Characterization of structural properties and biological activities of sulfated polysaccharides extracted from brown seaweed *Sargassum wightii* Greville and seagrass *Halophila ovalis* R. Br. Hook

Thesis submitted to Pondicherry University for the degree of

DOCTOR OF PHILOSOPHY

by

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INDIA

April 2013

PONDICHERRY UNIVERSITY

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCES

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CERTIFICATE

Certified that this thesis entitled "**Characterization of structural properties and biological activities of sulfated polysaccharides extracted from brown seaweed *Sargassum wightii* Greville and seagrass *Halophila ovalis* R. Br. Hook**" is a record of research work done by the candidate **Mr. N. Yuvaraj** during the period of his study in the Department of Biotechnology, School of Life Sciences, Pondicherry University, under my supervision and that it has not previously formed the basis of the award of any degree, diploma, associateship or fellowship.

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DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the guidance of Dr. V. Arul, Associate Professor, Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry, and this work has not been submitted elsewhere for any other degree.

Puducherry

Date: **(YUVARAJ. N)**

DEDICATED TO MY

BELOVED FAMILY……

ACKNOWLEDGEMENTS

Foremost, I would like to express my heartfelt gratitude to my Research Supervisor **Dr. V. Arul**, Associate Professor, Department of Biotechnology for providing me an opportunity to carry out my doctoral programme under his valuable guidance. I thank him for his constant motivation, support, patience, enthusiasm, and immense knowledge. His guidance helped me throughout my research and writing thesis.

Besides my supervisor, I am privileged to express my thanks to my doctoral committee members: **Prof. N. Sakthivel** and **Prof. N. Parthasarathy**, for their constant encouragement, insightful comments, and hard questions during the course of study.

I express my deep sense of gratitude to **Prof. N. Sakthivel**, Head, Department of Biotechnology for extending lab facilities and valuable suggestions.

Much indebted to **Dr. N. Arumugam**, **Dr. Lata Shukla**, **Dr. K. Prashanth**, **Dr. B. Sudhakar**, **Dr. A. Hannah Rachel Vasanthi**, **Dr. D. Arun Kumar**, **Dr. V. Venkateswara Sarma**, and **Mr. V. Balasubramanian** who have been influential in all my academic pursuits.

Obliged to my fellow lab mates: **Dr. P. Kanmani**, **Dr. R. Satishkumar**, **Dr. K.A. Paari**, **Mr. V. Pattukumar**, **Mr. P. Venkatesh**, **Mr. M. Balraj**, and **Mr. Ayyanah** for the stimulating discussions, immense co-operation, emotional support, and for all the fun we had.

I owe my deep sense of gratitude to **Mr. Jayaraj**, Rank Hatchery, Marakanam for constant help in providing sea water and brine shrimp and adult shrimp for our study.

I wish to express my sincere thanks to my colleagues **Mr. Dineshram, Mr. Senguttuvan, Mr. Vijaykumar, Mr. G. Raman, Mrs. Asha, Mrs. Pathma, Ms. Revathi, Mr. Jean Cletus, Mr. Kennedy, Mr. Saranathan, Mr. Sudakar pagal, Ms. Lalitha, Mrs. Subasri, Mrs. Veena, Mr. Rahul, Mr. Naresh, Mr. Anand, Mr. Aezaj, Mr. Wasim, Mr. Veeresh, Mr. Arun,**

Mr. Baskar, Mr. Sharath, Ms. Mamtha, Mr. Venkat, Ms. Prachi singh, Mrs. Sangeetha, Mr. Ashok, Mr. Suresh, Ms. Gokulapriya, and **Mr. Aravind** for their kind help and valuable support.

I also thank our office staffs, **Mr. Ramalingam**, **Mr. Vadivel**, **Mrs. Sarala**, **Mrs. Vinothini**, **Mr. Meiappan**, **Muthammal** and former staff **Mr. Balakrishnan** for their co-operation and help.

I am delighted to thank **Central Instrumentation Facility** (CIF), Pondicherry University for providing assistance in using necessary instruments whenever approached.

I gratefully acknowledge the financial support given by **Department of Biotechnology** (DBT), New Delhi in the form of Junior/Senior research Fellowship and **University Grants Commission** (UGC), New Delhi for University fellowship.

I wish to thank my intimate friends **Dr. K.A. Paari** and **Mr. J. Saravanan** and their families for providing a loving environment and constant support for me.

I wish to express my gratitude to my parents, **Mr. S. Neelakandan** and **Mrs. Mala Neelakandan,** my in laws **Mr. A. Souparayane** and **Mrs. Selvi Souparayane, brother, sister, nieces, and cousins** for showering with love and care throughout my life.

I extend my heartiest gratitude to my lovable and caring wife **Y. Suriya** for giving moral support and for everything, I achieved. For that, I thank almighty who made us to meet together. To her I dedicate this thesis. Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention personally each and everyone.

Puducherry **(YUVARAJ. N)**

Date:

CONTENTS

LIST OF ABBREVIATIONS

ABTS	(2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt))
bp	base pairs
CAT	Catalase
DMEM	Dulbecco modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	2' deoxynucleotide 5' triphosphate
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tetra acetic aicd
ELISA	Enzyme linked immune sorbent assay
FBS	Fetal Bovine Serum
g/l	gram/litre
GPX	Glutathione peroxidase
h	hour
HRS	Hydroxyl radical scavenging
IL-8	Interleukin-8
KDa	Kilo Dalton
LC-MS	Liquid Chromatography-Mass spectrometry
LPS	Lipopolysaccharides
µg	Microgram
µl	Microlitre
µM	Micromolar
mg/ml	Milligram per milli litre
ml	Milliliter
mm	Millimeter
mM	Milli molar
min	Minutes

MTT	3-(4, 5- Dimethylthiazol-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
N	Normality
nm	Nanometer
OD	Optical Density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
ProPO	Prophenol oxidase
rpm	rotation per minute
RPMI 1640	Rosewell park memorial Institute 1640
s	Second
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor- α
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

Ocean covers more than 70% of the world surface with more than 300000+ known species of flora and fauna (Hong, 2004; Jimeno *et al*., 2004; Kijoa & Swangwong, 2004). Seaweeds and seagrasses are the major plants found in the marine ecosystem apart from mangroves. Seaweeds are autotrophic, photosynthesize like vascular plants growing abundantly in the shallow waters, estuaries and backwaters. Seagrasses are marine vascular plants, which grow in seawater and survive the tides unscathed. For several decades, people along the coast have utilized seaweeds and seagrasses owing to their high nutritional and medicinal values. In the immense coastal areas, they play a major role in maintaining the marine ecosystem. Consumption of brown seaweed is higher than red and green seaweeds and the amount of seaweed harvesting is increasing every year worldwide (Barsanti & Gualtieri, 2006) mainly for food and for its cell wall polysaccharides.

Sargassum, a well known brown seaweed belonging to order Fucales, and class Phaeophyceae, is common to all oceans except Antartica. In India, it is the most diverse genus among Phaeophyta and is represented by 38 species. Of such genus, *Sargassum wightii* is prevalently distributed in southern coasts of Tamilnadu, and reported to be used as fertilizer, food ingredients, and animal feed as well as commercially exploited for alginates production. Concurrently, all the 15 seagrass species belong to seven genera were found in Tamilnadu. Seaweeds and seagrasses are indeed suitable natural agents in the biomedical area for producing bioactive substances, which show great potential as antimicrobial, antioxidant, anti-inflammatory, antiviral and anti-tumoral drugs (Nagai & Yukimoto, 2003; Smith, 2004; Gokce & Haznedaroglu, 2008; Yuvaraj *et al*., 2012). Besides the bioactive compounds, seaweeds and seagrasses possess dietary fibers and polysaccharides as treasures for hydrocolloid, human and animal health applications.

Antimicrobial resistance and its epidemiological consequences is a global growing problem with a significant impact on morbidity, mortality and healthcare-associated costs. Indiscriminate use of commercial antibiotics has resulted in the development of multidrug-resistant (MDR) bacterial strains all over the world and as expected, hospitals have become breeding grounds for human-associated microorganisms (Mainous & Pomeroy, 2001). According to an estimate by the National Institute of Health (NIH, USA), about 80% of all microbial infections are caused by bacterial biofilms (Yakandawala *et al*., 2006). Use of prophylactic antibiotics is detrimental to

human and animal health and for the environment (Cabello, 2006). The evolution of antibiotic-resistant pathogenic bacteria has led to the exploration of several alternative approaches from alternative sources including sources from the ocean. These features make the antibiotic of natural product resources as a powerful arsenal of molecules that could be the antimicrobial drugs of the new century as an innovative response to the increasing problem of MDR. Organic solvents extract of seaweeds and seagrasses were reported to exhibit antibacterial activity (Mahasneh *et al.,* 1995; Rengasamy *et al*., 2008) and have been investigated for several decades. However, very few authors purified the seaweed crude extracts into fractions and screened for antibacterial activity (Vlachos *et al*., 1997; Sree *et al*., 2005) and there is no report on the purification of seagrass extracts. In the present study, we delved in to purify the *S. wightii* and *H. ovalis* crude extract and identify the chemical constituents of purified fractions and thus could be exploited in pharmaceutical preparations.

In recent years, pharmacologists have turned their attention on sulfated polysaccharides of natural resource rather than secondary metabolites due to its extensive application in the food, cosmetic and pharmaceutical industries and in biotechnology (Wijesekara *et al*., 2011). Sulfated polysaccharides are complex group of macromolecules with a wide range of important biological properties and these polymers are prevalent in nature, taking place in a great variety of organisms. Chemical structures of polysaccharides from seaweeds have been investigated extensively in the past (Chizhov *et al*., 1998; Duarte *et al*., 2001; Fleury & Lahaye, 1991; Haug, 1964; Patankar *et al*., 1993; Percival & McDowell, 1967). In 2005, occurrence of sulfated polysaccharides in seagrasses has been reported for the first time in contrast with the land plants (Aquino *et al*., 2005). The presence of sulfated polysaccharide was determined in three seagrass species, of which the *Ruppia maritima* sulfated polysaccharide was purified and its structure was characterized. Alginates from most of the *Sargassum* species was extracted and its structural and biological activities have been reported worldwide (Dore *et al*., 2013; Chen *et al*., 2012; Khanavi *et al*., 2010). Similarly, crude alginate extract from *Sargassum wightii* have been documented for anti-inflammatory (Sarithakumari *et al*., 2012), and hepatoprotective (Josephine *et al*., 2008) activity. Nevertheless, there is a lack of published information on the heterofucan of *S. wightii* and galactan sulfate of *H. ovalis* to date. Recent reports showed that, chemical entities such as sulfate content, molecular weight, monosaccharide composition, and the structure of the main polymer chain had great influence on their biological activities (Vishchuk *et al*., 2012). Due to

the difficulties in identifying the chemical structure of sulfated polysaccharides, research approaches in establishing structure-function relationship found to be a mysterious one. Therefore, we ventured on determining the structural characteristics and functional properties of *S. wightii* and *H. ovalis* sulfated polysaccharides with commercial interest, before they recognized as important commodities.

Inflammation is known to contribute to physiological and pathological processes by the activation of the immune system, local vascular system, and various cells within the damaged tissue (Coussens & Werb, 2002). Perhaps, this is the foremost step in fighting infection and healing wounds. However, prolonged inflammation leads to various diseases and disorders. The classical characteristics of inflammation are pain, swelling, edema, redness and heat (Mantovani, 2010). During inflammation, macrophages play a central role in managing many different immunopathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as IL-1β, IL-6, NO, iNOS, COX-2 and TNF-α. Indeed a number of inflammatory stimuli, such as LPS and pro-inflammatory cytokines, activate immune cells to up regulate such inflammatory states, and these are therefore useful targets in the development of new anti-inflammatory drugs and exploration of the molecular anti-inflammatory mechanisms of the potential drug (Zeilhofer & Brune, 2006; Jachak, 2007). The administration of non-steroidal anti-inflammatory drugs is an important tool in the suppression of the inflammatory response in a clinical context and has the ability to inhibit initial or later manifestations. Fucan of *S. vulgare* (Dore *et al.,* 2013) and alginic acid of *S. wightii* (Sarithakumari *et al.,* 2012) have been documented for anti-inflammatory effects. However, there is no report on the antinociceptive and anti-inflammatory activity of *S. wightii* and *H. ovalis* sulfated polysaccharides to date. Hence, the present study exploited us to determine the antinociceptive and anti-inflammatory activity of heterofucan of *S. wightii* and galactan sulfate of *H. ovalis* in experimental animal models as well as the inhibitory action on proliferation of peripheral blood mononuclear cells and interleukin-8 (IL-8) secretion *in vitro*.

High levels of inflammatory cytokines and reactive oxygen species are proposed contributors to the pathophysiological mechanisms associated with various inflammatory disorders (Trouba *et al*., 2002). The free radicals and other reactive oxygen species, as oxidants and inhibitors of enzymes containing an iron-sulfur center, cause the oxidation of biomolecules such as amino acids, proteins, lipids and DNA, which leads to cell injury and death (Freidovich, 1999). The free

radicals at least concentrations, exert crucial actions such as signal transduction, and gene transcription in cells (Lander, 1997). However, it becomes deleterious when not being eliminated by the endogenous systems. Antioxidants delay the oxidation process by inhibiting the polymerization chain initiated by free radicals and successive oxidizing reactions. Consumption of products high in antioxidant compounds are thought to alleviate such deleterious effect brought about by the influence of reactive species (Schwartz, 1996, Halliwell & Gutteridge, 2007). Use of synthetic antioxidants is ruled out strictly in many countries due to its extensive side effects and toxicity. Hence, natural source received considerable attention as a safe, non-toxic antioxidative agent. Among the various natural resources, sulfated polysaccharides of seaweeds have received special attention as a source of natural antioxidant. Josephine *et al.,* (2007) reported that sulfated polysaccharides of *S. wightii* showed antioxidant effect *in vivo*. Similarly, sulfated polysaccharides from seagrass *H. wrightii* showed remarkable antioxidant activity in different *in vitro* assays (Silva *et al.,* 2012). There is no data about the antioxidant activity of sulfated polysaccahrides extracted from *S. wightii* and *H. ovalis in vitro*. Therefore, we attempted to investigate the ability of sulfated polysaccharides of *S. wightii* and *H. ovalis* to reduce the free radicals generation using different *in vitro* antioxidant assays.

Shrimp is the most important commodity among the mariculture with farming area of about 1.2 million to 1.4 million hectares in India. Farmed shrimps contribute about 50% of the India's total shrimp exports (Anon, 2002). White spot syndrome virus (WSSV), a rod-shaped double-stranded DNA virus of the genus Whispovirus, has emerged as one of the most prevalent as well as lethal for shrimp populations (Sanchez-Paz, 2010), and is responsible for huge economic loss in the shrimp culture industry worldwide (Flegel, 1997). Successive strategies generally employed to control the WSSV infection includes vaccination, immunostimulants, neutralization, quarantining and environmental management (Sanchez-Paz, 2010). Vaccination via intramuscular injection for all shrimps is not feasible under farming conditions. Immersion method is simple but requires large quantities of vaccine. Oral vaccination is the most practical method of administration to animals of all sizes. However, large amount of stimulants needed, thus increasing the cost and the uncertainty about the individual dosage. Unlike the vertebrates, crustaceans lack a true adaptive immune system, and are dependent on an innate immune system to protect and defend themselves against all intruding pathogens (Sarathi *et al*., 2007; Amparyup *et al*., 2009). Immuno-stimulants elevated the nonspecific defence mechanism of shrimp and

prevent losses from WSSV disease. However, it may suppress or alter certain biological pathways if used inappropriately (van de Braak, 2002). Environmental management, chemical or prophylactic interventions attempts have not provided a perfect solution for WSSV outbreaks in any part of the world, but seem to have helped reduce the impact. Although several therapeutic measures have shown some efficacy against WSSV under experimental conditions, it is worth noting that at present there is no treatment available to interfere with the unrestrained occurrence of WSSV and spread of the disease in the field. It has been reported that, fucoidan from brown seaweed *Sargassum wightii* exhibited protective effects against WSSV in black tiger shrimp *P. monodon* post larvae. However, there is no report on the effect of *S. wightii* heterofucan on immune response of adult shrimp *P. monodon*. Similarrly, there is no single report exists on using the galactan sulfate of seagrass as immunostimulant against WSSV. In this background, we also intended to study the efficacy of *S. wightii* heterofucan and *H. ovalis* galactan sulfate on immune response of *P. monodon* adult.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. The marine environment is the source of structurally unique secondary metabolites produced by different organisms such as sponges, tunicates, bryozoans, soft corals, mollusks, microorganisms and seaweeds (Blunt *et al*., 2011). Phytoplanktonic groups such as diatoms, dinoflagellates, and macrophytes including algae play a key role in productivity of the ocean, since it constitutes the base of marine food chain. Of such groups, marine algae, an inexhaustible treasure, produce a wide range of biologically active compounds. The secondary metabolites synthesized by these algae at the end of the growth phase and/or due to metabolic alterations induced by environmental stress conditions. Seaweeds or macroalgae belong to the lower plants, meaning that they do not have roots, stems and leaves. Instead, they are composed of a thallus (leaf-like), sometimes a stem, and a foot. Some species have gas-filled structures to provide buoyancy. In their natural environment, macroalgae grow on rock substrates and from stable, multilayered, perennial vegetation capturing almost all available photons. Seaweeds are used as a novel food with potential nutritional benefits in medicine and industry for multiple purposes (Shalaby, 2011).

Seaweeds are mainly produced for these end uses in Asian countries such as China, Philippines, North and South Korea, Japan, and Indonesia. Many countries such as USA, Canada and European countries like France, Germany and the Netherlands are attempting to establish large-scale seaweed cultivation (Perez, 1997; Buck & Buchholz, 2004; Reith *et al*., 2005). In Japan and China, they have been used traditionally in folk medicine and as healthy foods (Lee-Saung *et al*., 2003). India is one among 12 mega biodiversity countries and 25 hotspots of the richest and highly endangered ecosystem of the world (Myers *et al*., 2000). In 1927, Iyengar was the pioneer Indian algologist who gave a detailed descriptive account of Indian marine algae occurring on the south east coast, especially Krusadai Island. In all, 271 genera and 1153 species of marine algae including forms and varieties have been enumerated till date from the Indian waters (Anon, 2005). There has been a revival of interest in seaweeds due to increased awareness of the limited

ability of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from the seaweeds.

Seaweeds as antibacterial agents

Macroalgae, the first marine organisms explored for medicinal purposes (de Oliveira *et al*., 2012). Green algae (Chlorophyta, 13% marine) are the producers of isoprenoid derivatives, acetogenins, amino acid derivatives, carbohydrate and shikimate derivatives (Mcclintock & Baker, 2001). Red algae (Rhodophyta) which is about 98% marine are dynamic producers of halogenated compounds, ranging from acetogenins, indoles, peptides, phenols, polyketides and terpenes to volatile halogenated hydrocarbons (Cabrita *et al*., 2010; Fujii *et al*., 2011). Brown algae produce cyclic or acyclic short chain hydrocarbons, terpenoids, acetogenins, polyphenols, and terpenoid-aromatic compounds (Mcclintock & Baker, 2001, Toth & Pavia, 2006; Blunt *et al*., 2011). The exhibition of antimicrobial activities was considered as an effective indicator of the capacity of the seaweeds to synthesize bioactive secondary metabolites (del Val *et al*., 2001). Harder (1917) was the pioneer who observed the antimicrobial potential of seaweeds. Research towards antibacterial activity of Indian marine algae has been reported earlier (Sreenivasa rao & Parekh, 1981; Padma sridhar *et al*., 1984, Padmini sreenivasa rao *et al*., 1986; Padmakumar & Ayyakkannu, 1986). The extracts and active constituents of various seaweeds have shown antimicrobial activity *in vitro* against Gram-positive and Gram-negative bacteria (Han *et al*., 2005; Kim *et al*., 2008; Gupta *et al*., 2010). The chemical composition of macroalgae is not well known as the terrestrial plants. However, 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 known to be rich in proteins, minerals and polysaccharides as well as bioactive substances such as carotenoids, polyphenols, terpenoids, and tocopherols. Airanthi *et al*., (2011) documented the presence of carotenoid pigments such as fucoxanthin and astaxanthin. Polyphenols such as phlorotannins were observed in green and brown seaweeds (Yoshie *et al*., 2002; Sugiura *et al*., 2006; Zou *et al*., 2008; Lopez *et al*., 2011). In addition to that, bromophenols from red seaweeds have been reported as antimicrobial substance (Xu *et al*., 2003; Han *et al*., 2005; Oh *et al*., 2008). Fishes and shellfishes are susceptible to a wide variety of bacterial pathogens. Numerous investigations concerning the inhibiting activities of macrolagae

against fish pathogens were reported (Sridhar & Vidyavathi, 1991; Mahasneh *et al*., 1995; Liao *et al*., 2003, Sree *et al*., 2005). Though antimicrobial studies of several seaweeds have been reported earlier, cases of adding extracts to food products as a mean to improve the safety and shelf life are not much available. However, few studies reported that addition of seaweed extract has resulted in disease reduction in some marine animals used for human consumption (Kumaran *et al*., 2010; Kanjana *et al*., 2011).

Seagrass as antibacterial agents

Seagrasses or marine angiosperms exist as long ago as 100 million years, and examples of seagrass fossils from the Cretaceous were reported (den Hartog, 1970). Seagrasses have followed a relatively conservative evolutionary pathway, since there is no evidence of any massive diversification or major extinction events (Short *et al*., 2007). They are the richest and most productive systems in the ocean, have no link between marine algae and land-living higher plants but derived from land plants, which have secondarily recolonized marine habitats. Hence, seagrasses share Alismatales order primary and secondary metabolism features which live in land and freshwater habitats (Heglmeier & Zidorn, 2010). They are the only submerged marine plants with an underground root and rhizome system. The roots and rhizomes plays a crucial role in storm events and shoots provides a stable surface layer above the benthos, baffling currents and enhancing the settlement of sediments (Short & Short, 1984). Apart from that, seagrasses reduces problems of eutrophication, binding organic pollutants, maintaining genetic variability, carbon sequestration, removing CO_2 from atmosphere and binding it as organic matter (Short *et al*., 2007). There are 13 genera and 58 species available all over the world. Of these, seven genera (*Cymodocea, Enhalus, Halodule, Halophila, Syringodium, Thalassia* and *Thalassodendron*) are distributed in tropical seas. In India, seven genera, which comprise 15 species, exist along the southeast coast (Gulf of Mannar and Palk Bay) and in the lagoons of Islands from Lakshadweep and Andaman and Nicobar (Jagtap *et al*., 2003).

Extraction methods include separation of biologically active fractions from inactive/inert components by using selective solvents and extraction technology. Successful determination of biologically active lead from plant material is largely depends on the type of solvent used in the extraction procedure. Initial screening of plant material for possible antibacterial activities

typically begins by using crude extractions and can be followed by various organic solvent extraction methods. Earlier findings evidenced that, extraction techniques had definite effect on the isolation of bioactive principles (Shanmughapriya *et al*., 2008). Methanol extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate (Rosell & Srivatsava, 1987; Sastry & Rao, 1994; Paul & Puglisi, 2004). Whereas, Fables *et al*., (1995) reported that chloroform is a better solvent to extract active principles of seaweeds than methanol and benzene. Organic solvents always provide a higher efficiency in antimicrobial compound extraction compared to water based methods (Masuda *et al*., 1997; Lima-Filho *et al*., 2002).

It has been demonstrated that variation in extraction methods are usually depend on the length of the extraction period, solvent used, temperature, size of the particle and solvent-to-sample ratio. The basic principle is to grind the plant material finer, which increases the surface area for extraction thereby increasing the rate of extraction. Earlier studies showed that, sample to solvent ratio of 1:10 (w/v) has been used as ideal (Green, 2004). Two different methods are employed usually for the extraction of secondary metabolites from seaweeds and seagrasses. Serial exhaustive extraction is the first method, which involves successive extraction with solvents of increasing polarity from a non polar (n-hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compounds could be extracted (Green, 2004). Soxhlet extraction of dried plant material using organic solvents is another method (Kianbakht & Jahaniani, 2003). However, this method cannot be used for thermolabile compounds as prolonged heating may degrade compounds nature (de Paira *et al*., 2004). Considering their great economic diversity, investigations related to the search of new biologically active compounds from algae can be seen as an almost unlimited field.

Sulfated polysaccharides from seaweeds

Use of marine algae in Western countries has traditionally focused on the extraction of compounds used by cosmetics, food, and pharmaceutical industries (Ordonez *et al*., 2010). Polysaccharides exists in bacteria, fungi, algae, plants and animals play a pivotal role in biological functions such as cell-cell communication, cell adhesion, and molecular recognition (Dwek, 1996). In recent years, polysaccharides of marine source received much attention for wide range of biological activities. These anionic sulfated polysaccharides are present in not only

seaweeds but also in seagrasses, animals such as mammals and marine invertebrates (Mourao & Pereira, 1999; Mourao, 2007; Aquino *et al*., 2005). However, the sulfated polysaccharides of seaweeds are highly heterogeneous. Costa *et al*., (2010) reported that these polysaccharides vary according to the algal species. Ulvan is the water-soluble polysaccharide extracted from the cell wall of green algae. They are composed of disaccharide repetition moieties made up of sulfated rhamnose linked to either glucuronic or iduronic acid, or xylose (Lahaye & Robic, 2007). In addition, green seaweeds synthesize a variety of other forms of sulfated polysaccharides such as rhamnan sulfate (Lee *et al*., 2010) and heteroglycuronan (Ray, 2006). The sulfated polysaccharides of red seaweeds are classified as agarans and carrageenans based on their stereochemistry. Agarans are galactans with 4-linked α-D-galactose residues of the L-series, and those have a backbone of alternating 3-linked β- D-galactose are termed as carageenans (Knutsen *et al*., 1994). Carrageenans are classified into three categories such as Kappa (κ), iota (ι), and lambda (λ) based on their sulfation degree, solubility and gelling properties (Leibbrandt *et al*., 2010). However, Lahaye, (2001) reported that there are at least 15 different carrageenan structures. They are classified based on their structural characteristics, including their sulfation patterns and the presence or absence of 3,6-anhydro-α-galactopyranose units. The sulfated polysaccharides of brown seaweeds are categorized into two groups, i.e., reserve (laminarans) and structural polysaccharides (alginic acids and fucoidans). Laminarans are generally composed of 1,3-β-D-glucan (Zvyagintseva *et al*., 1999) with β(1,6) branching (Nelson & Lewis, 1974). The structure of laminarans varies between algal species. Alginates are composed of linear copolymers of (1→ 4) β-D-mannuronic acid (M) and (1 → 4) α-L-guluronic acid (G) units (Gomez *et al*., 2009). The structure of alginates varies according to the monomer position on the chain, forming either homopolymeric (MM or GG) or heteropolymeric (MG or GM) segments (Rioux *et al*., 2007). Fucoidans are complex sulfated polysaccharides, fucose rich besides other monosaccharide units (Holtkamp *et al*., 2009). These fucose containing sulfated polysaccharides composed of (1→3) and (1→4) linked α-L-fucopyranose residues, perhaps organized in stretches of (1→3) α-fucan or of alternating α (1→3) and α (1→4) bonded fucopyranose residues (Ale *et al*., 2011). Over the last decade, sulfated polysaccharides especially fucoidans of brown seaweeds have attracted much attention in the fields of pharmacology and biochemistry (Wijesinghe & Jeon, 2012).

Sulfated polysaccharides from seagrasses

Occurrence of polysaccharides in marine angiosperms, *Phyllospadix torreyi* (Woolard & Jones, 1978), *Halophila stipulecea* (Waldron *et al*., 1989), *Halophila ovalis* (Waldron *et al*., 1989), *Halodule uninervis* (Waldron *et al*., 1989), *Heterozostera tasmanica* (Webster & Stone, 1994), *Ruppia maritima* (Aquino *et al*., 2005), *Halophila decipiens* (Aquino *et al*., 2005), *Posidonia australis* (Torbatinejad *et al*., 2007), *Halodule wrightii* (Silva *et al*., 2012), *Zostera marina* (Khasina *et al*., 2003) have been reported (Table 1). The high amount of sulfated polysaccharides has been documented for the first time in marine angiosperms in contrast with the terrestrial and freshwater species (Aquino *et al*., 2005). The sulfated polysaccharides of seagrass species are composed of α- and β-D-galactose units as like red algae. However, these residues are not distributed in an alternating order as sulfated galactans found in red alga. The complete structure of seagrass *R. maritima* was determined (Aquino *et al*., 2005) and it was found to be a sulfated homogalactan composed of regular tetrasaccharide repeating unit: [3-β-D-Gal-2(OSO3)-1→4-α-D-Gal-1→4-α-D-Gal-1→3-β-D-Gal-4(OSO3)-1→]. Aquino *et al*., (2011) reported that, glycosyltransferases and sulfotransferases functions simultaneously during the biosynthesis of sulfated galactans, at least in *Ruppia maritima*. The activation and inhibition of glycosyltransferase genes modifies the composition of sulfated polysaccharides among the different phyla (Aquino *et al*., 2011).

Extraction and purification of sulfated polysaccharides from *Sargassum* species

In general, it is important to emphasize the relationship between chemical entities and biological activities. These polysaccharides are harvested by suitable extraction or precipitation method, followed by purification, characterization and biological studies. Chirinos *et al*., (2007) reported that, chemical nature, method of extraction and the existence of interfering substances influence extraction of bioactive components. Early reports with few exceptions showed that, treatment with dilute acids at ambient or slightly elevated temperature has been a preferred first step for the extraction of fucoidan or fucose containing sulfated polysaccharides from various brown seaweeds (Ale *et al*., 2011). Recent studies revealed that, enzyme-assisted extraction methodology could be employed as an alternative method to extract sulfated polysaccharides in

greater amounts for industrial use (Kang *et al*., 2011; Wijesinghe & Jeon, 2011). However, hot water mediated extraction was generally employed to extract sulfated polysaccharides from *Sargassum* species except few. The sulfated polysaccharide was extracted by hot water method from *Sargassum linifolium*, *S. stenophyllum*, *S. patens*, *S. horneri*, *S. fulvellum*, *S. kjellmanianum*, *S. pallidum*, *S. hemiphyllum*, *Sargassum sp., S. latifolium*, *S. siliquosum*, *Sargassum sp*. (**Table 1**). Whereas, the sulfated polysaccharide was extracted using 0.1 M HCl from *S. muticum* (Mabeau *et al*., 1990), *S. tenerrimum* (Sinha *et al*., 2010), and *S. hornery* (Ermakova *et al*., 2011).

Ale *et al*., (2012) extracted sulfated polysaccharides from *Sargassum* sp., using 0.03 M HCl. Dias *et al*., (2008) used 4M KOH to extract sulfated polysaccharides from *S. stenophyllum*. Modern extraction techniques such as supercritical CO_2 extraction and ultrasonic-aid extraction have been applied in *S. pallidum* polysaccharide preparation (Ye *et al*., 2008). Recently, proteolytic digestion mediated extraction was performed using Prolov 750 to extract sulfated polysaccharides from *S. filipendula* (Costa *et al*., 2011). Recently, Hwang *et al*., (2011) suggested that hot water process is an effective way to extract fucose-enriched sulfated polysaccharides from *Sargassum* species.

Over the decades, isolation and chemical characterization of sulfated polysaccharides from seaweeds have gained much attention. Lahaye, (1991) reported that seaweeds found to be a good source of bioactive polymer presenting great chemical, physico-chemical and rheological diversities. Zvyagintseva *et al*., (1999) developed a simple method for the separation of water-soluble polysaccharides of brown seaweeds based on hydrophobic chromatography. Abdel-Fattah *et al*., (1974) purified the sulfated heteropolysaccharide from *S. linifolium* by DEAE-cellulose and DEAE-Sephadex column. Gel filtration chromatography using Toyopearl HW-65F column was used to fractionate the crude polysaccharide of *S. thunbergii* (Zhuang *et al*., 1995). de Zoysa *et al*., (2008) purified the anticoagulant sulfated polysaccharides of *S. fulvellum* by two steps; DEAE-cellulose anion exchange chromatography followed by Sepharose 4B. Similarly, the sulfated polysaccharide from *S. patens* was purified using Sepharose 6B and DEAE-cellulose column using 0.1 M NaCl solution and obtained purified fractions (Zhu *et al*., 2006). Whereas, Asker *et al*., (2007) fractionated the sulfated polysaccharides of *S. latifolium* by DEAE-cellulose anion exchange chromatography and Sephacryl S-300 gel permeation chromatography. Preeprame *et al*., (2001) separated the crude polysaccharide of *S. horneri* into two fractions by

Sepharose 6B column using citrate buffer contains 0.1 M NaCl. Polysaccharide of *S. tenerrimum* was purified by Superdex 30 prep grade (size exclusion chromatography) column equilibrated with 500 mM sodium acetate buffer (Sinha *et al*., 2010). Similarly, Sephadex G-100 gel chromatography was employed to purify the sulfated polysaccharides of *S. pallidum* using 0.1 N NaCl solution (Ji *et al*., 2011). In recent study, Q sepharose fast flow column was employed to purify the sulfated polysaccharides from *S. plagiophyllum* (Suresh *et al*., 2012). Fucoidans of *S. hornery* was subjected to Macro-prep DEAE (anion exchange chromatography) column equilibrated with 0.1 M NaCl and successively eluted with linear gradient of NaCl from 0 to 2 M solution (Ermakova *et al*., 2011). Membrane separation technology with anion exchange chromatography was employed to purify the *S. pallidum* sulfated polysaccharides (Ye *et al*., 2008).

Table 1. Sulfated polysaccharides extracted from *Sargassum* species.

Species	Extraction method	Composition	Molecular range	Biological activity	References
Sargassum linifolium	Extracted with water for 3 h at 80°C	Mannose, galactose, xylose, uronic acid and fucose residues.	n.d.	Anticoagulant activity.	Abel-Fattah *et al*., 1974
S. fulvellum	Extracted with boiling water for 4 h and precipitated with ethanol 1 volume and repeated with 0.5 volume.	n.d.	33.4 KDa	Inhibited subcutaneous sarcoma-180 in mice.	Fujihara *et al*., 1984
S. kjellmanianum	Extracted with boiling water and precipitated with ethanol.	Fucose and ester sulfate.	n.d.	Anti-tumor activity against L-1210 leukemia cells.	Yamamoto *et al*., 1984
S. muticum	Extracted with Triton X-100, acid and alkali and precipitated with 80% ethanol.	Fucose, xylose, mannose, galactose, glucose and sulfate.	n.d.	n.r.	Mabeau *et al*., 1990

S. thunbergii	Extracted with hot water at 100°C for 4 h, repeated thrice, and precipitated with 3% cetylpyridinium chloride and finally precipitated with 99% ethanol.	Fucose, xylose, galactose, glucose and mannose.	10 KDa (SG II-1-B-b fraction)	No anti-tumor activity was observed.	Zhuang *et al*., 1995
	0.1 M HCl at 95°C for 4h, repeated thrice with 3% cetylpyridinium chloride and precipitated with 99% ethanol.	Fucose, xylose and and treatedgalactose	13-19 KDa (GIV-A and GIV-B fraction)	Anti-tumor activity in mice.	Zhuang *et al*., 1995
	5% sodium hydroxide at 28°C for 24 h, repeated twice and precipitated with 99% ethanol.	Fucose, xylose, galactose, glucose and mannose	15 – 20 KDa (GV-1-B, GV-2-B fr)	Fractions GIV-A and GIV-B showed antitumor activity in mice.	Zhuang *et al*., 1995
S. stenophylum	Extracted with 7% water for 12 h for three times, precipitation with ethanol and $CaCl_2$ and cetylpyridinium chloride.	Fucose, xylose, mannose galactose, glucose, and uronic acid.	n.d.	n.r.	Duarte *et al*., 2001
S. horneri	Extracted twice with boiling water for 1 h and precipitated with 80% ethanol.	n.d.	n.d.	Antiviral activity against HSV-1 *in vitro*.	Preeprame *et al*., 2001

S. patens	Extracted with hot water and precipitated with ethanol.	Fucose, galactose, mannose, xylose, glucose, and galctosamine.	424 KDa	Anti-herpes activity against HSV-1 and HSV-2.	Zhu *et al*., 2003, 2006
S. latifolium	Extracted with boiling water bath for 2 h and precipitated with ethanol.	Fucose, xylose, glucose, mannose, glucuronic acid and sulfate.	70–130 KDa	Fraction SP-III showed antiviral activity against HSV-1 and HAV *in vitro*.	Asker *et al*., 2007
S. stenophylum	Extracted with 4 M KOH, 10 mg $NaBH_4$ at room tem -perature followed by neutralization with acetone.	Fucose, mannose, galactose, xylose, glucose, glucuronic acid.	n.d.	Antiangiogenic and antitumor activity.	Dias *et al*., 2008
S. pallidum	Extracted with Huaan super critical fluid extractor at 55°C, 45 MPa, with flow rate of 20 l/h of CO_2 for 4 h.	n.d.	n.d.	SP-3-1 and SP-3-2 fractions showed antitumor activity against HepG2, A549 and MGC-803 cells.	Ye *et al.,* 2008
	Extracted by unltrasonic waves with JY98-cell breaker by incubating in water bath at 90°C for 5 h.	n.d.	n.d.		
Sargassum sp.,	Extracted with boiling water for 3 h and by autoclaving at 120°C for 3 h.	n.d.	n.d.	Antioxidant activity.	Yangthong *et al*., 2009

S. tenerrimum	Extracted with 0.1 M HCl twice at 25-30°C and precipitate with HCl.	Fucose, xylose, mannose, galactose, glucose and sulfate.	30 ± 5 KDa	Antiviral activity against HSV-1.	Sinha *et al*., 2010
Sargassum sp.	Extracted with 0.03 M HCl for 4 h at 90°C and precipitated with 60% ethanol.	Fucose, rhamnose, arabinose, glucose, galactose, mannose, xylose, uronic acid and sulfate.	n.d.	n.r.	Ale *et al*., 2012,
S. pallidum	Extracted in boiled water for 1 h twice, precipitated with 95% ethanol and 1% $CaCl_2$.	Xylose, mannose, arabinose, galactose, glucose, and others.	10–48 KDa	Sedative and hypnotic effect *in vivo*.	Ji *et al*., 2011
S. hemiphyllum	Extracted by hot water and precipitated with 4 V of 95% ethanol.	Myo-inositol, sorbitol, fucose, galactosamine, galactose, glucose, and mannose.	n.d.	*In vitro* anti-inflam matory activity against RAW 264.7 macrophage cells.	Hwang *et al*., 2011
S. filipendula	Extracted by proteolytic digestion by Prolav 750 and precipitation with acetone.	Fucose, glucose, galactose, mannose, xylose, glucuronic acid and sulfate.	n.d.	Antioxidant and antiproliferative activity against HeLa, PC3, HepG2 cells.	Costa *et al*., 2011
S. hornery	Extracted twice with 0.1 M HCl for 2 h at 60°C and precipitated with 96% ethanol.	Fucose, galactose, mannose, rhamnose, xylose, glucose and sulfate.	n.d.	Anticancer activity in human melanoma and colon cancer cells.	Ermakova *et al*., 2011

Sargassum	Extracted with boiling water at 80°C twice and precipitated with 95% ethanol (5 volumes).	Fructose, xylose, glucose, galactose, mannose, and rhamnose.	n.d.	Inhibit oxidative stress induced by infectious bursa virus.	Zhang *et al*., 2011
S. fusiforme	Extracted with 10 v of dis. water at 90°C for 3 h thrice and precipitated with 95% ethanol	n.d.	n.d.	*In vivo* and *in vitro* antitumor activity and immune response in mice.	Chen *et al*., 2012
S. siliquosum	Extracted with boiling water for 5 h, three times.	n.d.	n.d.	antioxidant and anti-cancer activity *in vitro*.	Ross *et al*., 2012
S. graminifolium	Extracted with hot water at 90°C for 3 h, repeated twice and precipitated with 95% ethanol.	n.d.	n.d.	Antioxidant and calcium oxalate crystallization inhibition activity.	Zhang *et al*., 2012

n.d. = not detected

n.r. = not reported

Extraction and purification of sulfated polysaccharides from seagrass species

Polysaccharides are abstracted by hot water, alkali and enzyme solutions from different seagrass species (**Table 1a**). The polysaccharide was extracted using hot water from seagrass *Phyllospadix torreyi* (Woolard & Jones, 1978). They used 5% ammonium oxalate and 7% sodium hydroxide for the extraction of polysaccharides from the same species. Aquino *et al*., (2005) extracted the sulfated polysaccharides from *R. maritima* and *H. decipiens* using papain enzyme. Similarly, Silva *et al*., (2012) used maxatase enzyme to extract polysaccharides from *H. wrightii*. Purification of seagrass sulfated polysaccharides has not been described in detail as like algal polysaccharides. The crude polysaccharide extracted from *P. torreyi* using hot water, 5% ammonium oxalate, and 7% sodium hydroxide was fractionated with hexadecyltrimethylammonium bromide obtained then four different fractions (Woolard & Jones, 1978). Aquino *et al*., (2005) partially purified the crude polysaccharide of *R. maritima* by Sephacryl 400 HR (gel chromatography) column equilibrated with 0.2 M sodium bicarbonate solution and then to a Mono Q-fast performance liquid chromatography (FPLC) column eluted with 1-4 M NaCl solution in a linear gradient method. Silva *et al*., (2012) used Sephadex G-75 to purify the sulfated polysaccharides of *H. wrightii*.

Structure-function correlation of sulfated polysaccharides

Seaweeds sulfated polysaccharides are structurally diverse and heterogeneous. Studying the structural properties remains mysterious and may have hindered their development as novel therapeutic agents to date. Infrared spectroscopy technique was applied to examine the structure of seaweeds sulfated polysaccharides (Lloyd *et al*., 1961). The discovery of Fourier transform algorithm in 1965 and processing of spectral data has been extensively applied in carbohydrate chemistry (Mathlouthi & Koenig, 1986). Chromatography techniques gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) methods are employed earlier to identify the monosaccharide compositions, types of glycosidic linkages and branching of polysaccharides.

Table 1a. Sulfated polysaccharides extracted from seagrass species.

