mm), and chloroform extract (24.33 mm) and ethylacetate extract showed a minimum zone of inhibition (23.33 mm) (Table 1). Methanol and ethyl acetate extract of *K. alvarezii* showed a maximum zone of inhibition 23.3 mm against *V. anguillarum*, followed by chloroform extract (23 mm), ethylacetate extract (23.33 mm) and acetone extract (22 mm) and minimum zone of inhibition were by diethylether extract (21 mm) (Figures 5, 6 and 7).

Diethylether extract of *G. edulis* showed maximum zone of inhibition against *V. parahaemolyticus* (23.33 mm) followed by acetone extract (23 mm), chloroform
extract (22.66 mm) and ethylacetate extract (21.66 mm) and minimum zone of inhibition was produced by methanol extract (16.66 mm). Methanol extract of S. wightii showed maximum zone of inhibition (32 mm) against V. parahaemolyticus, followed by diethylether extract (32 mm), acetone extract (30.66 mm), chloroform extract (31 mm) and acetone extract (30.66 mm) and minimum zone of inhibition produced by ethylacetate extract (29 mm) (Table 1). Methanol extract of K. alvarezii showed maximum zone of inhibition against V. parahaemolyticus, followed by chloroform extract (23.66 mm), diethylether extract (23 mm) and acetone extract (17 mm) and ethylacetate extract produced minimum zone of inhibition against V. parahaemolyticus (15.33 mm) (Figures 5, 6 and 7).

Diethylether extract of G. edulis produced a maximum zone of inhibition against V. harveyi (23 mm), followed by methanol extract (22.66 mm), ethylacetate extract (22.66 mm), acetone extract (22.33 mm) and minimum zone of inhibition was produced by chloroform extract (20.33 mm). Ethylacetate extract of S. wightii showed maximum zone of inhibition against V. harveyi (24.66 mm), followed by chloroform (24.33 mm), acetone extract (23.66 mm), diethylether (22.33 mm), minimum zone of inhibition was produced by methanol (22 mm) (Table 1). Chloroform extract of K. alvarezii showed a maximum zone of inhibition (24.66 mm) against V. harveyi, followed by ethylacetate extract (24.33 mm), methanol extract (23.33 mm), diethylether extract (22.33 mm), and minimum zone of inhibition was produced by acetone extract. No zone of inhibition was observed in aqueous extract of all the seaweeds aganist Vibrio sp. (Figures 5, 6 and 7).
5.3.2. Minimum inhibitory concentration

The lowest concentration of the extract showing no visible growth after incubation was taken as MIC of particular extract against the respective pathogen. Ethylacetate extract of *G. edulis* showed low MIC value of 0.625 mg ml\(^{-1}\) against *V. harveyi* followed by *V. vulnificus* and *V. anguillarum* (1.25 mg ml\(^{-1}\)) and highest MIC value against *V. parahaemolyticus* (1.25 mg ml\(^{-1}\)). Chloroform extract of *G. edulis* showed lowest MIC value against *V. parahaemolyticus* (0.312 mg ml\(^{-1}\)), followed by *V. vulnificus* and *V. anguillarum* (0.625 mg ml\(^{-1}\)), highest MIC value against *V. harveyi* (1.25 mg ml\(^{-1}\)). Methanol extract of *G. edulis* showed lowest MIC value against *V. vulnificus* and *V. anguillarum* (1.25 mg ml\(^{-1}\)) and highest MIC value against *V. parahaemolyticus* (5 mg ml\(^{-1}\)) (Table 2). Diethylether extract of *G. edulis* showed lowest MIC value against *V. harveyi* (0.312 mg ml\(^{-1}\)), followed by *V. vulnificus* (0.625 mg ml\(^{-1}\)) and high MIC value against *V. anguillarum* (2.5 mg ml\(^{-1}\)). Acetone extract of *G. edulis* showed lowest MIC value against *V. vulnificus*, *V. parahaemolyticus* and *V. anguillarum* (0.625 mg ml\(^{-1}\)) followed by *V. harveyi* (1.25 mg ml\(^{-1}\)).

