CHARACTERIZATION AND QUANTIFICATION OF MAJOR BIOCHEMICAL COMPONENTS AND SECONDARY METABOLITES OF SOME GREEN AND RED SEAWEEDS FROM THE KERALA COAST

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ABHILASH P. KAILAS
Reg. No. 3583

DEPARTMENT OF CHEMICAL OCEANOGRAPHY,
SCHOOL OF MARINE SCIENCES,
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI-682 016

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Characterization and Quantification of Major Biochemical Components and Secondary Metabolites of Some Green and Red Seaweeds from the Kerala Coast

Ph.D. Thesis under the Faculty of Marine Sciences

Author

Abhilash P. Kailas
Research Scholar
Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016
Email: abhilashpkailas@gmail.com

Supervising Guide

Dr. S. Muraleedharan Nair
Professor
Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016
Email: muralis@cusat.ac.in

Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016

October 2015

Dedication

To my respected teachers, beloved family, especially my parents, sister, spouse and daughter, and friends for always supporting, helping and standing by me.

.....

"The speed of your success is limited only by your dedication

&

what you're willing to sacrifice"

— Nathan W. Morris

Phone Off. 0484-2863402
Phone Res. 0484-2345357
Fax: 0484-2382131
Email: muralis@cusat.ac.in

DEPARTMENT OF CHEMICAL OCEANOGRAPHY
SCHOOL OF MARINE SCIENCES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI - 682 016, INDIA

Dr. S. Muraleedharan Nair
Professor

Certificate

I hereby declare that the thesis entitled ***"Characterization and quantification of major biochemical components and secondary metabolites of some green and red seaweeds from the Kerala coast"*** *submitted by Mr. Abhilash P. Kailas, Reg. No: 3583, is an authentic record of research work carried out under my supervision and guidance for the Ph.D degree in* ***Marine Environmental Chemistry****, in the Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree in any University.*

I further certify that the corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of Mr. Abhilash P. Kailas are incorporated in the thesis.

Kochi-16
Date: 01/10/2015

Dr. S. Muraleedharan Nair
Supervising Guide

Declaration

I hereby declare that the thesis entitled ***"Characterization and quantification of major biochemical components and secondary metabolites of some green and red seaweeds from the Kerala coast"*** *submitted by me is an authentic record of research work carried out under the supervision and guidance of Dr. S. Muraleedharan Nair, Professor, Department of Chemical Oceanography, Cochin University of Science and Technology and that no part of it has previous by formed the basis for award of any degree, diploma, associated fellowships or other similar recognition in any University or Institution.*

Kochi-16 ***Abhilash P. Kailas***
Date: 01/10/2015

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PREFACE

Sea is a huge source to meet our daily needs, which are in the form of marine organisms, sediments, water, air current etc. Of these, seaweeds are replenishable sources of many important biochemicals that are gifted to mankind for existence on earth. The phenomenal biodiversity of seaweeds, nurtures it into a rich natural resource of many biologically active compounds like the carotenoids, amino acids, terpenoids, chlorophylls, pycobilins, fatty acids, polysaccharides, vitamins, sterols, tocopherols, phycocyanins etc., which are formed due to its exposure into the complex habitats of extreme conditions of spatial, temporal and seasonal variations. Phytochemicals with economic importance have attracted a vast attention, mainly on their therapeutic use as reduction of oxidative stress. Huge quantities of seaweeds are being used up yearly in nutrition with appreciable vitamins, minerals, carotenoids and flavonoids contents. Sea vegetables (algae or seaweeds) are used as a staple food from ancient times, referred to over 10000 years ago. Nowadays as a nutraceutical and pharmaceutical product, it has been used commercially for the extraction of the phytocompounds like alginates, carrageenans, agars, carotenoids and poly unsaturated fatty acids.

Seaweeds have a greater biodiversity with red (Rhodophyceae), green (Chlorophyceae) and brown (Phaeophyceae) species. The recognition of seaweed as a rich source of nutrients, that are capable of fighting cardio vascular diseases has increased its potential use as food. They are also in use, as herbal medicines, fungicides, fertilizers, herbicides etc., and find application as functional foods and nutraceuticals due to their biochemical presence. In the human health point of view, the vegetative metabolites are upcoming onto possess an extreme importance by exhibiting anti-inflammatory, anti-mutagenic, anti-tumour, anti-diabetic and anti-hypertensive values. The adverse growth conditions of seaweeds result in the formation of many secondary metabolites

which are either classical or novel in nature. The variability of biochemical compositions has thus resulted in major researches onto this area.

The thesis entitled "Characterization and quantification of major biochemical components and secondary metabolites of some green and red seaweeds from the Kerala coast" is an attempt to characterize and quantify the major biochemical components such as proteins, carbohydrates, crude fibre, ash, lipids, calorific value, vitamins, carotenoids, phenolics, flavonoids, amino acids, saponins and polysaccharides. The antioxidant activity and antimicrobial activity are studied here. GC-MS profiling of the extracts is studied. In addition to this, the present study also isolated and characterized a sterone which haven't yet been reported elsewhere from seaweeds. Eight seaweeds species were selected based on the availability and abundance from the Kerala coast [Njarakkal (10°01'33.8" N 76°12'40.2" E) and Kayamkulam (9°08'31.4" N 76°27'37.5" E)].