Species	Extraction method	Composition	Molecular range	Biological activity	References
Phyllospadix torreyi	Extracted with hot water for 4 h at 90°C and precipitated with ethanol.	Apiose, arabinose, galactose, glucose, mannose, rhamnose, and xylose.	n.d.	n.r.	Woolard & Jones, 1978
P. torreyi	Extracted with 5% ammo--nium oxalate for 4 h at 90°C and precipitated with ethanol.	Apiose, arabinose, glucose, galactose, mannose, rhamnose, and xylose.	n.d.	n.r.	Woolard & Jones, 1978
P. torreyi	Extracted with 7% sodium hydroxide and precipitated with ethanol.	Apiose, arabinose, glucose galactose, mannose, rhamnose, and xylose.	n.d.	n.r.	Woolard& Jones, 1978
Halophila stipulacea	Hydrolyzed in 2 N TFA for 1 h at 120°C, dissolved in 0.5 ml water and dried under N_2 and desiccated over NaOH.	Rhamnose, arabinose, xylose mannose, galactose, glucose, and arabinose.	n.d.	n.r.	Waldron *et al*., 1989

Halophila ovalis	Hydrolyzed in 2 N TFA for 1 h at 120°C, dissolved in 0.5 ml water and dried under N_2.	Rhamnose, arabinose, xylose mannose, galactose, glucose, and arabinose.	n.d.	n.r.	Waldron *et al*., 1989
Halodule uninervis	Hydrolyzed in 2 N TFA for 1 h at 120°C, dissolved in 0.5 ml water and dried under N_2 and desiccated over NaOH.	Rhamnose, arabinose, xylose mannose, galactose, and glucose.	n.d.	n.r.	Waldron *et al*., 1989
Heterozostera tasmanica	Hydrolyzed with 72% H_2SO_4 for 1 h at 30°C and with 3% H_2SO_4 at 100°C for 4h.	Arabinose, rhamnose, fucose, xylose, mannose, galactose, glucose and apiose.	n.d.	n.r.	Webster & Stone, 1994
Ruppia maritima	Extracted with 0.1 M sodium acetate contains 2 g papain, 5 mM EDTA, 5 mM cysteine, incubated at 60°C for 24 h and preci--pitated with 95% ethanol.	n.d.	n.d.	n.r.	Aquino *et al*., 2005
Halophila decipiens	Extracted with 0.1 M sodium acetate contains 2 g papain, 5 mM EDTA, 5 mM cysteine, incubated at 60°C for 24 h and preci--pitated with absolute ethanol.	n.d.	n.d.	n.r.	Aquino *et al*., 2005

Posidonia australis	Hydrolyzed with 1 M H_2SO_4 and extracted with phosphate buffer at 100°C .	mannose, xylose, galactose, glucose, rhamnose, fucose, ribose and arabinose.	n.d.	n.r.	Torbatinejad *et al*., 2007
Halodule wrightii	Extracted with 0.25 M NaCl and 20 mg of maxatase, incubated for 18 h at 60°C under agitation and precipitated with 0.5 V of ice cold acetone at 4°C for 24 h.	Galactose, glucose, xylose.	11 KDa	antioxidant and anticoagulant activites.	Silva *et al*., 2012

n.d. = not detected

n.r. = not reported

Recently, Goa *et al*., (2011) employed liquid chromatography-tandem mass spectrometry to analyze the monosaccharide composition of marine polysaccharides. Fitton, (2005) reported that, biological activities of fucoidan was determined by their degree of sulfation, fine structure, monosaccharide composition, and molecular weight. Fucose containing sulfated polysaccharides might exhibit appreciable biological activities (Matsuhiro *et al*., 1996). The presence of sulfate group in various amounts in the seaweed polysaccharides determines the bioactivity. The anti-HSV activity of *S. tenerrimum* polysaccharide increased with increasing sulfate content (Sinha *et al*., 2010). Similarly, Costa *et al*., (2011) correlated the structural entities with antioxidant and antiproliferative activities of heterofucan derived from *S. filipendula*. Whereas, Ermakova *et al*., (2011) reported that, there is no correlation between amount of sulfate groups and anticancer activity. Over a decade, it is strongly believed that biological properties of sulfated polysaccharides determined by high degree of sulfation, fine structure and molecular weight (Zvyagintseva *et al*., 2003; Jiang *et al*., 2010; Wijesinghe & Jeon, 2011). Alasalvar *et al*., (2010) reported the strong correlation between structure of sulfated polysaccharides and their antioxidant potency. High sulfate content and low molecular size were studied to exert stronger radical scavenging activities. The molecular weight of most polysaccharides derived from *Sargassum* are more than 50000 Da (Ye *et al*., 2006). However, reports revealed the existence of low molecular weight sulfated polysaccharides between the range of 10000-48000 Da from *Sargassum* species (Fujihara *et al*., 1984; Zhuang *et al*., 1995; Sinha *et al*., 2010; Ji *et al*., 2011). It is believed that low molecular weight sulfated polysaccharides exerted potent antioxidant activity than high molecular weight sulfated polysaccharides (Sun *et al*., 2009). This is due to the fact that, low molecular weight polysaccharides could effectively incorporate into the cells and donate proton effectively than high molecular weight polysaccharides. Leiro *et al*., (2007) observed that immunostimulatory activity of ulvan-like sulfated polysaccharides extracted from *U. rigida* was decreased significantly after desulfation, suggesting the importance of the functional group in eliciting immune response.

Sulfated polysaccharides as anti-inflammatory and antinociceptive agent

Sulfated polysaccharides from seaweeds and seagrasses have received considerable attention as promising ingredients for pharmaceutical applications as a natural anti-inflammatory agent (Hwang *et al*., 2011; Guangling *et al*., 2011; Yuvaraj *et al*., 2012). The anti-inflammatory activities of organic solvents extract obtained from various *Sargassum* species have been investigated. Hong *et al*., (2011) reported that methanol extract of *Sargassum swartzii* showed acute anti-inflammatory activity in carrageenan induced paw edema and peritonitis models. Kang *et al*., (2008) reported that dichloromethane extract of *S. fulvellum* and ethanol extract of *S. thunbergii* inhibited ear edema. Na *et al*., (2005) reported that methanol extract of *S. hemiphyllum* reduced the IL-8, TNF-α and NF-κB secretion. Dar *et al*., (2007) showed that anti-inflammatory activity of *S. wightii* in carrageenan induced paw edema due to seasonal variations. Dichloromethane and hexane fractions of crude ethanol extract of *Sargassum micracanthum* suppressed the expression of pro-inflammatory cytokines, iNOS and COX-2 expression in LPS induced RAW 264.7 macrophages (Yoon *et al*., 2009). However, sulfated polysaccharides of seaweeds have gathered unprecedented attention in recent times being prolific source of natural anti-inflammatory agent. The complex heterogeneous polysaccharides from green, red, and brown seaweeds have broad-spectrum therapeutic properties (Patel, 2012). Sulfated polysaccharide isolated from green seaweed *Caulerpa cupressoides* exhibited anti-inflammatory effects by reducing neutrophil migration and potently reduced the paw edema induced by carrageenan and peritonitis models (Vanderlei *et al*., 2010). Rodrigues *et al*., (2012) reported that, Cc-SP2 fraction of *Caulerpa cupressoides* showed significant antinociceptive effect by inhibiting the number of writhes induced by acetic acid and reduced the licking time in the formalin test in a concentration dependent manner. In the hot-plate test, Cs-SP2 (9 mg/kg) increased the latency to thermal stimuli primarily at 60 min. Galactan from red seaweed *Gelidium crinale* inhibited dextran, histamine, and phospholipase A(2) induced paw edema and possible antinociceptive activity (de Sousa *et al*., 2011). Similarly, sulfated polysaccharide extracted from red seaweed *Gracilaria cornea* exhibited anti-inflammatory activity by reducing neutrophil migration in rat peritoneal cavity and

inhibiting paw edema induced by carrageenan (Coura *et al*., 2012). The antinociceptive effect of this sulfated polysaccharide was observed at 27 mg/kg in formalin test, hot plate test and acetic acid induced writhes. Fucoidans and sulfated fucans extracted from brown seaweed have been reported to possess anti-inflammatory activity (Cumashi *et al*., 2007; Medeiros *et al*., 2008). These sulfated polysaccharides could inhibit leukocyte migration to inflammation site. Preobrazhenskaya *et al*., (1997) reported that intravenous administration of fucoidan inhibited leukocyte recruitment to peritoneum in a peritoneal inflammation model and blocked the interaction of P-selectin with its carbohydrate ligand in a dose dependent manner. Similarly, Marques *et al*., (2012) showed that sulfated polysaccharides of brown seaweed *Padina gymnospora* reduced leukocyte influx (60%) into the mice peritoneal cavity. Senni *et al*., (2006) reported that fucoidan could act as inhibitor to heparanase and elastases, interfere with degradation of basement membrane in inflammation. Park *et al*., (2011) reported that fucoidan suppressed the phosphorylation of p38 and decreased the MCP-1 mRNA expression lead to decreased production of MCP-1 in a concentration dependent manner. In addition to that, fucodan inhibit NF- κB, Akt, and JNK pathways (Park *et al*., 2011). The sulfated polysaccharides of *Sargassum* have been documented for wide biological activities (Hwang *et al*., 2011; Zhang *et al*., 2011; Wang *et al*., 2006; Chen *et al*., 2009; Zhu *et al*., 2004; Ye *et al*., 2008). The hot water extracted fucose rich sulfated polysaccharides of *S. hemiphyllum* reduced the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, NO production, iNOS, COX-2 and NF-κB in LPS induced RAW 264.7 macrophages (Hwang *et al*., 2011).

Till date, seagrasses have received comparatively less bioassay attention than seaweeds. Hexane fraction of seagrass *Zostera japonica* inhibited proIL-1β expression in LPS induced J774A.1 murine macrophages (Hua *et al*., 2006). Fatty acids of hexane fraction H5 inhibited the expression of TNF-α, IL-1β, and IL-6 in a dose dependent manner (Hua *et al*., 2006). Yuvaraj *et al*., (2012) reported the presence of anti-inflammatory activity in crude methanol extract of seagrass *H. ovalis*. The crude extract suppressed the mitogen-induced proliferation of peripheral blood mononuclear cells in a dose dependent manner. There are no data about the anti-inflammatory activities of sulfated polysaccharides from

the vascular plants. However, Silva *et al*., (2012) determined the *in vitro* anticoagulant, and antioxidant activities of seagrass *H. wrightii* sulfated polysaccharides.

Sulfated polysaccharides as antioxidant agents

The term "antioxidant" is defined as a substance, present at low concentrations capable of delaying or inhibiting a specific oxidizing enzymes, or interacts with oxidizing agents prior to them causing damages to other molecules, or sequesters metal ions, or capable of repairing systems such as iron-transporting proteins (MacDonald-Wicks *et al*., 2006). In mechanistic terms, antioxidants are defined as a hydrogen donor or electron acceptor. Antioxidants play a pivotal role in preventing/slowing down the progression of various diseases (Schwarz *et al*., 2001). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the most commonly used synthetic antioxidants. These synthetic antioxidants cause pathological, enzyme and lipid alterations and have carcinogenic effects (Grillo & Dulout, 1995). Therefore, use of synthetic antioxidants was under strict regulation in food industries and products due to their adverse effects on human health (Hettiarachy *et al*., 1996). Thus, search for natural antioxidants was found to be mysterious one among researchers and food industries. Natural antioxidants received considerable attention in other fields also, such as in polymerization control in plastics, paint, rubber industries and for protection of clear plastics against ultraviolet light, and/or in the design of better automobile fuels and lubricating oils (Halliwell & Gutteridge, 1999). The antioxidant benefits from terrestrial and medicinal plants have long been accepted. However, there may be disadvantages to natural antioxidants, since carotenoids, vitamin E and some phenolic compounds are water-insoluble. Though vitamin C is water-soluble, it is heat-sensitive and easily degradable (Park *et al*., 2004). Traditionally, seaweeds have been consumed in Asian countries since ancient epoch, as they are rich in minerals, proteins, vitamins, dietary fibers, and functional polysaccharides and easier to use in food formulations and pharmaceuticals (Chapman & Chapman, 1980). Such macro and micro nutrients and bioactive compounds present in seaweeds, not only help in fighting disease but also help us to overcome age related disorders (Burtin, 2003; Li *et al*., 2009). Other than Asian countries, Europeans and

Americans use processed seaweeds as food additives (Boukhari & Sophie, 1998). This practice of seaweed diet has led to a large number of reports on the beneficial role of seaweed consumption in human health.

Seaweeds extract have been well known for potential antioxidant activity (Matsukawa *et al*., 1997; Le Tatour *et al*., 1998; Yan *et al*., 1998; Athukorala *et al*., 2003; Heo *et al*., 2003; Kuda *et al*., 2005; Yuan & Walsh, 2006; Iwai, 2008; Chew *et al*. 2008). Cornish & Garbary, (2010) reviewed the antioxidant properties of seaweeds and their potential applications in human health and nutrition. The antioxidant activity of extracts from brown seaweeds *S. polysystum* and *S. siliquastrum* was determined earlier (Anggadiredja *et al*., 1997; Lim *et al*., 2002). Park *et al*., (2004) evaluated the antioxidant activity of enzymatic extract from *S. horneri* using DPPH, hydroxyl and alkyl radicals. The ethanol and aqueous extracts of *Sargassum* species, significantly reduced the lipid peroxidation induced by acetaminophen, and was further concluded that the protective effect might due to their free radical scavenging activity (Raghavendran *et al*., 2005). Similarly, ethanol and water extract of *S. boveanum* examined for its antioxidant property and the results revealed that water extract of *S. boveanum* showed significant inhibition of peroxidation of linoleic acid and exhibited noticeable DPPH radical scavenging activity (Zahra *et al*., 2007). The aqueous extract prepared by boiling water for 3 h, and by autoclaving at 120°C for 3 h from four different algae was evaluated for their antioxidant property using various *in vitro* antioxidant systems. Of these, both extracts of brown seaweed *Sargassum sp.,* had the highest total phenolic content and displayed strong DPPH and hydroxyl radical scavenging activity (Yangthong *et al*., 2009). However, it relatively showed less scavenging effect on oxygen radicals. Patra *et al*., (2008) studied the DPPH and hydroxyl radical scavenging potential as well as inhibition of lipid peroxidation and glutathione-S-transferase activity of *Sargassum sp.,* methanol extract. Badrinathan *et al*., (2011) explored the various organic solvent extracts of *S. myriocystum* for antioxidant activity and the results revealed that methanol:chloroform extracts of Soxhlet showed highest reducing power, total antioxidant and hydroxyl scavenging activity. Similarly, Meenakshi *et al*., (2009) evaluated the *in vitro* antioxidant activity of methanol extract in *S. wightii*.

However, little emphasis has been placed on the merits of seaweeds sulfated polysaccharides for antioxidant benefits. In the last decade, seaweed polysaccharides have been reported for its antioxidant activities (Ruperez *et al*., 2002; Zhao *et al*., 2004; Qi *et al*., 2005; Rocha de Sousa *et al*., 2007; Wang *et al*., 2008; Ananthi *et al*., 2010). Several investigations have been focused on the extraction and characterization of functional properties of the polysaccharides derived from different *Sargassum* species, which revealed antioxidant activity (Jung *et al*., 2008; Zhou *et al*., 2008). Kim *et al*., (2007) determined the antioxidant activity of sulfated polysaccharides isolated from brown seaweed *S. fulvellum* and they observed that they are more potent NO scavenger than the commercial antioxidants such as butylated hydroxyanisole (BHA) and α-tocophorol. Gamal-Eldeen *et al*., (2009) tested the antioxidant capacity of sulfated polysaccharides extracted from *S. latifolium* against DPPH radicals. Costa *et al*., (2010) screened the sulfated polysaccharides of eleven species for antioxidant property. Brown seaweed *S. filipendula* emphasized the great antioxidant potentials such as total antioxidant capacity, reducing power and ferrous chelating (Costa *et al*., 2011). Similarly, sulfated polysaccharides isolated from *S. siliquosum* showed significant radical scavenging activity in DPPH radicals and suppressed nitric oxide production in LPS-induced promyelocytic leukemic cells (Ross *et al*., 2012). The antioxidant potential of *S. wightii* sulfated polysaccharides was documented earlier (Yan *et al*., 1998). Similarly, Josephine *et al*., (2008) showed that sulfated polysaccharides from *S. wightii* minimized the oxidative stress associated with cyclosporine A-induced nephrotoxicity by significantly preventing the oxidation of lipids, DNA and protein *in vivo*. Zhou *et al*., (2008) investigated the antioxidant properties of *S. fusiforme* acidic polysaccharide by traditional lipid peroxidation assay and scavenging effect on superoxide and hydroxyl radicals.

Ethnobotanical observations and literature survey revealed many inhibitory activities of seagrasses. Crude extract of seagrasses showed extensive biological activities. The metabolic contents such as polyphenols, sulfated polysaccharides are another buried treasure of the ocean. Kesraoui *et al*., (2011) reported that organic solvent extracts from *Posidonia oceanica* showed high reducing power and strong DPPH and oxygen radical scavenging activities. However, it showed relatively less hydroxyl scavenging activity.

Similarly, Gokce & Haznedaroglu, (2008) found that *P. oceanica* extract decreased lipid peroxidation and restored antioxidant enzymes in alloxan-induced rats. Ferrat *et al*., (2002) reported that *P. oceanica* contained high level of antioxidant enzymes, such as SOD, CAT and GPX. The antioxidant activity of *Zostera marina* L. was examined using DPPH and superoxide radical system (Kim *et al*., 2004). Compound 3 of *Z. marina* inhibited IL-1α and IL-6 production. In addition to that, Yuvaraj *et al*., (2012) found that methanol extract of seagrass *H. ovalis* showed good antioxidant activity in a dose dependent manner in various *in vitro* antioxidant systems. Silva *et al*., (2012) evaluated the *in vitro* antioxidant activity of sulfated polysaccharides extracted from seagrass *H. wrightii* employing different *in vitro* antioxidant systems such as total antioxidant, reducing power, ferric chelating activity, DPPH, superoxide and hydroxyl radical scavenging activities. The *in vivo* antioxidant activity of low etherified pectin extracted from seagrass *Zostera marina* was carried out in mice model (Khasina *et al*., 2003). Intragastral injection of pectin in the form of 1% gel at 100 mg/kg body weight significantly normalized the malonic dialdehyde level and the activity of glutathione peroxidase and reductase in the liver.

Sulfated polysaccharides as immunomodulatory agents

Sulfated polysaccharides of seaweeds have gained much attention in the biochemical and medical fields in stimulating the immune system and/or in controlling the macrophage activity to mitigate inflammation associated negative effects (Groth *et al*., 2009). The macrophage activation can be regulated by sulfated polysaccharides through specific membrane receptors such as pattern recognition receptors, toll-like receptors, competent receptor-3 (CR-3), cluster of differentiation 14 (CD14), and scavenging receptor (Chen *et al*., 2008). Teruya *et al*., (2009) reported that, intracellular signaling pathways and family of mitogen-activated protein kinases (MAPKs) mediate these receptors activation. It has been evidenced that sulfated polysaccharides have direct stimulatory effects on immune cells results in production of nitric oxide through induction of iNOS and pro-inflammatory cytokines (Leiro *et al*., 2007). Fucoidans are reported as biological response modifiers that improve various immune responses (Li *et al*., 2008) and directly

stimulate the innate immune system and find therapeutic use in autoimmune disorders and allergy (Choi *et al*., 2005; Kim & Joo, 2008; Do *et al*., 2010). In addition to that, fucoidan suppressed the development of tumor cells through enhancing body's immunomodulatory activity (Wijesinghe & Jeon, 2012). Hwang *et al*., (2010) reported the immunomodulatory activity of *Sargassum hemiphyllum*. Chotigeat *et al*., (2004) denoted that, polysaccharide extracts of different seaweeds had a great influence in immune response or disease resistance of cultured aquatic organisms. Immunostimulation is one of the successive strategies employed generally to control the WSSV infection (Xiang, 2001). The polysaccharide extracted from *Laminaria digitata* increased the ProPO activity in *P. monodon* (Sritunyalucksana *et al*., 1999). Oral administration of *Sargassum polycystum* polysaccharide enhanced the immune response against WSSV in *P. monodon* juveniles (Chotigeat *et al*., 2004). The polysaccharide extracted from *Sargassum fusiformi* enhanced the immune response and resistance to vibriosis in shrimp *Fenneropenaeus chinensis* (Huang *et al*., 2006). Similarly, hot water extract of *Sargassum glaucescens* significantly increased the immune responses of *Fenneropenaeus indicus* (Ghaednia *et al*., 2011). Fucoidan extracted from *S. wightii* enhanced the non specific immune response of *P. monodon* post larvae against WSSV infection (Immanuel *et al*., 2012).

CHAPTER 3

MATERIALS AND METHODS

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3.1. Materials

All fine chemicals, antibiotics, standard sugars, cell culture media, and enzymes used in this study were procured from Sigma-Aldrich Corporation, USA. Bacterial strains growth media, biochemicals, and standard dextrans were purchased from Hi-Media Laboratories (Mumbai), and SD-Fine chemicals, India. WSSV detection kit was purchased from Shrimpex Biotech Service.

3.1.1. Instruments

Instruments		Name of the Company/Firm
Autoclave	-	York Scientific Instruments, India.
CO_2 Incubator	-	Thermo Scientific, Inc., USA.
Deep freezer (-86°C)	-	Forma Scientific, USA.
Deionized water system	-	Milli-Q, Millipore water Corporation, USA.
Dialysis membrane (1000 Da)	-	Spectrum, USA.
Freeze drier	-	Virtis, USA.
FRP Tanks	-	Fibre Optics, India.
FT-IR Spectrophotometer	-	Thermo Nicolet Model: 6700, USA.
Gas Chromatography-Mass spectrometry	-	Agilent 6890 series, Agilent technologies (Agilent, USA).
Gel documentation system	-	Bio-Rad Laboratories Inc., USA.
Gel electrophoresis	-	Amersham Biosciences, USA
Glass filters G-3	-	Vensil Glass Works Ltd, Bangalore.
Hemocytometer	-	Sigma-Aldrich, USA.
High speed centrifuge	-	Sigma 6K15, USA.

Hot plate with magnetic stirrer	-	Remi Equipments, India.
Hydroplethysmometer	-	Panlabs, India.
Ice flaker	-	Scottman, Italy.
Incubators	-	Scigenics, India.
Laminar flow hoods	-	Matri Scientific Suppliers, India
LC-MS	-	Agilent technologies, Agilent, USA.
Micropipettes	-	LabCommerce, Inc. San Jose, USA.
Phase Contrast Microscope	-	Nikon Optiphot,
pH meter	-	STL instruments Pvt., Ltd., India.
Polymerase Chain reaction (PCR)		
Protein purification system	-	AKTA Prime plus (GE Healthcare, Germany)
Rotary evaporator	-	Heidolph Instruments, Germany
Sequant ZIC-pHILIC column for LC-MS	-	Merck, Germany
Shaker Cum Incubator	-	Scigenics, India.
Soxhlet Apparatus	-	Vensil Glass Works Ltd, Bangalore.
Speed Vac Vaccum drier	-	Thermo Scientific, Inc., USA.
Stereo Zoom Microscope	-	Novex, Holland
Table top centrifuge	-	Remi Instruments Ltd., India.
Thermal cycler	-	Eppendorf, Germany
UV – Vis Spectrophotometer	-	Hitachi, U-2000, Japan.
UV – Transilluminator	-	Fotodyne Inc, USA.
Water bath	-	Matri Scientific Suppliers, India.
Weighing balance	-	Sartorius AG, Germany

3.1.2. Bacterial strains

All bacterial strains were procured from Microbial Type Culture Collections (MTCC), IMTECH, Chandigarh, India. The details of microbial cultures procured from MTCC are listed in the following table (**Table 2**). Fish pathogen, *V. anguillarum* was procured from Central Institute of Brackish-water Aquaculture (CIBA), Chennai. The multidrug resistant bacteria *Acinetobacter baumannii* was obtained from Pondicherry Institute of Medical Sciences (PIMS), Pondicherry, India.

Table 2. List of bacterial strains used for screening of antibacterial activity of seaweeds and seagrasses

Bacterial strains	Source
Acinetobacter baumannii	PIMS, Pondicherry
Bacillus cereus 430	MTCC, Chandigarh, India
Escherichia coli 1687	MTCC, Chandigarh, India
Vibrio anguillarum	CIBA, Chennai
Vibrio fischeri 1738	MTCC, Chandigarh, India
Vibrio parahaemolyticus 451	MTCC, Chandigarh, India
Vibrio vulnificus 1145	MTCC, Chandigarh, India

3.1.3. Plant materials

In this study, a total of eleven seaweeds and four seagrasses were collected from different localities of Rameshwaram and Pondicherry coastal line during August 2009 to September 2010 (**Table 3**).

Table 3. Collection of seaweeds and seagrasses from different regions of Tamilnadu.

Seaweed/seagrass	Locality	Collection period
Seaweeds		
Ulva lactuca Linnaeus	Marakanam, Pondicherry	September, 2010
Ulva reticulata (Forsskal)	Sadaimunianvalasai, Yervadi	September, 2010
Gracillaria corticata J. Agardh	Gulf of Mannar, Rameshwaram	September, 2010
Gelidiella aceorsa (F. Minima & Sree., Rao)	Gulf of Mannar, Rameshwaram	September, 2010
Hypnea musciformis (Wulfen) J.V. Lamouroux	Gulf of Mannar, Rameshwaram	September, 2010
Gracillaria cylindrical Borgesen	Gulf of Mannar, Rameshwaram	September, 2010
Sargassum wightii Greville	Gulf of Mannar, Rameshwaram	September, 2009
Padina gymnospora (Kutzing) Sonder	Gulf of Mannar, Rameshwaram	September, 2010
Turbinaria conoides (J. Agardh) K	Gulf of Mannar, Rameshwaram	September, 2010
Dictyota dichotoma (Hudson) J.V. Lamouroux	Gulf of Mannar, Rameshwaram	September, 2010
Seagrasses		
Halophila ovalis (R. Br.) J.D. Hook	Nonankuppam, Pondicherry	August, 2009
H. ovalis sub sp. ramamurthiana (R. Br.) J.D. Hook	Marakanam, Pondicherry	September, 2010
Cymodocae serrulata (R.Br.) Asch. and Magnus	Gulf of Mannar, Rameshwaram	December, 2009
Syringodium isoetifolium (Asch.) Dandy	Gulf of Mannar, Rameshwaram	December, 2009

3.1.4. Animals

Male Wistar rats (180–220 g) were purchased from approved enterprises and maintained in Central Animal Facility of Pondicherry University, Pondicherry. Animals were housed in polypropylene cages at a room temperature of (21 ± 2°C), with 12 h light/12 h dark cycle and had free access to standard pellets and water *ad libitum*. The study was approved by the Institutional Animal Ethics Committee (PU/IAEC/12/05) to carry out the experiments as per the CPCSEA guidelines.

3.1. 5. Microbiological Media

3.1.5.1. Tryptone Soy Agar (Soybean Casein digest agar medium)

Casein enzyme hydrolysate	-	17.0 g
Papaic digest of soyabean meal	-	3.0 g
Sodium chloride	-	5.0 g
Dipotassium phosphate	-	2.5 g
Dextrose	-	2.5 g
Distilled water	-	1000 ml
Final pH (at 25°C) 7.3±0.2		

3.1.6. Reagents

3.1.6.1. 7 mM ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt).

0.378 g of ABTS was dissolved in 100 ml of distilled water.

3.1.6.2. $BaCl_2$-gelatin reagent

The $BaCl_2$-gelatin reagent for sulfate estimation was prepared by dissolving the gelatin (0.5%) in warm water (60-70°C). The solution was stored overnight at 4°C. The following day, 0.5% of $BaCl_2$ was added and the resultant cloudy solution was allowed to stabilize for 2-3 h at room temperature before using for sulfate estimation.

3.1.6.3. Brilliant green solution (0.435 mM)

20.8 mg of brilliant green was dissolved in 100 ml of distilled water to make 0.435 mM solution.

3.1.6.4. 2% Crystal Violet

2 g of crystal violet was dissolved in 100 ml of distilled water.

3.1.6.5. Genomic DNA isolation

3.1.6.5.1. GTE stock solution (Lysis buffer)

Glucose	-	50 mM
Tris	-	25 mM
EDTA	-	10 mM
Dis. water	-	100 ml
pH	-	8.0

3.1.6.5.2. SDS stock solution (10%)

SDS	-	10.0 g
Dis. water	-	100 ml

3.1.6.5.3. EDTA stock solution (0.5 M)

EDTA	-	14.61 g
Dis. water	-	100 ml

For proper dissolving, the EDTA was mixed with water at warm condition or autoclaving.

3.1.6.5.4. 10 mM Tris (pH 7.6)

Tris	-	0.12 g
Dis. Water	-	100 ml

3.1.6.5.5. Sodium acetate (3 M)

Sodium acetate	-	10.2 g
Dis. Water	-	25 ml

3.1.6.5.6. Tris-Saturated Phenol

To prepare Tris buffered phenol, distilled phenol is equilibrated first with equal volume of 1 M Tris-HCl (pH 8.0) and then with equal volume of 0.1 M Tris-Cl (pH 7.5), 8-hydroxyquinoline is added to a final concentration of 0.1% and stored at 4°C in dark bottle.

3.1.6.5.7. Phenol : Chloroform

Buffered phenol and chloroform were mixed in the ratio of 24:1 and stored in a brown bottle at 4°C along with 0.1 M Tris-Cl in aqueous phase.

3.1.6.5.8. RNase

RNase (10 mg/ml) was dissolved in 10 mM Tris-Cl (pH 7.5) and kept in boiling water bath for 15 min and cooled. It is stored at –20°C.

3.1.6.5.9. Tris- EDTA (TE) Buffer

1 M Tris HCl (pH 8.0)	-	1.0 ml
0.5 M EDTA (pH 8.0)	-	0.2 ml
Dis. Water	-	100 ml

3.1.6.5.10. Agarose gel electrophoresis

TAE Buffer (50 X)

Tris-base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5 M EDTA (pH 8.0)	-	100 ml

The final volume was made up to 1000 ml using distilled water.

Ethidium bromide (EtBr) stock solution

Ethidium bromide	-	10.0 mg
Dis. Water	-	1.0 ml

Gel loading dye (6X)

Bromophenol blue	-	0.25 g
Xylene cyanol	-	0.25 g
Glycerol	-	30 ml
Dis. Water	-	100 ml

3.1.6.6. Hemolymph anticoagulant solution (HAS)

Glucose (115 mM)	-	2.14 g
Sodium citrate (30 mM)	-	0.77 g
EDTA (10 mM)	-	0.29 g
NaCl (338 mM)	-	1.97 g
Distilled water	-	100 ml

3.1.6.7. Phosphate buffered saline (PBS)

Sodium diydrogen phosphate	-	1.4 g
Disodium hydrogen phosphate	-	0.02 g
NaCl	-	0.8 g
KCl	-	0.02 g
Dis. Water	-	100 ml
pH	-	7.0

3.1.6.8. Phenol solution (5%)

5 g of crystal phenol was dissolved in 100 ml of distilled water and stored in amber colored bottle until use**.**

3.1.6.9. Potassium ferricyanide (1%)

1 g of potassium ferricyanide was dissolved in 100 ml of distilled water.

3.1.6.10. Potassium persulfate solution (2.45 mM)

66 mg of potassium persulfate was dissolved in 100 ml of distilled water.

3.1.7. Protein estimation:

1. BSA stock solution (1mg/ml)
2. Analytical reagents:
(a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water).
(b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepare analytical reagents by mixing 2 ml of (b) with 100 ml of (a).
3. Folin - Ciocalteau reagent solution (1N). Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water).

3.1.8. Sodium phosphate buffer (0.2 M, pH 6.6)

Sodium phosphate monobasic	-	1.5 g
Sodium phosphate dibasic	-	2.01 g
Dis. Water	-	100 ml
pH	-	6.6

3.1.9. Trichloro acetic acid (10%)

10 g of trichloro acetic acid crystal was dissolved in 100 ml of distilled water as stock solution and used in different concentrations as per the methods used in this study.

3.1.10. Trifluroacetic acid preparation (4M)

Concentrated TFA is 12.98 M, 3.08 ml of TFA was used to every 6.92 ml of distilled water to make each 10 ml of 4 M TFA. The stock solution was prepared in sterile glass container.

3.1.11. Tris buffered saline (50 mM, pH 7.4)

Tris-HCl	-	0.66 g
Tris Base	-	0.09 g
NaCl	-	0.87 g
Distilled water	-	100 ml

50 mM tris-buffered saline was made as stock solution and from that 10 mM working solution was prepared.

3.2. Methods

3.2.1. Sterilization

All the media, buffers, glasswares and reagents were sterilized by autoclaving at 121°C for 20 min, unless otherwise stated. Antibiotic solutions were filter sterilized (0.22 µm, Millipore).

3.2.2. Maintenance and storage of test organisms

All the bacterial cultures were maintained at 4°C and regularly sub-cultured. The pathogenic bacteria were cultured individually on Tryptic soy broth at 37°C for 18 h, before inoculation for assay. Broth culture (100 µl), which contained 10^7-10^8 number of bacteria per ml was added to tryptic soy agar (TSA) (Hi-media, Mumbai), poured into sterile petri dishes and allowed to solidify.

3.2.2.1. Antibacterial assay

Growth inhibition of pathogens by different solvent extracts of seaweeds and seagrasses were assessed using the paper disc diffusion method. Briefly, sterile filter paper discs impregnated with crude extract (200 µg/ml), positive vehicle (Ampicillin 50 µg/ml), and

negative vehicle (solvent) were allowed to air dry and subsequently placed equidistantly onto the surface of the pathogen seeded tryptic soy agar plates. The plates were kept in an inverted position and incubated at 37°C for 18 h. The growth inhibition was assessed as the diameter (in mm) of the zone of inhibited microbial growth. The experiment was carried out in triplicate. Experimental data represent mean ± SD of each sample, unless otherwise stated.

3.2.2.2. Minimum inhibitory concentration (MIC) assay

A broth microdilution method was used to determine the Minimum Inhibitory Concentration (NCCLS, 2008; Mazzanti *et al*., 2000; Devienne & Raddi, 2002). Briefly, 100 µl of exponentially growing culture (about 10^8 colony-forming unit/ml) was loaded in 96 well plate and 100 µl of different concentrations of test solution was added. The plate was read immediately at 620 nm using an automatic ELISA plate reader (Dynex multimode detector). After incubation for 18 h, the wells were stirred and the plate was re-read at the same wavelength. The absorbances were compared to the values obtained before incubation to detect an increase in bacterial growth. The MIC value was determined using turbidity as an indication of growth and was expressed as the lowest concentration that produced a complete inhibition of turbidity. All experiments were performed in triplicate using ampicillin as antimicrobial standards.

3.2.2.3. Gas chromatography-mass spectrometry (GC-MS) analysis

After the initial thin layer chromatography, the purified active fractions were analyzed for chemical constituents using an Agilent 6890 series high-temperature gas chromatography-mass spectrometer (GC-MS), fitted with an auto injector. For GC-MS analysis, a high-temperature column (DB-5 ht, 30 m × 0.25 mm id × 0.25 µm film thickness) was purchased from Agilent Technologies (Agilent, USA). 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. 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 and maintained at this temperature for 40 min. The helium carrier gas was programmed to maintain a constant flow rate of 1 ml/min, and the mass spectra were acquired and processed using both Agilent ChemStation (Agilent, USA) and AMDIS32 software.

3.2.3. Infrared spectral analysis (Wang *et al*., 2004)

The infrared spectral analysis of sulfated polysaccharide was carried out in FT-IR spectrophotometer (Thermo Nicolet Model: 6700). Briefly, sulfated polysaccharide was ground with potassium bromide (KBr) powder and then pressed into a polymer film for FT-IR measurement in the frequency range of 4000–400 cm^{-1}.

3.2.3.1. Analysis of monosaccharide composition (Goa *et al*., 2011)

Monosaccharide composition analysis was carried out by Liquid Chromatography- Mass spectrometry (LC-MS) as per the method of Goa *et al*., (2011). Briefly, individual stock solutions of each monosaccharide were prepared at a concentration of 1 mg/ml with deionized water. Working standards were prepared by appropriate dilution of the stock solution with acetonitrile/water (80:20 v/v). For sugar analysis, 10 mg of sample was dissolved in 4 M trifluoroacetic acid (TFA). The solution was transferred into hydrolysis tube and was hydrolyzed at 120°C for 5 h. The hydrolyzed solution was concentrated in vacuo and the excess of acid was removed by repeated co–distillation with anhydrous ethanol. The resulting solution was reconstituted with 1 ml of acetonitrile/water (80:20 v/v) for LC/MS/MS analysis.

LC separations were performed on an Agilent system using SeQuant ZIC-pHILIC column (4.6 mm × 150 mm, 5 µm, Merck, Germany) at room temperature. Sample and standards (5 µl) were injected with Agilent auto-sampler and analyzed with 3200 Trap

MS/MS system with flow rate of 400 µl/min. The mobile phase was composed of 0.1% formic acid in acetonitrile: methanol: water (70:10:20,v/v).

3.2.3.2. Sugar analysis (Dubois *et al*., 1956)

Total sugars were estimated by the phenol-sulphuric acid method using fucose as standard. Briefly, 200 µl of sample was mixed with same amount of 5% phenol solution. Then, 1 ml of concentrated H_2SO_4 acid was added to the sample mixture and kept for 30 min after vortexing for 2 min. Absorbance was measured at 490 nm using spectrophotometer (Hitachi, U-2000, Japan).

3.2.3.3. Sulfate estimation (Dodgson & Price, 1962)

Sulfate content in crude and purified fractions was determined by the barium chloride-gelatin method. A known amount of crude and purified fractions was dissolved in deionized water and 2 ml of the sample was transferred into glass ampoules and dried under flow of nitrogen gas. To this ampoule, 1 ml of 1 N HCl was added and the sample was flushed with nitrogen. Thereafter, the ampoule was sealed and the sample was hydrolyzed at 105°C for 17 h. After hydrolyzis, the sample was cooled and the content was transferred to test tube containing 3.8 ml of 3% trichloro acetic acid (TCA) solution. To this mixture, 1 ml of $BaCl_2$-gelatin reagent was added and mixed thoroughly. The solution was kept at room temperature for 15-20 min and the turbidity formed was measured at 360 nm. Potassium sulfate dissolved in 1 N HCl was used as a standard for calibration. In order to estimate blanks in all the above estimations, deionized water was used in place of the samples and analyzed.

3.2.3.4. Protein estimation (Lowry *et al*., 1951)

Total protein content of crude and purified fractions was determined by Lowry's method (Lowry *et al*., 1951). Different dilutions of BSA solution were prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube. The final volume in each of the test

tubes was 5 ml. The BSA range was 0.05 to 1 mg/ml. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulfate reagent (analytical reagent). The solution was mixed well and incubated at room temperature for 10 mins. Then 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 min. Optical density was measured at 660 nm in a spectrophotometer. The absorbance against protein concentration was plotted to get a standard calibration curve. The concentration of the unknown sample was determined using the standard curve plotted.

3.2.3.5. Molecular weight determination (Pan & Mei, 2010)

The average molecular weight of crude and purified fractions of brown seaweed *S. wightii* and seagrass *H. ovalis* was determined by size exclusion chromatography using sephadex G-75 as a matrix. The column was packed with pre-swollen sephadex G-75 beads and connected to AKTA prime purification system (GE, Health Care, Germany). The column was stabilized with 50 mM NaCl solution at the flow rate of 1 ml/min. To determine the molecular weight of the samples, standard dextrans of different molecular weight 10 KDa, 15 KDa, and 20 KDa was chromatographed initially using 0.6 M sodium chloride solution as eluant, and the retention time was determined. The flow rate of the column was maintained at 1 ml/min. Crude sulfated polysaccharides and purified fractions (50 mg) of *S. wightii* and *H. ovalis* was chromatographed using the same parameters. The molecular weight of crude and purified fractions was calculated according to the retention time of standard dextrans.

3.2.4. Antinociceptive activity of sulfated polysaccharides

3.2.4.1. Formalin test (Hunskaar *et al*., 1985)

The formalin test was performed, which causes local tissue injury to the paw, used as a model for tonic pain and localized inflammatory pain. Wistar rats were injected intravenously with *S. wightii* fractions Sw Fr III, Sw Fr IV and *H. ovalis* fractions Ho Fr

III, Ho Fr IV (2.5, 5 or 10 mg/kg) or sterile saline (0.9%, w/v, NaCl). After 30 min of administration, 20 µl of 1% aqueous formalin was injected intraperitonially into the right hind paw and the licking time was then recorded from 0 to 5 min (phase 1, corresponding to the direct stimulation of nociceptors) and 20 to 25 min after formalin injection (phase 2, inflammatory). Indomethacin (5 mg/kg) was also administered subcutaneously 30 min before formalin injection and used as reference compound.

3.2.4.2. Hot plate test (Eddy & Leinback, 1953)

The hot plate test was performed to measure the analgesic activity as described previously. Each rat was placed twice on the heated plate (51 ± 1°C), with a 30-min inter-trial interval. The first trial familiarized the animal with the test procedure, and the second served as the control reaction time (licking the paw or jumping). Animals showing a reaction time greater than 10 s were excluded in subsequent analyses. Immediately after the second trial (control reaction time), group of animals (n = 6) received sterile saline (0.9%, w/v, NaCl), *S. wightii* fractions Sw Fr III, Sw Fr IV and *H. ovalis* fractions Ho Fr III, Ho Fr IV (2.5, 5 or 10 mg/kg; intravenously), Indomethacin (5 mg/kg, subcutaneously). The reaction times were measured at time zero (0 time) and 30, 60 and 90 min after drug administration with a cut-off time of 40 s to avoid paw lesions.