Ethyl acetate extract of *S. wightii* showed lowest MIC value against *V. vulnificus* and *V. anguillarum* (0.312 mg ml\(^{-1}\)) followed by *V. harveyi* (0.625 mg ml\(^{-1}\)) and highest MIC value against *V. vulnificus* (2.5 mg ml\(^{-1}\)). Chloroform extract of *S. wightii* showed lowest MIC value against *V. vulnificus*, *V. parahaemolyticus* and *V. harveyi* (0.625 mg ml\(^{-1}\)) followed by highest MIC value against *V. anguillarum* (2.5 mg ml\(^{-1}\)). Methanol extract of *S. wightii* showed lowest MIC value against all the four *Vibrio* sp (*V. vulnificus*, *V. anguillarum*, *V. parahaemolyticus* and *V. harveyi* - 3.12 mg ml\(^{-1}\)). Diethylether extract of *S. wightii* showed lowest MIC value against *V. vulnificus* and *V. harveyi* (0.312 mg ml\(^{-1}\)) followed by *V. anguillarum* and
*V. parahaemolyticus* (0.625 mg ml\(^{-1}\)). Acetone extract of *S. wightii* showed lowest MIC value against *V. anguillarum* and *V. harveyi* (0.312 mg ml\(^{-1}\)) followed by *V. vulnificus* (0.625 mg ml\(^{-1}\)) and highest MIC value against *V. parahaemolyticus* (1.25 mg ml\(^{-1}\)) (Table 2).

Ethyl acetate extract of *K. alvarezii* were showed lowest MIC value against *V. harveyi* and *V. parahaemolyticus* (1.25 mg ml\(^{-1}\)), highest MIC value against *V. anguillarum* (2.5 mg ml\(^{-1}\)). Chloroform extract of *K. alvarezii* showed lowest MIC value against *V. anguillarum* and *V. parahaemolyticus* (0.625 mg ml\(^{-1}\)), highest MIC value against *V. harveyi* (5 mg ml\(^{-1}\)). Methanol extract of *K. alvarezii* showed lowest MIC value against *V. anguillarum* *V. parahaemolyticus* and *V. harveyi* (0.625 mg ml\(^{-1}\)) (Table 2). Diethylether extract of *K. alvarezii* was showed lowest MIC value against *V. anguillarum* and *V. parahaemolyticus* (0.312 mg ml\(^{-1}\)), highest MIC value against *V. harveyi* (2.5 mg ml\(^{-1}\)). Acetone extract of *K. alvarezii* showed lowest MIC value against *V. anguillarum, V. parahaemolyticus* and *V. harveyi* (0.312 mg ml\(^{-1}\)).

Based on the minimum inhibitory concentration methanol extract of *S. wightii* showed a better result when compare with other seaweed extracts. It was taken for further studies.

### 5.3.3. TLC and PTLC

In the TLC, 8:2 ratio of chochlum: methanol mixture separated the compound in the TLC. TLC plated showed a 2 separated fraction. Retention factors (Rf) value of the separated spots were calculated as 0.87 and 0.75. Methanol extract of *S. wightii* cured extract were run in the PTLC plate methanol and chloroform proportions at 8:2 ration solvent showed 2 spot in the PTLC plate. The PTLC fraction 1 and 2 were checked for antimicrobial activity against the tested organisms (Table 3). The PTLC
purified second fractions showed lowest MIC value to all the tested microorganisms after 24 h of incubation. The PTLC second fraction was analysed by FTIR and GCMS.

5.3.4. FTIR analysis

The TLC 2\text{nd} fraction was analysed by FTIR. The FTIR spectrum indicated several intense characteristic bands related with functional groups presented in the TLC fraction of \textit{Sargassum wightii}. FTIR frequency ranges of 594.08 cm\textsuperscript{-1} intensities was medium (C-C stretch), indicating the presence of chloro compound, 673.16 cm\textsuperscript{-1} intensities was medium (CH bending), indicating the presence of arenes, 1267.23 cm\textsuperscript{-1} intensities was medium (C-C-C), indicating the presence of aldehydes and ketones, 1396.46 cm\textsuperscript{-1} intensities was medium (CH\textsubscript{3}) indicating the presence of alkanes, 2353.16 cm\textsuperscript{-1} intensities was strong (P-H r Si-H) indicating the presence of phosphine or silane, 2931.83 cm\textsuperscript{-1} intensities was strong (CH\textsubscript{3})it indicating the presence of alkanes and 3452.21 cm\textsuperscript{-1} intensities was Strong (-N=C=N-) indicating presence of the carbodiimides (Table 4).