The thesis is divided into seven chapters,

Chapter1: A general introduction about seaweeds and its uses along with the aim, scope and the selected samples of this study is discussed here.

Chapter 2: The general biochemical characterizations of the seaweeds and their contribution to the nutritional recommended daily intake (RDI) levels are discussed here.

Chapter 3: The selection of extraction solvent systems, quantification of vitamins, phenolics, flavonoids and carotenoid contents, and their contribution to nutritional recommended daily intake (RDI) levels are dealt in this chapter.

Chapter 4: This chapter deals with the 16 amino acids and L-dopa contents and their contribution to nutritional recommended daily intake (RDI) levels.

Chapter 5: This chapter covers the quantification of saponins contents, the characterization of the stage wise solvent extracts, its corresponding saponification and iodine values, antioxidant activity, antimicrobial activity and GC-MS profiling.

Chapter 6: Quantification of total isolatable polysaccharides and their characterization studies are discussed in this chapter.

Chapter 7: Isolation and characterization of the steroid isolated from the abundantly available algal species *G. foliifera* species is discussed in this chapter.

Summary: Provides a brief conclusion on the achievements of the study and indicates the scope of future work.

All the chapters have a brief introduction, materials and methods, discussions and conclusion sections. Chapters end with the respective references.

Contents

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List of Abbreviations

$[\alpha]_D^{20}$	Specific Rotation Measured in Sodium D Line (589 nm) at 20 ^oC
$[\alpha]_D^{25}$	Specific Rotation Measured in Sodium D Line (589 nm) at 25 ^oC
^{13}C	Carbon ^{13}C Isotope NMR
^{1}H	Proton NMR
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate
Acetyl-CoA	Acetyl coenzyme A
AcN	Acetonitrile
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
ASTA	American Spice Trade Association
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BC	Before Christ
BHA	Butylatedhydroxyanisole
BHT	Butylatedhydroxytoluene
CF	Chloroform Fraction
con.	Concentrated
COSY	Correlation Spectroscopy
CRN	Council for Responsible Nutrition
D-	Dextrorotatory
DANSYL-Cl	5-dimethylaminonaphthalene-1-sulfonyl chloride
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
Dwt	dry weight
E-	Entegen Isomer
EAA	Essential Amino Acids
EAF	Ethyl Acetate Fraction
ECFR	Electronic Code of Federal Regulations
ed.	Edition
Eds.	Editors
EPA	eicosapentaenoic acid
FAMEs	Fatty Acid Methyl Esters
FAO	Food and Agriculture Organization
FRAP	Ferric Reducing Antioxidant Power
FID	Flame Ionizing Detector

FMOC-Cl	9-flouroenylmethyl-chloroformate
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
h	hour
H_2O	Water
HMBC	Heteronuclear Multiple Bond Correlation
HP	High Performance
HPLC	High Performance Liquid Chromatography
HPLC-RI	High Performance Liquid Chromatography-Refractive Index
HSQC	Heteronuclear Single Quantum Correlation
HTA	hexadecatrienoic acid
L-	Levorotatory
L-EMBA	Levin-Eosin Methylene Blue Agar
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
L-dopa	Levorotatory Dopamine
LPGE	Low Pressure Gradient Elution
MA	Massachusetts
MeOH	Methanol
Met E	B12 Independent Methionine Synthase
Met H	B12 Dependent Methionine Synthase
MHz	Megahertz
min	minute
MS	Mass Spectrometry
MSA	Mannitol Salt Agar
MUFA	Monounsaturated Fatty Acid
MUFAs	Monounsaturated Fatty Acids
n	Number of Entity
NCTC	National Collection of Type Cultures
NEAA	Nonessential Amino Acids
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
OPA	o-phthalaldehyde
PEF	Petroleum Ether Fraction
PITC	Phenylisothiocyanate
pp.	Pages
psi	pounds per square inch
PUFA	Polyunsaturated Fatty Acid

PUFAs	Polyunsaturated Fatty Acids
RDI	Recommended Daily Intake
RID	Refractive Index Detector
ROS	Reactive Oxygen Species
rpm	rotations per minute
SD	Standard Deviation
SF	Saponins Fraction
SFA	Saturated Fatty Acid
SFAs	Saturated Fatty Acids
SLF	Seaweed Liquid Fertilizer
sp.	Species
SPSS	Statistical Package for the Social Sciences
sq.	Square
t-BHQ	tertiary-butylatedhydroxyquinone
TEA	Triethylamine
TFA	Triflouroacetic acid
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
UFLC	Ultra Fast Liquid Chromatography
UNU	United Nations University
US	United States
USA	United States of America
USDA	United States Department of Agriculture
USFDA	United States Food and Drug Administration
UV	Ultraviolet
Vis	Visible
v/v	Volume/Volume
v/v/v	Volume/Volume/Volume
var.	Variety
w/w	Weight/Weight
WHO	World Health Organization
XLDA	Xylose Lysine Deoxycholate Agar
µm	Micrometer
ω	Omega Fatty Acids