Anti-inflammatory activity of sulfated polysaccharides

3.2.4.3. Carrageenan-induced rat paw edema (Winter *et al*., 1962)

The rats (n = 6 per group) were pretreated with *S. wightii* fractions Sw Fr III, Sw Fr IV and *H. ovalis* fractions Ho Fr III, Ho Fr IV at doses of 2.5, 5 or 10 mg/kg (0.1 ml/100 g body weight; subcutaneously) or sterile saline (0.9%, w/v) 1 h before carrageenan injection. Carrageenan (500 µg/paw, 100 µl) was injected intraperitonially into the right hind paw. In a control experiment, diclofenac (1 mg/kg), a synthetic glucocorticoid with potent anti-inflammatory and immunosuppressant properties was administered

subcutaneously 1 h before carrageenan administration. Paw volume was measured immediately before (zero time) the stimulus and at selected time intervals (1, 2, 3 and 4 h) using a plethysmometer (Panlab, Spain). The results are expressed as the variation in paw volume (ml), calculated as the difference from the basal volume (zero time) and the volume at the selected time interval.

3.2.4.4. Freund's adjuvant induced arthritis (Newbould, 1963)

Male wistar albino rats weighing 180 ± 20 g were used for the chronic inflammation study. The animals were divided into five groups of six animals each. Group I served as control (without treatment), Group II served as arthritic control (negative control), Group III is treated with diclofenac sodium at 5 mg/kg (positive control), Group IV received Sw Fr IV (10 mg/kg), and group V received Ho Fr IV (10 mg/kg). After 30 min of drug treatment, arthritis was induced by injecting a 0.1 ml (0.1% w/v) of Complete Freund's Adjuvant (CFA) into the left hind paw. The drug treatment was continued from the initial day i.e. from the day of adjuvant injection (0 day), and continued till 14th day. The paw volume of all the groups was measured on 0, 3, 7 and 14 day of drug treatment by using plethysmometer. The mean changes in injected paw edema with respect to initial paw volume, were calculated on respective days and % inhibition of paw edema with respect to untreated group was calculated.

3.2.5. Antioxidant activity of sulfated polysaccharides

3.2.5.1. Scavenging ability on hydroxyl radicals (Barahona *et al*., 2011)

Hydroxyl radicals were generated by the Fenton's reaction at 20°C as described by Barahona *et al.,* (2011). The absorbance at 624 nm of aqueous solutions of 0.435 mM brilliant green (BG), 0.25 mM solution of $FeSO_4$, and varying concentrations of purified fractions (0.5–2.5 mg/ml) were measured as time function immediately after the addition of H_2O_2. The scavenging effect was expressed as $\Delta Abs/(Abs)_{initial} \times \Delta 100$, where ΔAbs is

the difference between the absorbance of BG in the presence and in the absence of polysaccharide, measured at 30 min reaction time. $(Abs)_{initial}$ is the initial absorbance in the absence of the polysaccharide.

3.2.5.2. Scavenging ability on ABTS radicals (Re *et al*., 1999)

The radical scavenging activity of purified fractions of *S. wightii* and *H. ovalis* was measured by ABTS radical cation ($ABTS^+$) test with some modifications. $ABTS^+$ was produced by reacting 7 mM of ABTS solution with 2.45 mM of potassium persulphate and the mixture kept in the dark at room temperature for 16 h. In the moment of use, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample (0.2 ml) with various concentrations (0.5–2.5 mg/ml) were added to 2 ml of $ABTS^+$ solution and mixed vigorously. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured. The $ABTS^+$ scavenging effect was calculated by the following formula: $ABTS^+$ scavenging effect (%) = $A_C – A_S / A_C \times 100$, where A_C was the absorbance of the control and A_S was the absorbance of the test sample.

3.2.5.3. Ferric- reducing antioxidant power (FRAPs) assay (Oyaizu, 1986)

Total reducing capacity of purified fractions of *S. wightii* and *H. ovalis* was determined according to the method of Oyaizu, (1986). The polysaccharide solution of 1 ml at different concentrations (0.5–2.5 mg/ml) was mixed with 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixtures were incubated at 50°C for 20 min followed by the addition of 2.5 ml of 10% trichloroacetic acid. The reaction mixtures were then centrifuged at 1000 × g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml), $FeCl_3$ (1.5 ml, 0.1%) and the absorbance was measured at 700 nm. An increase in absorbance was used as a measure of the reducing power.

3.2.6. Immune parameters

3.2.6.1. Total hemocyte count (THC)

For THC assay, 10 µl of anticoagulant mixed hemolymph were immediately diluted with 0.5% tryphan blue in 2.6% NaCl and the hemocytes were counted using hemocytometer (Neubauer chamber), connected with the Nikon Optiphot phase contrast microscope and calculated the number of cells (Total hemocytes/mm^3). Total protein concentration of each group was estimated spectrophotometrically as described by Lowry *et al*., (1951) using bovine serum albumin as standard.

3.2.6.2. Prophenoloxidase assay (Chang *et al*., 2003)

Phenoloxidase activity in supernatant of hemolymph from control, treated and infected shrimps was spectrophotometrically determined as described previously with minor modifications. Briefly, freshly sampled shrimp hemolymph was kept on ice in to a microtube and hemocytes were broken by sonication for 10 s. Phenoloxidase activity in haemolymph samples was determined using L-Dihydroxyphenylalanine (L-DOPA) as a substrate. Tris buffered saline (TBS) at 30 µl was added to the cuvette containing 30 µl of haemolymph sample. Then, 90 µl of L-Dopa buffer (3 mM L-Dopa in 10 mM TBS, pH 7.4) was added and mixed. After incubation at 37°C for 20 min, the amount of dopachrome produced in the reaction mixture was determined as the optical density at 490 nm. An increase in the optical density at 490 nm of 0.001 every minute under this condition was defined as one unit of PO activity. The L-Dopa buffer only was used as a control. The assay was carried out in triplicate.

3.2.6.3. Superoxide anion analysis (NBT reductase assay) (Liu & Chen, 2004)

Superoxide anion was quantified using a microplate reader and expressed as NBT reduction in 10 µl of haemolymph. Briefly, 100 µl of hemolymph were collected from control, WSSV infected and polysaccharide treated groups in anticoagulant solution and

deposited in triplicate on microplates previously coated with 100 µl poly L-lysine solution (0.2%) to improve cell adhesion. Microplates were centrifuged at 500 × *g* for 20 min at 4°C (Sigma 6K15). The supernatant was decanted and 100 µl zymosan (0.1% in Hank's Balanced Salt Solution) was added and allowed to react for 30 min at room temperature. 100 µl of NBT solution (0.3%) was added and stained for 30 min at room temperature and then 100 µl of methanol was added. The mixed solution was discarded, and the microplates were washed three times with 100 µl of 70% methanol and air-dried. The formazan was dissolved by addition of 120 µl of 2 M KOH and 140 µl of DMSO (Dimethyl sulphoxide). The samples were read at 630 nm and the superoxide anion was measured in all the tested groups.

3.2.7. Statistical analysis

All data were expressed as mean ± standard deviation. Statistical differences between the experimental groups were determined by one way ANOVA, and differences were considered to be statistically significant if $P < 0.05$. All computations were done by employing statistical software (SPSS Version 7.5).

CHAPTER 4

ANTIBACTERIAL ACTIVITY OF SEAWEEDS AND SEAGRASSES

CHAPTER 4
ANTIBACTERIAL ACTIVITY OF SEAWEEDS AND SEAGRASSES

4.1. Introduction

Seaweeds are one of the important renewable resources in the marine environment and have been a part of human civilization from time immemorial. Reports on the uses of seaweeds have been cited as early as 2500 years ago in Chinese literature (Tseng, 2004). Among the Asian countries, India is perhaps the only one that has a long record of inventories of coastal and marine biodiversity dating back to at least two centuries (Venkataraman & Wafer, 2005). In 1927, Iyengar was the pioneer Indian algologist who gave a detailed descriptive account of Indian marine algae occurring on the south east coast, especially Krusadai Island. The seaweed flora of India is widely diversified and comprises mostly of tropical species, but boreal temperate and subtropical elements have also been reported.

Seagrasses are submerged marine angiosperms that grow successfully in tidal and subtidal marine environments except in polar regions. Applied research on seagrass has been concerted in few areas of the world with many existing archetype expanded from even fewer species and areas (Duarte & Chiscano, 1999). The global distributions of seagrasses have been explored recently (Short *et al*., 2007). Seagrasses are well documented for the presence of potent diverse secondary metabolites (Puglisi *et al*., 2007). The possibility of collecting organisms directly from the ocean with the use of SCUBA (self-contained under water breathing apparatus) opened a new gate to a largely untapped resource with a wide range of unique structures and novel compounds.

Vibrio spp., especially luminous *Vibrio harveyi* Johnson & Shunk (Vibrionaceae) and *Vibrio parahaemolyticus* Fujino *et al*. (Vibrionaceae) have been implicated as the main bacterial pathogens of shrimp in, hatcheries as well as farms (Swain *et al*., 2009). Problems including solubility, palatability, toxicity, cost, delivery, and governmental restrictions have limited the available antibiotics, especially in food fish culture (Choudhury *et al*., 2005). Decreased efficacy and increased resistance of pathogens to

antibiotics has necessitated the development of new alternative drugs/compounds (Smith *et al*., 1994). *Acinetobacter baumannii* has emerged as an important and problematic human pathogen as it is the causative agent for several types of infections including pneumonia, meningitis, septicemia, and urinary tract infections. Clinical impact of *A. baumannii* infection has been a matter of continuing debate. Since pathogens gaining resistance to drugs is common due to haphazard use of antibiotics, much attention is needed to kill or control the pathogens using bioactive substances.

The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from alternative sources including sources from the ocean. Recently, a great deal of interest has developed to isolate bioactive compounds from marine resources because of their numerous beneficial health effects (Wijesekara *et al*., 2011). Though literature speaks diverse studies of bioactivity of marine flora against several pathogens, our work on testing the antibacterial efficacies of seaweeds, and seagrasses mainly on human multidrug resistant pathogens *A. baumannii* and also on fish pathogens *V. parahaemolyticus*, *V. fischeri*, *V. anguillarum*, *V. vulnificus*, *B. cereus*, and *E. coli* is relatively on a new concept as few attempts had been made earlier in this line. Hence, analyses of secondary metabolites towards seaweeds and seagrasses possessing antimicrobial properties are found to be imperative to emphasize. In the present study, different solvents extract of seaweeds and seagrasses were tested against Gram-positive and Gram-negative pathogens. The TLC purified fractions of crude extract were analyzed by GC-MS for the identification of chemical constituents accountable for antibacterial activity.

4.2. Materials and methods

4.2.1. Collection of Plant materials

A total of ten seaweeds, *Ulva lactuca* Linnaeus, *Ulva reticulata* Forsskal, *Gracilaria cylindrica* Borgesen, *Gracilaria corticata* J. Agardh, *Gelidiella acerosa* F. Minima and Srinivasa Rao, *Hypnea musciformis* (Wulfen) J.V. Lamouroux, *Sargassum wightii* Greville, *Padina gymnospora* (Kutzing) Sonder, *Turbinaria conoides* (J. Agardh) K,

Dictyota dichotoma (Hudson) Lamouroux and two seagrasses, *Cymodocea serrulata* (R. Br.) Asch. and Magnus, and *Syringodium isoetifolium* (Asch.) Dandy were collected from Mandapam, Rameshwaram in Tamilnadu, India during August 2008. Fresh seagrass samples of *Halophila ovalis* (R.Br.) J.D. Hook, and *Halophila ovalis subsp. ramamurthiana* were collected in low tide from Chunnambar estuary, and Marakanam backwaters (Pondicherry), India during September 2009. Plant specimens were identified by Prof. N. Parthasarathy, Salim Ali School of Ecology, Pondicherry University. Specimens were preserved in 5% formalin solution for identification.

4.2.2. Preparation of extracts

The seaweed and seagrass samples were rinsed with seawater three times and then successively with tap water and distilled water to remove the epiphytes and other wastes. The plant materials were then desiccated under the shade for two weeks. The dried plant material (10 g dry weight) was ground to fine powder and extracted with 100 ml of solvents increasing polarity from a non-polar to polar (hexane, chloroform, ethylacetate, and methanol) for 24 h by cold extraction method and the extract was filtered through a Buchner funnel with Whatman No. 1 filter paper and fresh solvent was added to the residue for another 24 h. This was repeated three times for the complete extraction of compounds, and all the three solvent extracts were pooled. The solvent was evaporated from crude extract by rotatory evaporator. The dried extracts (1 g) were dissolved in 2 ml of respective solvent and stored at 4°C until use.

4.2.3. Antibacterial and minimum inhibitory concentration (MIC) assay

The antibacterial activity of seaweeds and seagrasses extract against Gram-positive and Gram-negative pathogens were carried out as described in general material and methods section **3.2.2.1.** The minimum inhibitory concentration (MIC) assay of active extract was carried out as described in methods section **3.2.2.2.**

4.2.4. Purification of fractions by thin layer chromatography

To investigate the chemical constituents, concentrated crude methanol extract of seaweeds and seagrass was purified by thin layer chromatography using ethylacetate-hexane, and methanol-chloroform as solvent systems. The crude methanol extracts of green seaweed *C. glomerata* and seagrass *H. ovalis* were separated in a pre coated aluminum TLC sheet with silica gel G 60 as stationary phase, and ethyl acetate-hexane mixture in the ratio of 9.5:0.5 as mobile phase. Whereas, crude methanol extracts of brown seaweed *S. wightii* was separated using methanol-chloroform mixture in the ratio of 10:1 as mobile phase. The eluted spots, representing various fractions were visualized under UV transilluminator at 254 nm, and also in the iodine chamber. TLC resolved spots of methanol extract at various R_f values were scrapped from the TLC plate, and the scrapped spots were dissolved in methanol, mixed well and centrifuged at 12,000 × *g* for 5 min. A total of six different fractions from *H. ovalis*, *S. wightii*, and five fractions from *C. glomerata* were collected and the supernatant (40 µl) of each fraction was used to check the antibacterial activity against pathogens using the disc diffusion method in triplicate. Ampicillin was used as positive control. Experimental data represent mean ± SD of each sample, unless otherwise stated.

4.2.5. Gas chromatography-mass spectrometry (GC-MS) analysis

The chemical constituents of seaweeds and seagrass purified fractions were identified by GC-MS as described in general materials and methods section **3.2.2.3.**

4.3. Results

4.3.1. Antibacterial activity of seaweeds and seagrasses

The antibacterial activity of different solvent extracts of seaweeds and seagrasses were summarized in (**Table 4-7**). The results of the present study showed that methanol extract

(200 µg/ml) of green seaweed *C. glomerata*, brown seaweed *S. wightii*, and seagrass *H. ovalis* exhibited appreciable antibacterial activity against the Gram-positive and Gram-negative pathogens than other solvent extracts. In this study, the methanol extract of *C. glomerata* showed high inhibiting activity of 15 mm against *A. baumannii* followed by 12 mm against *V. anguillarum*, and 11 mm against *V. vulnificus*. Similarly, brown seaweed *S. wightii* exhibited a good antibacterial activity of 18 mm against *B. cereus* followed by 15 mm against *A. baumannii*. The crude methanol extract of seagrass *H. ovalis* displayed a good antibacterial activity of 17 mm against *B. cereus* followed by 14 mm against *A. baumannii* and *H. ovalis sub. sp ramamurthiana* showed antibacterial activity of 15 mm against *A. baumannii* (**Table 4**). The ethylacetate extract of all seaweeds and seagrasses showed antibacterial activity of more than 10 mm against Gram-negative *A. baumannii* and Gram-positive *B. cereus*. Whereas, *V. parahaemolyticus*, *V. vulnificus*, *V. anguillarum*, *V. fischeri*, and *E. coli* showed inhibition zone of less than 10 mm against the seaweeds and seagrasses extracts tested (**Table 5**). Similarly, chloroform extract of all seaweeds and seagrasses exhibited antibacterial activity of more than 10 mm against *A. baumannii* and *B. cereus* except green seaweed *U. lactuca*, and seagrass *S. isoetifolium*. However, the growth of *V. parahaemolyticus*, *V. vulnificus*, *V. anguillarum*, *V. fischeri*, and *E. coli* was not inhibited effectively by chloroform extracts of seaweeds and seagrasses (**Table 6**). The hexane extract of seaweeds and seagrasses did not showed antibacterial activity against the pathogens tested except Gram-positive pathogen *B. cereus* (**Table 7**). The brown seaweed *S. wightii* only showed antibacterial activity of more than 10 mm against *B. cereus*.

4.3.2. Minimum inhibitory concentration assay

The minimum inhibitory concentration (MIC) of the methanol extracts of *C. glomerata*, *S. wightii*, and *H. ovalis* showed maximum activity against the pathogens was depicted (**Table 8**). In the present study, the MIC of methanol extract of *C. glomerata* was found to be 75 µg/ml against *B. cereus*, *E. coli* and 100 µg/ml against Gram-negative pathogens *A. baumannii* and Vibrio spp. Similarly, the growth of *B. cereus* was inhibited at a minimum inhibitory concentration of 50 µg/ml followed by Gram-negative pathogens at

100 µg/ml by brown seaweed *S. wightii*. Whereas, *E. coli* growth was inhibited at a minimum inhibitory concentration of 110 µg/ml. The seagrass *H. ovalis* inhibited the growth of *B. cereus* at MIC of 50 µg/ml followed by Gram-negative pathogens at 75 µg/ml except *V. vulnificus* (100 µg/ml).

4.3.3. Purification of antibacterial fractions

The crude methanol extract of green seaweed *C. glomerata* and seagrass *H. ovalis* were purified by thin layer chromatography using ethyl acetate-hexane as solvent systems in the ratio of 9.5:0.5 as mobile phase. Whereas, brown seaweed *S. wightii* was purified using methanol-chloroform (10:1) as mobile phase. A total of five different fractions were obtained from *C. glomerata*, and six different fractions from brown seaweed *S. wightii* and seagrass *H. ovalis*. In the present study, the second fraction of green seaweed *C. glomerata* showed good antibacterial activity of 11 mm against fish pathogens *V. fischeri*, and 10 mm against *V. vulnificus*. Similalry, the third and fifth fractions of *C. glomerata* exhibited good antibacterial activity against fish pathogens *V. fischeri* (10 mm), *V. vulnificus* (10 mm), and human pathogen *A. baumannii* (10 mm) respectively (**Table 9**). The antibacterial activity of TLC purified fractions of seaweed *S. wightii* was depicted in **Table 10**. Purified fraction III showed the inhibition zone of 11 mm against *B. cereus* and 10 mm against the other pathogens tested except *E. coli* (6 mm). Similarly, purified fraction VI showed the maximum inhibition zone of 13 mm against *B. cereus, A. baumannii* (12 mm), *V. anguillarum, V. parahaemolyticus* (11 mm), and *V. fischeri, V. vulnificus* (10 mm). Whereas, diameter of less than 10 mm was observed against *E. coli* (8 mm). In this study, the purified fractions of seaweed *S. wightii* displayed good antibacterial activity against Gram-positive as well as Gram-negative pathogens. Fractions III and VI of *S. wightii* extract efficiently inhibited *B. cereus, A. baumannii, V. parahaemolyticus* and *V. anguillarum.* Whereas, Purified fractions V and VI of seagrass *H. ovalis* showed the inhibition zone of more than 10 mm against *Vibrio parahaemolyticus* (12 mm), *Acinetobacter baumannii* (12 mm), *V. anguillarum* (11 mm), and *V. fischeri* (11 mm) whereas diameter of less than 10 mm was observed in other fractions against the pathogens tested (**Table 11**).

4.3.4. Gas Chromatography-Mass Spectrometry analysis

The TLC purified active fractions of *C. glomerata*, *S. wightii*, and *H. ovalis* were dissolved in hexane and subjected to GC-MS to analyze the chemical constituents. GC-MS analyses of active fractions of *C. glomerata* were summarized. In the second active fraction, Pentadecane, 8-hexyl-was found to be a major compound (7.02%) followed by heptadecane, 9-hexyl- (7.02%) and tridecane, 8-hxyl-(4.93) (**Table 12**). Similarly, pentadecane, 8-hexyl-(8.76%) was also found as major compound followed by the hydrocarbon compounds heptadecane, 9-hexyl-(6.48%) and octacosane (4.07%) in third fraction (**Table 13**). Octacosane (11.66%) was found to be major compound followed by pentadecane, 8-hexyl- (10.01%), Tridecane, 7-hexyl-(7.27%) and heptadecane, 9- hexyl-(6.93%) in fifth fraction (**Table 14**). The results obtained by the GC-MS analysis of the purified fractions III and VI of *S. wightii* are presented in the **Table 15** and **16**. As determined from the GC-MS analysis, the major compounds were 9- Octadecenamide (11.40%), Eicosane (6.47%), Hexanoic acid, pentadecyl ester (5.57%), 1H-Indole, 6-methyl-(5.43%), Estra-1, 3, 5 (10)- trien- 17 beta-ol (3.52%) and Butane, 2, 3-bis (trimethylsiloxy)-(3.29%) in purified fraction III. Whereas, fraction VI revealed the presence of 26-Nor-5- cholesten-3.beta.-ol-25-one (14.55%), 1, 4, 7, 10, 13, 16-Hexaoxanonadecane, 18- (2-propenyl)-(10.52%), Methylenebis (2, 4, 6-triisopropylphenylphosphine) (9.47%), 9- Octadecenamide (7.95%), 1, 4, 7, 10, 13, 16-Hexaoxacyclooctadecane (5.98%), 7- hydroxyl-3-(1,1-dimethylprop-2-enyl) coumarin (5.50%), 18-crown-6, (2-bromophenyl)- (5.26%), 1, 2- Benzenedicarboxylic acid, butyl 1 octyl ester (3.54%).

Similarly, GC-MS analysis revealed the presence of saturated fatty acids, and aromatic carboxylic acid in the purified fractions of *H. ovalis* (**Table 17, 18**). The major component was 9-octadecenoic acid (27.01%) followed by hexadecanoic acid (21.63%), and octadecanoic acid (10.42%) in fraction V. Whereas, benzoic acid (11.11%) was found to be a major chemical constituent followed by tetradecanoic acid (6.12%), and hexadecane (3.47%) in fraction VI.

Table 4. Antibacterial activity of methanol extract against pathogens. Values are means of growth inhibition of three replicates.

Pathogens / Test Samples	*V. parahaemolyticus*	*V. vulnificus*	*V. anguillarum*	*V. fischeri*	*E. coli*	*A. baumannii*	*B. cereus*
1	6.66±0.28	8.83±1.04	6.66±0.76	7.00±0.50	0.00±0.00	9.66±0.28	9.50±0.86
2	7.33±0.28	9.33±1.52	6.33±1.15	6.83±0.28	0.00±0.00	10.16±0.76	8.00±0.50
3	7.00±0.50	7.16±0.28	6.83±0.28	0.00±0.00	0.00±0.00	8.83±0.28	7.66±0.28
4	7.16±0.28	7.00±0.50	7.00±0.50	0.00±0.00	0.00±0.00	10.16±0.76	7.66±0.28
5	7.33±0.28	7.66±0.28	6.50±0.50	6.66±0.28	7.33±0.28	10.83±0.28	9.33±1.25
6	7.16±0.28	6.50±0.50	6.16±0.28	6.33±0.28	8.00±0.50	10.16±0.28	9.50±0.50
7	10.83±0.76	12.16±1.04	10.16±1.04	11.83±0.76	8.33±0.76	15.16±1.25	17.83±0.76
8	12.33±1.25	7.16±0.76	8.50±0.86	10.00±1.32	8.33±0.76	9.83±0.76	10.66±0.76
9	13.00±1.41	7.25±0.35	9.25±0.35	7.25±0.35	7.00±0.00	9.00±0.00	11.25±1.06
10	7.50±0.50	8.16±0.76	7.00±0.50	7.10±0.28	8.00±1.32	11.16±0.57	10.16±0.76
11	10.16±0.28	10.36±0.75	10.16±0.28	10.00±0.00	8.53±0.51	13.83±1.04	17.16±0.28
12	9.50±0.50	8.16±0.28	8.33±0.28	9.16±0.28	7.83±1.04	15.16±0.76	11.5±0.50
13	0.00±0.0	0.00±0.00	0.00±0.00	7.16±0.28	8.33±0.28	6.50±0.50	0.00±0.00
14	6.50±0.50	8.66±0.76	8.33±0.28	7.16±0.28	0.00±0.00	8.33±0.76	9.33±0.76
15	9.00±1.53	11.00±2.00	12.00±1.00	9.00±2.52	9.00±0.58	15.00±1.58	9.00±0.58
16	12.00±0.57	12.16±0.28	10.00±0.00	13.00±0.50	12.00±0.50	10.16±0.28	10.16±0.28

1. *U. lactuca* 2. *U. reticulata* 3. *G. cylindrica* 4. *G. corticata*
5. *G. acerosa* 6. *H. musciformis* 7. *S. wightii* 8. *P. gymnospora*
9. *T. conoides* 10. *D. dichotoma* 11. *H. ovalis* 12. *H. ovalis sub sp.*
13. *C. serrulata* 14. *S. isoetifolium* 15. *C. glomerata* 16. *Ampicillin.*

Table 5. Antibacterial activity of ethylacetate extract against pathogens. Values are means of growth inhibition of three replicates.

Pathogens / Test Samples	*V. parahaemolyticus*	*V. vulnificus*	*V. anguillarum*	*V. fischeri*	*E. coli*	*A. baumannii*	*B. cereus*
1	7.66±0.76	7.16±0.57	7.66±0.28	8.00±0.50	7.50±0.86	11.50±0.50	10.00±0.86
2	7.66±0.28	7.83±0.57	7.66±0.28	7.33±0.28	7.83±0.28	11.16±0.28	11.00±0.50
3	8.33±0.28	7.16±0.28	7.83±0.57	7.16±0.78	7.66±0.76	11.66±1.75	10.66±0.76
4	8.00±0.50	7.66±0.57	7.50±0.50	7.33±0.28	8.00±0.50	11.66±0.52	10.83±0.76
5	7.83±0.76	7.83±0.57	8.33±0.28	8.16±0.76	8.16±1.04	11.16±0.76	11.50±0.50
6	8.16±0.28	7.00±0.50	7.66±0.76	7.33±0.28	8.16±0.28	12.66±0.57	11.00±0.86
7	7.33±1.25	8.16±0.76	8.66±0.76	8.16±0.28	8.33±0.28	11.50±0.50	11.50±1.80
8	7.50±0.86	7.33±0.28	7.83±0.28	8.33±1.25	7.16±0.76	11.50±1.32	9.83±1.60
9	7.50±0.86	8.33±0.76	8.16±0.28	8.00±0.50	7.83±0.28	10.50±0.50	9.83±1.25
10	8.00±0.50	7.83±0.76	8.16±0.76	8.33±0.28	8.00±1.32	10.33±0.57	10.16±1.25
11	8.16±1.04	7.66±0.28	8.33±0.28	7.66±0.76	8.00±0.50	14.16±0.76	12.83±2.02
12	7.83±0.76	7.66±0.76	8.00±0.86	7.66±0.76	7.16±0.28	12.83 1.60	11.16±1.04
13	7.00±0.00	8.16±0.28	7.66±0.28	8.16±0.76	8.66±0.28	10.66±0.57	9.83±1.04
14	7.50±0.86	8.33±0.57	7.33±0.28	8.00±0.50	8.50±0.86	10.33±1.89	9.16±1.60
15	7.83±0.57	7.16±0.78	7.66±0.28	8.00±0.50	8.00±0.50	12.18±0.28	10.00±0.50
16	12.00±0.5	12.16±0.28	10.00±0.00	13.00±0.50	12.00±0.50	10.16±0.28	10.16±0.28

1. *U. lactuca* 2. *U. reticulata* 3. *G. cylindrica* 4. *G. corticata*
5. *G. acerosa* 6. *H. musciformis* 7. *S. wightii* 8.*P. gymnospora*
9. *T. conoides* 10. *D. dichotoma* 11. *H. ovalis* 12. *H. ovalis sub sp.*
13. *C. serrulata* 14. *S. isoetifolium* 15. *C. glomerata* 16. *Ampicillin*

Table 6. Antibacterial activity of chloroform extract against pathogens. Values are means of growth inhibition of three replicates.

Pathogens / Test Samples	*V. parahaemolyticus*	*V. vulnificus*	*V. anguillarum*	*V. fischeri*	*E. coli*	*A. baumannii*	*B. cereus*
1	6.33±0.28	6.66±0.28	7.00±0.50	6.16±0.28	7.50±0.50	9.50±0.50	9.16±0.76
2	6.50±0.50	6.66±0.76	7.33±0.76	7.33±0.28	8.16±0.76	10.33±0.76	10.5±0.86
3	8.66±0.76	8.00±1.80	8.33±0.57	6.50±0.50	8.33±0.28	12.33±1.04	11.50±1.32
4	8.16±0.28	7.66±0.28	8.33±0.28	7.00±0.50	8.16±0.76	12.16±2.02	11.33±0.76
5	7.50±0.50	8.00±0.50	8.16±0.57	8.66±0.28	9.33±0.76	11.33±0.76	12.33±0.76
6	7.83±0.28	7.66±0.76	8.16±1.25	6.66±0.76	8.00±0.86	12.83±0.28	11.16±0.76
7	7.33±1.04	9.50±0.50	9.50±1.32	9.00±0.50	8.00±0.50	13.66±0.76	12.00±0.50
8	7.16±0.76	7.5±0.50	8.16±0.28	7.66±0.76	7.83±1.25	10.16±0.76	10.83±0.76
9	7.33±0.28	8.50±0.86	8.33±0.28	8.16±1.04	8.00±0.50	10.00±0.50	10.33±0.76
10	8.00±0.50	8.16±0.57	8.66±0.28	8.33±0.28	8.16±0.57	12.16±0.28	10.66±0.76
11	8.66±0.76	8.33±0.28	8.66±1.04	9.16±1.04	8.83±0.57	12.50±1.50	11.33±1.04
12	7.66±0.76	7.10±0.76	7.66±0.28	8.00±0.50	7.33±0.76	11.00±0.50	9.66±1.04
13	7.00±0.50	8.50±0.50	8.16±0.50	8.00±0.50	8.50±1.32	10.00±0.50	11.66±1.60
14	7.50±0.50	8.66±0.76	7.66±0.28	8.00±0.50	9.00±0.50	10.16±0.76	9.33±0.16
15	6.16±0.28	7.00±0.50	7.33±0.76	7.50±0.50	8.33±0.28	12.00±0.50	2.16±0.28
16	12.00±0.57	12.16±0.28	10.00±0.00	13.00±0.50	12.00±0.50	10.16±0.28	10.16±0.28

1. *U. lactuca* 2. *U. reticulata* 3. *G. cylindrica* 4. *G. corticata*

5. *G. acerosa* 6. *H. musciformis* 7. *S. wightii* 8. *P. gymnospora*

9. *T. conoides* 10. *D. dichotoma* 11. *H. ovalis* 12. *H. ovalis sub sp.*

13. *C. serrulata* 14. *S. isoetifolium* 15. *C. glomerata* 16. *Ampicillin*

Table 7. Antibacterial activity of hexane extract against pathogens. Values are means of growth inhibition of three replicates.

Pathogens / Test Samples	*V. parahaemolyticus*	*V. vulnificus*	*V. anguillarum*	*V. fischeri*	*E. coli*	*A. baumannii*	*B. cereus*
1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.16±0.28
2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.66±0.28
3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	8.00±0.50
4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	8.50±0.50
5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.33±0.28
6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.50±0.50
7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	10.5±0.50
8	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	8.00±0.50
9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	8.33±0.28
10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.16±0.28
11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	9.66±0.76
12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.66±0.28
13	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.33±0.28
14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	8.00±0.50
15	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	9.00±0.50
16	12.00±0.57	12.16±0.28	10.00±0.00	13.00±0.50	12.00±0.50	10.16±0.28	10.16±0.28

1. *U. lactuca* 2. *U. reticulata* 3. *G. cylindrica* 4. *G. corticata*
5. *G. acerosa* 6. *H. musciformis* 7. *S. wightii* 8. *P. gymnospora*
9. *T. conoides* 10. *D. dichotoma* 11. *H. ovalis* 12. *H. ovalis sub sp.*
13. *C. serrulata* 14. *S. isoetifolium* 15. *C. glomerata* 16. *Ampicillin*

Table 8. Minimum inhibitory concentration of methanol extracts of seaweeds *C. glomerata*, *S. wightii*, and seagrass *H. ovalis* against pathogens. Values are means of inhibitory concentration of three replicates.

Sl. No	Pathogens	Minimum inhibitory concentration (µg/ml)		
		C. glomerata	*S. wightii*	*H. ovalis*
1	*V. parahaemolyticus*	100 ± 0.00	100 ± 0.00	75 ± 0.00
2	*V. anguillarum*	100 ± 0.00	100 ± 0.00	75 ± 0.00
3	*V. fischeri*	100 ± 0.00	100 ± 0.00	75 ± 0.00
4	*V. vulnificus*	125 ± 0.00	100 ± 0.00	100 ± 0.00
5	*E. coli*	75 ± 0.00	110 ± 0.00	75 ± 0.00
6	*B. cereus*	75 ± 0.00	50 ± 0.00	50 ± 0.00
7	*A. baumannii*	100 ± 0.00	100 ± 0.00	75 ± 0.00

Table 9. Antibacterial screening of *Cladophora glomerata* purified fractions against pathogens (inhibition zone was measured to the nearest millimeter)

Test organism	Inhibition zone in mm (mean ± SD)					Ampicillin
	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	(50µg/ml)
V. parahaemolyticus	07.00 ± 0.29	07.00 ± 0.25	00.00 ± 0.00	08.00 ± 0.25	07.00 ± 0.66	12.00 ± 0.50
V. anguillarum	00.00 ± 0.00	09.00 ± 0.62	09.00 ± 0.15	08.00 ± 0.40	09.00 ± 0.92	10.00 ± 0.00
V. fischeri	08.00 ± 0.58	11.00 ± 0.42	10.00 ± 0.60	06.00 ± 0.92	06.00 ± 0.66	13.00 ± 0.50
V. vulnificus	06.00 ± 0.58	10.00 ± 0.26	07.00 ± 0.26	06.00 ± 0.60	10.00 ± 0.15	12.16 ± 0.28
E. coli	00.00 ± 0.00	05.00 ± 0.40	00.00 ± 0.00	00.00 ± 0.00	05.00 ± 0.40	12.00 ± 0.50
B. cereus	08.00 ± 0.58	08.00 ± 0.55	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	10.16 ± 0.28
A. baumannii	08.00 ± 0.58	08.00 ± 0.30	12.00 ± 0.15	09.00 ± 0.15	10.00 ± 0.15	10.16 ± 0.28

Values are means of growth inhibition of three replicates, Fr= fraction

Table 10. Antibacterial screening of purified fractions of methanol extract of seaweed *S. wightii* against pathogens (inhibition zone was measured to nearest millimeter).

Pathogens	Inhibition zone in mm (mean ± SD)						Ampicillin
	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6	(50µg/ml)
V. parahaemolyticus	00.00 ± 0.00	00.00 ± 0.00	10.00 ± 0.57	06.00 ± 0.57	08.00 ± 0.57	11.00 ± 0.57	12.00 ± 0.50
V. anguillarum	00.00 ± 0.00	00.00 ± 0.00	10.00 ± 0.16	00.00 ± 0.00	08.00 ± 0.57	11.00 ± 1.52	10.00 ± 0.00
V. fischeri	00.00 ± 0.00	00.00 ± 0.00	10.00 ± 0.57	06.00 ± 0.57	09.00 ± 0.16	10.00 ± 0.57	13.00 ± 0.50
V. vulnificus	08.00 ± 0.57	00.00 ± 0.00	10.00 ± 0.57	07.00 ± 0.00	08.00 ± 0.57	10.00 ± 0.57	12.16 ± 0.28
E. coli	00.00 ± 0.00	00.00 ± 0.00	06.00 ± 0.57	00.00 ± 0.00	00.00 ± 0.40	08.00 ± 0.57	12.16 ± 0.28
B. cereus	06.00 ± 0.57	07.00 ± 0.57	11.00 ± 0.57	07.00 ± 0.57	08.00 ± 0.57	13.00 ± 0.57	10.16 ± 0.28
A. baumannii	00.00 ± 0.00	06.00 ± 0.57	10.00 ± 0.57	00.00 ± 0.00	00.00 ± 0.00	12.00 ± 0.57	10.16 ± 0.28

Values are means of growth inhibition of three replicates, Fr= fraction

Table 11. Antibacterial screening of purified fractions of seagrass *H. ovalis* against pathogens (Inhibition zone was measured to nearest millimeter). Values are means of growth inhibition of three replicates.

Pathogens	Inhibition zone in mm (mean ± SD)						Ampicillin
	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6	(50 µg/ml)
V. parahaemolyticus	10.00 ± 0.57	10.00 ± 0.57	09.00 ± 0.15	10.00 ± 0.57	12.00 ± 0.57	12.00 ± 0.57	12.00 ± 0.57
V. anguillarum	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	08.00 ± 0.57	11.00 ± 1.52	00.00 ± 0.00
V. fischeri	10.00 ± 0.57	09.00 ± 0.57	08.00 ± 0.57	10.00 ± 1.00	11.00 ± 0.66	10.00 ± 0.57	13.00 ± 0.50
V. vulnificus	08.00 ± 0.57	00.00 ± 0.00	06.00 ± 0.57	09.00 ± 0.57	08.00 ± 0.57	08.00 ± 0.57	12.16 ± 0.28
E. coli	00.00 ± 0.00	05.00 ± 0.40	00.00 ± 0.00	00.00 ± 0.00	05.00 ± 0.40	06.00 ± 0.57	12.00 ± 0.50
B. cereus	00.00 ± 0.00	07.00 ± 0.57	05.00 ± 0.57	08.00 ± 0.00	08.00 ± 0.57	06.00 ± 0.57	10.16 ± 0.28
A. baumannii	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	12.00 ± 0.58	10.00 ± 0.57	10.16 ± 0.28

Values are means of growth inhibition of three replicates, Fr= fraction

Table 12. GC–MS analysis of major compounds of *C. glomerata* purified fraction II

Rt (min)	Compound	Area (%)
27.32	Pentadecane, 8-hexyl-	7.02
28.41	Tridecane, 8-hexyl-	4.93
29.33	Heptadecane, 9-hexyl-	7.02
29.66	Triacontane	4.34
30.35	Octacosane	4.38

Table 13. GC–MS analysis of major compounds of *C. glomerata* purified fraction III

Rt (min)	Compound	Area (%)
25.77	Tridecane, 7-hexyl-	3.43
27.77	Heptadecane, 3-methyl-	3.18
28.41	Pentadecane, 8-hexyl-	8.76
29.34	Heptadecane, 9-hexyl-	6.48
30.35	Octacosane	4.07
31.53	Heptadecane, 9-octyl-	3.21

Table 14. GC–MS analysis of major compounds of *C. glomerata* purified fraction V

Rt (min)	Compound	Area (%)
27.32	Pentadecane, 8-hexyl	10.01
27.77	Pentadecane, 2, 6, 10- trimethyl-	3.44
28.41	Tridecane, 7-hexyl-	7.27
29.34	Heptadecane, 9-hexyl-	6.93
30.36	Octacosane	11.66

Table 15. GC–MS analysis of major compounds of *S. wightii* purified fraction III

Rt (min)	Compound	Area (%)
30.57	Eicosane	06.47
30.78	Hexanoic acid, pentadecyl ester	05.57
31.28	Butane, 2, 3-bis (trimethylsiloxy)-	03.29
31.37	1H-Indole, 6-methyl-	05.43
32.57	Estra-1, 3, 5 (10)- trien- 17.beta.-ol	03.52
32.87	9- Octadecenamide	11.40

Table 16. GC–MS analysis of major compounds of *S. wightii* purified fraction VI

Rt (min)	Compound	Area (%)
18.90	1, 2- Benzenedicarboxylic acid, butyl 1 octyl ester	03.54
30.84	26-Nor-5- cholesten-3.beta.-ol-25-one	14.55
31.38	18-Crown-6	05.26
31.83	1, 4, 7, 10, 13, 16- Hexaoxacyclooctadecane	05.98
32.45	1, 4, 7, 10, 13, 16-Hexaoxanonadecane, 18- (2-propenyl)-	10.52
32.58	7- hydroxyl-3-(1,1-dimethylprop-2-enyl) coumarin	05.50
32.88	9- Octadecenamide	07.95
33.27	Methylenebis (2, 4, 6-triisopropylphenylphosphine)	09.47

Table 17. GC–MS analysis of major compounds of *H. ovalis* purified fraction V

Rt (min)	Compound	Area (%)
18.24	Hexadecanoic acid	21.63
21.33	9, 12-Octadecadienoic acid	10.32
21.49	9- Octadecenoic acid	27.01
21.58	10, 13-Octadecadienoic acid	07.56
21.92	Octadecanoic acid	10.42

Table 18. GC–MS analysis of major compounds *H. ovalis* purified fraction VI

Rt (min)	Compound	Area (%)
10.83	Phenol, 2,4-bis (1,1-dimethylethyl)	03.81
11.01	Benzoic acid	11.11
11.99	Hexadecane	03.47
15.69	Tetradecanoic acid	06.12

Rt = Retention time

4.4. Discussion

Natural products are considered as an important source of new antibacterial agents. Many chemically unique compounds of marine origin with different biological activities have been isolated and a number of them are under investigation and or are being developed as new pharmaceuticals (Faulkner, 2000 a,b; Kim *et al*., 2008). In recent years, pharmaceutical firms have started gazing towards marine organisms, including seaweeds, for new drugs from natural products (Smith, 2004). Several species of seaweeds and seagrasses have caused an emerging avenue in the biomedical field due to their great potential as antimicrobial, anti-inflammatory,

antiviral and anti-tumoral drugs (Smith, 2004; Blunden, 1993; Kumar *et al*., 2008; Hua *et al.*, 2006; Premnathan *et al*., 1992).