5.3.5. GCMS analysis.

In TLC, the active compound separated from \textit{S. wightii} fraction was analysed by GCMS. The GCMS data of \textit{S. wightii} active fraction was showed in figure 10 and the relative percentage of identified compounds is summarized in Table 5. n-Hexadecanoic acid (59.44) followed by tetradecanoic acid (7.98\%), 3-Eicosene, (E) (7.18412\%), 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester( 4.7968\%), Dibutyl phthalate (3.30\%), 9-Eicosene, (E) (2.19\%), 3-Eicosene (3.10\%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (1.83), Hexadecanoic acid, methyl ester(1.42), 1-(3-Isopropylidene-5,5-dimethyl-bicyclo [2.1.0]pent-2-yl)-ethanone (1.06\%), n-Decanoic
acid (0.81%), 2-Dodecene, (Z) (0.34%), 6-Methyl-6-(5-methyl-furan-2-yl)-hept-3-en-2-one (0.22%), Cyclohexanol, 2,4-dimethyl (0.15%) and Pentanoic acid, 2-(aminoxy) (0.08%).
5.4. DISCUSSION

Salama et al. (2010) reported that different solvents have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent. In this present study also phytoconstituents extracted from three seaweeds using different solvents exhibited different spectrum of activities against Vibrio sp. Rangaiaha et al., (2010) also reported that seaweed extracts in different solvents exhibited different antimicrobial activities. The high and low effect of organic extract against microorganisms could be related to the presence of different bioactive metabolites (Kolanjinathan and Stella, 2009; Manivannam et al., 2011). In the present study Sargassum wightii had a good antimicrobial activity against Vibrio sp.

Kandhasamy and Arunachalam (2008) reported that extracts prepared from seaweed with methanol showed the best activity. In the present study, methanol extract of seaweeds showed a good antibacterial activity against the Vibrio sp. Manilal et al. (2009) and Rangaiah et al. (2010) reported that methanol extraction yielded higher antimicrobial activity than other solvent.

Wefky and Ghobrial, (2008) and Fareed and Khairy (2008) reported that acetone is the suitable solvent for the extraction of bioactive compounds from seaweeds. In the present study, acetone extract of S. wightii showed maximum zone of inhibition against V. vulnificus and V. anguillarum as well. Osman et al. (2010) reported that, acetone was the best solvent for extraction of the bioactive compounds: Meanwhile it gave the highest antimicrobial activity against the selected pathogens.
Arputha Bibiana et al. (2012) reported that the maximum activity of diethyl ether extract of S. wightii and K. alvarezii against were test pathogens. In this study, diethylether extract of G. edulis showed maximum zone of inhibition against V. parahaemolyticus and V. harveyi.

Kanjana et al. (2011) classified plant extract as antimicrobial agents based on minimal inhibitory concentration (MIC) values of their extracts. Extracts with minimal inhibitory concentration values at less than 100 microgram ml$^{-1}$ are classed as strong inhibitors, at 100-500 mg ml$^{-1}$ as moderate inhibitors, at 500-1000 mg ml$^{-1}$ as weak inhibitors and at more than 1000 mg ml$^{-1}$ as inactive According to this classification the low MIC value are found in G. edulis, S. wightii and K. alvarezii which is the indication of seaweed extracts efficacy against the test organisms. Saxena et al. (1994) documented MIC varying from 12.5 to 1,000 μg ml$^{-1}$ when testing different concentrations of Rhus glaba extracts aganist, Gram negative and Gram positive bacteria.

Neveen et al., (2008) reported that minimal inhibitory concentration (MICs) (μg ml$^{-1}$) of ethyl acetate extracts for Anabaena variabilis and Anabaena circinalis against Aeromonas species. In our present study, ethylacetate extract of seaweeds showed better Low MIC value against the Vibrio sp. Most of active antimicrobial phytoconstituents was soluble in chloroform and consequently displaying the highest relative antimicrobial activity (Salama and Marraiki, 2010). Chloroform extract of G. edulis, S. wightii and K. alvarezii showed lowest MIC value against Vibrio pathogen.