Chapter 1

INTRODUCTION

Contents

1.1 Introduction

Origin of life on earth is estimated to be millions of years ago. Sea was the mother of all the living organisms and still it continues to be in one or the other form. It is a natural habitat for many plants, animals and microorganisms. Quest for foods that are rich in nutritive values are still in its higher research (El-Shafay, 2014). The source in search is mainly for an output of lower spatial/temporal/seasonal variation which could supply the inclined demand and also support the commercial sector as a main stream raw material. Sea is the dependable resource for meeting these needs. The phenomenal biodiversity faced by the marine world nurtures it into a rich natural resource of many biologically active compounds like the carotenoids, terpenoids, xanthophylls, chlorophylls, phycobilins, poly unsaturated fatty acids, polysaccharides, vitamins, sterols, tocopherols, phycocyanins etc. (Francavilla et al., 2013). These compounds are formed due to the exposure of the marine organisms to the complex habitats of extreme conditions of spatial,

temporal and seasonal variations (Francavilla et al., 2013). Phytochemicals from the marine environment possessing economical importance have attracted a vast attention, mainly on their therapeutic use and for reduction of oxidative stress (Southon, 2000). The first plant form on earth was the marine algae, which upon evolutions developed into the modern plant forms (bryophytes, pteridophytes, gymnosperms, angiosperms etc.). Until the date, sea withholds the primitive forms of life with marine algae constituting the major share among the marine plants (Dring, 1982). This generation history remarks the marine algae as a rich resource of various outputs that could fulfill the modern requirements.

Many marine organisms (both flora and fauna) are found to be edible, but as a huge source of biochemical constituents are not observed. Marine algae fill up the space for it (Watanabe et al., 1983). Seaweeds or marine macro algae are photosynthetic organisms which exhibit greater diversity and have gained potential interest. They are replenishable sources gifted to mankind for existence on earth. They flourish on all sources of attachments like the rocks, corals or any natural or manmade substrata (Manivannan et al., 2009), which make them a versatile product that has been widely used as a food that is intended for direct consumption (Ghosh et al., 2012). Seaweeds are a remarked resource of bioactive compounds which led to an unpredicted bloom in the recent researches. The enormous amount of seaweeds available in the world is not completely used up commercially which might be due to lack of its potentiality attention or harvesting negligence (Marsham et al., 2007).

Seaweeds vary greatly in their colour, quality, consistency and biochemical constituents. Seaweeds are mainly classified on the presence of carotenoids as red (Rhodophyceae), green (Chlorophyceae) and brown (Phaeophyceae) (Dawczynski et al., 2007; Shahnaz & Shameel, 2009). Red

algae are rich in phycocolloids - agars, carrageenans and phycobiliproteins and so known as agarophytes or carrageenophytes. They are also rich in vitamin B and carotenoids (Mabeau & Fleurence, 1993; MacArtain et al., 2007). Brown algae are rich in hydrocolloids, iodine, fucoxanthins and chlorophyll a & b. Abundance of phlorotannins and carotenoids in brown algae is responsible for its anti-obesity and anti-diabetic properties (Miyashita et al., 2010; O'Sullivan et al., 2010). Green algae contain chlorophylls and polysaccharides (O'Sullivan et al., 2010; Kim, 2011). In the past years, nutritional evaluation of seaweeds is mainly concentrated on red than brown or green seaweeds (Wong & Cheung, 2000; Marinho-Soriano et al., 2006; Kumari et al., 2010; Siddique et al., 2013).

1.2 Application of Seaweeds

1.2.1 In Food

Seaweeds have been in traditional use for centuries as a food, supplement and medicine (Holdt & Kraan, 2011). Centuries back, seaweeds were included in the food chain of humans living along the coastlines all around the world. Sea vegetables (algae or seaweeds) are used as a staple food from ancient times, which are referred to over 10,000 years ago (Fitzgerald et al., 2011; Venugopal, 2011). Uses of seaweeds were observed in ancient history in the diets of Pacific and Asian cultures which extended to Iceland, Wales, Canada and US (Yuan, 2008). Recently, seaweed biomass is gaining a great attention due to the assured increase in biomass demand as a result of overpopulation and feasibility in cultivation (Ross et al., 2008; Anastasakis et al., 2011). Ireland has a rich tradition of using algae in soups. They are also consumed raw in salads, soups, cookies and meals (Aguilera-Morales et al., 2005; Dawczynski et al., 2007). In many other countries, it is used as an animal fodder. Henceforth, the cultivation was promoted with special

considerations on red and brown species (Mouritsen et al., 2013). Seaweeds were used as food in countries such as China, Japan, South Korea, Thailand, Vietnam, Taiwan etc. (Dawes, 1998; Wong & Cheung, 2000; McHugh, 2003; Bocanegra et al., 2009; Mišurcová et al., 2014).