The antimicrobial susceptibility test is an essential technique in many disciplines of science. It is used in pathology to determine resistance of microbial strains to antimicrobials, and in ethanopharmacology research, to determine the efficacy of novel antimicrobials against microorganisms, essentially those of medical importance. This test is the first step towards new anti-infective drug development. Extraction methods involve separation of active portions of plant tissues from the inactive/inert components by using appropriate solvents. Successful determination of biologically active compounds from plant materials is largely dependent on the type of solvent ranging from low to high polarity used in the extraction procedure. The most commonly used solvents for investigations of antimicrobial activity in plants are methanol, ethanol, and water (Parekh *et al*., 2005; Lourens *et al*., 2004; Rojas *et al*., 2006). However, other solvents such as n-hexane, ethyl acetate, dichloromethane, and acetone were also used (Thillairajasekar *et al*., 2009; Chiao-Wei *et al*., 2011; Demirel *et al*., 2009). In this study, methanol, chloroform, ethyl acetate, and hexane were used in which their polarity indexes (PI) varied from low (n-hexane), intermediate (chloroform, and ethyl acetate), to high (methanol). Among the solvents used for the extraction and investigation of antimicrobial activity, methanol extract of green seaweed *C. glomerata*, brown seaweed *S. wightii*, and seagrass *H. ovalis* exhibited higher antibacterial activity against pathogens tested (Yuvaraj *et al*., 2011; Yuvaraj *et al*., 2012) when compared with the chloroform, ethyl acetate, and hexane extracts. Similar observation was made in methanol extract of green seaweed *Ulva lactuca* (200 µg/ml) which showed high inhibiting activity against *Staphylococcus aureus* (Kandhasamy & Arunachalam, 2008). It was reported that, n-hexane extracts of *Sargassum polycystum*, exhibited higher antibacterial activity than dichloro-methane and methanol extracts (Chiao-Wei *et al*., 2011). In contrast, the present study showed that methanol extract of *S. wightii* exhibited good antibacterial activity against an array of pathogens tested. Similarly, methanol extract of *S. wightii* and *T. ornata* inhibited the growth of human pathogens (Vijayabaskar & Shiyamala, 2011). Previous reports showed that, ethanol and methanol extracts of seagrasses showed better zone of inhibition against bacterial pathogens (Thirumaran *et al*., 2009). The methanol and diethyl methyl formamide extracts of seagrass sp., was found to be active against Gram-positive pathogens (Shelat, 1979). We also observed that

crude methanol extract of seagrass *H. ovalis* exhibited good antibacterial activity against Gram-positive as well as to Gram-negative pathogens. The results of the present study consisted with some preceding studies of seagrass antibacterial activity (Rengasamy *et al*., 2008). Similarly, extracts from *Cymodocea rotundata* Ehrenbers & Hemprich ex Ascherson (Cymodoceaceae) was effective against *Bacillus* species (Bernard & Pesando, 1989). Earlier reports showed that Gram-positive bacteria were controlled more effectively by the extracts of algae used in their study in comparison to Gram- negative bacteria (Taskin *et al*., 2001; Tuney *et al*., 2006). This may be probably due to the hydrophobic lipopolysaccharide in the outer membrane of Gram-negative bacteria, which provides protection against different agents (Caccamese & Azzolina, 1979; Pesando & Caram, 1984; Paz *et al*., 1995; Vlachos *et al*., 1997). In addition to that, the resistance displayed by the pathogens might be due to masking of antibacterial activity by the presence of some inhibitory compounds or factors in the extract (Sastry & Rao, 1994). In the present study, we observed that methanol extracts of *C. glomerata*, *S. wightii*, and *H. ovalis* was active against Gram-negative bacteria. The presence of antibacterial substances could be varied from algal species to species (Lustigman & Brown, 1991). Conflicting reports were observed on the presence of bioactive compounds in the seaweeds and seagrass related to the seasonal variation, as well as the method of extraction and organic solvents used for extraction of bioactive compounds and differences in assay methods (Yuvaraj *et al*., 2011).

Minimum inhibitory concentration (MIC) assay was conducted to assess the bacteriostatic concentration of the seaweeds and seagrass extract against the respective pathogenic bacterial strains. The lower the MIC value, the higher the antibacterial potential of the plant extracts. In the present study, methanol extract of *C. glomerata* inhibted the growth of *B. cereus, E. coli* at a minimum concentration of 75 µg/ml and Gram-negative pathogens of 100 µg/ml except *V. vulnificus* 110 µg/ml. Whereas, methanol extract of *S. wightii* inhibited the growth of *B. cereus* at a minimum concentration of 50 µg/ml and Gram-negative pathogens at 100 µg/ml except *E. coli* 110 µg/ml. However, the crude methanol extract of seagrass *H. ovalis* exhibited bacteriostatic activity against *B. cereus* at 50 µg/ml and Gram-negative pathogens at 75 µg/ml except *V. vulnificus* 100 µg/ml. Similarly, crude methanol extract of *S. wightii,* and *H. ovalis* exhibited promising bacteriostatic agents against *B. cereus* (MIC = 200 µg/ml), *Pseudomonas*

syringae, and *Micrococcus luteus* at MIC of 50 µg/ml (Chiao-Wei *et al*., 2011; Kumar *et al*., 2008; Rengasamy *et al*., 2008).

The crude methanol extracts of *C. glomerata*, *S. wightii*, and *H. ovalis* were purified by thin layer chromatography using hexane:ethyl acetate, and methanol:chloroform as solvent systems. Five different fractions were obtained from *C. glomerata* and six fractions from *S. wightii*, and *H. ovalis*. All the TLC purified fractions were assayed for antibacterial activity against an array of pathogens. In the present study, the second fraction of green seaweed *C. glomerata* showed good antibacterial activity against fish pathogens *Vibrio fischeri* and *V. vulnificus*. Similarly, the third and fifth fractions of *C. glomerata* exhibited good antibacterial activity against fish pathogens *V. fischeri*, *V. vulnificus* and human pathogen *A. baumannii* respectively. Whereas, brown seaweed *S. wightii* purified fractions III and VI showed good antibacterial activity against the pathogens. Similarly, Fractions V and VI of *H. ovalis* extract effectively inhibited *V. parahaemolyticus, V. vulnificus*, and *A. baumanni.* The antibacterial activity of marine algae and mangrove plants were screened against fish pathogens and reported that fractions of methanol extract of red seaweed *Gracilaria corticata* J. Agardh (Gracilariaceae) showed good activity against fish pathogens *Pseudomonas aeruginosa* and *V. alginolyticus* (Sree *et al*., 2005). Similarly, purified fractions of selected South African seaweeds showed broad spectrum activity against Gram-positive as well as Gram-negative pathogens than crude extracts of the same seaweeds (Vlachos *et al*., 1997). In this study, we found that, purified fractions of green seaweed *C. glomerata*, brown seaweed *S. wightii*, and seagrass *H. ovalis* showed broad spectrum activity against human and fish pathogens (Yuvaraj *et al*., 2011, 2012).

To find out the chemical constituents present in the active purified fractions, Gas Chromatogrpahy-mass Spectrometry analysis were carried out. There are numerous reports of compounds derived from macro algae with a broad range of biological activities such as antibiotics (Scheuer, 1990), hydrocarbons (Nor Qhairul Izzreen & Vijaya Ratnam, 2011), and triacylglycerols in model diatoms (Eizadora *et al*., 2009). Our results are in accordance with the reported investigations (Blumer *et al*., 1971; Youngsblood *et al*., 1971). Straight chain paraffins (n-alkanes), branched chain paraffins (alkyl-alkanes) and unsaturated hydrocarbons (alkenes) were already reported from many marine algae (Gelpi *et al*., 1970; Weber & Leaf, 1991). Hydrocarbon distribution pattern mainly in *C. glomerata* is closely similar to prokaryotic *Anacystis montane* and *Btryococcus braunii* belonging to Cyanophycophyta and

Chrysophycophyta respectively. Similar group of hydrocarbons heptadecane and hexadecane have been reported as common major volatile components in many other algae (Tellez *et al*., 2001). Distribution of hydrocarbons among the studied algae showed a very interesting pattern in respect to geographical variations. The presence of methyl and hexyl groups could be a result of alkylation of hydrocarbons with methanol and hexane, which was used in extraction and purification process in the present study. The GC-MS analysis of active fractions of *S. wightii* revealed the presence of major groups of compounds from hydrocarbon, esters, ketones and other miscellaneous compounds. Similar group of compounds were reported in brown seaweeds *Kappaphycus alvarezii* (Doty) and *S. polycystum* C. Agardh (Nor Qhairul Izzreen & Vijaya Ratnam, 2011) and in essential oils of brown algae (Demirel *et al*., 2009), and *Allium atroviolaceum* Boiss (Alliaceae) flowers (Dehpour *et al*., 2011). Similarly, the GC-MS analysis of active fractions of *H. ovalis* in this study revealed the presence of triacylglycerols such as hexadecanoic acid, tetradecanoic acid, 9-octadecenoic acid, octadecanoic acid, and hydrocarbon, hexadecane. Similar group of triacylglycerols were reported in Zosteraceae species *Zostera japonica* Aschers. & Graeben (Hua *et al*., 2006), and *Zostera marina* L. (Sanina *et al*., 2004), respectively. The presence of hydrocarbon, hexadecane in the *H. ovalis* extract corroborated well with earlier reports in brown algae *Cystoseira barbata* (Good et Woodw.) J. Agardh (Cystoseiraceae), and Dictyotaceae species *Dictyopteris membranaceae* (Stackhouse) batters (Ozdemir *et al*., 2006). The present study warrants that hydrocarbons, ketones and triacylglycerols from seaweeds and seagrass showed appreciable antibacterial activity and could be a source of biopharmaceuticals. Nevertheless, it is imperative to elucidate the chemical structure of the active compounds.

CHAPTER 5

CHARACTERIZATION OF STRUCTURAL PROPERTIES OF SULFATED POLYSACCHARIDES EXTRACTED FROM *SARGASSUM WIGHTII* AND *HALOPHILA OVALIS*

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CHARACTERIZATION OF STRUCTURAL PROPERTIES OF SULFATED POLYSACCHARIDES EXTRACTED FROM *SARGASSUM WIGHTII* AND *HALOPHILA OVALIS*

5.1. Introduction

Sulfated polysaccharides are complex group of macromolecules with a wide range of important biological properties and these polymers are prevalent in nature, taking place in a great variety of organisms. During the last decade, numerous bioactive polysaccharides with interesting functional properties have been identified from macroalgae. Seaweed species belonging to chlorophyta, rhodophyta, and phaeophyta have been recognized as crucial sources of sulfated polysaccharides. Green seaweeds are known to produce ulvan like polysaccharides. Whereas, red seaweeds produces agar, carrageenan, and galactan sulfates. Agar is an unbranched polysaccharide built on a disaccharide repeating unit of 3-linked β-D-galactopyranosyl (G) and 4-linked 3,6-anhydro-α-L-galactopyranosyl (LA) residues (Araki, 1966). Brown seaweeds (Phaeophycophyta) are known to be a source of structurally and functionally unique polysaccharides namely alginic acids, laminarans, and fucoidans (Ermakova *et al*., 2011). Alginates constitute a family of linear copolymers of (1 → 4) β-D- mannuronic acid (M) and (1 → 4) α-L-guluronic acid (G) units (Gomez *et al*., 2009). The structure varies according to the monomer position on the chain, forming either homopolymeric (MM or GG) or heteropolymeric (MG or GM) segments (Rioux *et al*., 2007). Laminaran structure varies between algal species and are generally composed of (1, 3)-β-D-glucan (Zvyagintseva *et al*., 1999) with β (1, 6) branching (Nelson & Lewis, 1974). Fucoidans are sulfated complex polysaccharides, extracted often from macroalgae i.e. seaweeds, containing fucose residues in various amounts besides many other monosaccharides (Holtkamp *et al*., 2009). The structure and chemical composition of fucoidans varies depending on habitat, area of cultivation, harvesting period, extraction methods and other parameters (Rioux *et al*., 2009). The high amount of sulfated polysaccharides in marine angiosperms documented for the first time. They are composed of repeating units, which vary in a species-specific manner (Aquino *et al*., 2005;

Mourao, 2004; Mourao, 2007). The chemical structure of seaweed polysaccharides have been depicted extensively (Percival, 1979; Rinaudo, 2007). However, only few reports with respect to the chemical characterization of seagrass polysaccharides are existing. To date, the occurrence of sulfated polysaccharides in three seagrass species has been documented (Silva *et al*., 2012). In recent years, the search for similar biological activities from seagrass polysaccharides has received considerable attention. Previous reports showed that the sulfate content, molecular weight, monosaccharide composition, and the structure of the main polymer chain of sulfated polysaccharides had great influence on their biological activities (Vishchuk *et al*., 2012). Hence, the aim of the present study was to delineate the chemical motifs such as, molecular weight, sulfate content, and monosaccharide composition of sulfated polysaccharides extracted from brown seaweed *S. wightii* and seagrass *H. ovalis*, which might reflect its biological activities.

5.2. Materials and methods

5.2.1. Extraction of polysaccharides from brown seaweed *Sargassum wightii*

Polysaccharide was extracted according to the method of Foley *et al.,* (2011), with slight modifications. Briefly, the milled algal biomass (100 g) was treated with EtOH (80% v/v) at room temperature for 12 h and sub sequentially at 70°C for 12 h to extract mannitol and some salts (S1 and S2) to leave a depigmented algal powder (DAP, 85 g). Extraction of depigmented algal powder (10 g) with Milli–Q H_2O (w/v: 1:10) at room temperature for 7 h, at 70°C for 7 h, and at 70°C for 4 h under constant stirring for three times (S3, S4, and S5). All the three fractions were pooled and treated with 2 M $CaCl_2$ at room temperature for 5 h in order to precipitate alginates. The precipitated alginates were then removed by centrifugation at 10,000 rpm for 30 min followed by filtration through glass filter (G-3), obtained then polysaccharide in the supernatant. The residue was briefly washed with additional Milli–Q H_2O and the wash was collected to maximize the polysaccharide recovery. Dialysis was carried out (MWCO 1000 Da, Spectrum, USA) at 4°C extensively against deionized water over a 48 h period to decrease salinity, and Milli–Q H_2O was changed every 12 h and lyophilized. The recovered material was re-dissolved in water then precipitated with ethanol (4 vols.) overnight. This

process was repeated twice for complete recovery of polysaccharide. The final pellet was dissolved in Milli–Q H_2O and lyophilized to yield the water-extracted polysaccharide (SwWE, 1.11 ± 0.1 g).

5.2.2. Extraction of polysaccharides from seagrass *Halophila ovalis*

The seagrass *H. ovalis* was collected in low tide from Chunnambar estuary (Pondicherry), India, during September 2011. The samples were rinsed with seawater and then successively with tap water and distilled water to confiscate the epiphytes and other wastes. Finally, the sample material was shade dried to remove the moisture. The dried material (20 g) was ground to fine powder, suspended in 400 ml 0.1 M sodium acetate (pH 6.0), containing 2 g papain, 5 mM ethylenediamine tetra-acetic acid (EDTA), and 5 mM cysteine. After 24 h incubation at 60°C, separation of the residue from the extract was performed by centrifugation at 8000 rpm for 10 min followed by filtration through glass filter (G-3), obtained then polysaccharide in the supernatant. The sulfated polysaccharides in the solution were precipitated with 800 ml absolute ethanol. After 24 h, the precipitate formed was collected by centrifugation (2560 × *g* for 20 min at 4°C). The final precipitate was dried at 60°C for 12 h. Approximately 1.3 g (dry weight) of crude polysaccharide was obtained after these procedures.

5.2.3. FT-IR analysis

The presence of different functional groups in brown seaweed *S. wightii* and seagrass *H. ovalis* sulfated polysaccharides was determined by FT-IR spectral analysis as described in general materials and methods section **3.2.3**.

5.2.4. Monosaccharide composition analysis

The presence of individual monosaccharide in seaweed *S. wightii* and seagrass *H. ovalis* was determined by liquid chromatography-mass spectrometry analysis. The LC parameters were given in detail in general materials and methods section **3.2.3.1**.

5.2.5. Purification of biopolymer by anion exchange chromatography

Purification process is based on successive solubilizations and precipitations of the biopolymer in aqueous medium. Water and enzyme extracted polysaccharide was applied to a DEAE–cellulose column (20 × 2 cm) equilibrated with 50 mM sodium chloride solution. Thereafter, the column was then eluted successively with 50 mM (fraction I), 0.2 M (fraction II), 0.6 M (fraction III), and 2 M (fraction IV) sodium chloride in a stepwise manner. The flow rate of the column was 0.2 ml/min and the fractions were collected through an auto collector. The collected fractions were dialyzed extensively against deionized water for 48 h and then lyophilized. The lyophilized samples were stored at 4°C for further characterization.

5.2.6. Total sugars, sulfate and protein estimation

Total sugar, sulfate and protein contents of crude and purified fractions of *S. wightii* and *H. ovalis* sulfated polysaccharides were estimated as described in general materials and methods section **3.2.3.2** to **3.2.3.4**.

5.2.7. Molecular weight determination

Crude sulfated polysaccharide and purified fractions (50 mg) of *S. wightii* and *H. ovalis* was chromatographed on a sephadex G-75 column (20 × 2 cm) using 0.6 M sodium chloride solution as eluant. The flow rate of the column was 1 ml/min. Different molecular weight of dextrans 10, 15, and 20 KDa (Himedia, Mumbai) was used as standards. The molecular weight was calculated according to the calibration curve of standard dextrans as described in general materials and methods section **3.2.3.5**.

5.3. Results

5.3.1. Extraction of sulfated polysaccharides from brown seaweed *Sargassum wightii* and seagrass *Halophila ovalis*

The sulfated polysaccharide was extracted from marine brown alga *S. wightii* using hot water method (Fig. 1).

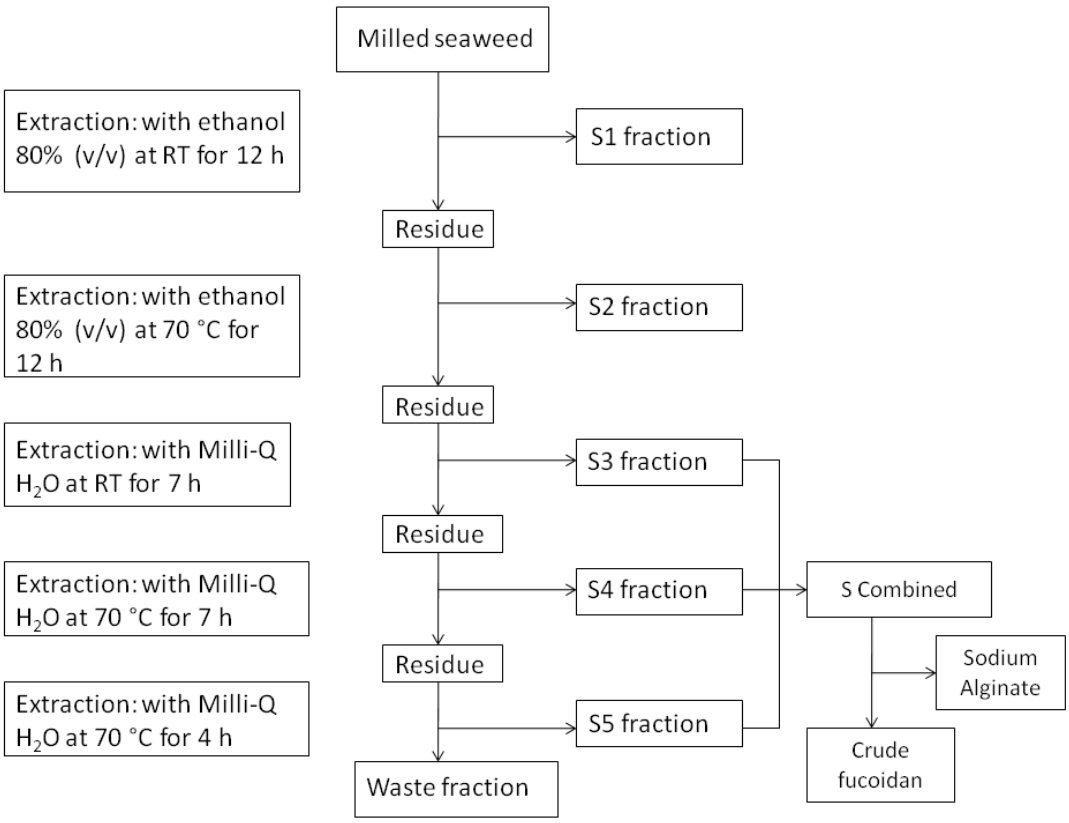


Fig. 1. Scheme for the isolation of fucose containing sulfated polysaccharide from brown seaweed *S. wightii.*

The enzymatic extraction method was employed for the extraction of sulfated polysaccharides from seagrass *H. ovalis* (Fig. 2).

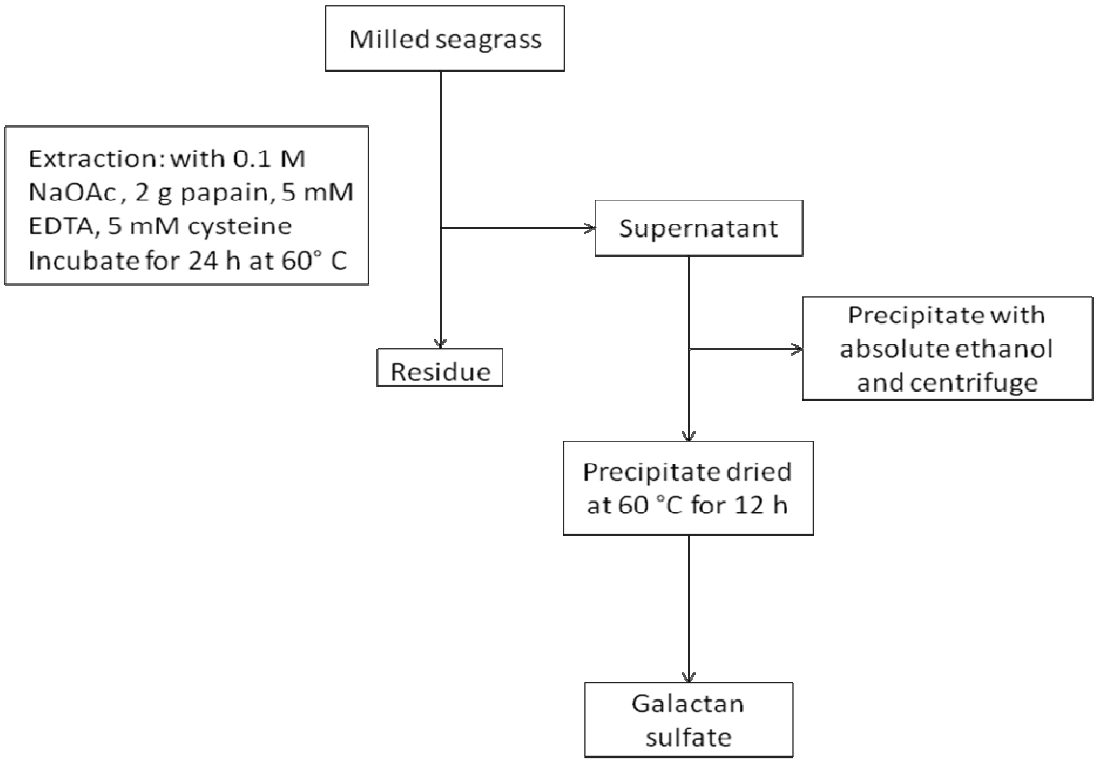


Fig. 2. Scheme for the isolation of galactan sulfate from the seagrass *H. ovalis.*

5.3.2. FT-IR spectra analysis

The FT-IR spectrum of *S. wightii* crude sulfated polysaccharide was depicted in Fig. 3. The broad stretching intense characteristic peak at 3400 cm^{-1} indicated the presence of hydroxyl group and C–H stretching at 2928 cm^{-1} respectively. A strong signal at 1620 cm^{-1} was attributed to the asymmetric stretch vibrations of COO^- of uronic acid and at 1430 cm^{-1} was due to the symmetric stretch vibrations of COO^- and the stretch vibration of C–O within –COOH. The absorption at 1251 cm^{-1} common to all sulfate esters and an additional sulfate absorption band at 821 cm^{-1} (C–O–S, secondary equatorial sulfate) indicated that majority of sulfate groups occupy positions C–2 and/or C–3 of fucopyranose residues as in other sulfated fucans.

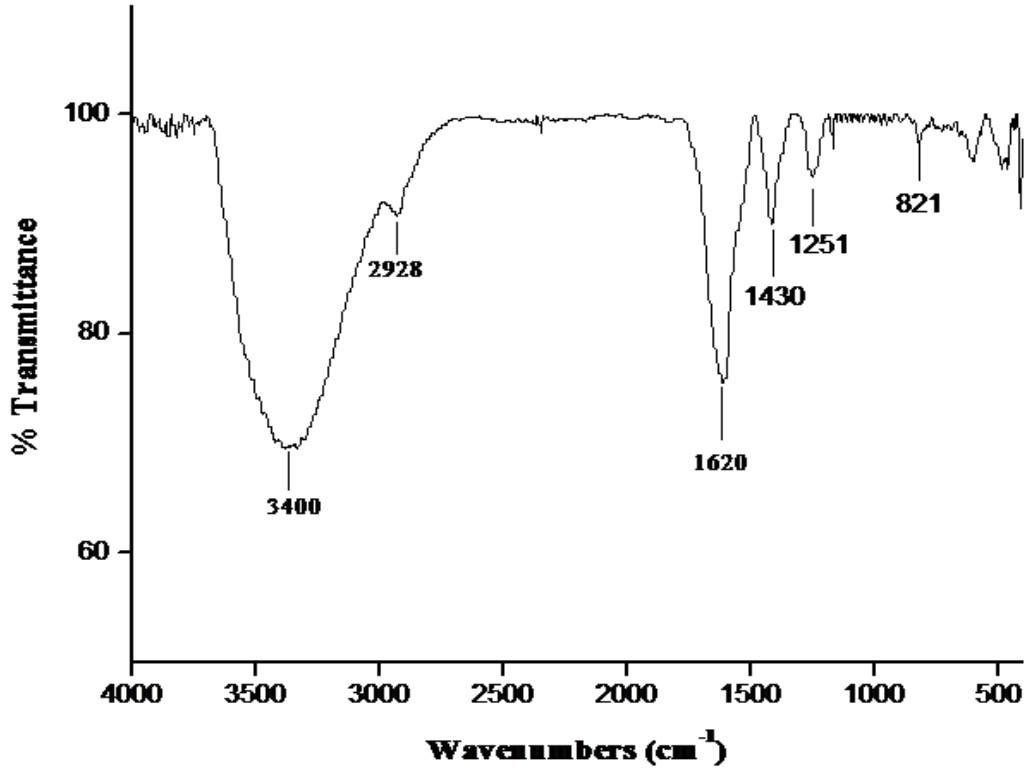


Fig. 3. FT-IR spectrum of crude sulfated polysaccharide extracted from the brown seaweed *S. wightii*.

The FT-IR spectrum of *H. ovalis* galactan sulfate was depicted in Fig. 4. The broad stretching intense characteristic peak at 3420 cm^{-1} indicated the presence of hydroxyl group and C–H stretching at 2920 cm^{-1} respectively. The presence of O–acetyl group was indicated by vibration at 1720 cm^{-1}. A strong signal at 1620 cm^{-1} was attributed to the asymmetric stretch vibrations of COO^- group and at 1420 cm^{-1} was due to the symmetric stretch vibrations of COO^- and the stretch vibration of C–O within –COOH. The absorption at 1254 cm^{-1} common to all sulfate esters and an additional sulfate absorption band at 823 cm^{-1} (C–O–S, secondary equatorial sulfate) indicated that majority of sulfate groups are located at positions 2 and/or 3. Signals at 1050 cm^{-1} correspond to stretching vibrations of C–O respectively.

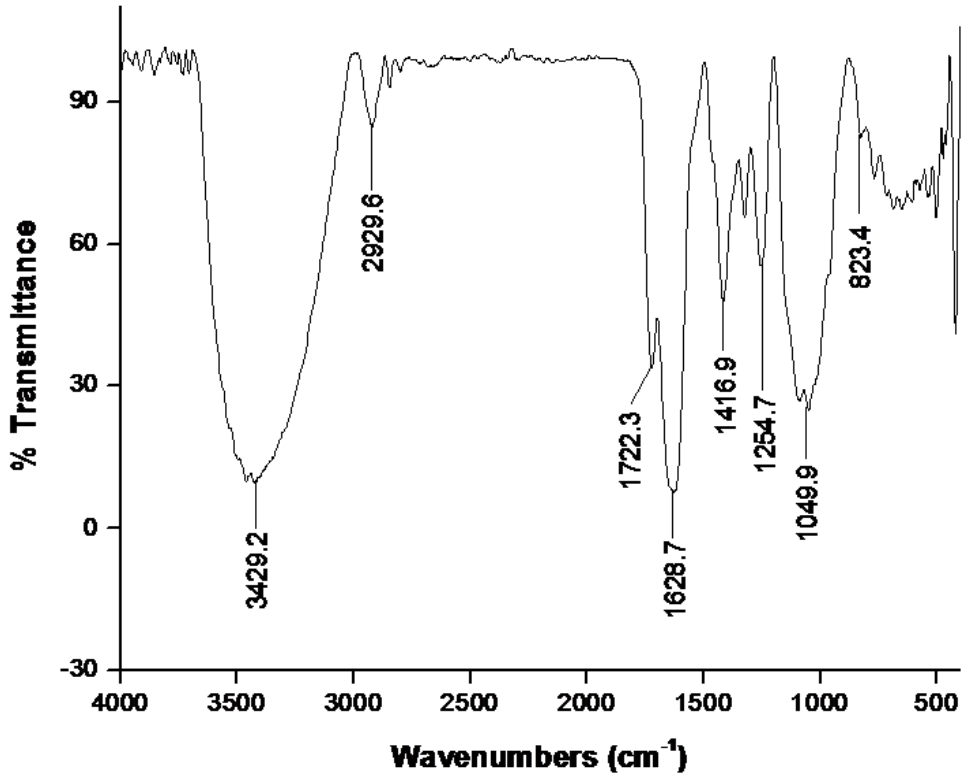


Fig. 4. FT-IR spectrum of sulfated polysaccharide extracted from seagrass *H. ovalis*.

5.3.3. Monosaccharide composition analysis

The monosaccharide composition of crude sulfated polysaccharides obtained from *S. wightii* and *H. ovalis* was analyzed by LC-MS using different standards after hydrolysis and neutralized with NaOH (**Table 19-25**). Sugar compositional analysis revealed the presence of high amount of fucose (66.13%), followed by xylose (7.67%), arabinose (7.67%), mannose (6.87%), fructose (5.95%), galactose (2.91%), glucose (2.77%). Whereas, monosaccharide composition of *H. ovalis* revealed the presence of high galactose content (82.43%) followed by xylose (7.61%), fructose (4.06%), mannose (2.03%), fucose (1.62%), glucose (1.21%), and arabinose (1.01%).

Table 19. LC-MS analysis for Galactose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Galactose1.0 ppm	Standard	126659	1.00	2.32	0.815	81.5
2	Galactose2.5 ppm	Standard	288311	2.50	2.32	2.86	114
3	Galactose5.0 ppm	Standard	500682	5.0	2.32	5.54	111
4	Galactose7.5 ppm	Standard	671168	7.5	2.33	7.69	103
5	Galactose 10 ppm	Standard	783136	10.0	2.33	9.10	91.0
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	6957839	N/A	2.32	81.21	N/A
8	*S. wightii* (SW)	Unknown	237077	N/A	2.32	2.21	N/A

Table 20. LC-MS analysis for Fucose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Fucose 1.0 ppm	Standard	123696	1.00	2.40	0.839	83.9
2	Fucose 2.5 ppm	Standard	274189	2.50	2.40	2.79	112
3	Fucose 5.0 ppm	Standard	489318	5.0	2.41	5.58	112
4	Fucose 7.5 ppm	Standard	634349	7.5	2.43	7.47	99.6
5	Fucose 10 ppm	Standard	776992	10.0	2.43	9.32	93.2
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	181443	N/A	2.44	1.63	N/A
8	*S. wightii* (SW)	Unknown	3956653	N/A	2.42	50.6	N/A

Table 21. LC-MS analysis for Xylose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Xylose 1.0 ppm	Standard	224984	1.00	2.34	0.827	82.7
2	Xylose 2.5 ppm	Standard	488582	2.50	2.34	2.84	114
3	Xylose 5.0 ppm	Standard	837604	5.0	2.34	5.51	110
4	Xylose 7.5 ppm	Standard	1107789	7.5	2.36	7.57	101
5	Xylose 10 ppm	Standard	1326938	10.0	2.36	9.25	92.5
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	1105788	N/A	2.40	7.50	N/A
8	*S. wightii* (SW)	Unknown	885255	N/A	2.36	5.87	N/A

Table 22. LC-MS analysis for Mannose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Mannose 1.0 ppm	Standard	172460	1.00	2.29	0.900	90.0
2	Mannose 2.5 ppm	Standard	365802	2.50	2.29	2.77	111
3	Mannose 5.0 ppm	Standard	616563	5.0	2.29	5.20	104
4	Mannose 7.5 ppm	Standard	814236	7.5	2.30	7.12	94.9
5	Mannose 10 ppm	Standard	974645	10.0	2.30	8.68	86.8
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	287355	N/A	2.28	2.01	N/A
8	*S. wightii* (SW)	Unknown	617803	N/A	2.27	5.22	N/A

Table 23. LC-MS analysis for Glucose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Glucose 1.0 ppm	Standard	1494420	1.00	2.31	0.779	77.9
2	Glucose 2.5 ppm	Standard	3453311	2.50	2.31	2.96	118
3	Glucose 5.0 ppm	Standard	5853189	5.0	2.31	5.62	112
4	Glucose 7.5 ppm	Standard	7541364	7.5	2.32	7.50	100
5	Glucose 10 ppm	Standard	9017722	10.0	2.32	9.14	91.4
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	1878778	N/A	2.32	1.21	N/A
8	*S. wightii* (SW)	Unknown	2747369	N/A	2.31	2.17	N/A

Table 24. LC-MS analysis for Arabinose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Arabinose1.0 ppm	Standard	127142	1.00	2.39	0.818	81.7
2	Arabinose2.5 ppm	Standard	286803	2.50	2.39	2.91	117
3	Arabinose5.0 ppm	Standard	479658	5.0	2.39	5.44	109
4	Arabinose7.5 ppm	Standard	624803	7.5	2.40	7.35	98
5	Arabinose 10 ppm	Standard	786787	10.0	2.41	9.47	94.7
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	166270	N/A	2.43	1.10	N/A
8	*S. wightii* (SW)	Unknown	508197	N/A	2.40	5.82	N/A

Table 25. LC-MS analysis for Fructose

S. No	Sample Name	Sample Type	Analyte Peak Area (Counts)	Analyte Concentration (ng/ml)	Analyte Retention Time (min)	Calculated Concentration (ng/ml)	Accuracy (%)
1	Fructose 1.0 ppm	Standard	18958	1.00	2.36	0.796	79.6
2	Fructose 2.5 ppm	Standard	45095	2.50	2.35	2.88	115
3	Fructose 5.0 ppm	Standard	80814	5.0	2.35	5.72	114
4	Fructose 7.5 ppm	Standard	101984	7.5	2.36	7.41	98.8
5	Fructose 10 ppm	Standard	124283	10.0	2.36	9.19	91.9
6	BLANK	Unknown	0	N/A	0.00	No Peak	N/A
7	*H. ovalis* (HO)	Unknown	60128	N/A	2.40	4.08	N/A
8	*S. wightii* (SW)	Unknown	66144	N/A	2.35	4.56	N/A

5.3.4. Purification of sulfated polysaccharides by anion exchange chromatography

The crude extract was initially purified by anion exchange chromatography (DEAE–cellulose) separated the crude polysaccharide into four fractions (Sw FrI, Sw FrII, Sw FrIII, and Sw FrIV) eluted with increasing concentration of sodium chloride (Fig. 5). Similarly, crude sulfated polysaccharide of seagrass *H. ovalis* was separated into four fractions (Ho FrI, Ho Fr II, Ho FrIII, and Ho Fr IV) using step wise gradient method (Fig. 6).

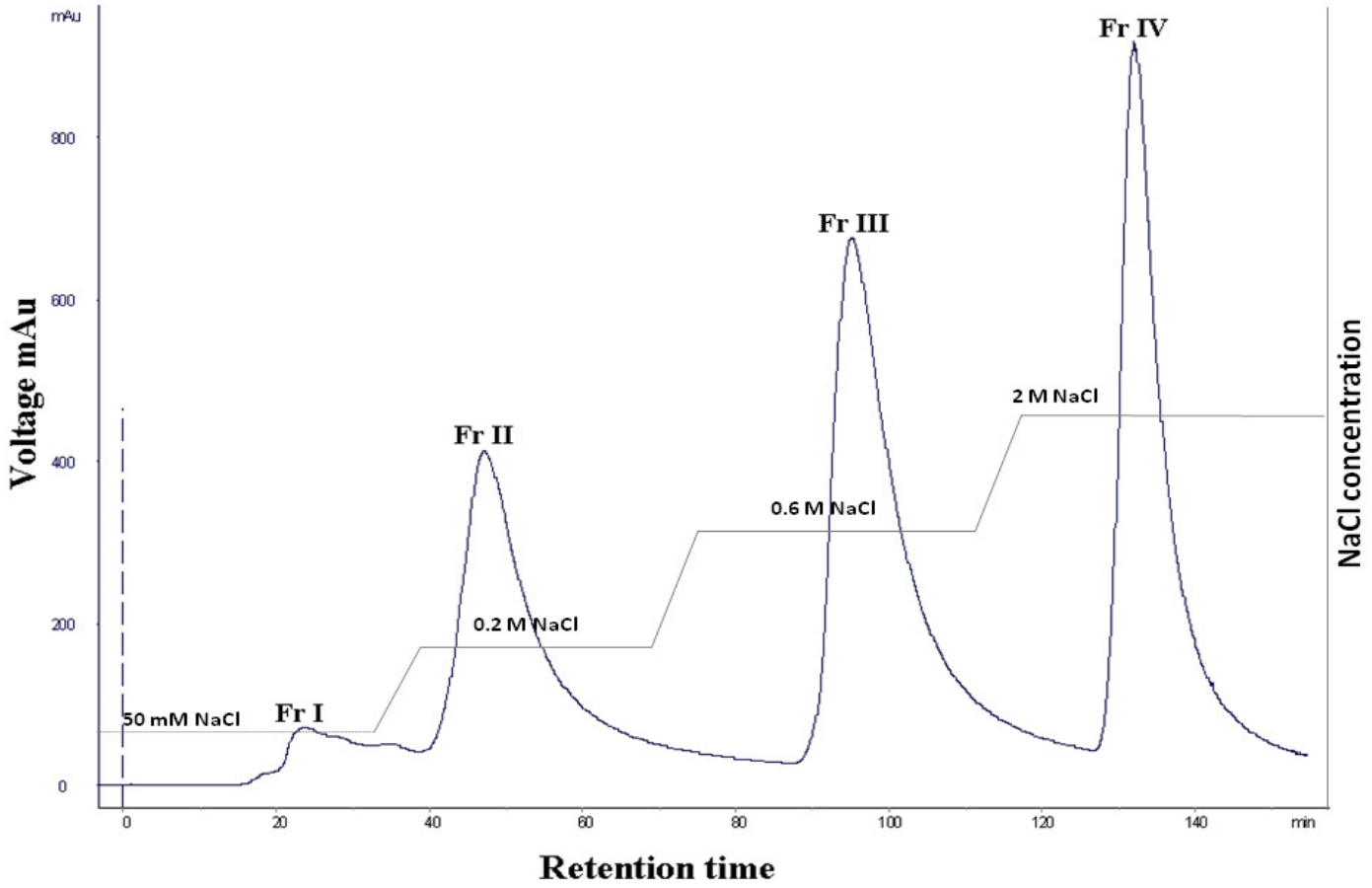


Fig. 5. Anion exchange chromatography of *S. wightii* biopolymer on DEAE–cellulose column. The column was equilibrated with 50 mM NaCl solution. The fractions were eluted with sodium chloride solution at different concentrations (50 mM, 0.2 M, 0.6 M and 2M).

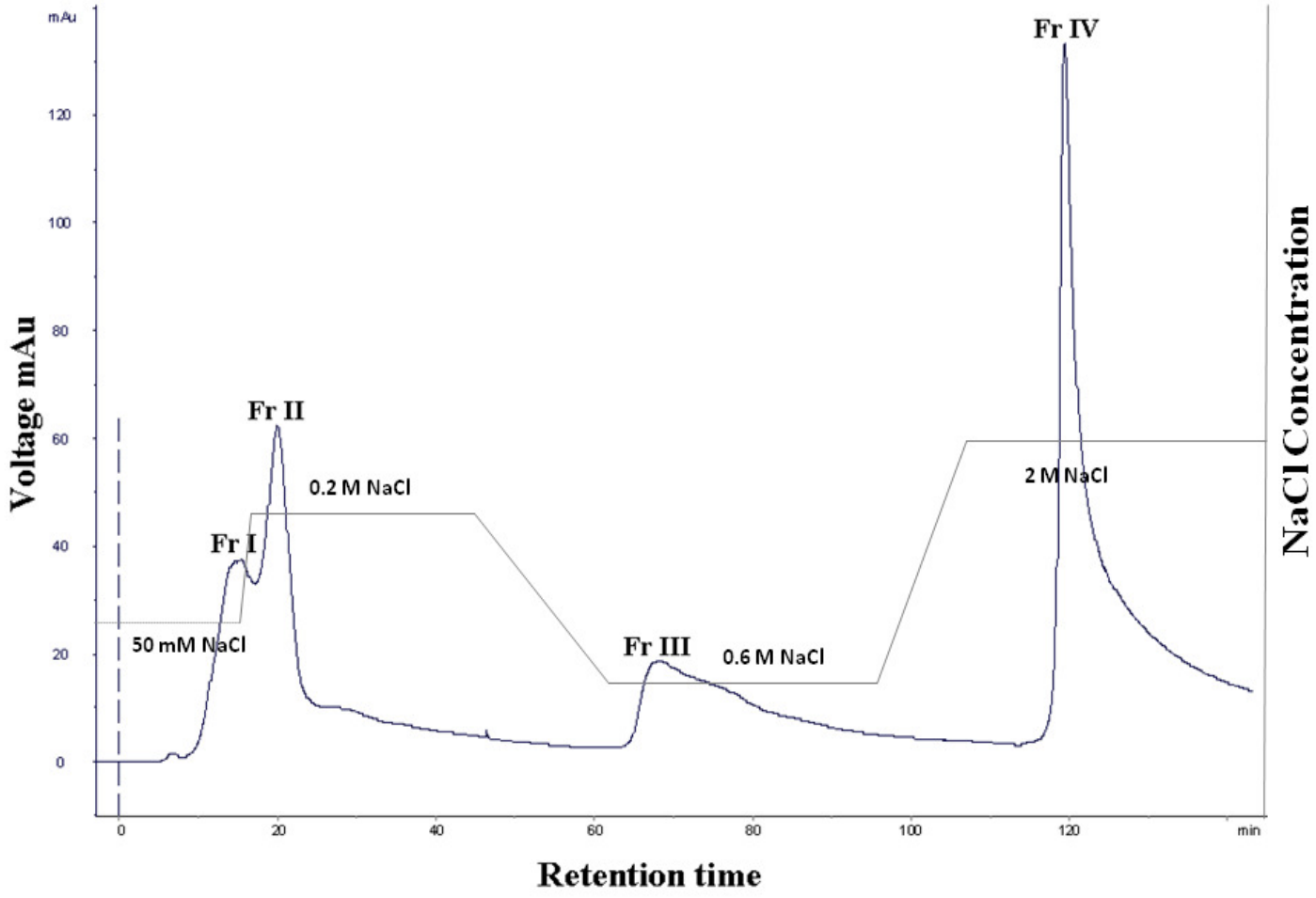


Fig. 6. Anion exchange chromatography of *H. ovalis* sulfated polysaccharide on DEAE–cellulose column. The column was equilibrated and washed with 50 mM NaCl solution. The fractions were eluted with NaCl solution at different concentrations (50 mM, 0.2 M, 0.6 M and 2M).