Methanol extract of G. edulis showed a Low MIC value against V. vulnificus and V. anguillarum, Whereas Methanol extract of S. wightii showed Low MIC value against all the Vibrio sp tested. K. alvarezii showed lowest MIC value against V.
anguillarum, V. parahaemolyticus and V. harveyi (0.625 mg ml⁻¹). The Methanolic extract of Ecklonia cava and Ecklonia kurome showed MIC value against Staphylococcus epidermidis was 2.5 mg ml⁻¹ and Staphylococcus latiuscula was 0.63 mg ml⁻¹ (Choi et al., 2011).

Diethylether extract of G. edulis showed MIC value against V. harveyi (0.312 mg ml⁻¹), S. wightii showed MIC value against V. vulnificus and V. harveyi (0.312 mg ml⁻¹) and K. alvarezii showed MIC value against V. anguillarum, and V. parahaemolyticus (0.312 mg ml⁻¹). Xavier et al. (2012) reported that acetone extract of S. wightii showed better MIC value than other solvents. The present study suggests that acetone extract had a good antimicrobial activity against the pathogens. In our present study also supported that acetone extract of G. edulis, S. wightii and K. alvarezii showed moderated MIC values when compare with other solvent.

Zubia et al. (2008), suggested that the great variation observed in the potential antimicrobial components in seaweeds could be due to the external environmental factors such as herbivory, light, depth, salinity and nutrients of their growing environment. All of these factors could act on the spatiotemporal regulation on metabolic expression of the active compounds leading to marked qualitative and quantitative variations among their similar species at a smaller scale than different species. Thus, this might be some of the reasons that led to the higher bacteriostatic activity in Sargassum polycystum.

However, crude seaweeds extracts are mixed with many compounds and their active portion may be very low. FTIR major peak showed that it has Phenol, Aldehyde and Ketone groups as a major compound in the seaweeds. Phenolic compounds are exhibited good antioxidant and antimicrobial activities (Kostic et al.
2012; Barros et al., 2007). Further investigations should focus on attempts to purify active compounds and to elucidate their chemical structure. The most active extract resulted noncytotoxicity to fish. The extracts from S. wightii could be a source of antibacterial compounds with potential use in aquaculture, in order to control fish infections and as fish feed component.

A wide range of lipophilic antibacterial compounds have been isolated from gastropod molluscs, including polyunsaturated fatty acids and alkaloids (Benkendorff, 2010). Polat and Ozogul (2008) reported that palmitic acid (Hexadecanoic acid) and oleic acid were the major fatty acids found in the seaweeds that they examined. Fatty acids and sterols content determined in red alga Chondrus crispus showed that the main fatty acids were palmitic, palmitoleic, oleic, arachidonic and eicosapentanoic acids (Tasende, 2000). Besides halogenated compounds, fatty acids have been identified as antimicrobial substances in algae (Rosell and Srivastava, 1987). Bansemir et al. (2006) reported that C. rubrum contains several fatty acids with antimicrobial activities.

It is well known that fatty acids are a vital constituent of both terrestrial and marine plants (Saravanakumar et al., 2008). Antimicrobial properties of fatty acids were reported as early as 1960; Synthesis of fatty acids in seaweed is controlled by both biotic and abiotic factors (Nichols, 1965). Evidence supporting bioactivity of fatty acids was earlier demonstrated in certain microalgae and mangrove plants (Agoramoorthy et al., 2007).

The remarkable difference between our results and the results obtained in previous studies may be due to several factors. One of the main reasons is the seasonal variation of the seaweeds and another important reason could be due to
difference in the extraction procedure to recover the active metabolites and differences in assay methods that would result in different susceptibilities of the target strains. The volatile compounds observed from Mandapam costal area were the credible evidence that algae maintain effective antimicrobial chemical defences. From the present study, it can be concluded that *Sargassum wightii* are potential sources of bioactive compounds.