Seaweeds are not only edible but also tasty and healthy, with large and fairly unexplored potential use in the cuisine. Direct human consumption of a broad range of seaweeds is increasing over Asia. Their food applications are mainly due to the vitamins, proteins, minerals, fibres and antioxidant contents (Fleurence, 1999; Ortiz et al., 2006; Marsham et al., 2007). Seaweeds are known for its food additive effects too (Lim et al., 2002; Athukorala et al., 2003). Irrespective of its classification, they are found to function as an important dietary input which has led to its commercial cultivation (McHugh, 2003). A huge quantity (> 22,000 tons) is being used up yearly (Anonymous, 2003) with its major use in nutrition (Abbott, 1996).

1.2.2 In Nutraceuticals

Nutraceuticals connected that part of food that provides medical or health benefits which may be isolated from the food sources (DeFelice, 1995; Shahidi, 2009; Mendis & Kim, 2012). In the human health point of view, the vegetative metabolites are upcoming onto possess an extreme importance by exhibiting anti-inflammatory, antimutagenic, antitumor, antidiabetic and antihypertensive properties (Andrade et al., 2013). The recognition of seaweed as a rich source of nutrients, that are capable of fighting cardio vascular diseases has increased its potential use as food (Venugopal, 2011). Nutritive values and availability of many bioactive properties classify seaweeds as a functional food and an important constituent in nutraceuticals (Mišurcová et al., 2014). The western world increased the seaweed utilization in terms of seaweed extracts as an application in

nutraceuticals (Mouritsen et al., 2013). Seaweeds were in use as a treatment aid to chronic diseases from ancient times (1534 BC) (Teas, 1981). The potential effects of marine macro algae were also confirmed with animal model studies (Teas et al., 1984). The extending consumer demands for bioactive food ingredients of natural source explored the possibilities of seaweeds in catering the elongated needs (Kanazawa et al., 2008; Sangeetha et al., 2008; Granado-Lorencio et al., 2009; Miyashita, 2009).

Seaweeds are generally exposed to extreme conditions like salt variations, nutrient variations, temperature variations etc., and thus can result in the formation of free radicals and oxidizing chemicals. The endogenous protective activities are important for seaweeds to protect them from oxidative stress associated with the adverse dwelling areas. This is seen to be supported by vitamins, carotenoids, tocopherols etc., which are low in winter and spring, but higher in summer and monsoon (Yuan et al., 2009). The natural defense mechanism of the organism can thus create up an adaptive mechanism by the production of secondary metabolites which protects them from the oxidative stress (Garcia-González et al., 2009). Seaweeds find its application in nutraceuticals as antioxidants which are dietary in nature for human health in preventing ageing and degenerating diseases like the cardiovascular and cancerous growths through radical scavenging activities (Steinmetz & Potter, 1996; Dillard & German, 2000; Prior & Cao, 2000; Wargovich, 2000; Virgili & Scaccini, 2003; Li et al., 2008a). Natural antioxidants from vegetative sources, especially seaweeds, have a remarkable importance in the development of preventive medicines to attack the foresaid effects (Cahyana et al., 1992; Yan et al., 1999; Kaur & Kapoor, 2002; Lim et al., 2002). Isolated polysaccharides from seaweeds have antioxidant and anticoagulation (Athukorala et al., 2007; Li et al., 2012) properties. Carotenoids such as the lutein and zeaxanthin in seaweeds

express the biosynthesis of two compounds of lycopene - α-carotene and β-carotene respectively. Lutein and zeaxanthin have recently acquired a greater view in nutraceutical industries in paste and granular forms. The needs for natural colorants like carotenoids are now in greater considerations to replace toxic chemicals. These beneficial nutrients make the use of seaweeds in pharmaceutical activities (Lahaye, 1991).

1.2.3 In Industries

Seaweeds directly or indirectly supply organic molecules like alginates, carrageenans, carotenoids and agars that are being used in medicine and pharmacy for multiple decades (Gressler et al., 2010). The phycocolloids obtained from seaweeds are used in cosmetics (McHugh, 2003). In addition to this, the presence of sulfated polysaccharides have brought value additions in the food industry due to its gelling and thickening properties (Jiao et al., 2011a; Jiao et al., 2011b; Souza et al., 2012; El-Shafay, 2014). Use of seaweeds in agricultural and industrial sectors has increased nowadays (Diniz et al., 2011). Seaweeds are being used as biofuels (Tarwadi & Chauhan, 1987; Menetrez, 2012; Reznik & Israel, 2012; Murphy et al., 2013; Milledge et al., 2014). Biodegradable plastics produced from seaweeds could act as a replacement to the conventional non degradable forms (Rajendran et al., 2012; Gade et al., 2013). Seaweeds are used for water filtration and depuration activities (McHugh, 2003). Seaweeds are used as fish feeds and so finds the application in integrated aquaculture (McHugh, 2003). They are cultivated in large farms (Horn, 1989; Fleming et al., 1996) and millions of tons are harvested annually (Anonymous, 2003). The commercial exploitation of seaweed resources due to its various applications has enlarged the scenario of its research (Qasim, 1991).