5.3.5. Chemical composition analysis.

The chemical motifs such as total sugar, sulfate and protein contents of crude and purified fractions was depicted in Table 26.

Table 26.

Sample	NaCl (M)	Total sugars[a] (%)	Sulfate[a] (%)	Protein[a] (%)
S. wightii				
Crude	-	76.2	19.0	3.4
Sw FrI	0.05	71.4	16.8	2.9
Sw FrII	0.2	70.5	19.4	2.3
Sw FrIII	0.6	69.3	19.8	4.6
Sw FrIV	2.0	74.5	21.2	4.0
H. ovalis				
Crude	-	74.9	21.0	3.4
Ho FrI	0.05	68.3	10.9	5.7
Ho FrII	0.2	68.7	12.4	4.7
Ho FrIII	0.6	71.6	18.7	2.9
Ho FrIV	2.0	75.2	21.3	2.0

[a] weight % of fraction dry weight

The results showed that, purified fraction Sw FrIV contained relatively high amount of total sugars (74.5%) and sulfate content (21.2%) based on dry weight when compared with other fractions. Similarly, the purified fraction Ho FrIV contained 75.2% of neutral sugar and 21.3% sulfate on the basis of dry weight followed by 71.6% neutral sugar, 18.7% sulfate (Ho FrIII), 68.7% neutral sugar, 12.4% sulfate (Ho FrII), and 68.3% neutral sugar, 10.9% sulfate (Ho FrI). Additionally, all the fractions showed protein contamination ranging from 2.0 to 5.7%.

5.3.6. Molecular weight determination

The average molecular weight of crude and purified fractions of sulfated polysaccharides extracted from brown seaweed *S. wightii* and seagrass *H. ovalis* was determined by size exclusion chromatography using different molecular weight standard dextrans (**Fig. 7-14**).

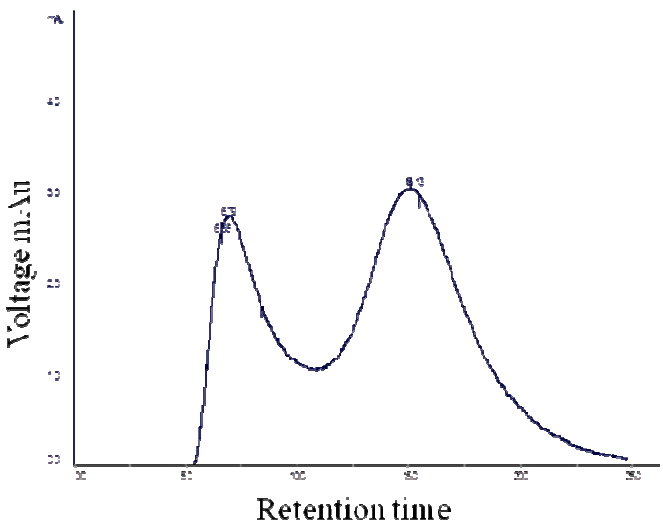


Fig. 7. Standard dextran 15-20 KDa

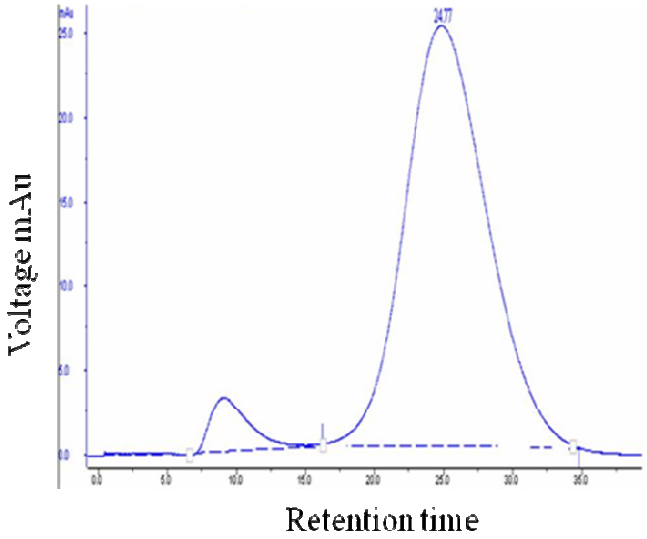


Fig. 8. Standard dextran 10 KDa

Fig. 7 & 8. Molecular mass determination of standard dextrans on sephadex G-75 column using 0.6 M NaCl solution as eluant.

The results suggested that the average molecular weight of crude sulfated polysaccharides of *S. wightii* and *H. ovalis* was found to be 15 KDa and 20 KDa (**Fig. 9, 12**). Whereas, the purified fraction III of *S. wightii* and *H. ovalis* was more than 10 KDa (**Fig. 10, 13**) and purified fraction IV of *S. wightii* and *H. ovalis* was found to be 10 KDa in size (**Fig. 11, 14**).

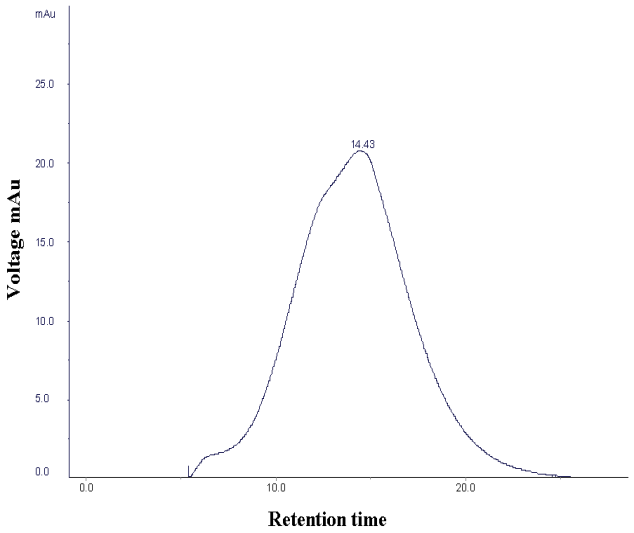


Fig. 9. *S. wightii* crude polysaccharide

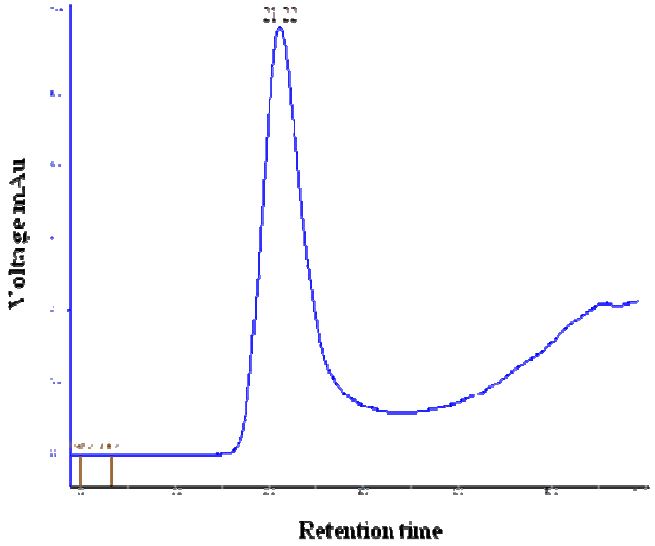


Fig.10. *S. wightii* purified fraction III

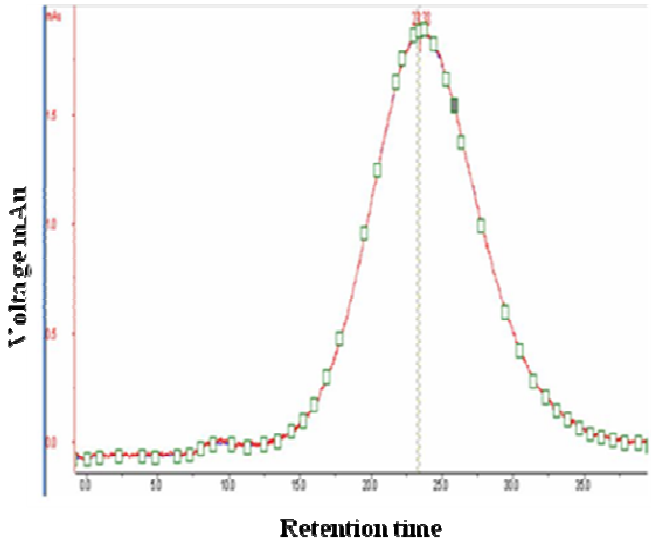


Fig. 11. *S. wightii* purified fraction IV

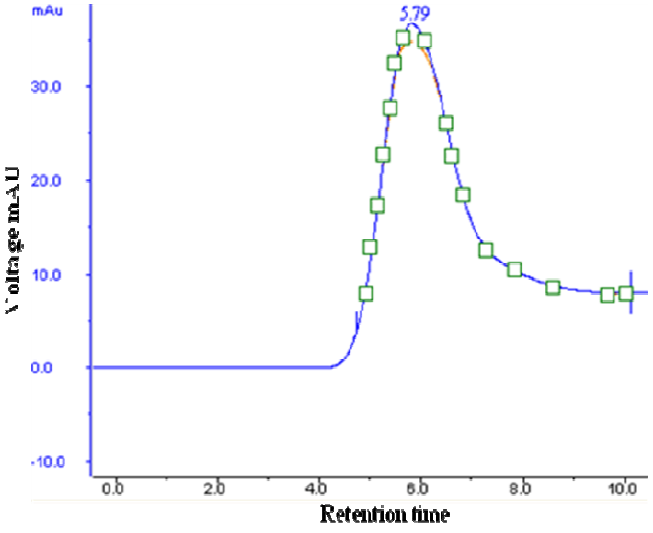


Fig. 12. *H. ovalis* crude Polysaccharide

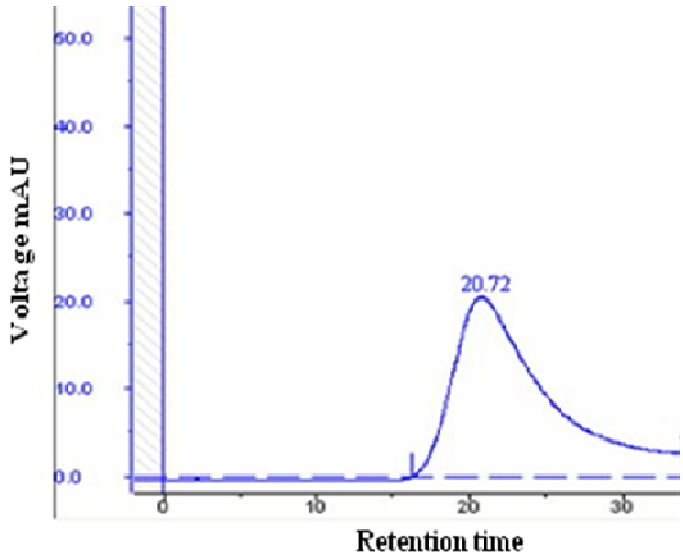


Fig. 13. *H. ovalis* purified fraction III

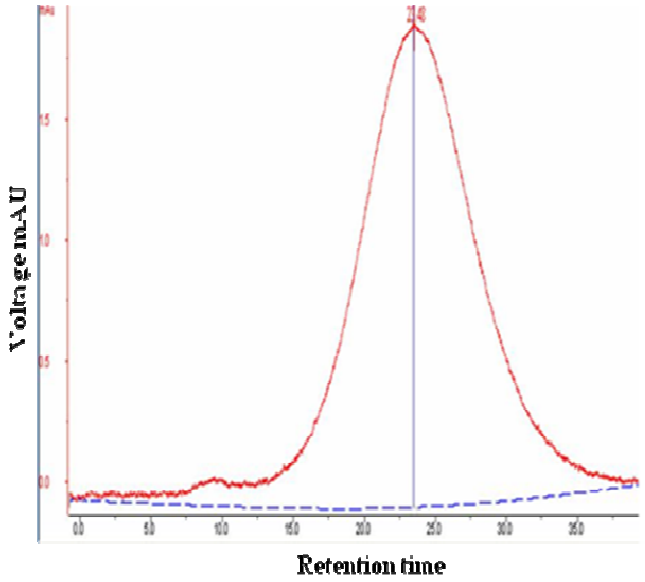


Fig. 14. *H. ovalis* purified fraction IV

5.4. Discussion

Marine natural products became more sophisticated nowadays as it contains wide biological active components with different structures and interesting functional properties. Of such active components, polysaccharides of seaweeds expanded the global industries. Over the last decade, seaweed polysaccharides are used in food, pharmaceutical and other products for human consumption. Recently, several fucoidan structures have been isolated and their biological activities have been elucidated (Li *et al*., 2008). Earlier reports showed that sulfated polysaccharides from brown seaweeds can be extracted by acid, alkali, and hot water treatment methods. The acid hydrolysis step might itself have contributed a substantial amount of sulfate. Consequently, the use of H_2SO_4 clearly biased the interpretation of the compositional analysis. If HCl used for extraction, uroic acid will come along with fucoidan due to alginate contamination. Classical extraction steps involve long, repetitive, multi-step acid and alkaline treatments, which may be detrimental to its structural makeup, yield, and compositional attributes and thus may influence its bioactive properties. Typical extraction of sulfated polysaccharides from brown seaweeds involves a harsh processing condition and several

purification steps. The preservation of the structural integrity of the sulfated polysaccharides essentially depends on the extraction methodology, which has a crucial, but partly overlooked, significance for obtaining the relevant structural features required for specific biological activities. Minimal extraction step of sulfated polysaccharides will preserve the polysaccharides structural integrity, thus increase the yield, and improve its biological properties. In this study, we extracted the sulfated polysaccharides from brown seaweed *S. wightii* by hot water extraction method as described by Foley *et al*., (2011). Whereas, the sulfated polysaccharide from seagrass *H. ovalis* was extracted by enzyme digestion method. Recently, various methods were employed to extract polysaccharides from natural materials such as supercritical CO_2 extraction, ultrasonic-aid extraction, microwave assisted extraction and membrane separation technology to harvest sulfated polysaccharides from the seaweeds. Rodriguez-Jasso *et al*., (2011) employed microwave-assisted extraction methodology to extract fucoidan from brown seaweed *F. vesiculosus*. Precipitation is a universal technique for isolation of polysaccharides from crude extract (Yang *et al*., 2008). Earlier studies showed that, ethanol at different concentrations were in use to precipitate the polysaccharides (McCleary, 2007; Zhao *et al.,* 2008; Xia *et al.,* 2010). However, in the present study, absolute ethanol was used to precipitate sulfated polysaccharides from *S. wightii* and *H. ovalis*. FTIR spectroscopy was employed to determine the presence of different functional groups, glucosidic bonds, and monosaccharide types of polysaccharides by investigating the vibrations of molecules and polar bonds between the different atoms (Mathlouthi & Koenig, 1986; Zhang, 1994). The FT-IR spectrum showed that, the major absorption bands were observed at 3429 cm^{-1} (O–H stretching), 1049 cm^{-1} (hemiacetal stretching), 1254 cm^{-1} (S=O asymmetric stretching), and band at 823^{-1} could be assigned to the sulfate group in the axial position of the C-6 of galactose (Silva *et al*., 2012; Mazumder *et al*., 2002). In addition to that, the presence of O–acetyl group was indicated by vibration at 1720 cm^{-1} which is a characteristic feature of sulfated fucans (Chizhov *et al*., 1999). A strong signal at 1620 cm^{-1} was attributed to the asymmetric stretch vibrations of COO^- group and at 1420 cm^{-1} was due to the symmetric stretch vibrations of COO^- and the stretch vibration of C–O within –COOH. The present result showed that algae and marine angiosperms have a tendency to present sulfate groups in the C-2 or C-6 (Pomin, 2010). The IR spectra of fucoidans showed that most sulfate groups were in axial positions, showed a strong band at 842 cm^{-1} and the remainder were in equatorial

positions, showed a shoulder band at 820 cm^{-1} in the spectra (Zvyangintseva *et al.,* 1999). In the present investigation, the sulfate groups were present in equatorial position, showed a shoulder peak at 821 cm^{-1} along with sulfate esters 1250 cm^{-1} in brown seaweed *S. wightii*. In seagrass *H. ovalis*, we are reporting the presence of fucose for the first time as it was evidenced by the presence of O-acetyl group vibration at 1720 cm^{-1}, which is a unique characteristic feature of sulfated fucans (Chizhov *et al*., 1999). A more plausible explanation for the presence of fucose in the sulfated galactans is that, evolutionary distant organisms that share the marine environment is a convergent adaptation due to environmental selective pressure (Aquino *et al*., 2005). Several reports have shown that monosaccharide composition, chemical components, and molecular weight of biopolymer are the important factors for its biological properties (Zhou *et al*., 2004). The monosaccharide composition analysis by LC-MS revealed that fucose was found to be major sugar along with glucose, fructose, galactose, xylose, and mannose in brown seaweed *S. wightii*. Our results were highly encouraged by (Zhang *et al.,* 2011) who reported the similar kind of neutral sugars from brown alga *S. tenerrimum*. Similarly, galactose was found to be major sugar followed by xylose, fructose, mannose, fucose, glucose, and arabinose in seagrass sulfated polysaccharides. In *Posidonia australis* Hook.f. (Posidoniaceae), glucose, galactose and mannose was found to be the major sugar components (Torbatinejad *et al*., 2007). Whereas, *Halodule wrightii* contains glucose, galactose, and xylose as major sugars (Silva *et al*., 2012). In the present study, galactose, xylose, fructose, and mannose were found to be the major sugar components in *H. ovalis*. The presence of individual monosaccharide varies in their composition according to the species. In order to correlate the structural properties and its biological activities of sulfated polysaccharides, the hot water and enzyme extracted crude polysaccharides of *S. wightii* and *H. ovalis* were purified by anion exchange chromatography as described by (Adhikari *et al.,* 2006) obtained then four different fractions in the increasing concentration of eluant. Considering total sugars, sulfate and protein content, total percentage varies from 91.1 to 99.7% (Sw FrI – Sw FrIV), and 88.2 to 99.9% (Ho FrI – Ho FrIV) respectively. The sum of the three components found in the purified fractions does not approach 100%. This may be because these polymers are very hygroscopic, absorbing water from the atmosphere very rapidly after lyophilization. Furthermore, because of the negative loads of sulfate clusters and glucuronic acids, metals are not eliminated even after dialysis. In addition to that, these polymers exhibit in aqueous solutions, which may capture

cations within their structures (Costa *et al.,* 2011). The average molecular weight of crude and purified fractions of sulfated polysaccharides was determined using standard dextrans. We found that, the average molecular weight of sulfated polysaccharide extracted from brown seaweed *S. wightii* was determined to be 15×10^3 Dalton. Whereas, the purified fraction Sw FrIII was found to be $> 10 \times 10^3$ Dalton and Sw FrIV was 10×10^3 Dalton. Similar kind of low molecular weight polysaccharides were reported from brown seaweeds *Sargasssum fulvellum* (de Zoysa *et al.,* 2008). Notably, the molecular weight of biopolymer of the present study was lower than the other brown seaweed *Sargassum tenerrimum* (Sinha *et al.,* 2010). However, the low molecular weight biopolymer of *S. wightii* well reflected the already well-known low molecular weight polymers reported in Sargassacea family. Similarly, average molecular mass of *H. ovalis* polysaccharide was determined to be 20×10^3 Dalton, and its fractions Ho FrIII (> 10 KDa), Ho FrIV (10 KDa) in size. Similarly, a 11 KDa sulfated polysaccharide rich fraction was obtained from seagrass *Halodule wrightii* Asch. Cymodoceaceae (Silva *et al*., 2012).

In the present study, we have extracted and purified the heterofucan from brown seaweed *S. wightii* and galactan sulfate from seagrass *H. ovalis* and characterized its structural properties such as functional groups, monosaccharide composition, molecular weight, and chemical composition such as total sugars, sulfate and protein content. The functional properties such anti-inflammatory, antioxidant, and immunomodulatory activity of low molecular weight heterofucan and galactan sulfate were investigated *in vitro* and *in vivo* in forthcoming chapters.

CHAPTER 6

STUDIES ON ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF *SARGASSUM WIGHTII* AND *HALOPHILA OVALIS* SULFATED POLYSACCHARIDES *IN VIVO* AND *IN VITRO*

CHAPTER 6
STUDIES ON ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF *SARGASSUM WIGHTII* AND *HALOPHILA OVALIS* SULFATED POLYSACCHARIDES *IN VIVO* AND *IN VITRO*

6.1. Introduction

Inflammation is a part of the normal immune response to tissue injury, infection, and stress, which leads to increased blood supply, enhanced vascular permeability and migration of immune cells to damaged sites (Yoon *et al*., 2009). In this process, nitric oxide, prostaglandin E_2 (PGE_2), and cytokines such as Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α secreted in higher amounts play important roles in pro-inflammatory responses (Hwang *et al*., 2011). These substances activate macrophages in rheumatoid arthritis in addition to cell and tissue damage (Kasama *et al*., 2005; Cheon *et al*., 2006). During inflammation, macrophages play a central role in managing many different immunopathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators (Yoon *et al*., 2009). Although activated macrophages produce a variety of inflammatory cytokines, it has been reported that IL-8 production is presumed to be a sensitive marker of macrophage activation and useful for the evaluation of biological response modifiers in various diseases. IL-8, a C–X–C chemokine family, produced by a variety of cells in response to stimulation with lipopolysaccharides and proinflammatory cytokines such as IL-1 and TNF-α.

Tissue damage, inflammation or injury to the nervous system may result in chronic neuropathic pain characterized by increased sensitivity to painful stimuli, the perception of innocuous stimuli as painful and spontaneous pain (Moalem & Tracey, 2006). The classical characteristics of inflammation are pain, swelling, edema, redness and heat. Prolonged inflammation, known as chronic inflammation, is caused by a variety of factors, including microbial pathogen infection, physical, chemical, and surgical irritation, and/or wounding. Epidemiological studies have also revealed that chronic inflammation is associated with various human diseases, including cerebrovascular, cardiovascular, joint, cutaneous, pulmonary, blood, liver and intestinal diseases as well as diabetes. The suppression of inflammatory response by non-

steroidal anti-inflammatory drugs administration is an important tool and has the ability to inhibit initial or later manifestations.

Seaweed polysaccharides received extensive attention due to their intriguing potential as novel anti-inflammatory and analgesic drugs (Cardozo *et al*., 2007; Rodrigues *et al*., 2012). The sulfated polysaccharides from *Sargassum vulgare* (Dore *et al*., 2013), *S. hemiphyllum* (Hwang *et al*., 2011), and alginic acid from *S. wightii* (Sarithakumari *et al*., 2012) have been explored for possible anti-inflammatory effects. The solvent extract of *S. swartzii* (Hong *et al*., 2011), *S. micracanthum* (Yoon *et al*., 2009), *S. wightii* (Dar *et al*., 2007), *S. fulvellum* and *S. thunbergii* (Kang *et al*., 2008) reported to have anti-inflammatory and analgesic activity. We reported the existence of anti-inflammatory activity in seagrass *Halophila ovalis* methanol extract (Yuvaraj *et al*., 2012). However, there is no single report about the biological activities of sulfated polysaccharides from seagrass *H. ovalis* until to date. Inhibition of secretion of IL-8 by sulfated polysaccharides can aid in the development of a useful therapeutic strategy for allergic inflammatory diseases. Therefore, the present study was undertaken to determine the anti-inflammatory and analgesic activity of sulfated polysaccharides from brown seaweed *S. wightii* and seagrass *H. ovalis* in experimental animal models and its inhibitory effect on peripheral blood mononuclear cells and IL-8 secretion.

6.2. Materials and methods

6.2.1. Formalin test

The peripheral analgesic activity of *S. wightii* and *H. ovalis* sulfated polysaccharide fractions was evaluated by formalin test as described in general materials and methods section **3.2.4.1.**

6.2.2. Hot plate test

The central analgesic activity of *S. wightii* and *H. ovalis* sulfated polysaccharide fractions was evaluated by hot plate test as described in general materials and methods section **3.2.4.2.**

6.2.3. Carrageenan-induced paw edema

The acute anti-inflammatory activity of *S. wightii* and *H. ovalis* fractions was studied using carrageenan-induced paw edema test as described in general materials and methods section **3.2.4.3.**

6.2.4. Freund's adjuvant induced arthritis

The chronic anti-inflammatory activity of *S. wightii* and *H. ovalis* fractions was studied using adjuvant induced arthritic model as described in general materials and methods section **3.2.4.4.**

6.2.5. Isolation of peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from heparinized venous blood by Histopaque–1077 (Sigma) density gradient centrifugation as described earlier (Bignold & Ferrante, 1987). The blood collected (5 ml) aseptically in a 22 gauge syringe was mixed gently with heparin and carefully layered over 5 ml of Ficoll reagent (1:1 ratio) and centrifuged at 2000 rpm for 45 min at room temperature. PBMCs from the buffy layer at the interface were collected, washed thrice with the RPMI-1640 medium without serum and centrifuged at 2000 rpm for 15 min. The pellet was suspended in RPMI-1640 medium containing 10% fetal bovine serum and 10 µl of the suspension was mixed with trypan blue and loaded in the Neubauer's chamber to count the cells and assess for cell viability.

6.2.6. Mitogen induced lymphocyte proliferation and its inhibition by anti-inflammatory agents

PBMCs (2×10^5 cells/well) in a volume of 200 µl in RPMI medium containing 10% FCS and 1 µg/ml of phytohemagglutinin (PHA) was seeded in a 96-well U bottom plate followed by the compounds at different concentrations. The culture was then incubated at 37°C for 24 hours in CO_2 incubator containing 5% CO_2 and 90% humidity. Assays were conducted in triplicate for

each concentration of purified fractions. Experimental data represent mean ± SD of each compound, unless otherwise stated.

6.2.7. Cytotoxic studies by MTT assay

To confirm that the suppressive effect of crude and purified fractions of *S. wightii* and *H. ovalis* sulfated polysaccharide on lymphocyte proliferation, 2 µl of the compound was added with various concentrations (1, 10, 50, 100 µg/ml). Triton X was used as negative control, deionized water was used as solvent control. Following the removal of medium from the wells, 10 µl of MTT (5 mg/ml resuspended in PBS) to each well was added. After 4 h of incubation at 37°C the floating cells were carefully removed and 50 µl of DMSO was added to each well to lyse the cells and the absorbance was measured at 570 nm. The percentage of cell viability was calculated using the formula

Cell viability (%) = (Absorbance of test sample/Absorbance of control) × 100.

6.2.8. Cell lines and culture

The human colon cancer cell line HT-29 was procured from National Centre for Cell Sciences, Pune and maintained with Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 100 ng/ml, each, of penicillin and streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

6.2.9. Measurement of IL-8 cytokine level

For cytokine determination, HT-29 cells were cultured in the presence of different concentrations of Sw Fr IV and Ho Fr IV (0.001-0.1 mg/ml) in a 24-well microtiter plate (2×10^5 cells/well) in a total volume of 1 ml. IL-8 secretion was induced by TNF-α (1 µg/ml). Cells were incubated for 24 h, and the supernatant was collected and used to determine cytokine production. Immunoreactive IL-8 present in the culture supernatants was

determined in triplicate using an IL-8 specific enzyme-linked immunosorbent assay (ELISA) system (Biolegend) as recommended by the manufacturer. Briefly, anti-human IL-8 pre-coated 96-well strip microplate (Biolegend) was incubated at 4°C for 16 h. The plate was washed for four times with wash buffer, 200 µl of 1 X assay diluent A was added, and incubated at room temperature for 1 h with shaking at 200 rpm. After washing the plate with wash buffer four times, culture supernatant and standards were added to appropriate well and incubated at room temperature for 2 h with shaking. Unbound material was washed off and detection antibody solution was added to each well and incubated for 1 h at room temperature with shaking. Bound antibody was detected by addition of avidin-HRP (100 µl) for 30 min followed by addition of the substrate solution F for 15 min in dark. Absorbance at 450 nm was taken 10 min after the addition of substrate solution F. A standard curve was constructed using different dilutions of recombinant human IL-8 in 1 X assay diluent A. The amount of cytokine in the culture supernatants was determined by extrapolation of absorbances to the standard curve.

6.3. Results

6.3.1. Formalin test

The antinociceptive effect of *S. wightii* and *H. ovalis* sulfated polysaccharide purified fractions was characterized by decrease in licking time in 1% formalin administered rats (**Fig. 15-18**). There was no significant reduction of licking time was observed during the first phase following 1% formalin administration (neurogenic) with 2.5 or 5 mg/kg of *S. wightii* and *H. ovalis* purified fractions respectively. Whereas, there was a considerable reduction of licking time by 51.8% (Sw FrIII), 59.9% (Sw FrIV), 54.3% (Ho FrIII), and 61.9% (Ho FrIV) with 10 mg/kg dose (**Fig. 15-18, panel A**). In addition, purified fractions of *S. wightii* and *H. ovalis* (10 mg/kg) injected intravenously 30 min prior to the formalin injection elicited a dose-dependent inhibition of the formalin response during the second phase (inflammatory) by 73.5% (Sw FrIII), 74.7% (Ho FrIII), 79.3% (Sw FrIV), and 82.7% (Ho FrIV) respectively (**Fig. 15-18, panel B**). Indomethacin (5 mg/kg, subcutaneous injection) did not inhibit the first phase but caused significant reduction 65.2% in licking time during the second phase when compared with the saline treated control group.

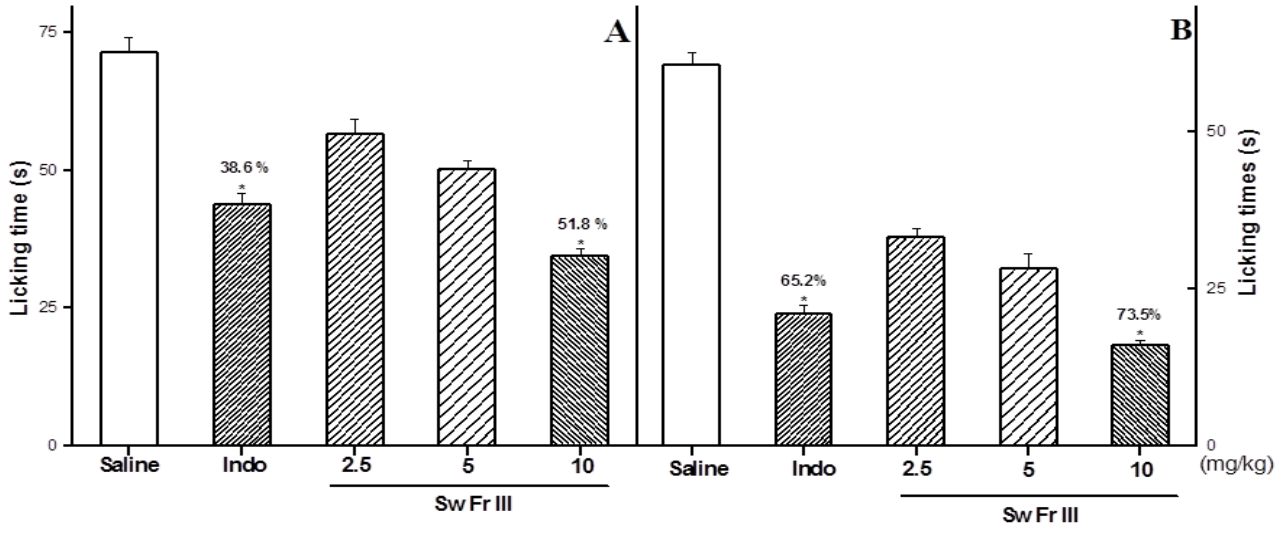


Fig. 15. Effect of *S. wightii* fraction III on the chemical stimuli (formalin test). Sw FrIII (2.5, 5 or 10 mg/kg) or saline were given intravenously 30 min before the formalin and the licking time was determined during the first 5 min (phase I, panel A) and during 20-25 min (phase II, panel B) after a 1% formalin injection in rats. Data are expressed as the mean ± SEM of six animals for each group. * $P < 0.05$ indicates significant difference from indomethacin.

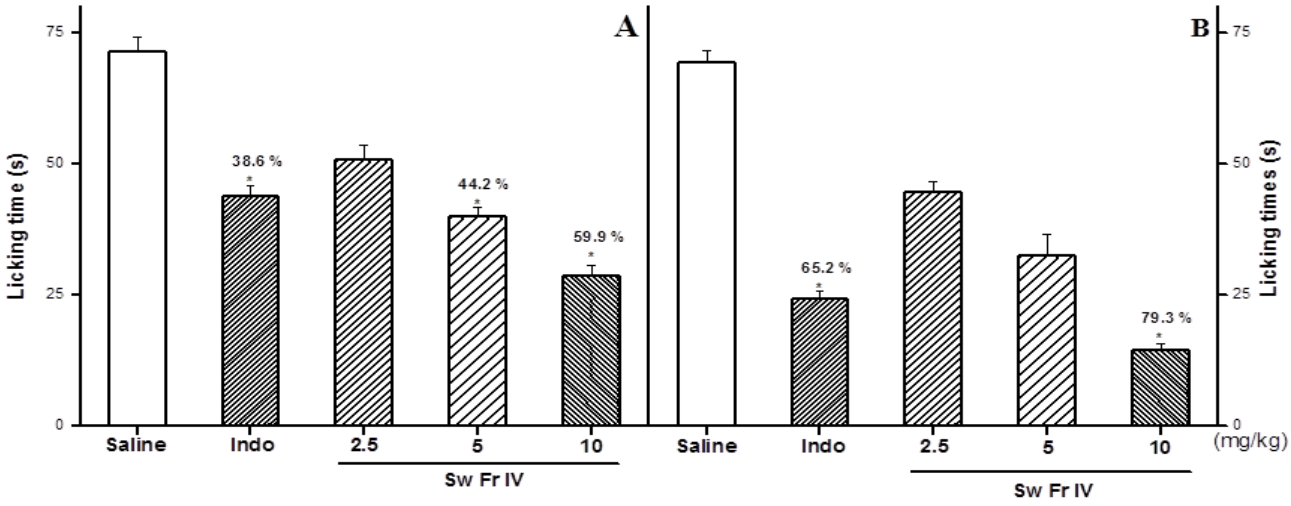


Fig. 16. Effect of *S. wightii* fraction IV on the chemical stimuli (formalin test). Sw FrIV (2.5, 5 or 10 mg/kg) or saline were given intravenously 30 min before the formalin and the licking time was determined during the first 5 min (phase I, panel A) and during 20-25 min (phase II, panel B) after a 1% formalin injection in rats.

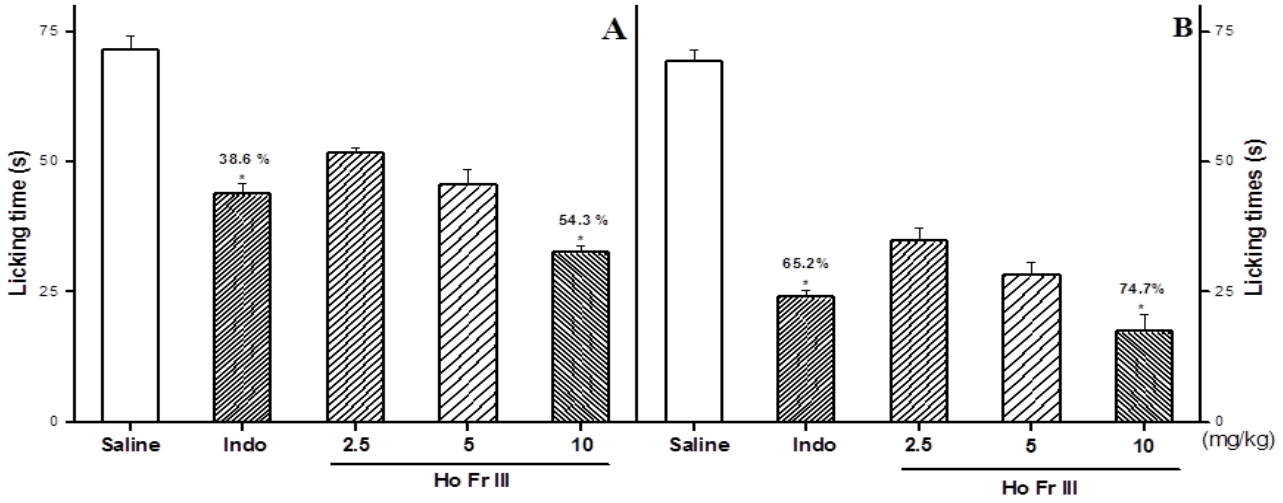


Fig. 17. Effect of *H. ovalis* fraction III on the chemical stimuli (formalin test). Sw FrIII (2.5, 5 or 10 mg/kg) or saline were given intravenously 30 min before the formalin and the licking time was determined during the first 5 min (phase I, panel A) and during 20-25 min (phase II, panel B) after a 1% formalin injection in rats.

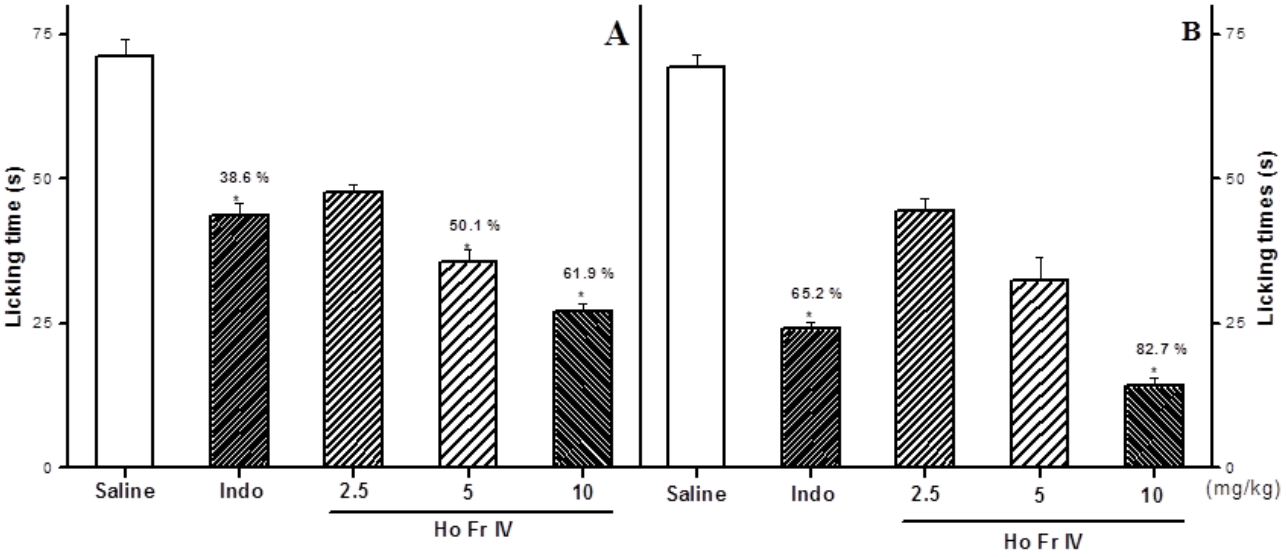
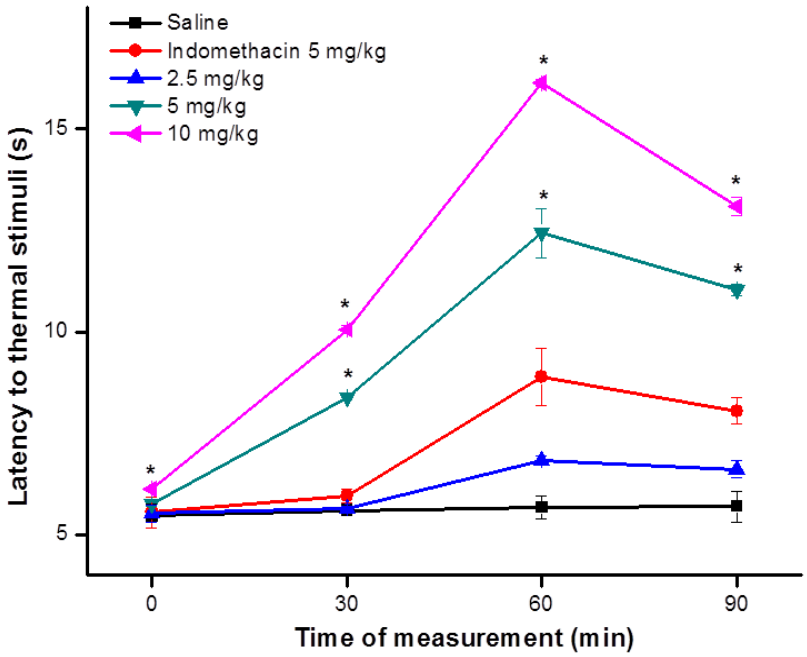


Fig. 18. Effect of *H. ovalis* fraction IV on the chemical stimuli (formalin test). Sw FrIV (2.5, 5 or 10 mg/kg) or saline were given intravenously 30 min before the formalin and the licking time was determined during the first 5 min (phase I, panel A) and during 20-25 min (phase II, panel B) after a 1% formalin injection in rats.

6.3.2. Hot plate test

The treatment of rat with purified fractions Sw FrIII and Sw FrIV of *S. wightii* (5 or 10 mg/kg, intravenously) after the second hot-plate trial produced significant antinociceptive effects, as measured by the reaction time during, the 90 min. of observation (**Fig. 19 A and B**). The minimal dose of purified fractions Sw FrIII and Sw FrIV (2.5 mg/kg) were ineffective at inducing antinociception at all time intervals. The systemic administration of *S. wightii* fractions Sw FrIII and Sw FrIV (5 or 10 mg/kg) significantly increased latency in the hot-plate test at 30, 60, and 90 min. interval. At 10 mg/kg, fractions Sw FrIII and Sw FrIV showed the maximum latency (16.12 ± 0.08, 21.95 ± 0.87 s) to thermal stimuli at 60 min. after administration when compared to indomethacin treated control.

A



B

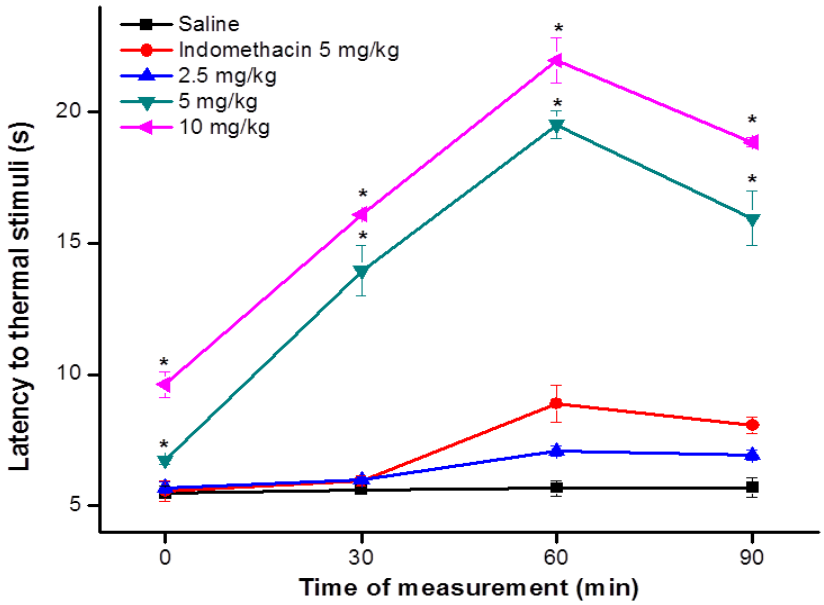


Fig. 19. Effect of *S. wightii* sulfated polysaccharide fractions III (**Fig. 19 A**) and IV (**Fig. 19 B**), indomethacin on the latency period to thermal stimuli (hot-plate) induced in male Wistar rats. Animal received indomethacin (5 mg/kg, subcutaneously). Saline, Sw FrIII or Sw FrIV (2.5, 5 or 10 mg/kg) was injected intravenously. Data are expressed as mean ± SEM of six rats for each group. * $P < 0.05$ indicates significant difference from the saline group.

Similarly, intravenous administration of purified fractions Ho FrIII and Ho FrIV of *H. ovalis* (5 or 10 mg/kg) produced significant antinociceptive effects after the second hot-plate trial, as measured by the reaction time during, the 90 min. of observation (**Fig. 20 A and B**). The minimal dose of purified fractions Ho FrIII and Ho FrIV (2.5 mg/kg) were ineffective at inducing antinociception at all time intervals. The rat injected with purified fraction Ho FrIII and Ho FrIV (5 or 10 mg/kg) significantly increased latency to thermal stimuli at 30, 60, and 90 min. interval time. At 10 mg/kg, fractions Ho FrIII and Ho FrIV showed the maximum latency (16.34 ± 0.41, 22.89 ± 0.40 s) to thermal stimuli at 60 min. after administration when compared to indomethacin treated control.

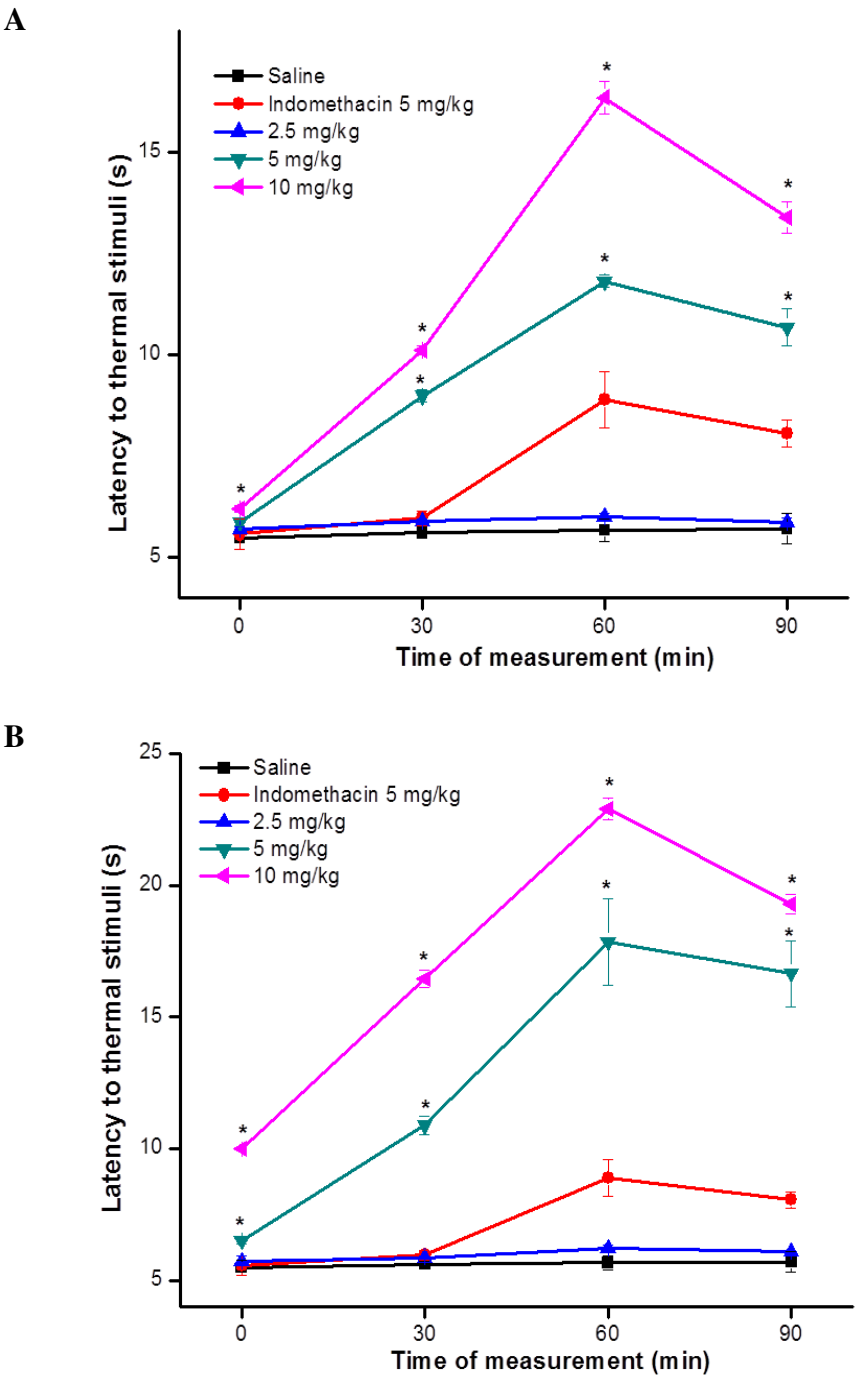


Fig. 20. Effect of *H. ovalis* sulfated polysaccharide fractions III (**Fig. 20 A**) and IV (**Fig. 20 B**), indomethacin on the latency period to thermal stimuli (hot-plate) induced in male Wistar rats. Animal received indomethacin (5 mg/kg, subcutaneously). Saline, Ho FrIII or Ho FrIV (2.5, 5 or 10 mg/kg) was injected intravenously. Data are expressed as mean ± SEM of six rats for each group. * $P < 0.05$ indicates significant difference from the saline group.

6.3.3. Carrageenan induced paw edema

Acute anti-inflammatory activity of *S. wightii* and *H. ovalis* purified fractions (2.5, 5 or 10 mg/kg) was evaluated by carrageenan induced paw edema in rats (**Fig. 21 and 22**). Carrageenan (500 µg, intraperitonially) showed intense paw edema and reached a maximum level (0.59 ± 0.03 ml) at 3 h after administration, and then decreased over the subsequent hour. *S. wightii* fraction Sw FrIV at 10 mg/kg showed significant inhibition in carrageenan induced acute paw edema after 1 h administration at all time intervals, first (0.16 ± 0.02), second (0.18 ± 0.02), third (0.23 ± 0.02), and four (0.14 ± 0.01) hour when compared to diclofenac treated groups (**Fig. 21 B**).

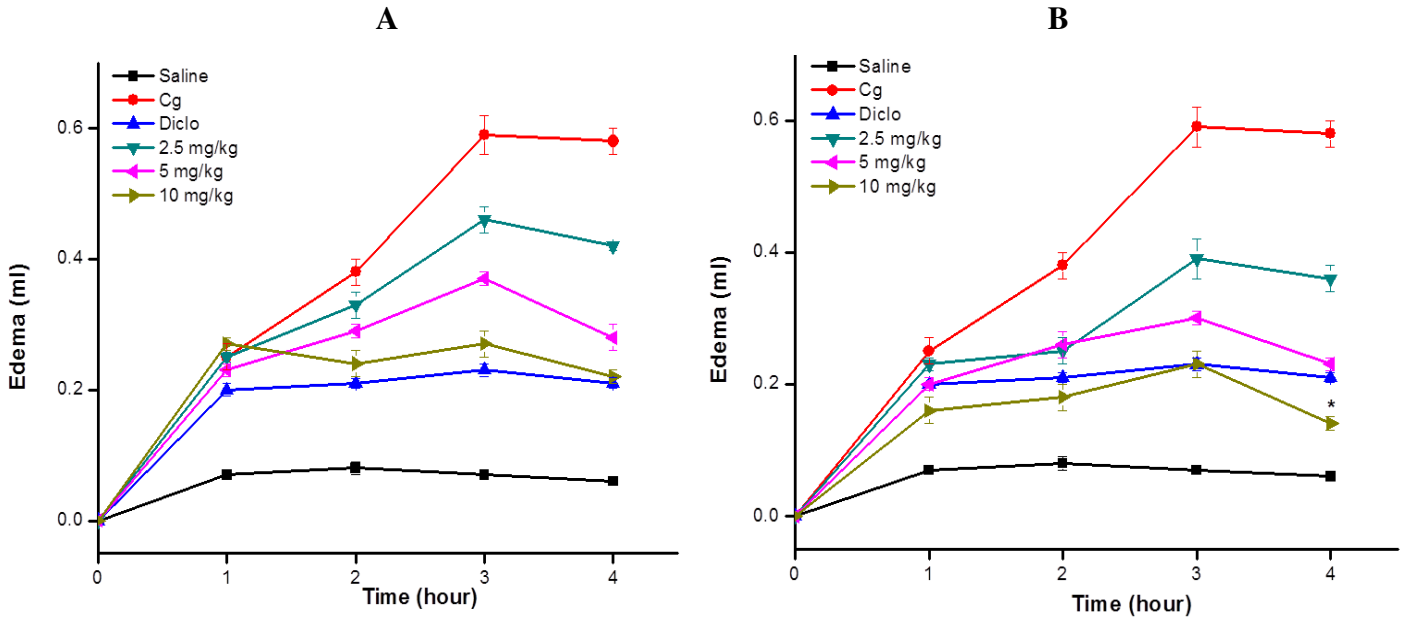


Fig. 21. Effect of *S. wightii* fractions III (**A**) and IV (**B**) on paw edema induced by carrageenan in rats. Before receiving a 0.1 ml injection of carrageenan (Cg, 500 µg/paw) intraperitonially, rats received saline or Sw SP (2.5, 5 or 10 mg/kg). Diclofenac sodium (5 mg/kg) was injected subcutaneously. Another group received only saline without Cg. Data are expressed as mean ± SEM of six rats for each group. * $P < 0.05$ indicates significant difference from the saline group.

Similarly, *H. ovalis* fraction Ho FrIV at 10 mg/kg reduced the carrageenan induced paw edema at all time intervals, first (0.18 ± 0.01), second (0.19 ± 0.01), third (0.22 ± 0.01), and four (0.13 ± 0.02) hour than diclofenac treated groups (**Fig. 22 B**). Whereas, there was no significant inhibition in the carrageenan induced paw edema was observed in *S. wightii* fractions Sw FrIII and Sw FrIV (2.5 or 5 mg/kg) treated groups at all time intervals than diclofenac treated groups (**Fig. 21 A, B**). Similarly, *H. ovalis* fractions Ho FrIII (2.5 or 5 mg/kg), and Ho FrIV (2.5 mg/kg) did not show significant inhibition in the carrageenan induced paw edema than diclofenac treated groups. However, fraction Ho FrIII at 10 mg/kg, and Ho FrIV at 5 mg/kg showed significant inhibition in the carrageenan induced paw edema during fourth hour (**Fig. 22 A, B**).

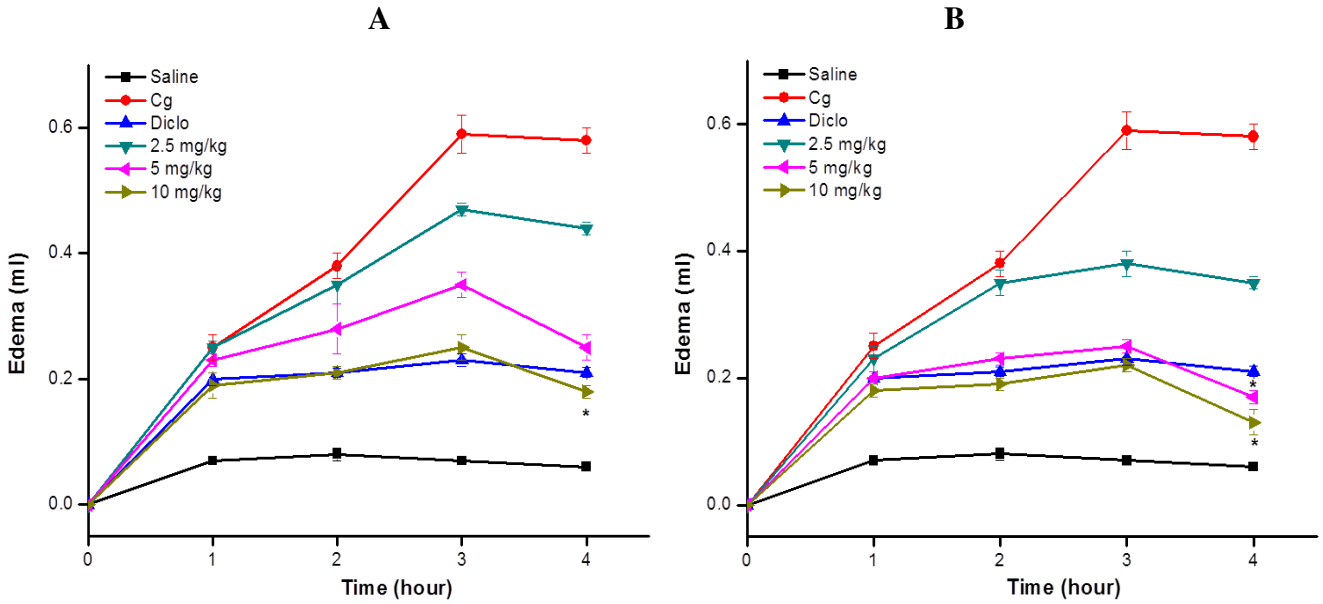


Fig. 22. Effect of *H. ovalis* fractions III (**A**) and IV (**B**) on paw edema induced by carrageenan in rats. Before receiving a 0.1 ml injection of carrageenan (Cg, 500 µg/paw) intraperitonially, rats received saline or Sw SP (2.5, 5 or 10 mg/kg). Diclofenac sodium (5 mg/kg) was injected subcutaneously. Another group received only saline without Cg. Data are expressed as mean ± SEM of six rats for each group. * $P < 0.05$ indicates significant difference from the saline group.

6.3.4. Freund's adjuvant induced arthritis

The chronic anti-inflammatory activity of *S. wightii* and *H. ovalis* fractions at 10 mg/kg was evaluated by FCA induced arthritis model in male Wistar albino rats during 0, 3, 7, 11, and 14 day intervals (Table 27). There is a significant increase in rat paw volume in FCA injected arthritic control rats when compared to saline treated rats at all day intervals. Whereas, fractions of *S. wightii* (Sw FrIII, Sw FrIV) and *H. ovalis* (Ho FrIII, Ho FrIV) at 10 mg/kg showed significant reduction in rat paw volume when compared with the arthritic group. On the 14th day, the percentage inhibition of FCA induced paw edema exhibited by Sw FrIV and Ho FrIV (10 mg/kg) were 73.0 and 77.1% respectively. However, the inhibition was not significant when compared to diclofenac (5 mg/ kg; 68.2% inhibition) treated groups.

Table 27. Effect of *S. wightii* and *H. ovalis* purified fractions on rat paw edema in Freund's adjuvant arthritic rats.

Treatment groups	Increase in paw volume (ml) ± S.E.M (% inhibition)				
	Day 0	**Day 3**	**Day 7**	**Day 11**	**Day 14**
Saline	0.14±0.005	0.16±0.005	0.18±0.005	0.16±0.01	0.12±0.005
Arthritic control	0.39±0.01	0.54±0.02	0.76±0.02	0.81±0.01	0.97±0.005
Diclofenac	0.23±0.01*	0.35±0.01*	0.52±0.0*	0.48±0.005*	0.31±0.02*
(5 mg/kg)	(15.1%)	(22.8%)	(29.5%)	(40.4%)	(68.2%)
Sw Fr III	0.27±0.02*	0.39±0.03*	0.63±0.005*	0.56±0.02*	0.37±0.01*
(10 mg/kg)	(10.9%)	(15.4%)	(23.9%)	(31.0%)	(61.7%)
Sw Fr IV	0.32±0.005*	0.39±0.01*	0.52±0.01*	0.47±0.01*	0.26±0.02*
(10 mg/kg)	(17.6%)	(26.5%)	(31.7%)	(42.4%)	(73.0%)
Ho Fr III	0.25±0.01*	0.35±0.01*	0.61±0.005*	0.55±0.02*	0.35±0.02*
(10 mg/kg)	(13.4%)	(23.4%)	(24.7%)	(31.8%)	(63.8%)
Ho Fr IV	0.23±0.01*	0.34±0.01*	0.51±0.01*	0.46±0.01*	0.22±0.01*
(10 mg/kg)	(15.9%)	(25.9%)	(30.0%)	(42.8%)	(77.1%)

6.3.5. Mitogen induced lymphocyte proliferation

To find out whether the purified fractions obtained from brown seaweed *S. wightii* do possess anti-inflammatory properties, their effect at various concentrations on the inhibition of proliferation of mitogen induced PBMCs was studied preliminarily and the results were shown (**Fig. 23 A**). There was significant increase in proliferation of PBMCs on induction with PHA, but this response was considerably inhibited by purified fractions Sw FrIV (IC_{50} 62.45 µg/ml) and Sw FrIII (IC_{50} 72.37 µg/ml). The cytotoxic effect of purified fractions by tryphan blue exclusion test showed that, after two days of treatment, no microscopically visible alteration of normal cells morphology was observed and the purified fractions did not affect cell viability at the various concentrations (**Fig. 23 B**).

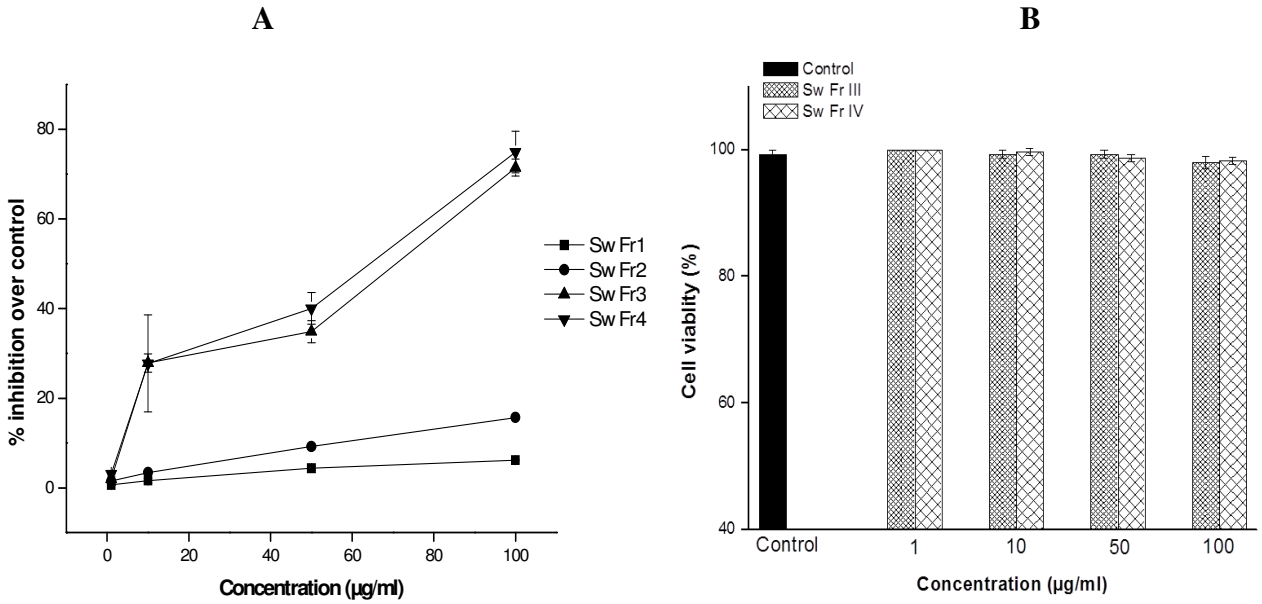


Fig. 23 A. Inhibitory effect of purified fractions isolated from *S. wightii* on mitogen induced proliferation. Mitogen PHA (1 µg/ml) induced PBMC (2×10^5 cells/well) was treated with different concentration of purified fractions and the % inhibition of lymphocyte proliferation was determined. **B**. Effect of *S. wightii* purified fractions Sw FrIII and Sw Fr IV on cell viability. Values are mean ± SD of three determinations.

Similarly, the anti-inflammatory properties of *H. ovalis* purified fractions was studied on the inhibition of proliferation of mitogen induced PBMCs at various concentrations and the results were shown (**Fig. 24 A**). A 50% inhibition (IC_{50}) of proliferation of PBMCs was observed at 43.85 and 61.28 µg/ml of purified fractions Ho FrIV and Ho FrIII. The present study showed that there was a significant increase in proliferation of PBMCs on induction with PHA. However, this response substantially inhibited by the purified fractions Ho FrIV than other fractions. The tryphan blue exclusion test revealed that, purified fractions Ho FrIV and Ho FrIII have non-cytotoxic effect at tested concentrations (**Fig. 24 B**). No microscopically visible alteration of normal cells morphology and destruction of cell layer were observed.

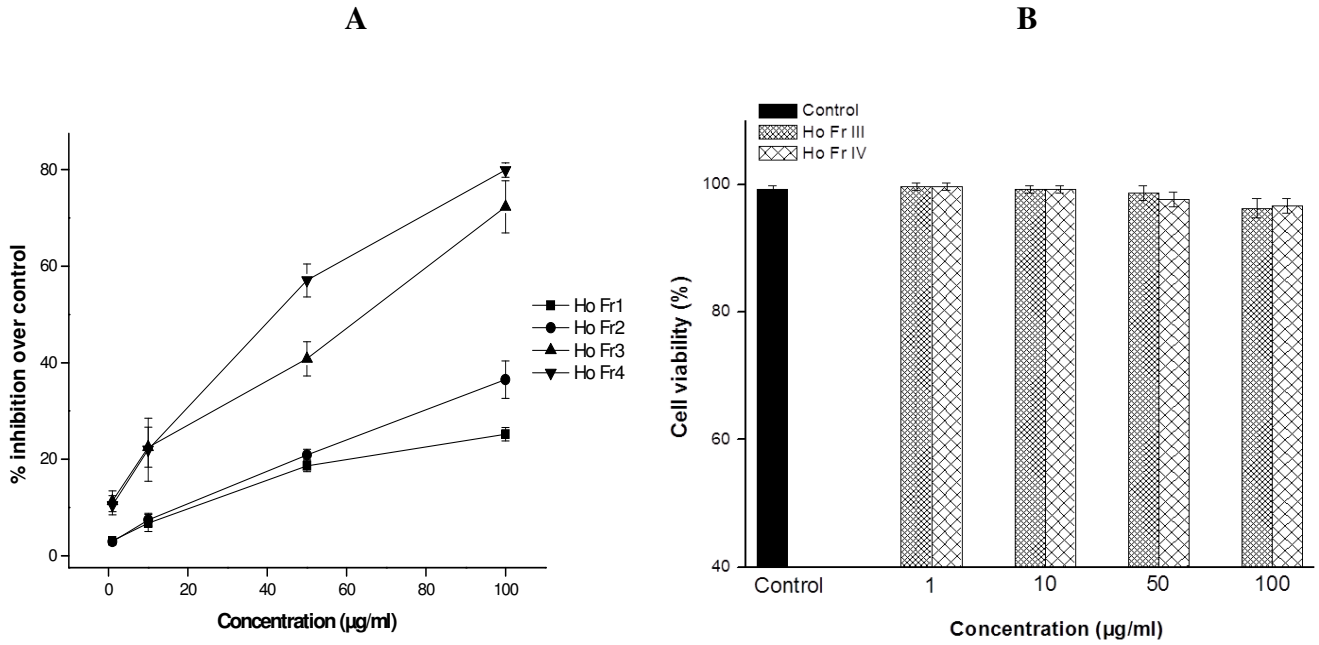


Fig. 24 A. Inhibitory effect of purified fractions isolated from *H. ovalis* on mitogen induced proliferation. Mitogen PHA (1 µg/ml) induced PBMC (2×10^5 cells/well) was treated with different concentration of purified fractions and the % inhibition of lymphocyte proliferation was determined. **B**. Effect of *H. ovalis* purified fractions Ho FrIII and Ho Fr IV on cell viability. Values are mean ± SD of three determinations.

6.3.6. Effect of sulfated polysaccharides on IL-8 secretion

Il-8 is a sensitive marker in the early stages of inflammation and infection. The effect of purified fraction Sw FrIV and Ho FrIV on TNF-α induced epithelial interleukin IL-8 secretion was analyzed in the human HT-29 colonic epithelial cell line (**Fig. 25 A & B**). The increase in IL-8 secretion was observed in TNF-α treated HT-29 cells. Whereas, IL-8 secretion was significantly ($P < 0.05$) decreased in TNF-α treated HT-29 cells with purified fractions Sw FrIV and Ho FrIV in a concentration dependent manner.

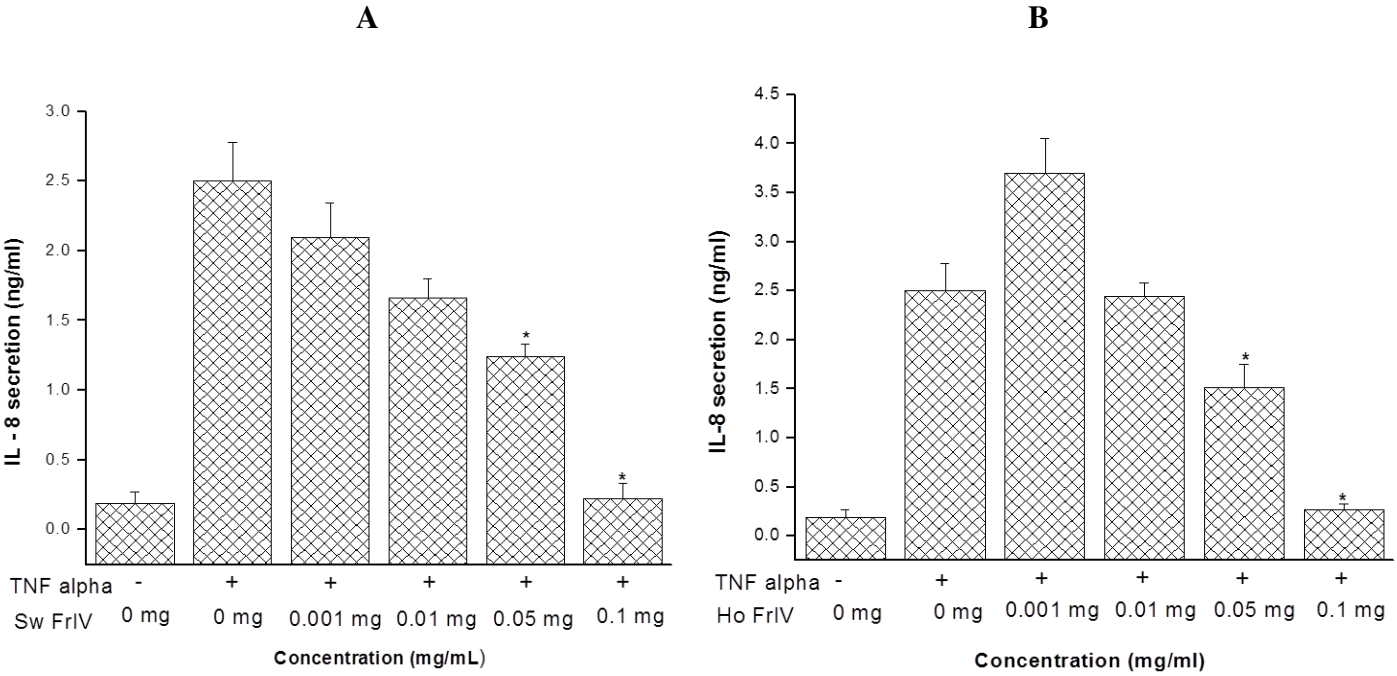


Fig. 25. Dose dependent effect of purified fraction Sw FrIV (**A**) and Ho FrIV (**B**) on TNF-α induced IL-8 secretion in colonic epithelial cell line HT-29. Each datum represents mean ± SD of three determinations. * $P < 0.05$, significantly different from the control value. Blank, none (phosphate buffered saline).

6.4. Discussion

In recent times, there is a strong interest in searching for new anti-inflammatory agent from natural products due to indiscriminate use of commercially available analgesic and non-steroidal anti-inflammatory drugs. Currently, sulfated polysaccharides of different seaweed species warrant new analgesic and anti-inflammatory agent (Rodrigues *et al*., 2012; Coura *et al*., 2012; Vanderlei *et al*., 2010). To the best of our knowledge, no reports have been documented for the anti-inflammatory activity of sulfated polysaccharides extracted from seagrass species. This study demonstrated that sulfated polysaccharide active fractions obtained from the brown algae *Sargassum wightii* and seagrass *Halophila ovalis*, produces antinocicpetive and anti-inflammatory effects in models of nociception (formalin test, and hot plate test), acute inflammation (carrageenan induced paw edema test), and chronic inflammation (FCA induced arthritis test).

The formalin test is the most predictive model of tonic pain and is useful for the screening of novel compounds, since it encompasses inflammatory, neurogenic, and central mechanisms of nociception (Shield *et al*., 2010). Injection of 1% formalin into the paw is a biphasic event. The first phase (neurogenic pain) caused by the direct chemical stimulation of nociceptors. The second phase (inflammatory pain) is triggered by a combination of stimuli, including inflammation of the peripheral tissues and mechanism of central sensitization. Substances that inhibit both phases are acts as central analgesics, while inhibit only the second phase are peripheral analgesics (Deraedt *et al*., 1980), suggesting that its nocicpetive effect may be the result of inhibition of inflammatory mediators released in the effected tissue (Rosland *et al*., 1990). In the present study, fraction Sw FrIII and Sw FrIV of *S. wightii* showed 51.8% and 59.9% inhibition with 10 mg/kg during first phase. Similarly, fraction Ho FrIII and Ho FrIV of *H. ovalis* showed 54.3% and 61.9% inhibition with 10 mg/kg during first phase. Rodrigues *et al*., (2012) observed 52.1% inhibition with 27 mg/kg of *Caulerpa cuppressoides* fraction 2 (Cc-sp2) during first phase. Similarly, Coura *et al*., (2012) observed 56.1% inhibition with 9 mg/kg administration of red seaweed *Gracilaria cornea* total sulfated polysaccharide (Gc-TSP) during first phase.

Whereas, fraction I of red seaweed *Solieria filiformis* (SP-Sf) did not reduce the licking time during first phase of formalin injection (de Araujo *et al*., 2011). However, it showed 86.4% inhibition during second phase at the test dose of 9 mg/kg. Similarly, Cc-SP2 and GC-TSP at 9 mg/kg showed 82.34% and 94.7% inhibition during second phase. In this study, fractions Sw FrIII and Sw FrIV of *S. wightii* showed 73.5% and 79.3% inhibition with test dose of 10 mg/kg during second phase. Similarly, fractions Ho FrIII and Ho FrIV of *H. ovalis* showed 74.7% and 82.7% inhibition at 10 mg/kg during second phase. Thus, the present study showed that fractions of *S. wightii* and *H. ovalis* act as central analgesics by inhibiting both phases.

In order to discriminate between central and peripheral antinociceptive action, we examined the effect of *S. wightii* and *H. ovalis* sulfated polysaccharide fractions using the hot plate test. This test is known to evaluate the possible specific central action in which analgesic effects exerted through opioid agents via supra spinal and spinal receptors (Nemirovsky *et al*., 2001). The present study showed that fractions of *S. wightii* and *H. ovalis* increased the latency to thermal stimuli in a dose dependent manner. At 10 mg/kg, fractions Sw FrIV and Ho FrIV showed maximal latency period (21.95 ± 0.87; 22.89 ± 0.40s) at 60 min. Similarly, Cc-SP2 at 9 mg/kg showed maximal effect (26.7 ± 1.2s) at 60 min (Rodrigues *et al*., 2012). Whereas, GC-TSP and SP-Sf at 9 mg/kg were ineffective at inducing antinociception in the hot plate test at all time intervals (Coura *et al*., 2012; de Araujo *et al*., 2011). Therefore, the results of the present study revealed that the antinociceptive action of *S. wightii* and *H. ovalis* sulfated polysaccharide fractions occur via a central-acting mechanism.

There is a well-established link between the antinociceptive action and inflammatory pain. In order to evaluate this correlation, the anti-inflammatory activity of *S. wightii* and *H. ovalis* sulfated polysaccharide fractions were investigated in the paw edema model. Carrageenan has been used for decades to induce inflammation and to study the mediators of inflammation and the effectiveness of anti-inflammatory mediators (Moyana & Lalonde, 1990). The edema induced by carrageenan (500 µg, intraperitonially) was temporal and multi-mediated, with release of diverse set of mediators, such as histamine, serotonin, bradykinin, nitric oxide, and prostaglandins, and characterized by an intense neutrophil infiltrate (Morris *et al*., 2003).

In this test, edema evoked by carrageenan was significantly inhibited by purified fractions of *S. wightii* and *H. ovalis* in a dose dependent manner. At 10 mg/kg, Sw FrIV and Ho FrIV showed marked reduction in edema induced by carrageenan than diclofenac treated group at all time intervals. Similarly, sulfated polysaccharide fractions from *Padina tetrastromatica* and crude polysaccharide from *Turbinaria ornata* showed significant reduction in edema at increasing concentration (Sulaiman *et al*., 2011; Ananthi *et al*., 2010). Whereas, sulfated galactan of *Champia feldmannii* and SP-Sf of red seaweed *S. filiformis* at 9 mg/kg demonstrated edematogenic activity (de Araujo *et al*., 2011; Assreuy *et al*., 2008). In contrast, galactan sulfate of seagrass *H. ovalis* inhibited edema induced by carrageenan in a concentration dependent manner.

Paw swelling is an index, employed to measure the antiarthritic activity of various drugs. Freund's adjuvant induced arthritis in rats is a classical model to study the chronic inflammatory disease, characterized by infiltration of synovial membrane in association with destruction of joints resembles rheumatoid arthritis in humans (Astusi *et al*., 2005). Alginic acid extracted from *S. wightii* inhibited the paw edema of about 65% in arthritic rats at 100 mg/kg on day 14 (Sarithakumari *et al*., 2012). In the present study, purified fractions Sw FrIV and Ho FrIV (10 mg/kg) exhibited significant paw edema inhibition of about 73% and 77.1% in arthritis-induced rats on day 14, respectively, which is higher than that of standard drug, diclofenac. The reduction in edema formation in adjuvant-induced arthritis attributed to its inhibitory action on prostaglandin synthesis. The Loss of body weight has been used to assess the course of arthritis and the response to therapy of anti-inflammatory drugs (Walz *et al*., 1971). Yoshikawa *et al*., (1985) reported that there was significant weight loss during the course of experimental period, but thereafter continued to show normal weight gain in rats. In this study, we observed the initial weight loss during arthritic condition and recovered normal weight on day 14. Similarly, Somasundaran *et al*., (1983) reported the loss of body weight during arthritic condition on alterations in the metabolic activity of diseased rats. Thus, the present study indicates that there is a close relationship between the extent of inflammation and loss of body weight. Reducing the local inflammatory response through blockade of the activation, proliferation of inflammatory cells and production of inflammatory cytokines is one such anti-inflammatory goal (Arai *et al*., 1990).

Peripheral blood mononuclear cells (PBMCs) consist of B cells, T cells, and monocytes/macrophages. Phytohemagglutinin (PHA) is a potent mitogen for T lymphocytes, which binds to *N*-acetylgalactosamine glycoproteins expressed on the surface of T cells and induce the cells to proliferate (Charles *et al*., 1997). PBMC is considered as the best *in vitro* model for studying the immunomodulatory and anti-inflammatory properties of plant drugs (Chen *et al*., 2007; Selvakkumar *et al*., 2007; Yuvaraj *et al*., 2012). Most of earlier data on anti-inflammatory potential of algae were *in vivo* method, the present study focused on *in vitro* anti-inflammatory effect on PBMC's as preliminary approach. In the present study, we have shown that Sw FrIV and Ho FrIV fractions has a profound inhibitory effect on PHA activated PBMC in a concentration dependent manner. However, the inhibitory effect of *S. wightii* and *H. ovalis* sulfated polysaccharide fractions on PBMC was not related to direct cytotoxicity as the viabilities of activated PBMC were not significantly decreased. Similarly, Chen *et al*., (2007) reported that suberosin from root of *Plumbago zeylanica* Linn. a chinese traditional medicine inhibited the PHA activated PBMC proliferation in a non cytotoxic range of concentration. It has been reported that, sulfated polysaccharide from brown seaweeds *S. filipendula, S. fusiformi, Dictyopteris delicatula,* and *Dictyota menstruallis* have an excellent antiproliferative effects on several cancer cell lines (Chen *et al*., 2012; Costa *et al*., 2010). The present result suggests that sulfated polysaccharide fractions of *S. wightii* and *H. ovalis* do possess immunopharmacological activities. Chen *et al*., (2007) hypothesized that decreased production of cytokines as one of the inhibitory mechanisms on PHA-activated PBMC proliferation.

Epidemiological studies have revealed that, chronic inflammation is usually linked to various diseases, including cerebrovascular, cardiovascular, joint, cutaneous, pulmonary, blood, liver and intestinal diseases as well as diabetes. One chemokine implicated in chronic inflammation is IL-8 (Harada *et al*., 1996) and observed in elevated level in inflammatory diseases such as bowel disease, rheumatoid arthritis and psoriasis. Futhermore, increased risk of malignancy is linked with the chronic inflammation caused by chemical and physical agents (Gulumian, 1999), autoimmune and inflammatory reactions of unknown etiology (Ekbom *et al*., 1990).

There is now evidence that inflammatory cytokines and chemokines, which can be produced by the tumor cells/ or tumor-associated leucocytes and platelets, may contribute directly to malignant progression. The chemotactic cytokine IL-8 secreted by monocytes, fibroblasts and endothelial cells in response to stimulation with IL-1, TNF-α or lipoploysaccharides. However, reports showed that human tumors also frequently secrete IL-8 (Balkwill & Mantovani, 2001) and it induces proliferation and migration of melanoma cells with a related chemokine called "gro". Hence, abrogation of IL-8 activity represents a candidate therapeutic strategy for chronic inflammatory diseases. In this study, we induced the human colonic cancer cell line HT-29 by TNF-α and the effect of purified fractions Sw FrIV and Ho FrIV on IL-8 secretion was investigated. The present study interestingly showed that IL-8 secretion was significantly ($P > 0.05$) reduced in a concentration dependent manner in TNF-α induced HT-29 cell line. Similarly, recent studies revealed that mollugin, a Chinese herb inhibited TNF-α induced inflammatory responses and chemotaxis in HT-29 cells through inhibition of NF-κB activation and decreased MCP-1, IL-8 and ICAM-1 expression (Kim *et al*., 2009). The naturally occurring sulfated polysaccharides are mostly complex and heterogeneous. Such polysaccharides have the potential to bind to diverse physiologically important proteins and thereby harbor an intrinsic risk of inducing off-target effects and cytotoxicity (Ghosh *et al*., 2010). Similarly, Bouhlal *et al*., (2011) showed that galactans from red seaweeds lacked cytotoxic effects and displayed a broad spectrum of antiviral activity against HSV-1 and HSV-2. In the present study, we found that hetrofucan and galactan sulfate lacked cytotoxic effects and reduced IL-8 secretion in a non-cytotoxic range of concentration. Further study is in need to find out whether the sulfated polysaccharides decreases IL-8 secretion through NF-κB activation and/or by blocking the cross linking of chemokines with Fas receptors. Earlier studies revealed that free radicals/reactive oxygen species (ROS) involved in the pathogenesis of rheumatoid arthritis (Jasin, 2005). The increase in ROS levels leads to lipid peroxidation with production of malondialdehyde (MDA) and cause tissue injury (Khansari *et al*., 2009). Thus, the present study necessitates the study of antioxidant properties of *S. wightii* and *H. ovalis* sulfated polysaccharides.