1.2.4 As a Biochemical Storehouse

Seaweeds function as a healthy storehouse of many bioactive compounds (Patel, 2012). Advanced researches on seaweeds show a huge source of bioactive compounds with potent therapeutic values (Fujihara & Nagumo, 1993; Konig et al., 1994). A number of different compounds were isolated and characterized from seaweeds which include phlorotannins, carotenoids and sterols (Rupérez et al., 2002; Gin-Nae et al., 2007; Miyashita & Hosokawa, 2008; Toyosaki & Iwabuchi, 2009) which exhibited appreciable antioxidant effects (Velioglu et al., 1998). Recent studies that are concentrated upon marine algae are done in order to find the antioxidants that would meet the requirements of the pharmaceutical and chemical sectors (Duan et al., 2006; Li et al., 2007; Li et al., 2008b). This search resulted in the isolation of many novel compounds from marine systems and this discovery is increasing tremendously (Blunt et al., 2012).

Seaweeds have a huge bio availability of vitamins, amino acids, phenolic compounds, carotenoids, fibres and carbohydrates which are supported by secondary metabolites like sterols and fatty acids that could provide health benefits by reducing the risk of diseases. Their biochemical richness has been clinically proved to have health benefits (Athukorala et al., 2003; Yuan, 2008) and so is getting exploited due to its high economical value. Algae are important sources of nitrogen which highlights its presence in the form of protein and amino acids (Fleurence, 1999; Kumar & Kaladharan, 2007). The protein content was seen as 10-30 % of dry matter in the seaweeds (Ramos et al., 2000). This highly rich biochemical composition provides a large scope of nutrition for the entire animal kingdom including human beings (Chapman & Chapman, 1980; Indergaard & Minsaas, 1991; Ventura & Castanon, 1998; Norziah & Ching, 2000; Wong & Cheung, 2000; McHugh, 2003; Sánchez-Machado et al., 2004; Marsham et al., 2007). Seaweeds were generally observed with low calorific value and rich in vitamins, minerals,

dietary fibers (Ito & Hori, 1989), carotenoids and flavonoids (Watanabe et al., 1999; Yamada et al., 1999; Yon & Hyun, 2003).

Seaweed cell walls are made of interconnecting polysaccharides with higher amounts of active compound diversity. This extended diversity is difficult to degrade and are present as hydrogen bonded crystalline fibers with self imposed sulfated and branched polysaccharides that are attached to proteins and metals like calcium and potassium (Shanmugham et al., 2013). Algal sulfated polysaccharides are a renewable source that is widespread in nature (Kaliaperumal et al., 1995; Hooper et al., 1996) possessing numerous biological activities with structural diversity and pharmacological features that finds therapeutic benefits.

1.2.5 In Agricultural Sector

The influence of seaweeds as a leading biological factor on the field crops increased in recent years (Matysiak et al., 2011). The extracts of seaweeds were being used as a seaweed liquid fertilizer (SLF) due to the immense quantity of biochemical composition (Bai et al., 2007; Mohanty et al., 2013; Dwivedi et al., 2014). Seaweeds are also used in stimulating plant growth (Verkleij, 1992; Zodape, 2001; Craigie, 2011). They also function as an organic compost (Haq et al., 2011) and pesticide (Sultana et al., 2011). Use of seaweeds as fuel were already remarked (McHugh, 2003). They are also used as a sheep feed and goat feed (Arieli et al., 1993; Ventura & Castanon, 1998). Hansen et al. (2003) showed that due to the high protein content, the *Laminaria* sp. could be used as a feed source for small ruminants.

1.2.6 As a Dynamic Resource of Bioactive Compounds

Seaweeds function as a potential source of new and novel compounds. Their biochemical compositions vary with varying growth conditions and possess direct positive correlations. Chemical compositions are influenced by spatial and

temporal changes occurring in the environment which composes of light, temperature, nutrients, salinity and biotic interactions (Marsham et al., 2007; Stengel et al., 2011; Khairy & El-Shafay, 2013). Inter-specific, inter-annual or intra-annual variations are also observed in seaweeds (Fujiwara-Arasaki et al., 1984; Haroon, 2000). Protein contents vary with seasonal and nutritional differences which depend directly on the nitrogen content (Renaud & Luong-Van, 2006; Hernández-Carmona et al., 2009; Gerasimenko et al., 2010; Stengel et al., 2011). This variation also reflects on the individual amino acid contents too (Fleurence, 1999; Galland-Irmouli et al., 1999; Hernández-Carmona et al., 2009). The temperature variations were seen to possess direct relation which affected the growth rate and chemical contents of Rhodophytes (Rosell & Srivastava, 1985; McKee et al., 1992; Mishra et al., 1993; Castro-Gonzalez et al., 1994; Honya et al., 1994). Constituents are also correlated with depth (Dere et al., 2003) and factors involved in the environmental growth conditions (Jurković et al., 1995; Rupérez & Saura-Calixto, 2001).