CHAPTER 7

ANTIOXIDANT PROPERTIES OF *SARGASSUM WIGHTII* AND *HALOPHILA OVALIS* SULFATED POLYSACCHARIDE FRACTIONS *IN VITRO*

CHAPTER 7

ANTIOXIDANT PROPERTIES OF *SARAGSSUM WIGHTII* AND *HALOPHILA OVALIS* SULFATED POLYSACCHARIDE FRACTIONS *IN VITRO*

7.1. Introduction

Reactive oxygen species (ROS), collectively called as a group of oxidants, which are either free radicals or molecular species capable of generating free radicals. In normal physiological conditions, typically low concentration of ROS is essential for gene expression, cellular growth and defense against infection as well as act as the stimulating agents for biochemical processes within the cell (Kunwar *et al*., 2011; Droge, 2002). ROS induced oxidative damage depending upon their reactions with different biological important macromolecules such as DNA, protein and lipid, produce different types of secondary radicals. Under pathological conditions, excessive generation of reactive oxygen species (ROS) initiates biomolecular oxidations and creates oxidative stress, which leads to age related degenerative diseases and disorders such as stroke, diabetes, cancer, myocardial infarction, septic and hemorrhagic shock, Alzheimer's and Parkinson's diseases (Yangthong *et al*., 2009). The negative effects of oxidative stress may be mitigated by antioxidants (Larson, 1995). An antioxidant is a substance that is present at low concentrations and significantly delays or prevents oxidation of the oxidizable substrate. They donate their own electron to unstable radical and make it as stable one thereby neutralizing the adverse effects of ROS. The antioxidant systems are classified as (i) enzymatic antioxidants and (ii) non enzymatic antioxidants. Cells are equipped with different defense mechanisms for protection against ROS mediated oxidative damage and to maintain the redox homeostasis of cell. Enzymatic antioxidants are endogenous include catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD) that acts as body's first line defense against ROS by converting it into less reactive or inert species. Non-enzymatic antioxidants such as glutathione (GSH), α-tocopherol, bilirubin, vitamin C and E scavenge the ROS directly or prevent the production of ROS through sequestration of redox active metals like iron and copper (Kunwar *et al*., 2011; Chanda & Dave, 2009).

The importance of reactive oxygen species and free radicals has attracted increasing attention over the past decade. The environment in which seaweeds and seagrasses grow is harsh as they are exposed continuously to a combination of light and high oxygen concentrations. Such factors can lead to the formation of free radicals and other strong oxidizing agents. However, seaweeds and seagrasses seldom suffer any serious photodynamic damage during metabolism. This fact implies that seaweeds and seagrasses have some protective mechanism and compounds. In order to reduce the damages caused by free radicals, natural antioxidants from seaweeds and seagrasses are being investigated (Nagai & Yukimoto, 2003; Yuvaraj *et al*., 2012). In recent years, fucoidan and alginic acid, which are not found in higher land plants, were reported to be an effective non-toxic candidate as free radical scavengers *in vitro* and prevent the oxidative damage in cellular system (So *et al*., 2007). The antioxidant capacity of algal polysaccharides has been studied by different *in vitro* methods including hydrogen peroxide, superoxide anion, hydroxyl radical scavenging, antioxidant capacity by ABTS, and ferric reducing power assays. In recent years, sulfated polysaccharides have received considerable attention as free radical scavengers for the prevention of oxidative damage in living organisms (Hu *et al*., 2010). Hence, in the present study, we evaluated the antioxidant properties of sulfated polysaccharide and its purified fractions of brown seaweed *Sargassum wightii* and seagrass *Halophila ovalis* using different *in vitro* antioxidant systems.

7.2. Materials and methods

7.2.1. Hydroxyl radical scavenging activity

The scavenging activity of sulfated polysaccharide purified fractions of brown seaweed *S. wightii* and seagrass *H. ovalis* on hydroxyl radicals was determined according to the method of Barahona *et al*., (2011) as described in general materials and methods section **3.2.5.1.**

7.2.2. ABTS radical scavenging activity

The scavenging ability of purified fractions of *S. wightii* and *H. ovalis* sulfated polysaccharides on ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)) radicals were examined in the concentration range of (0.5–2.5 mg/ml) as described in general materials and methods section **3.2.5.2**.

7.2.3. Ferric reducing power assay

The total reducing power of sulfated polysaccharide purified fractions extracted from *S. wightii* and *H. ovalis* was investigated according to the method reported by Oyaizu (1986), described in general materials and methods section **3.2.5.3**.

7.3. Results

7.3.1. Scavenging activity on hydroxyl radicals

The hydroxyl radical scavenging ability of purified fractions Sw FrI, Sw FrII, Sw FrIII, and Sw FrIV was monitored using brilliant green as scavenging probe. In the present study, the scavenging effect of purified fractions on hydroxyl radicals increases in a concentration dependent manner (**Fig. 26**). As shown in the Fig. 26, the purified fraction Sw FrIV exhibited significant hydroxyl scavenging effect ($P < 0.05$) between concentrations and IC_{50} was observed at 1.29 mg/ml. Indeed, the scavenging effect was inferior to those of the standard ascorbic acid at 1 mg/ml but superior at 2 mg/ml. Whereas, the scavenging ability of other fractions Sw FrI, Sw FrII, and Sw FrIII on hydroxyl radical was statistically not significant ($P > 0.05$) and inferior to commercial counterpart at all concentrations.

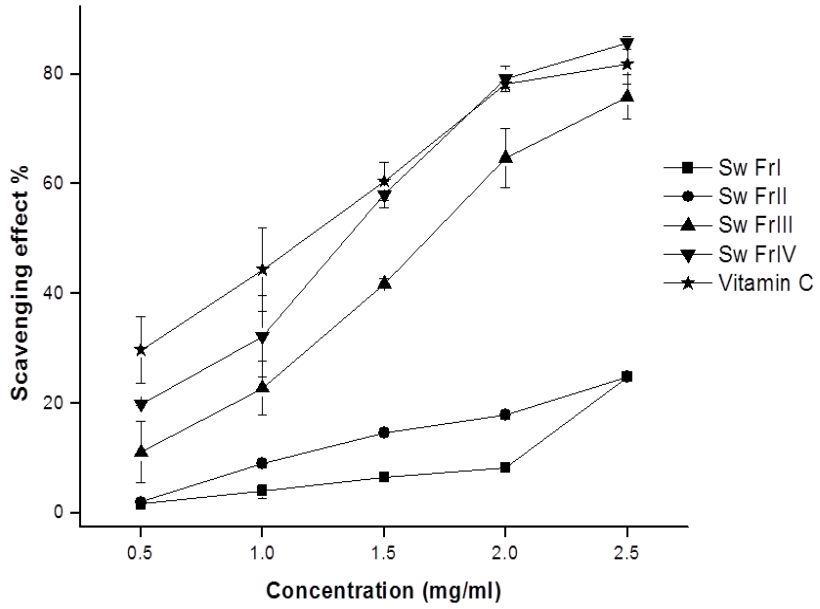


Fig. 26. Scavenging effect (%) of *S. wightii* sulfated polysaccharide purified fractions on hydroxyl radical measured at 30 minutes after H_2O_2 addition. Values are mean ± SD of three determinations.

The hydroxyl radical generated through the Fenton reaction in this system was scavenged by seagrass polysaccharide samples. The scavenging ability of purified fractions Ho FrI, Ho FrII, Ho FrIII and Ho FrIV was portrayed (**Fig. 27**). The results indicated that the scavenging ability of test samples and vitamin C increased in a concentration dependent manner. As shown in the **Fig. 27**, the purified fraction Ho FrIV exhibited significant hydroxyl scavenging effect ($P < 0.05$) between concentrations and IC_{50} was observed at 1.04 mg/ml. In fact, the scavenging effect was superior to those of the commercial counterpart such as vitamin C at every concentration point. However, the hydroxyl scavenging ability of other three fractions Ho FrI, Ho FrII and Ho FrIII was statistically not significant ($P > 0.05$) and inferior to ascorbic acid at all concentration points.

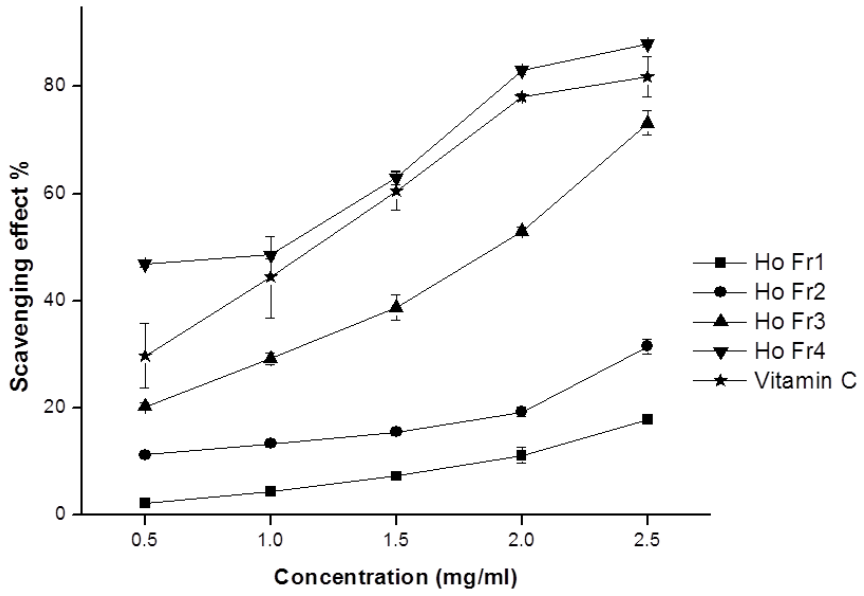


Fig. 27. Scavenging effect (%) of *H. ovalis* sulfated polysaccharide purified fractions on hydroxyl radical measured at 30 min after H_2O_2 addition. Values are mean ± SD of three determinations.

7.3.2. Scavenging activity on ABTS radicals

ABTS assay was often employed to evaluate the total antioxidant power of test samples at a specific absorbance (734 nm). In this study, $ABTS^{\cdot+}$ cation radical was employed as an oxidant. The scavenging ability of *S. wightii* purified fractions on ABTS radicals was portrayed (**Fig. 28**). The results showed that purified fraction Sw FrIV scavenged the ABTS radicals significantly ($P < 0.05$) between the concentrations and IC_{50} was observed at 1.55 mg/ml respectively but not significant ($P > 0.05$) when compared with commercial counterpart. Similarly, the purified fractions Sw FrI, Sw FrII, and Sw FrIII was not significantly ($P > 0.05$) scavenging the ABTS radicals and found to be inferior to vitamin C at all concentrations.

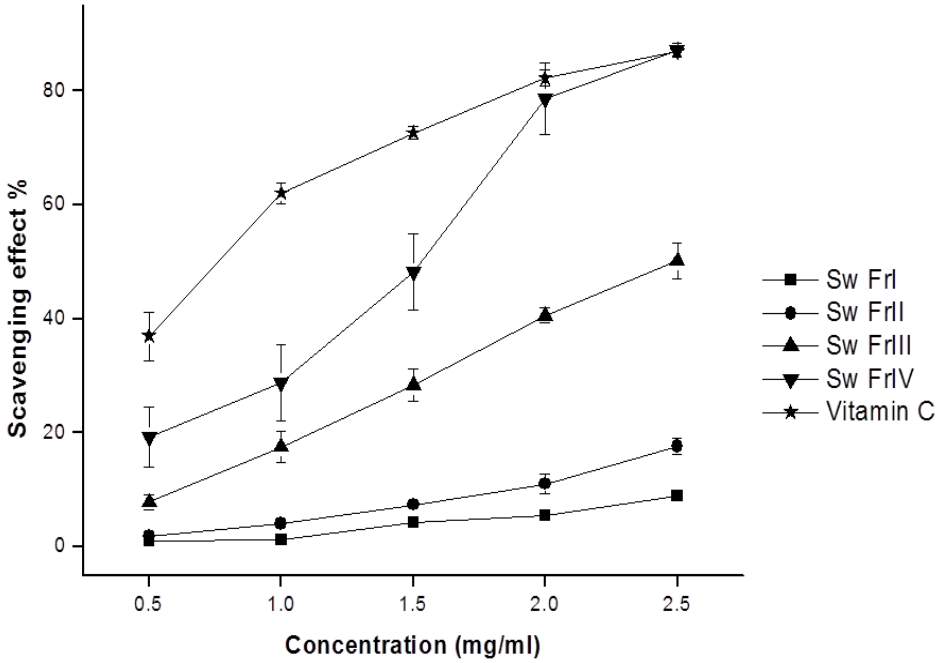


Fig. 28. Scavenging effect (%) of *S. wightii* sulfated polysaccharide purified fractions on ABTS radical. Values are mean ± SD of three determinations.

The antioxidant capacity of seagrass sulfated polysaccharides was measured using an assay based on electron transfer. In this study, $ABTS^{\cdot+}$ cation radical was employed as an oxidant. The scavenging ability of purified fractions Ho FrI, Ho FrII, Ho FrIII and Ho FrIV on ABTS radicals was portrayed (**Fig. 29**). Purified fraction Ho FrIV showed significant ABTS radicals scavenging activity ($P < 0.05$) and IC_{50} was observed at 0.55 mg/ml. Whereas, purified fraction Ho FrIII scavenged ABTS radicals at IC_{50} of 2 mg/ml respectively. The results indicated that purified fraction Ho FrIV has strong scavenging ability on ABTS radicals than commercial counterpart. However, the other three purified fractions was statistically not significant ($P > 0.05$) and exhibited less scavenging effect than vitamin C at every concentration point.

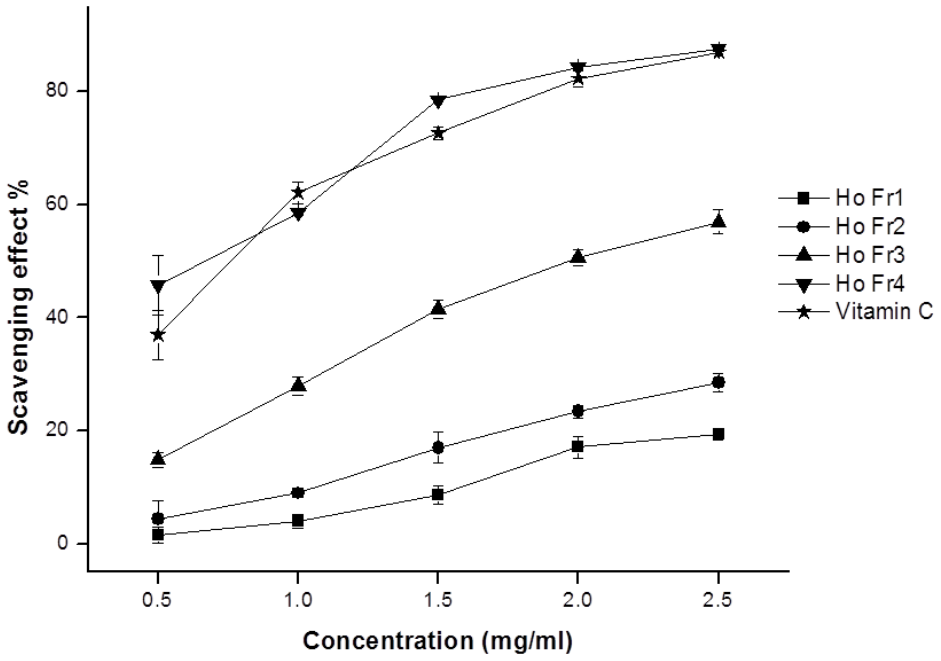


Fig. 29. Scavenging effect (%) of *H. ovalis* sulfated polysaccharide purified fractions on ABTS radical. Values are mean ± SD of three determinations.

7.3.3. Reducing power assay

The reducing power of purified fractions of *S. wightii* was depicted in **Fig. 30**. In the present study, the reducing power correlated well with increasing concentration of purified fractions. In fact, the reducing power of purified fraction Sw FrIV was found to be statistically significant ($P < 0.05$) and superior to the standard ascorbic acid at every concentration point. Whereas, the reducing power of other three fractions Sw FrI, Sw FrII, and Sw FrIII was statistically not significant ($P > 0.05$) and inferior to vitamin C at all concentrations.

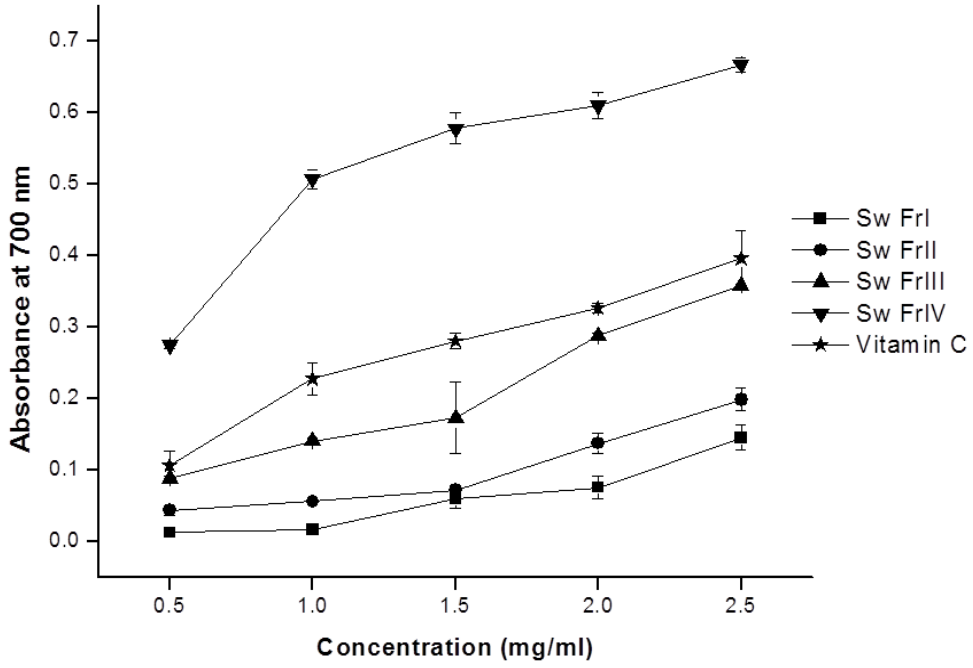


Fig. 30. Reducing power of *S. wightii* sulfated polysaccharide purified fractions. Values are mean ± SD of three determinations.

The reducing power of purified fractions Ho FrI, Ho FrII, Ho FrIII and Ho FrIV was depicted in **Fig. 31**. As shown in the Figure 31, the reducing power was found to be increasing in concentration dependent. Indeed, the reducing power of purified fraction Ho FrIV was found to be close to the standard ascorbic acid at every concentration point and exceeded at 2.5 mg/ml than the commercial counterpart. However, the reducing power of other purified fractions Ho FrI, Ho FrII and Ho FrIII was inferior to that of positive control at all the concentration point. The reducing capacity of purified fractions was statistically not significant ($P > 0.05$) as compared to commercial counterpart. However, both the test sample and standard showed significant difference between the concentrations ($P < 0.05$). This property is correlated with the presence of reductones that were reported to be terminators of free radical chain reaction by donating a hydrogen atom.

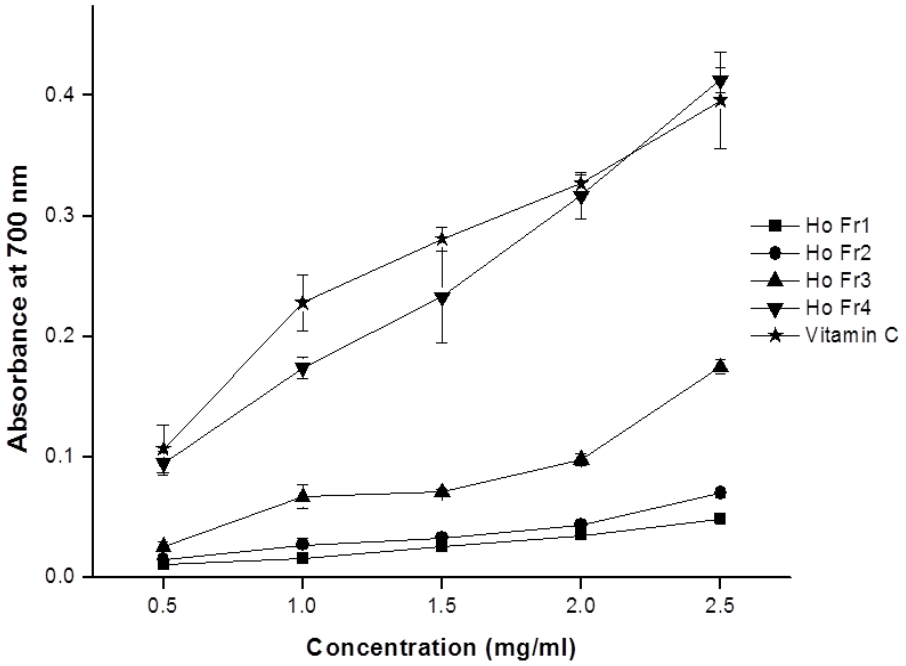


Fig. 31. Reducing power of *H. ovalis* sulfated polysaccharide purified fractions. Values are mean ± SD of three determinations.

Discussion

In the search of new antioxidants from marine environment, marine algae and seagrasses are now being considered as a rich and new source of antioxidants. In recent years, seaweeds and seagrasses are reported to be an effective non-toxic antioxidant candidate. In the present study, the scavenging ability of sulfated polysaccharides and purified fractions of *Sargassum wightii* and *Halophila ovalis* was investigated in a comprehensive manner employing a variety of *in vitro* methods including hydroxyl radical, ABTS, and reducing power assays as free radical scavengers for the prevention of oxidative damage in living organisms.

Hydroxyl radical is the most reactive and toxic that reacts with unsaturated fatty acids of membrane phospholipids to generate free radicals, in turn react with oxygen and forms peroxides quickly (So *et al*., 2007). The peroxides which then acts as free radicals and initiate an autocatalytic chain reaction, leads to loss of unsaturated fatty acids and

extensive damage of cell membrane. Among the most used antioxidant measurement activity of polysaccharides, hydroxyl radicals are those based on the competitive reaction with a probe, which scavenge these radicals (Barahona *et al*., 2011). Hydroxyl scavenging ability of tested samples, measured as the change of the probe absorbance at 30 min, shows complex concentration dependence. The Sw FrIV fraction, with high sulfate content, exhibited strong hydroxyl scavenging activity in concentration dependent, whereas fractions Sw FrI and Sw FrII displayed weak activity. Similarly, purified fraction Ho FrIV exhibited strong hydroxyl scavenging potential than commercial standard vitamin C and other fractions. Whereas, sulfated polysaccharides extracted from brown seaweed *Dictyopteris delicatula* J. V. Lamour. (Dictyotaceae) and seagrass *Halodule wrightii* showed relatively less scavenging activity on hydroxyl radicals (Silva *et al*., 2012; Alves & Rocha, 2011). Low molecular weight sulfated polysaccharides has the potential ability to stop free radical chain reactions.

Reaction with $ABTS^{\bullet+}$ radical is a single electron transfer process takes place faster than DPPH radicals (Lekameera *et al*., 2008). Decolorization of $ABTS^{\bullet+}$ radical by donating electrons or hydrogen atoms and inactivates radical species reflects the potential of antioxidant species (Re *et al*., 1999). Therefore, change in the $ABTS^{\bullet+}$ absorbance at 1 min in the presence of polysaccharide at different concentrations indicated the antioxidant capacity. In the present study, fraction Sw FrIV of *S. wightii* heterofucan and Ho FrIV of *H. ovalis* galactan sulfate scavenged the $ABTS^{\bullet+}$ radicals in a concentration dependent manner. Similarly, low molecular weight chitosan polysaccharide from crustacean shells showed efficient scavenging of $ABTS^{\bullet+}$ (Tomida *et al*., 2009). Similarly, sulfated galactan from red seaweed *Schizymenia binderi* (J. Agardh ex Kutzing) J. Agardh (Schizymeniaceae), and green variant of *Gigartina skottsbergii* Setchell & N. L. Gardner (Gigartinaceae) exhibited ABTS radical scavenging activity than commercial carrageenan (Barahona *et al*., 2011; Barahona *et al*., 2012). This finding suggests that the chemical structure of the polysaccharides play some role on the hydrogen abstraction reaction by the $ABTS^{\bullet+}$ cation radical as reported earlier (Barahona *et al*., 2011).

The antioxidant activity has been reported to have positive correlation with reducing power (Costa *et al*., 2010; Osman *et al*., 2004). The reducing properties are generally

associated with the presence of reductones, which have shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. The reducing capacity of heterofucan and galactan sulfate would result in reduction of Fe^{3+} / ferricyanide complex to the ferrous form (Fe^{2+}) and depending on the reducing power of sulfated polysaccharide, the yellow color of the test solution changes into various shades of green and blue colours (Zhao *et al*., 2006). Similar results have also been reported in *Sargassum filipendula* (Costa *et al*., 2011). Whereas, there was no activity was observed in the sulfated polysaccharides of seagrass *Halodule wrightii* (Silva *et al*., 2012). Our data on the reducing power suggested that, sulfated polysaccharide with high donating-hydrogen was likely to contribute towards the observed antioxidant effect. This findings warrant use as potential natural antioxidant in the processed and/or functional food and pharmaceutical industries. However, it is strongly believed that antioxidant potential depends on the ecology and metabolism of seaweeds (Cornish & Garbary, 2010).

CHAPTER 8

EFFECT OF HETEROFUCAN AND GALACTAN SULFATE ON ENHANCEMENT OF IMMUNE RESPONSE AND DISEASE RESISTANCE OF BLACK TIGER SHRIMP, *PENAEUS MONODON* (FAB) AGAINST WSSV

CHAPTER 8

EFFECT OF HETEROFUCAN AND GALACTAN SULFATE ON ENHANCEMENT OF IMMUNE RESPONSE AND DISEASE RESISTANCE OF BLACK TIGER SHRIMP, *PENAEUS MONODON* (FAB) AGAINST WSSV

8.1. Introduction

White spot syndrome virus (WSSV), is pathogenic to decapod crustaceans including marine and freshwater shrimp, crab, crayfish and lobsters (Flegel, 2006). Indiscriminate use of chemicals and antibiotics in intensive shrimp culture practices lead to environmental degradation (Bachere, 2000). It has been reported that environmental factors play a major role on the severity of disease outbreaks of aquatic animals (Snieszko, 1974). Aquaculture experts tried to solve the problem of the diseases by enhancing the non-specific immune response, which is a central defense mechanism in shrimp. Seaweeds and medicinal plant extracts have been utilized for long time to control WSSV infection and enhance the immune response in shrimps (Immaneul *et al*., 2012; Immanuel *et al*., 2010).

Use of immunostimulants was found to be another approach to increase the immune potential of shrimp to defend against diseases. Recent reports showed that, oral administration of peptidoglycan in shrimp *Penaeus japonicus* acts as a protective immunostimulant against WSSV (Itami *et al*., 1998) and β–1, 3-glucan in *P. monodon* (Chang *et al*., 2003). The non specific immune system of shrimp has found to be induced by feeding with several dietary polysaccharides such as peptidoglycan (Boonyaratpalin & Boonyaratpalin, 1995; Itami *et al*., 1998; Purivirojkul *et al*., 2006), lipopolysaccharide (Takahashi *et al*., 2000), glucan (Chang *et al*., 2003), sodium alginate (Cheng *et al*., 2004), fucoidan (Chotigeat *et al*., 2004; Immanuel *et al*., 2012). Mandal *et al*., (2007) reported that the possible antiviral activity of fucoidan is to inhibit viral sorption so as to inhibit viral-induced syncytium formation. In addition to that, probiotics and medicinal plant extracts acts as potent immunostimulant against WSSV infection in shrimp (Peraza Gomez *et al*., 2011; Peraza Gomez *et al*., 2009; Rameshthangam *et al*., 2007; Balasubramanian *et al*., 2008; Citarasu *et al*., 2006). However, most of these agents

improve the immunity of shrimp against WSSV based on the non-specific recognition between immune factors and the virus.

Haemocytes play a crucial role in the non-specific innate immune system of crustaceans. The haemocyte count varies among crustacean species and is known to be affected by a variety of factors such as infection and environmental stress. Upon activation and degranulation of the haemocytes, the inactive proPO is coverted to the active phenoloxidase (PO) by prophenoloxidase activating enzyme. The PO enzyme thus catalyses the stepwise oxidation of phenols to quinines, followed by several intermediate steps that lead to the formation of melanin. The measurement of PO activity has been proposed as a procedure to evaluate the immune response of shrimps. Similarly, measurement of superoxide anion has been accepted as a precise method for measuring respiratory burst, since superoxide anion (O_2^-) is the first product to be released from the respiratory burst. In recent years, fucoidans from brown seaweed *Sargassum wightii* exhibited protective effects and reduce the impact of WSSV in black tiger shrimp *P. monodon* post larvae (Dechamag *et al*., 2006; Immanuel *et al*., 2010; Immaneul *et al*., 2012). However, there is no report on the effect of *S. wightii* fucoidan on immune response of adult shrimp *P. monodon* documented. Similalrly, the effect of seagrass polysaccharides on immune parameters of *P. monodon* was not evaluated to date. In this background, the present study aimed to investigate the effects of heterofucan from brown alga *S. wightii* and galactan sulfate from *Halophila ovalis* on immune response of adult black tiger shrimp *P. monodon*. The polysaccharide was administered orally at different concentrations and alteration in immune parameters such as, total hemocyte count (THC), protein concentration, prophenoloxidase activity (PPO), and respiratory burst activity (NBT assay) were measured.

8.2. Materials and methods

8.2.1. Preparation of polysaccharide extract

The crude polysaccharide was extracted from brown seaweed *S. wightii* and seagrass *H. ovalis* as described in materials and methods section of chapter 5.

8.2.2. Preparation of test diets

Three test diets containing different concentrations of heterofucan and galactan sulfate from *S. wightii* and *H. ovalis* were prepared by following the procedure described by Yeh *et al*., (2008) (**Table 28**). The basal diet contained 0.2% cellulose (without polysaccharide) served as the control diet. The extracted polysaccharides added individually to the test diets at different concentrations such as 0.25, 0.5, and 1.0 g per kg diet with corresponding decrease in the amount of cellulose. To prepare the diets, ingredients were mixed thoroughly, 2.5% gelatin solution containing active principles with different concentration was added along with oil ingredients, and water was added until a still dough resulted. The resulting dough was cold extruded through a palletizer with appropriate size. The pelletized feed was dried by using hot air oven at 40°C. After drying, the feeds were stored at 4°C in airtight containers until use.

8.2.3. Collection and maintenance of experimental animals

Healthy *Penaeus monodon*, weighing approximately 15 ± 2 g were collected from grow-out ponds at Marakanam, Tamilnadu, India. They were stocked in a fibre glass tank (3000 L capacity) and fed with commercial pelleted feed manufactured by CP AQUACUTURE, INDIA (Crude protein 41%, fat 6%, fiber 2%, ash 13% and moisture 11%) for acclimatization. Before starting the experiment, shrimps were randomly selected and screened by PCR for WSSV infection as described by Yoganandhan *et al*., (2003).

Table 28. Composition of basal diet (g/kg) for *Penaeus monodon*

S. No.	Ingredients	Control	Experimental diets (g/kg)		
		0	0.25	0.5	1.0
1	Fish meal	560	560	560	560
2	Yeast meal	25	25	25	25
3	Soybean meal	60	60	60	60
4	Squid meal	30	30	30	30
5	α- Starch	250	250	250	250
6	Cellulose	2	1.75	1.5	1
7	Heterofucan/ Galactan sulfate	0	0.25	0.5	1
8	Vitamins and Mineral mix	24	24	24	24
9	Cod liver oil	24	24	24	24
10	Binder	25	25	25	25

Source: Yeh *et al*., (2008) modified

8.2.4. Feeding experiment

Uniform size of *P. monodon* were selected from the acclimatized stock and transferred in to individual experimental FRP tanks (0 g – control and 0.25, 0.5, and 1.0 g per kg experimental diets). The shrimps were maintained with continuous flow-through water and constant aeration system to provide optimal oxygen level. The water quality parameters such as temperature (28 ± 1°C), salinity (32 ± 1 ppt), and pH (8.2 ± 0.1) were maintained every day. The shrimp were fed *ad libitum* thrice a day at 7.00, 15.00 and

23.00 h at a rate of 10% body weight. Uneaten food and waste matters were removed before feeding and 25% water was exchanged daily during the experimental period. The feeding experiment was carried out for the period of 25 days. Simultaneously, triplicates were maintained in each group.

8.2.5. Preparation of WSSV inoculum

WSSV-infected black tiger shrimp, *Penaeus monodon*, with typical white spot disease, were collected from a Shrimp farm near Marakanam, Tamilnadu, India. Gills and hepatopancreas were homogenized in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4) at 1:10 (w/v) and centrifuged at 8000 g for 10 min at 4°C. The supernatant obtained was then filtered through 0.22 µm filter and stored at –20°C until use. The presence of WSSV in the tissue sample was checked by nested PCR assay (Yoganandhan *et al*., 2003; Immanuel *et al*., 2010).

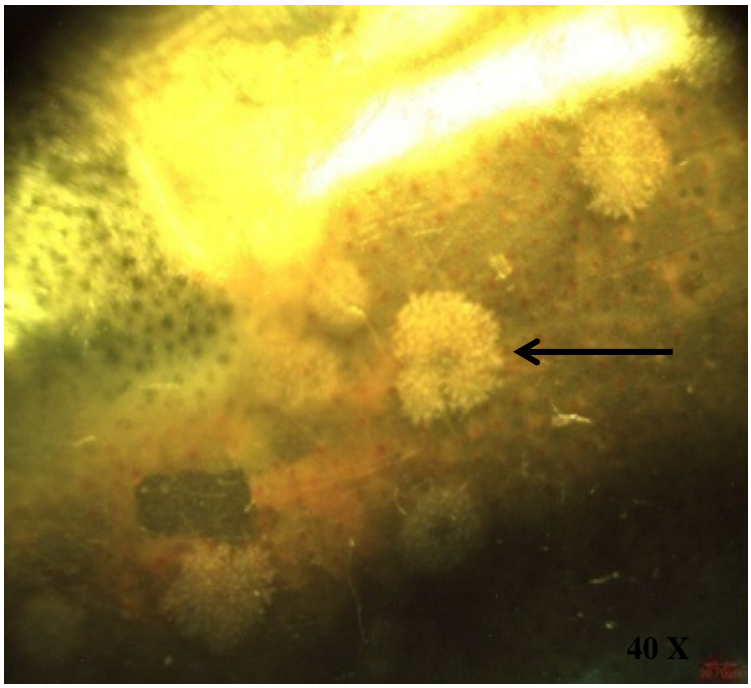


Fig. 32. Stereozoom microscopic image of white spots in the cephalothorax region of WSSV infected shrimp indicated by arrow.

8.2.6. WSSV challenge test

After 25 days of feeding experiment, 25 shrimps from each dietary group in triplicate ($n = 25 \times 3 = 75$) were collected and reared separately in sterile 1000 L FRP tanks with sterilized aerated water. The tanks were covered properly to avoid contamination. The shrimp were fed on the polysaccharide supplemented diets and control group was fed with unsupplemented diet. The WSSV filtrate prepared from infected shrimps were intramuscularly injected at a dose of 0.01 ml in the second abdominal segment per shrimp. Similarly, a negative control group of shrimp was injected with 0.01 ml saline per shrimp. After WSSV injection, the survival of *P. monodon* was monitored at regular intervals of 6 h until all the shrimp had succumbed. The dead shrimp were removed from the respective tanks during each observation intervals. The results obtained in every 6 h intervals were pooled and presented as per day interval up to 21 days. The immunological parameters such as total hemocyte count (THC), prophenoloxidase activity (PPO), superoxide anion activity (NBT) were analyzed in the haemolymph samples of shrimps before injection of WSSV (0 day), 7 days and after 21st days of challenge experiment (Chang *et al*., 2003).

8.2.7. Immunological assays

8.2.7.1. Hemolymph collection

Hemolymph (0.5 ml) was withdrawn from the heart of control, treated and WSSV infected shrimp (triplicates) using a 26-guage needle fitted to a tuberculin syringe containing 100 µl of ice cold hemolymph anticoagulant solution (HAS) (115 mM glucose, 30 mM sodium citrate, 10 mM EDTA, and 338 mM NaCl).

8.2.7.2. Total hemocyte count (THC)

The total hemocyte count in control and polysaccharide supplemented diets fed shrimp was calculated using hemocytometer as described in general materials and methods section **3.2.6.1**.

8.2.7.3. Total protein concentration

The protein concentration in the hemolymph of control and polysaccharide supplemented diets fed shrimp was measured by Lowry's method as described in general materials and methods section **3.2.3.4**.

8.2.7.4. Prophenoloxidase assay

Phenoloxidase activity in supernatant of hemolymph from control, treated and infected shrimps was spectrophotometrically determined as described in general materials and methods section **3.2.6.2**.

8.2.7.4. Superoxide anion analysis (NBT reductase assay)

Superoxide anion was quantified in control, polysaccharide supplemented diets fed groups using a microplate reader and expressed as NBT reduction in haemolymph as described in general materials and methods section **3.2.6.3**.

8.2.8. Statistical analysis

All data obtained in the present study were expressed as mean ± standard deviation. Statistical differences between the experimental groups were analyzed using students '*t*' test with a *post hoc* multiple comparison of Tukey's test at a significant level of $P < 0.05$. All computations were done by employing statistical software (SPSS Version 7.5).

8.3. Results

8.3.1. Survival and WSSV prevalence

The survival of control and experimental groups of *P. monodon* after challenged with WSSV is given in Fig. 33.

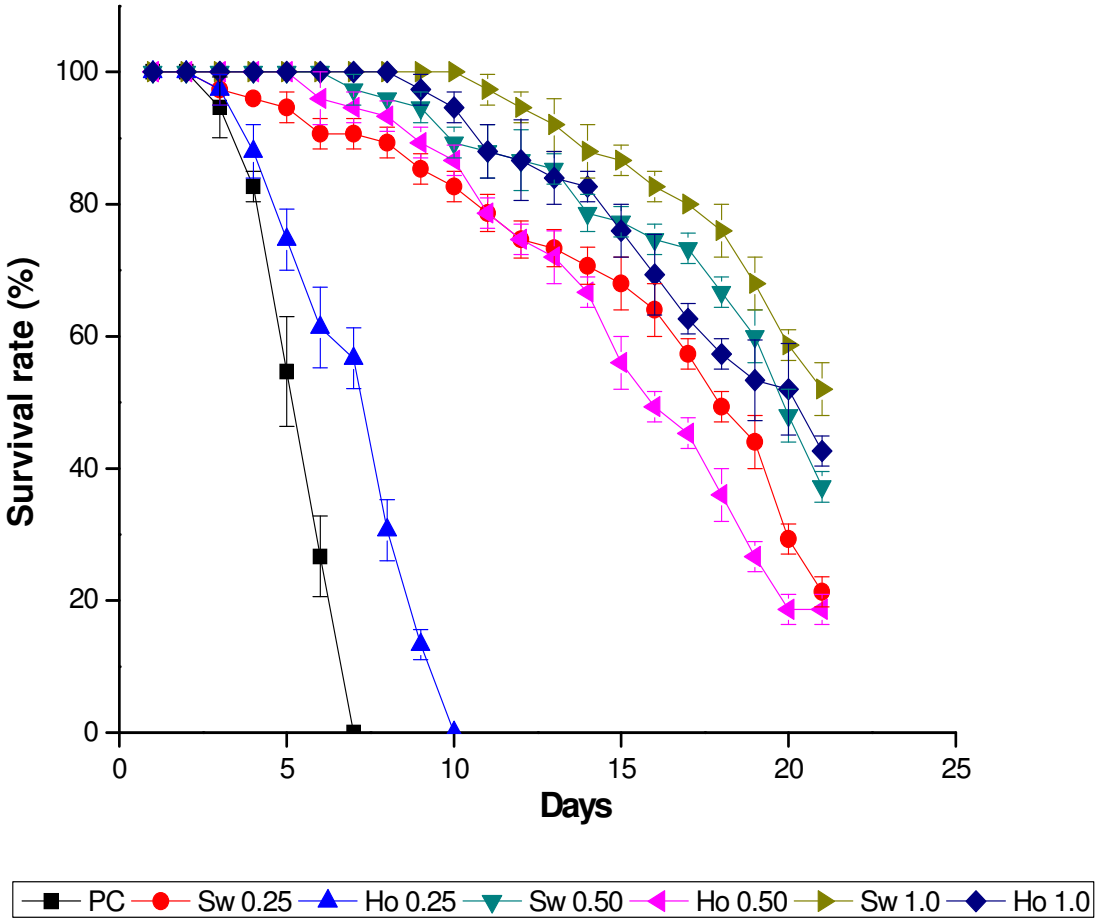


Fig. 33. Survival rate of shrimp *P. monodon* fed on different concentrations of heterofucan and galactan sulfate supplemented diets during challenge experiment with WSSV in different days interval. Values are mean ± SD of three determinations. PC- Positive control, Sw- *S. wightii*, Ho- *H. ovalis*.

The shrimp were succumbed to death started from 3rd day of challenge test with 94% survival in control group. Whereas, the survival rate recorded was 97% on 3rd day in 0.25 g/kg heterofucan of *S. wightii* and galactan sulfate of *H. ovalis* supplemented diets fed shrimps. In 0.5 g/kg of heterofucan and galactan sulfate supplemented diets fed shrimp, the survival rate was started to decrease from 7th day and 6th days of challenge test. But in the highest concentration (1 g/kg) of heterofucan and galactan sulfate, the survival rate was started to decrease from 10th and 9th days of challenge test. When the duration of the challenge experiment increased, the survival rate was decreased gradually and 100% mortality was observed in control group within 7 days, whereas, in the experimental groups, the survival was prolonged up to 21 days of challenge experiment. Within 21 days, 21, 37, and 52% survival was recorded in 0.25, 0.5, and 1.0 g/kg concentrations of heterofucan supplemented diets fed shrimps and 0, 18, and 42% survival in 0.25, 0.50, and 1.0 mg/kg concentrations of galactan sulfate supplemented fed shrimps.