1.3 Advancements in the Seaweed Research Sector

The latest biological properties studied on seaweeds are related to food and pharmacological applications (Jiménez-Escrig et al., 2001; Smit, 2004; Cardozo et al., 2007; Chu, 2011; Gupta & Abu-Ghannam, 2011; Holdt & Kraan, 2011; Jiao et al., 2011a; Jiao et al., 2011b; Lordan et al., 2011; Thomas & Kim, 2011; Wijesekara et al., 2011; Mohamed et al., 2012). Recent studies on macro algae reviewed the commercialization by compositional studies, bioactivity and extraction methods (Herrero et al., 2006; Plaza et al., 2008). Seaweeds are also used as herbal medicine, fungicide, fertilizer and herbicide (Aguilera-Morales et al., 2005; Cardozo et al., 2007). Seaweeds could be used as a medium of toxic element absorbance from the drainage system as a resort to depuration activities (Chojnacka et al., 2012).

1.4 Status of Seaweeds in India

The coastline of India extends around 8000 km with the peninsular region of India having the luxurious growth of seaweeds. Out of the total of 20,000 marine algal species reported worldwide, 844 have been reported from the Indian coastline (Oza & Zaidi, 2001). Among the 844 species, more than 200 species of seaweeds have been reported from the southern coastal area (Renganathan, 2015). These include the red, green and brown seaweeds which contribute to the productions of agars, carrageenans and alginins. Around 1,00,000 tons wet weight of seaweeds are estimated as the standing stock of seaweeds, with around 6,000 tons of agar yielding seaweeds, 16,000 tons of alginin yielding seaweeds and 8,000 tons of carrageenan yielding seaweeds (Kaladharan & Kaliaperumal, 1999). The rest of more than 70,000 tons are edible green seaweeds (Kaladharan & Kaliaperumal, 1999). The rich growths of seaweeds are seen along the Tamil Nadu coastline to Mumbai and in Andaman-Nicobar and Lakshadweep archipelagos. The Indian phyco industries depend on this area for their major share of production. At present, agar and sodium alginate are the main products of this industry.

Many research works on seaweeds from the Indian coast have been reported. They constituted of general biochemical constituent determinations (Premalatha et al., 2011; Eahamban & Antonisamy, 2012; Thinakaran & Sivakumar, 2012; Balakrishnan et al., 2013, Flora & Rani, 2013; Subathraa & Poonguzhali, 2013), amino acids determinations (Kumar & Kaladharan, 2007), biodiesel productions (Sharmila et al., 2012), agar productions (Kaliaperumal et al., 1992; Sahu & Sahoo, 2013), antioxidant activity studies (Chakraborty & Paulraj, 2010; Sachindra et al., 2010), antimicrobial activity studies (Vallinayagam et al., 2009; Premalatha et al., 2011; Selvin et al., 2011; Priyadharshini et al., 2012; Saritha et al., 2013; Muthuraman et al., 2014; Sivakumar et al., 2014; Singh &

Raadha, 2015), antiviral activity studies (Chattopadhyay et al., 2007), anti-inflammatory activities (Raju et al., 2015), hemolytic activities (Priyadharshini et al., 2012), *in vitro* cytotoxicity (Muthuraman et al., 2014), and heavy metal bio monitoring studies (Kalesh & Nair, 2005; Kalesh & Nair, 2006; Manivannan et al., 2008; Devi et al., 2009; Jothinayagi & Anbazhagan, 2009). Despite of all the research works being done, the use of seaweeds in India is still not in any sort of appreciable levels. The declining cultivatable land and increasing preference of humans towards vegetarian food habits have increased the need for an alternative mode of cultivation. India having a long coastal belt, has the opportunities for marine agriculture. Effective utilization of marine agriculture could meet the vast demanding needs.

1.5 Status and Significance of Seaweed Studies in Kerala

Kerala is situated between the Arabian Sea to the west and the Western Ghats to the east. It is bordered by Karnataka state to the north and north east, Tamil Nadu state to the east and south, and the Lakshadweep archipelago to the west. The total land area is 38,863 sq. km which constitutes about 1.18 % of the India's total land area. The state has three climatically distinct regions with the eastern highlands (rugged and cool mountainous terrain), the central midlands (rolling hills), and the western lowlands (coastal plains). The state lies in between the north latitudes 8°17'30" N and 12°47'40" N and the east longitudes 74°27'47" E and 77°37'12" E. The Kerala's climate is mainly wet and maritime tropical, heavily influenced by the seasonal heavy rains brought by the monsoon. The coastline extends to 580 km in length which is almost 8 % of the total Indian coastline. The coasts north of Kozhikode district and south of Kollam district are mainly rocky, while the central part is mainly sandy. The sandy shores are protected from soil erosions through the construction of seawalls.