8.3.2. Immunological assays

8.3.2.1. Total hemocyte count (THC)

The total hemocyte count (THC) in the hemolymph of control group was 1.23×10^7 cells/ml at the beginning of the challenge experiment. Whereas, the counts were significantly ($P < 0.05$) increased with increasing concentration of heterofucan and galactan sulfate. At 0.25 g/kg, the THC was 1.48 and 1.24×10^7 cells/ml, whereas it was 1.69, 1.39×10^7 cells/ml in 0.50 g/kg and 1.91, 1.51×10^7 cells/ml in 1.0 g/kg of heterofucan and galactan sulfate supplemented diets fed shrimp respectively. The THC in all the groups of shrimp decreased considerably during 7th day with respect to WSSV challenge. In control group it was 0.91×10^7 cells/ml, whereas 1.39, 1.19×10^7 cells/ml (0.25 g/kg), 1.48, 1.27×10^7 cells/ml (0.50 g/kg), and 1.55, 1.40×10^7 cells/ml (1.0 g/kg) respectively in heterofucan and galactan sulfate supplemented diets fed shrimp.

However, the THC was recovered and increased with increasing concentration of test diets at the end of the challenge experiment (21st day). For instance, the THC recorded was 1.75, 0.00 × 10^7 cells/ml (0.25 g/kg), 2.23, 1.44 × 10^7 cells/ml (0.50 g/kg), and 2.57, 1.81 × 10^7 cells/ml (1.0 g/kg) in heterofucan and galactan sulfate supplemented diets fed shrimp respectively (Fig. 34).

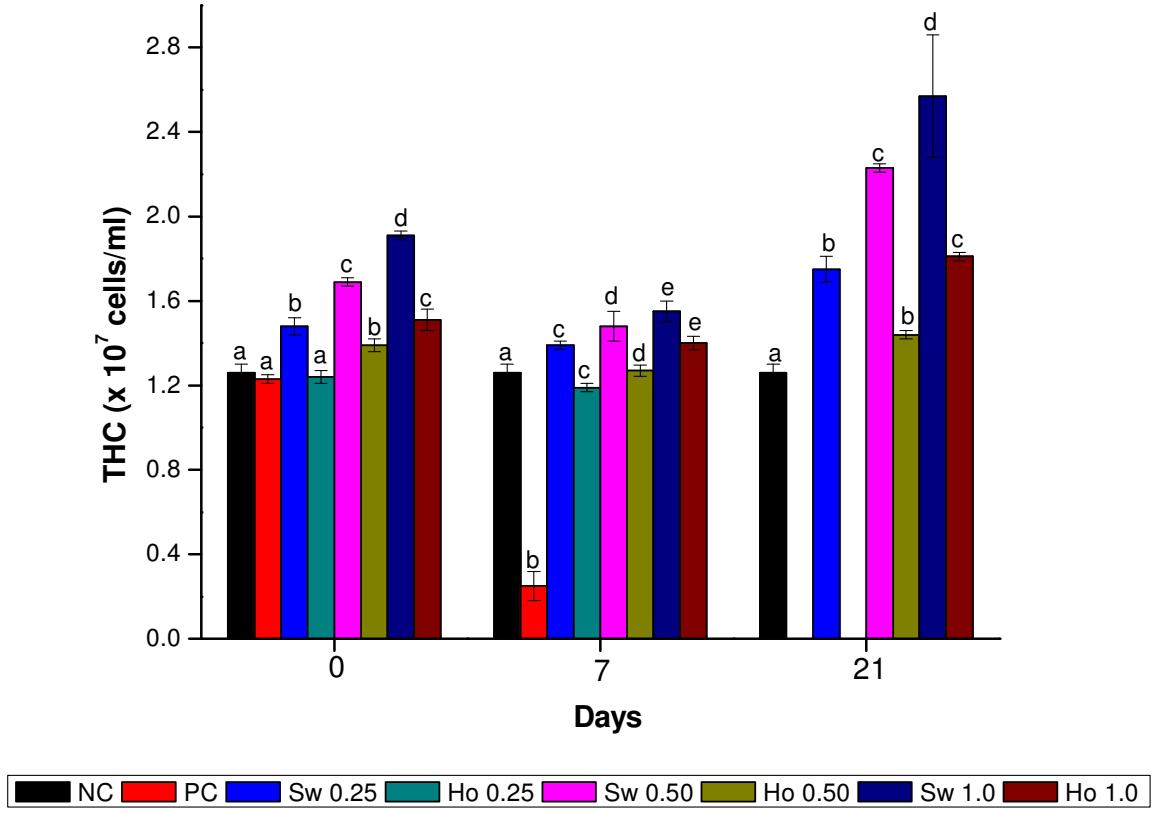


Fig. 34. Total hemocyte count (THC) of shrimp *P. monodon* fed on different concentrations of heterofucan and galactan sulfate supplemented diets during challenge experiment with WSSV in different days interval. Values are mean ± SD of three determinations; bars with different letters are statistically significant from each other (t-test; $P < 0.05$ subsequent post hoc multiple comparison with Tukey's test) (NC- Negative control, PC- Positive control, Sw- *S. wightii*, Ho- *H. ovalis*).

8.3.2.2. Total protein concentration

At the initial day of the challenge experiment (0 day), the protein concentration of control group was 110 mg/ml, whereas it was significantly ($P < 0.05$) increased (121–125 mg/ml) in the experimental groups fed with different concentrations (0.25–1.0 g/kg) of heterofucan supplemented diets. Similarly, protein concentration was increased (116–133 mg/ml) significantly ($P < 0.05$) in the experimental groups fed with different concentrations of galactan sulfate of *H. ovalis*.

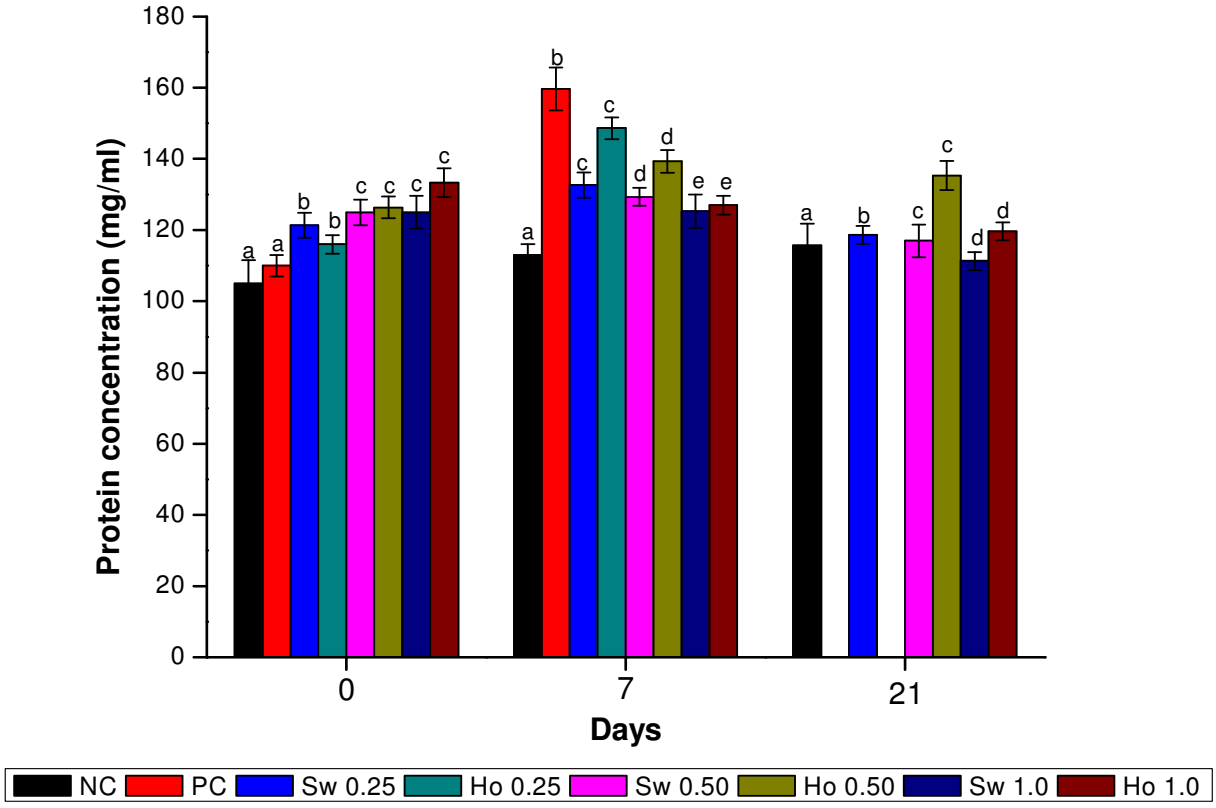


Fig. 35. Total protein concentration of shrimp *P. monodon* fed on different concentrations of heterofucan and galactan sulfate supplemented diets during challenge experiment with WSSV in different days interval. Values are mean ± SD of three determinations; bars with different letters are statistically significant from each other (t-test; $P < 0.05$ subsequent post hoc multiple comparison with Tukey's test) (NC- Negative control, PC- Positive control, Sw- *S. wightii*, Ho- *H. ovalis*).

The protein concentration was increased considerably in both control and experimental groups during 7th day with respect to WSSV challenge. In control group, the protein concentration was at maximum (159 mg/ml). Further the protein concentration was significantly ($P < 0.05$) low (132, 129, 125 mg/ml) in the experimental groups fed with 0.25, 0.50, 1.0 g/kg of heterofucan. Similarly, the protein concentration was low (148, 139, 127 mg/ml) in the experimental groups fed with 0.25, 0.50, 1.0 g/kg of galactan sulfate. However, at the end of the challenge study, the protein concentration was recovered (118–111 mg/ml), and (135–119 mg/ml) respectively in 0.25–1.0 g/kg heterofucan and galactan sulfate supplemented diets fed shrimp (Fig. 35). The variation between the protein concentration of hemolymph in all the experimental groups is statistically significant ($P < 0.05$).

8.3.2.3. Prophenoloxidase activity

The prophenoloxidase activity (PO) among the experimental groups fed with different concentrations (0.25–1.0 g/kg) of heterofucan and galactan sulfate showed a significant ($P < 0.05$) increase when compared with the control group. At the beginning of the challenge experiment (0 day), the PO activity of control group was 0.1476 (OD), whereas it was significantly ($P < 0.05$) increased (0.1763–0.2100 OD), and (0.1623–0.1980 OD) in the experimental groups fed with different concentrations (0.25–1.0 g/kg) of heterofucan and galactan sulfate supplemented diets fed shrimp. Whereas, the PO activity notably decreased in both control (0.0263 OD) and experimental groups (0.0563–0.0786 OD), and (0.0520–0.0770 OD) fed with different concentrations (0.25–1.0 g/kg) of heterofucan and galactan sulfate supplemented diets fed shrimp when the duration of WSSV challenge experiment increased. Invariably at the end of challenge experiment, the PO activity steadily increased (0.1790–0.2180 OD) in heterofucan, and (0.1740–0.1813 OD) galactan sulfate (except 0.25 g/kg) supplemented diets fed shrimp, respectively (Fig. 36).

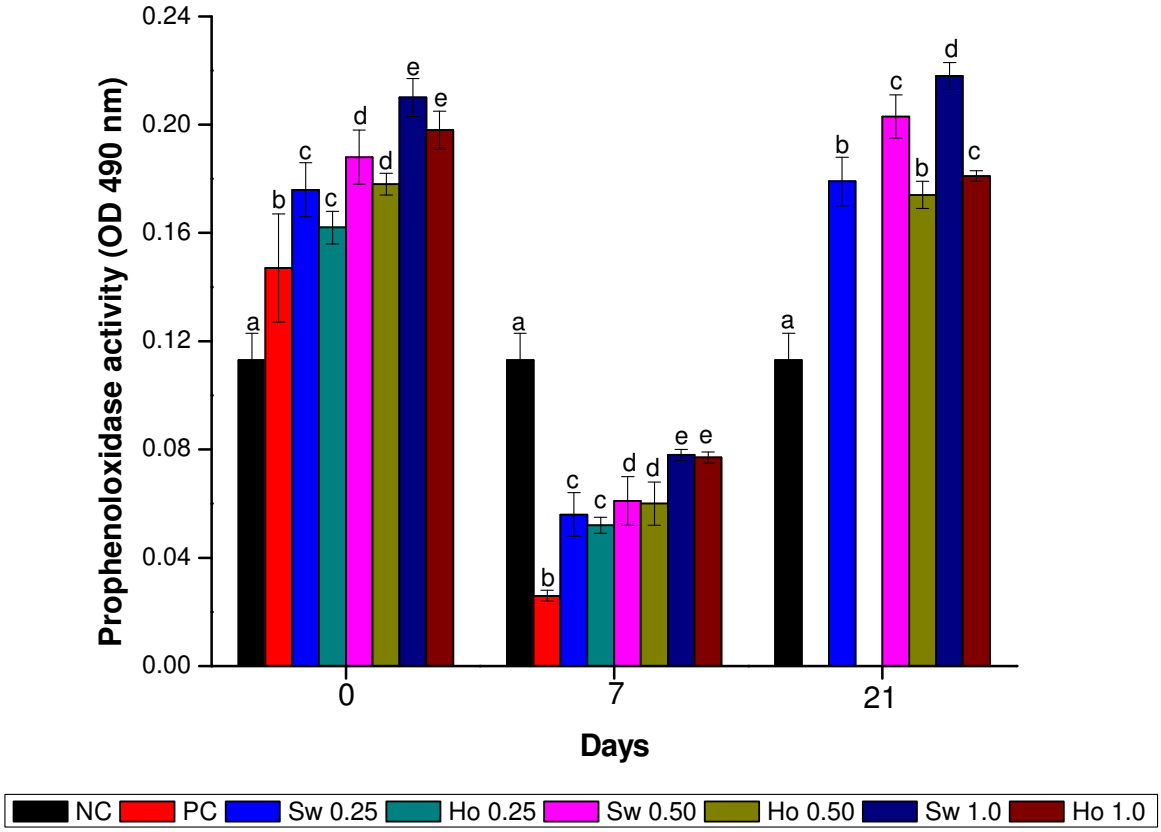


Fig. 36. Prophenoloxidase activity (PO) of shrimp *P. monodon* fed on different concentrations of heterofucan and galactan sulfate supplemented diets during challenge experiment with WSSV in different days interval. Values are mean ± SD of three determinations; bars with different letters are statistically significant from each other (t-test; $P < 0.05$ subsequent post hoc multiple comparison with Tukey's test) (NC- Negative control, PC- Positive control, Sw- *S. wightii*, Ho- *H. ovalis*).

8.3.2.4. Respiratory burst activity (NBT assay)

Shrimp infected with WSSV by intramuscular injection and oral administration showed a significant ($P < 0.05$) increase in respiratory burst activity in comparison with the control group (Fig. 37). The respiratory burst activity of control group was (0.340 OD) on 0 day,

but it was increased significantly ($P < 0.05$) to (0.540–0.930 OD), (0.413–0.816 OD) in heterofucan and galactan sulfate supplemented diets fed shrimp in concentration dependent manner. On 7th day of challenge experiment, the respiratory burst activity was significantly increased ($P < 0.05$) in experimental groups fed with heterofucan (0.583–0.950 OD), and galactan sulfate (0.493–0.850 OD), whereas in control group, it was decreased (0.090 OD). At the end of challenge experiment (21st day), the respiratory burst activity was gradually decreased (0.440–0.877 OD) in heterofucan, and (0.67–0.816 OD) in galactan sulfate (except 0.25 g/kg) supplemented diets fed shrimp, respectively.

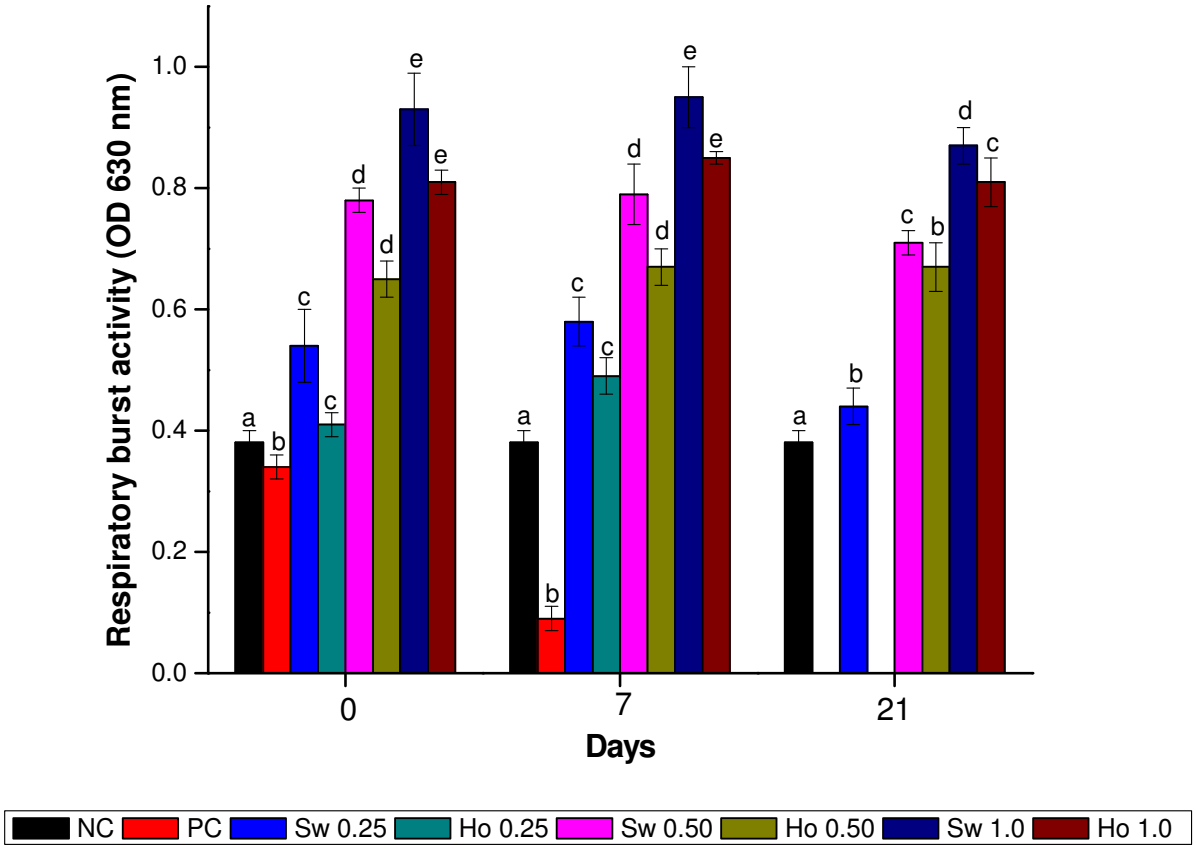


Fig. 37. Respiratory burst activity (NBT assay) of shrimp *P. monodon* fed on different concentrations of heterofucan and galactan sulfate supplemented diets during challenge experiment with WSSV in different days interval. Values are mean ± SD of three determinations; bars with different letters are statistically significant from each other (t-test; $P < 0.05$ subsequent post hoc multiple comparison with Tukey's test) (NC- Negative control, PC- Positive control, Sw- *S. wightii*, Ho- *H. ovalis*).

8.4. Discussion

Seaweeds have been extensively used for studying their antiviral activities (Naqvi *et al*., 1980; Caccamese *et al*., 1980). Antivirals isolated from seaweeds included a sulfated polysaccharide from *Sargassum horneri* (Hoshino *et al*., 1998), *S. patens* (Zhu *et al*., 2006), water soluble polysaccharide from *S. latifolium* (Asker *et al*., 2007) has been reported. Extensive research revealed that *Halophila ovalis* has a broad range of pharmacological effects such as anti-inflammatory, antioxidant, antibacterial, antifungal, and immunomodulatory activities (Bernard & Pesando, 1989; Hua *et al*., 2006; Yuvaraj *et al*., 2012). In the present study, heterofucan from *S. wightii* and galactan sulfate from seagrass *H. ovalis* extracted was investigated on reduction of WSSV viral particles and immune response in shrimp *P. monodon*. The heterofucan extracted from *S. wightii* contains 76.2% of total sugars and 19.0% of sulfate content. The total sugar and sulfate contents of *H. ovalis* galactan sulfate were 74.9 and 21.0% respectively. Similarly, Immanuel *et al*., (2012) have reported that the fucoidan of *S. wightii* contain 70.61 and 45.06% of total sugars and sulfate. The chemical composition of fucoidans varies depending on habit, area of cultivation, harvesting period, extraction methods and other parameters (Rioux *et al*., 2009; Ponce *et al*., 2003; Bilan *et al*., 2006).

The heterofucan and galactan sulfate was supplemented with pellet diets at three different concentrations (0.25, 0.50, and 1.0 g/kg) and fed to shrimp *P. monodon* for 25 days. After feeding experiment, the WSSV challenge test was performed and the survival rate of experimental groups of shrimp increased from 21 to 52% (heterofucan supplemented), and 0 to 42% (galactan sulfate supplemented) over control groups after 21 days of infection. The increase in survival rate of all the tested groups increased with increasing concentrations of heterofucan and galactan sulfate. Similarly, Chotigeat *et al*., (2004) have reported that the oral administration of fucoidan from *S. polycystum* increased the survival rate against WSSV infection in *P. monodon*. Similarly, oral administration of partially purified fucoidan (PPF) from *Cladosiphon okamuranus* increased the survival rate of *P. japonicus* against WSSV infection (Takahashi *et al*., 1998). They reported the survival rate of 82.4% in 100 mg/kg PPF-fed groups of shrimp (12.3 g in size) after 10days of WSSV challenge experiment. However, in the present study, the survival rate

of 82.6% in 250 mg/kg crude heterofucan fed groups of shrimp after 10 days of WSSV challenge experiment. This result suggests that the possible mechanism of inhibition of heterofucan and galactan sulfate against virus is that the negative charges of the sulfate group of the polysaccharide bind with positive charges of amino acids at V3 loop of viral envelope glycoprotein (Immanuel *et al*., 2012).

Medicinal herbs and seaweeds have long been used as dietary supplements, because they contain numerous biologically active compounds, such as polysaccharides that enhance immune function, and increase host resistance to bacterial, viral, and parasitic infections (Harikrishnan *et al*., 2011; Immanueal *et al*., 2012; Citarasu *et al*., 2006). In the present study, effect of heterofucan and galactan sulfate on immunological parameters was analyzed during WSSV challenge experiment. Hemocytes play an important role in immune defence processes such as clotting, non-self recognition, phagocytosis, melanization, encapsulation, cytotoxicity and cell-to-cell communication (Johansson *et al*., 2000). Thus, hemocyte number can help in monitoring shrimp health as lower than normal numbers of circulating hemocytes in crustacean correlate well with a reduced resistance to pathogens. In this study, at the initial day of WSSV challenge experiment, the THC was significantly ($P < 0.05$) increased with increasing concentration of heterofucan and galactan sulfate in 0.25–1.0 g/kg supplemented diet fed shrimp than control group (1.23×10^7 cells/ml). When the WSSV challenge duration prolonged, the THC decreased considerably during 7th day in all the groups. The THC was recovered and increased with increasing concentrations of heterofucan and galactan sulfate at the end of WSSV challenge experiment. Similarly, Pholdaeng & Pongsamart, (2010) have reported the effect of polysaccharide gel (PG) extracted from *Durio zibethinus* on THC in shrimp *P. monodon* against WSSV challenge. They observed that the THC level of 1–3% PG-supplemented diets fed shrimp showed higher values than that of the positive control group. The results of our study are consistent with existing reports. The increase in hemocytes after heterofucan and galactan sulfate administration was attributed to accelerated maturation of hemocyte precursors in the hematopoietic tissue followed by release of new cells into the circulation (Soderhall *et al*., 2003).

Crustaceans have an open circulatory system in which the hemolymph carries out several physiological functions. The protein concentration in hemolymph of crustaceans was found to be high due to WSSV infection (Lo *et al*., 1997). At the beginning (0 day) of WSSV challenge test the protein concentration of experimental shrimp was significantly ($P < 0.05$) increased with increasing concentration of heterofucan and galactan sulfate (0.25–1.0 g/kg) supplemented diet fed group than control group. When the WSSV challenge duration prolonged, the protein concentration increased considerably during 7th day in all the groups. The increase of protein content in WSSV infected shrimp is due to the action of proteases that melt the tissues and that the proteins of melted tissues incorporated into hemolymph of shrimp (Beckage, 1996). At the end of challenge experiment (21st day), the shrimp recovered normal protein concentration in heterofucan and galactan sulfate supplemented diets fed shrimp. Similarly Lo *et al*., (1997) and Citarasu *et al*., (2006) have reported the high concentrations of protein in the hemolymph of crustaceans due to WSSV heavy load.

Phenoloxidase (PO) is a key enzyme, in the synthesis of melanin, occurs in hemolymph as an inactive proenzyme prophenoloxidase (proPO). proPO is activated to form PO when it reacts with zymosan, LPS, urea, calcium ions, trypsin, or heat. The activated proPO system is involved with some important molecules that are released to perform crucial immune responses, including non-self recognition, melanin formation, adhesion, and cell-to-cell communication (Immaneul *et al*., 2012). In this study, PO activity of experimental shrimp was significantly ($P < 0.05$) increased in concentration dependent manner than control group during WSSV challenge test. Whereas, the PO activity decreased considerably when the duration of WSSV challenge experiment increased (7th day). But at the end of challenge experiment, the shrimp recovered normal PO activity in fucoidan and galactan sulfate supplemented diets fed shrimp. Similarly, dietary effect of β- 1, 3-glucan and fucoidan showed higher PO activity of *P. monodon* challenged with WSSV than control group at the end of challenging experiment (Chang *et al*., 2003; Immanuel *et al*., 2012). Felix *et al*., (2004) observed the highest prophenoloxidase activity of 0.62 unit/min/mg. proteins on the 12th day with experimental diet contains 10 g/kg of *S. wightii* powder. In addition to that, Sung *et al*., (1996) found that

P. monodon fed with peptidoglycan showed better growth and phenoloxidase activity compared to those fed with normal diet.

Reactive oxygen intermediates (ROIs) including superoxide anion are formed by phagocytic cells during respiratory bursts of phagocytosis, which represent a defence mechanism against microbial infection. Thus, the production of O_2^- (respiratory burst) has been reported as an accurate method to measure the effectiveness of potential immunostimulants (Song & Hsieh, 1994; Munoz *et al*., 2000). However, the excessive accumulation of ROIs is extremely toxic to host cells, and the damaging effects of ROIs are neutralized by the antioxidant defense system (Halmblad & Soderhall, 1999). In the present study, the respiratory burst activity of experimental groups of shrimp at the beginning (0 day) was significantly ($P < 0.05$) higher than control group after WSSV challenge test. On the 7th day of challenge experiment, the respiratory burst activity was decreased in control group, whereas in experimental group, it was increased with increasing concentration. Nevertheless, at the end of challenge experiment (21st day), the shrimp recovered normal respiratory burst activity in heterofucan and galactan sulfate supplemented diets fed shrimp. Song *et al*., (1997) have reported that the relative in vivo intracellular O_2^- production of hemocytes increased 15.7 times following oral administration of BG for 24 days in brooder *P. monodon*. Similarly, Balasubramanian *et al*., (2008) have reported that the effect of *Cynodon dactylon* extract on respiratory burst activity of *P. monodon* challenged with WSSV. The present study imperatively emphasized that oral administration of heterofucan and galactan sulfate supplemented diets fed shrimp act as immunostimulant via enhancing the immune response has a positive effect of increasing disease resistance.

CHAPTER 9
SUMMARY

In the present study, sulfated polysaccharides extracted from brown seaweed *Sargassum wightii* and seagrass *Halophila ovalis* was characterized for its functional properties such as anti-inflammatory, antioxidant, and immunostimulant *in vitro* and *in vivo*. Antimicrobial susceptibility test is the primitive step towards new anti-infective drug development in the ethanopharmacology research. Hence, a total of eleven seaweeds and four seagrasses collected from the east coast of India in Tamilnadu were screened for antibacterial activity. The cleaned samples were dried and crude bioactive compound was extracted using different polar and non-polar solvents. All the solvent extracts were screened for antibacterial property using disc diffusion method. Of these, methanol extract of green seaweed *Cladophora glomerata*, brown seaweed *S. wightii* and seagrass *H. ovalis* showed appreciable antibacterial activity against human pathogen *A. baumannii* and *B. subtilis*. The crude compound was purified by thin layer chromatography using different solvent systems and the fractions were screened for antibacterial activity. The purified active fractions were subjected to GC-MS for the identification of chemical constituents compared with NIST library. We found that hydrocarbons in green seaweed *C. glomerata*, ketones and esters in brown seaweed *S. wightii* and triacylglycerols in seagrass *H. ovalis* are the chemical constituents. Of these, three samples, *S. wightii* and *H. ovalis* preferred over *C. glomerata* for further study. Sulfated polysaccharides from these species were extracted by hot water and enzymatic method. FT-IR spectral analysis revealed the presence of different functional groups. The presence of sulfate groups in the polysaccharide identified by stretching vibrations at 1251 cm^{-1} common to all sulfate esters and additional sulfate groups at 821 cm^{-1} (C–O–S, secondary equatorial sulfate). Monosaccharide composition of *S. wightii* and *H. ovalis* crude sulfated polysaccharide was determined by LC-MS after hydrolysis with 4 M TFA. Sulfated polysaccharide of *S. wightii* contained fucose and galactan sulfate of *H. ovalis* revealed galactose as the major sugar along with other sugars in minor amounts. The crude polysaccharide was purified by anion-exchange chromatography using NaCl solution in a step-wise gradient

method. Four different fractions were obtained from each species. All four fractions were collected and dialyzed extensively against distilled water and lyophilized. Analyses were performed to determine the total sugars, protein, and sulfate contents in crude as well as purified fractions. Fraction IV of *S. wightii* and *H. ovalis* contained high sulfate content than other fractions. Molecular mass of crude and purified fractions were determined by size exclusion chromatography using standard dextrans of different molecular weights. Molecular mass of *S. wightii* and *H. ovalis* crude sulfated polysaccharide was identified to be 15×10^3 Da and 20×10^3 Da respectively. Purified fractions III of *S. wightii* and *H. ovalis* were identified to be more than 10 KDa. Whereas, fraction IV of *S. wightii* and *H. ovalis* was found to be 10 KDa in size. Fraction III and IV of *S. wightii* and *H. ovalis* relatively contained high sugar and sulfate content. Therefore, these two fractions were preferred over fraction I and II for antinociceptive and anti-inflammatory study *in vivo*. The antinociceptive effect was determined by formalin and hot-plate test. Formalin test revealed that fractions III and IV of *S. wightii* and *H. ovalis* act as central analgesics by inhibiting both phases in a dose dependent manner. Similarly, both fractions of *S. wightii* and *H. ovalis* increase the latency to thermal stimuli via a central-acting mechanism. The link between antinociception and anti-inflammatory pain was determined by carrageenan-induced paw edema and Freund's adjuvant induced arthritis method. At 10 mg/kg fraction IV of *S. wightii* and *H. ovalis* showed marked reduction in edema induced by carrageenan than diclofenac treated group at all time intervals. Similarly, fraction IV of *S. wightii* and *H. ovalis* (10 mg/kg) exhibited significant paw edema inhibition of about 73% and 77.1% in arthritis-induced rats on day 14, respectively. Furthermore, inhibition of proliferation of inflammatory cells and production of inflammatory cytokines was determined *in vitro*. We observed that fraction IV of *S. wightii* and *H. ovalis* has profound inhibitory effect on PHA activated PBMC in a concentration dependent manner. In order to determine the effect of sulfated polysaccharide fraction IV of *S wightii* and *H. ovalis* on chemokine IL-8 secretion, the human colonic cancer cell line HT-29 was induced by TNF-α and the effect of purified fraction IV on IL-8 secretion was investigated. The present study interestingly showed that IL-8 secretion was reduced in a concentration dependent manner in TNF-α induced HT-29 cell line. Free radicals/reactive oxygen

species (ROS) involved in the pathogenesis of inflammation. Therefore, fractions of *S. wightii* and *H. ovalis* sulfated polysaccharide were investigated for antioxidant activity using different *in vitro* methods. In this study, fractions with low molecular weight and high sulfate content showed good antioxidant activity by scavenging different radicals tested in a concentration dependent manner than fractions with low sulfate content. Furthermore, immune response of heterofucan of *S. wightii* and galactan sulfate of *H. ovalis* was studied on adult black tiger shrimp *P. monodon* infected with white spot syndrome virus. The heterofucan and galacatan sulfate supplemented diets were fed to shrimp for 25 days, then challenged with WSSV and the survival rate was recorded daily up to 21 days. The control group showed 100% mortality within 7 days during the challenge test. The experimental groups showed increase in survival rate within 21 days in heterofucan, galactan sulfate diets fed shrimp. Immune parameters such as THC, protein concentration, prophenoloxidase activity, respiratory burst activity showed significant difference than control groups during the WSSV challenge experiment. These results demonstrated that oral administration of heterofucan and galactan sulfate supplemented diets fed shrimp act as immunostimulant via enhancing the immune response has a positive effect of increasing disease resistance. Overall, study demonstrated the antinociceptive and anti-inflammatory activity of brown seaweed *S. wightii* and seagrass *H. ovalis* sulfated polysaccharides and its use as potential natural antioxidant in the processed and/or functional food and pharmaceutical industries. The sulfated polysaccharides of above seaweed and seagrass could be employed as immunostimulant in aquaculture practices.

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PUBLICATIONS

Accepted for publication in Journal of Medicinal Food – March 2013

Antinociceptive and anti-inflammatory activities of *Sargassum wightii* and *Halophila ovalis* sulfated polysaccharides in experimental animal models

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ABSTRACT

The present study investigated the effects of sulfated polysaccharides from brown seaweed *Sargassum wightii* (Sw-SP) and seagrass *Halophila ovalis* (Ho-SP) in nociceptive and inflammatory models. In the formalin test, Sw-SP and Ho-SP significantly reduced licking time in both phases of the test at a dose of 10 mg/kg. In the hot plate test, the antinociceptive effect was observed only in animals treated with 10 mg/kg of Sw-SP and 5, 10 mg/kg of Ho-SP, suggesting that the analgesic effect occurs through a central action mechanism at the higher dose. Sw-SP and Ho-SP (10 mg/kg) significantly inhibited paw edema induced by carrageenan, especially at 3 hr after treatment and potentially decreased neutrophil migration by 53 and 52% respectively. In Freund's adjuvant induced arthritic rats, there was significant increase in rat paw volume and decrease in body weight, but in Sw-SP and Ho-SP treated groups (10 mg/kg) a significant reduction in paw volume and normal gain in body weight was observed. The present results indicate that Sw-SP and Ho-SP possess antinociceptive and anti-inflammatory effects and have potential usefulness for development as therapeutic agents.

Keywords: ***antinociception· anti-inflammatory· H. ovalis· sulfated polysaccharides· S. wightii***

Pharmaceutical Biology, 2011, 1–10, Early Online

ISSN 1388-0209 print/ISSN 1744-5116 online
DOI: 10.3109/13880209.2011.611948

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RESEARCH ARTICLE

Seagrass as a potential source of natural antioxidant and anti-inflammatory agents

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Abstract

Context: *Halophila* spp. is a strong medicine against malaria and skin diseases and is found to be very effective in early stages of leprosy. Seagrasses are nutraceutical in nature and therefore of importance as food supplements.

Objective: The antibacterial, antioxidant, and anti-inflammatory activities of *Halophila ovalis* R. Br. Hooke (Hydrocharitaceae) methanol extract were investigated and the chemical constituents of purified fractions were analyzed.

Materials and methods: Plant materials were collected from Pondicherry coastal line, and antimicrobial screening of crude extract, and purified fractions was carried out by the disc diffusion method and the minimum inhibitory concentration (MICs) of the purified fractions and reference antibiotics were determined by microdilution method. Antioxidant and anti-inflammatory activities were investigated *in vitro*. Chemical constituents of purified fractions V and VI were analyzed by gas chromatography–mass spectrometry (GC–MS), and the phytochemicals were quantitatively determined.

Results: Methanol extract inhibited the growth of *Bacillus cereus* at a minimum inhibitory concentration of 50 µg/mL and other Gram-negative pathogens at 75 µg/mL, except *Vibrio vulnificus*. Reducing power and total antioxidant level increased with increasing extract concentration. *H. ovalis* exhibited strong scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radicals at IC_{50} of 0.13 and 0.65 mg/mL, respectively. Methanol extract of *H. ovalis* showed noticeable anti-inflammatory activity at IC_{50} of 78.72 µg/mL. The GC–MS analysis of *H. ovalis* revealed the presence of triacylglycerols as major components in purified fractions. Quantitative analysis of phytochemicals revealed that phenols are rich in seagrass *H. ovalis*.

Discussion and conclusion: These findings demonstrated that the methanol extract of *H. ovalis* exhibited appreciable antibacterial, noticeable antioxidant, and anti-inflammatory activities, and thus could be use as a potential source for natural health products.

Keywords: Antibacterial, GC–MS, *Halophila ovalis*, MIC, triacylglycerols

Introduction

Seagrasses are submerged marine angiosperms that grow successfully in tidal and subtidal marine environments except in polar regions. The possibility of collecting organisms directly from the ocean with the use of SCUBA opened a new gate to a largely untapped resource with a wide range of unique structures and novel compounds. Seagrasses are well documented for the presence of potent diverse secondary metabolites (Puglisi et al., 2007).

Vibrio spp., especially luminous *Vibrio harveyi* Johnson & Shunk (Vibrionaceae), and *Vibrio parahaemolyticus* Fujino et al. (Vibrionaceae), have been implicated as the main bacterial pathogens of shrimp in hatcheries as well as farms (Sandip et al., 2009). Problems including solubility, palatability, toxicity, cost, delivery, and governmental restrictions have limited the available antibiotics, especially in food fish culture (Choudhury et al., 2005). Decreased efficacy and increased resistance of pathogens to antibiotics has necessitated the development of new alternative drugs/compounds (Smith et al., 1994).

Actnetobacter baumannii has emerged as an important and problematic human pathogen as it is the causative agent for several types of infections including pneumonia, meningitis, septicemia, and urinary tract infections. Clinical impact of *A. baumannii* infection has been a matter of continuing debate. Since pathogens gaining resistance to drugs is common due to haphazard

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(Received 27 January 2011; revised 29 July 2011; accepted 06 August 2011)

World Journal of Fish and Marine Sciences 3 (1): 51-57, 2011
ISSN 2078-4589

Extraction, Purification and Partial Characterization of *Cladophora glomerata* Against Multidrug Resistant Human Pathogen *Acinetobacter baumannii* and Fish Pathogens

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Abstract: The antibacterial effect of the crude methanol extracts and purified fractions of *Cladophora glomerata* (Linnaeus) Kützing (Cladophoraceae) against multidrug resistant human pathogen *Acinetobacter baumannii* and fish pathogens were investigated. *Cladophora glomerata* demonstrated appreciable activity against the human pathogen *A. baumannii* and fish pathogens *Vibrio fischeri*, *V. vulnificus*, *V. anguillarum*, *V. parahaemolyticus, E. coli* and *B. cereus*. TLC Purified fractions III and V of green seaweed *C. glomerata* inhibited the human pathogen *A. baumannii* and fish pathogens *V. fischeri* and *V. vulnificus*. Purified fraction II of the same seaweed inhibited only *V. fischeri* and *V. vulnificus*. Methanol extract of *C. glomerata* inhibited *E. coli* and *B. cereus* growth at a minimum inhibitory concentration of 75 µg/ml and other species at 100 µg/ml. Whereas, *V. vulnificus* growth was inhibited at a minimum concentration of 125 µg/ml. GC-MS analysis revealed the presence of hydrocarbon compounds in active fractions II, III and V of *C. glomerata*. These findings demonstrate that the methanol extract and their purified fractions of *C. glomerata* exhibited appreciable antimicrobial activity and thus have great potential as a source for natural health products.

Key words: Seaweeds • Methanol extracts • Antibacterial activity and GC-MS

INTRODUCTION

Seaweeds are one of the important renewable resources in the marine environment and have been a part of human civilization from time immemorial. They offer a wide range of therapeutic possibilities both for internal and external applications. Although terrestrial biodiversity is the foundation of pharmaceutical industry, the oceans have enormous biodiversity and potential to provide novel compounds with commercial value [1, 2]. It has been reported that seaweeds serve as an important source of bioactive natural substances [2]. Many marine macro algae produce a variety of secondary metabolites [3]. These metabolites are mainly terpenes, acetogenins, alkaloids and polyphenolics, with many of these compounds being halogenated [4]. Specific studies on seaweeds, carried out in the Atlantic, Pacific and Indian oceans, have demonstrated antibacterial, antifungal and antiviral activities [5-10]. Extracts of marine algae were reported to exhibit antibacterial activity and has been investigated most widely [11-13]. *Acinetobacter baumannii* has emerged as an important and problematic human pathogen as it is the causative agent of several types of infections including pneumonia, meningitis, septicaemia and urinary tract infections. *Acinetobacter* infections ranked second after *Pseudomonas aeruginosa* among the nosocomial, aerobic, non-fermentative, Gram negative bacilli pathogens [14, 15]. Clinical impact of *A. baumannii* infection has been a matter of continuing debate. Many studies reported high overall mortality rates in nosocomial patients susceptible to *A. baumannii* bacteraemia or pneumonia [16, 17]. *A. baumannii* is attracting much attention owing to the increase in antibacterial resistance and occurrence of strains that are resistant to virtually all available drugs [18]. Further *Vibrio* spp., especially luminous *Vibrio harveyi* and *V. parahaemolyticus* have been implicated as the main bacterial pathogens of shrimp farms [19]. These *Vibrio* species are resistant to every antibiotic used, including chloramphenicol, oxytetracycline and streptomycin, (and are more virulent than in previous years). Consequently, the problem of giving treatment against resistant pathogenic bacteria is becoming increasingly difficult [20]. Since pathogens gaining resistance to drugs, is common due to indiscriminate use of antibiotics, much attention is needed to kill or control the pathogens using bioactive substances. Though literature speaks diverse studies of bioactivity of marine flora against several pathogens, our

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