Seaweeds were observed to flourish all along the 580 km long Kerala coastline, irrespective of the growing conditions. Seaweeds were observed on the rocky shores, sandy shores and manmade seawalls. Bioactivities of these easily available vegetative resources are literally unattended. A few studies on seaweeds were reported from the Kerala coast. Quantification of amino acids in seaweeds such as *Sargassum wightii*, *Ulva lactuca*, *Kappaphycus alvarezii*, *Hypnea musciformis*, *Acanthophora spicifera* and *Gracilaria corticata* collected from Quilon (Kumar & Kaladharan, 2007), free radical scavenging activities of isolated sesquiterpenoids from *Ulva fasciata* (Chakraborty & Paulraj, 2010), trace and heavy metal monitoring on the seaweeds such as *Ulva lactuca, Enteromorpha intestinalis, Chaetomorpha antennina, Gracilaria corticata, Centroceras clavulatum* and *Grateloupia filicina* collected from Ettikulam and *Enteromorpha intestinalis, Chaetomorpha antennina, Centroceras clavulatum* and *Grateloupia filicina* collected from Njarakkal (Kalesh & Nair, 2005; Kalesh & Nair, 2006) and antimicrobial activity of the extracts of *Ulva fasciata* collected from Quilon (Selvin et al., 2011) are some among the reported studies. None of the reports were observed to have an elaborate description of the biochemical composition and bioactivity properties. The current study was done to mentor this limitation.

In addition to the above, as per the current scenario, the declinations of agricultural lands are observed high in the Kerala state, with the major declination being observed in the districts of Ernakulam and Alleppey. Thus, the pace for a search of an alternative source of food is increasing. The present study has been undertaken to characterize and quantify the major biochemical components and secondary metabolites of some green and red seaweeds collected from the above coastal regions. The peculiarities of the highly populated and naturally grown seaweeds were determined through compositional and bioactivity studies.

1.6 Sampled Algal Materials

Based on the quest for maximum selection and sampling of seaweeds, the samples were collected from the Njarakkal location (10°01'33.8" N 76°12'40.2" E) of the Ernakulam district and the Kayamkulam location (9°08'31.4" N 76°27'37.5" E) of the Alleppey district. Both the locations are sandy beach with rocky man made seawalls built in order to protect the banks from soil erosion. The seaweeds grew on these rocks. Nine species of the marine macro algae belonging to two different systematic groups were chosen for the present study (Fig. 1.1; Fig. 1.2). Taxonomy of these species is given in Table 1.1. Samples were handpicked from their natural habitat during the period of low tides, sorted out and washed thoroughly in seawater followed by tap water. Samples were then washed with milli-Q water and stored in freezer at -18 ^{o}C until analysis. Half of the sample was air dried under shade, then dried in vacuum oven at 35 ^{o}C until moisture was < 2 %, powdered and stored in sealed glass bottles for further analysis.

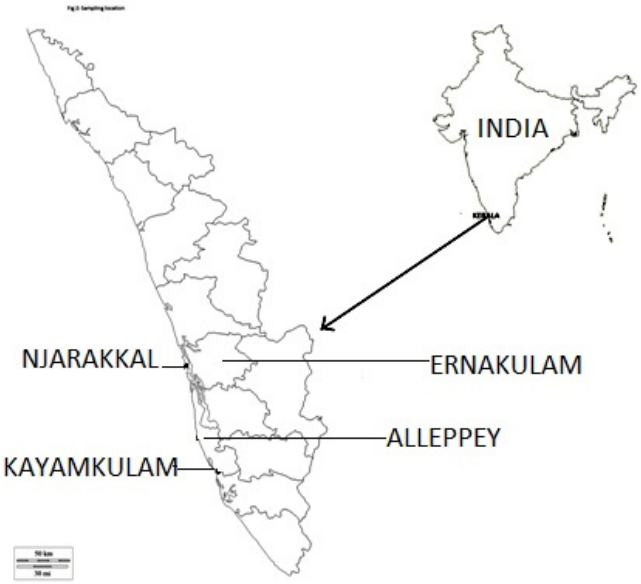
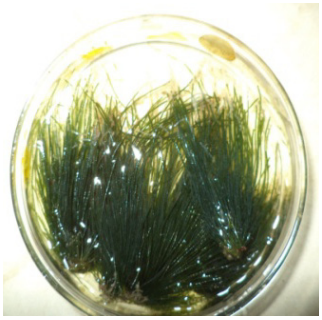
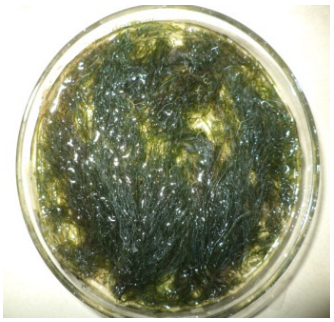


Fig. 1.1 The location map of sampling sites.

Chlorophyceae



Chaetomorpha antennina



Enteromorpha prolifera

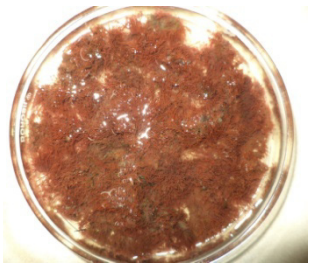
Ulva fasciata

Rhodophyceae

Acanthophora spicifera

Gelidium pusillum

Gracilaria corticata



Gracilaria corticata var. *cylindrica*

Gracilaria foliifera

Fig. 1.2 Selected seaweed species.

Table 1.1 Taxonomic classification of seaweed species collected from the Kerala coast

❖ Kayamkulam

○ Chlorophyceae

- *Enteromorpha prolifera* (O.F.Müller) J.Agardh
  - **Empire** Eukaryota
  - **Kingdom** Plantae
  - **Class** Ulvophyceae
  - **Order** Ulvales
  - **Family** Ulvaceae
  - **Genus** *Enteromorpha*
  - **Species** *prolifera*
- *Ulva fasciata* Delile
  - **Empire** Eukaryota
  - **Kingdom** Plantae
  - **Class** Ulvophyceae
  - **Order** Ulvales
  - **Family** Ulvaceae
  - **Genus** *Ulva*
  - **Species** *fasciata*

❖ Njarakkal

○ Chlorophyceae

- *Chaetomorpha antennina* (Bory de Saint-Vincent) Kützing
  - **Empire** Eukaryota
  - **Kingdom** Plantae
  - **Class** Ulvophyceae
  - **Order** Cladophorales
  - **Family** Cladophoraceae
  - **Genus** *Chaetomorpha*
  - **Species** *antennina*
- *Enteromorpha prolifera* (O.F.Müller) J.Agardh
  - **Empire** Eukaryota
  - **Kingdom** Plantae
  - **Class** Ulvophyceae
  - **Order** Ulvales
  - **Family** Ulvaceae
  - **Genus** *Enteromorpha*
  - **Species** *prolifera*

- **Rhodophyceae**
  - *Acanthophora spicifera* (M.Vahl) Børgesen
    - **Empire** Eukaryota
    - **Kingdom** Plantae
    - **Class** Florideophyceae
    - **Order** Ceramiales
    - **Family** Rhodomelaceae
    - **Genus** *Acanthophora*
    - **Species** *spicifera*
  - *Gelidium pusillum* (Stackhouse) Le Jolis
    - **Empire** Eukaryota
    - **Kingdom** Plantae
    - **Class** Florideophyceae
    - **Order** Gelidiales
    - **Family** Gelidiellaceae
    - **Genus** *Gelidium*
    - **Species** *pusillum*
  - *Gracilaria corticata* (J.Agardh) J.Agardh
    - **Empire** Eukaryota
    - **Kingdom** Plantae
    - **Class** Florideophyceae
    - **Order** Gracilariales
    - **Family** Gracilariaceae
    - **Genus** *Gracilaria*
    - **Species** *corticata*
  - *Gracilaria corticata* var. *cylindrica* Umamaheswara Rao
    - **Empire** Eukaryota
    - **Kingdom** Plantae
    - **Class** Florideophyceae
    - **Order** Gracilariales
    - **Family** Gracilariaceae
    - **Genus** *Gracilaria*
    - **Species** *corticata* var. *cylindrica*
  - *Gracilaria foliifera* (Forsskål) Børgesen
    - **Empire** Eukaryota
    - **Kingdom** Plantae
    - **Class** Florideophyceae
    - **Order** Gracilariales
    - **Family** Gracilariaceae
    - **Genus** *Gracilaria*
    - **Species** *foliifera*

Chaetomorpha antennina, a green seaweed was collected from Njarakkal location. *C. antennina* has been reported previously to have an appreciable phytochemical content, which included flavonoids, triterpenoids, alkaloids, coumarins, quinones and saponins (Subathraa & Poonguzhali, 2013), possessing anti-inflammatory activities (Raju et al., 2015), antimicrobial and antioxidant activities (Premalatha et al., 2011), capable of biodiesel productions (Sharmila et al., 2012) and in acting as heavy (Kalesh & Nair, 2005) and trace (Kalesh & Nair, 2006) metal bio indicators. *Enteromorpha prolifera*, a green seaweed was collected from both Njarakkal and Kayamkulam location. *E. prolifera* has been reported for its utilization as a biofuel (Wang et al., 2013), for heavy metal depuration activities (Jiang et al., 2013), and as an algicidal agent (Renjun et al., 2013). The biochemical composition data are less studied. *Ulva fasciata,* a green seaweed was collected from Kayamkulam location. *U. fasciata* has been reported earlier to be used as an antiviral (Mendes et al., 2010), antibacterial (Stirk et al., 2007; Premalatha et al., 2011; Selvin et al., 2011; Priyadharshini et al., 2012; Sivakumar et al., 2014), antioxidant (Chakraborty & Paulraj, 2010) and antifungal (Stirk et al., 2007) agent. *U. fasciata* is being used to alleviate inflammatory diseases (Kim et al., 2013).

Acanthophora spicifera, a red seaweed was collected from Njarakkal location. *A. spicifera* has been reported to possess antimicrobial and cytotoxicity properties (Muthuraman et al., 2014) along with some commercially important organic molecules such as octanol, piperazine, benzoic acid and octadecenoic acid (Flora & Rani, 2013). Antioxidant activities (Sachindra et al., 2010), hypolipidaemic effects (Hetta et al., 2009) and amino acids content (Kumar & Kaladharan, 2007) were also reported previously. *A. spicifera* was also been reported to be used as a heavy metal

bioindicator (Devi et al., 2009). *Gelidium pusillum*, a red seaweed was obtained from Njarakkal location. *G. pusillum* has been reported earlier to have appreciable levels of dietary fibre, lipids, ash contents, protein contents and amino acids (Siddique et al., 2013). *Gracilaria corticata*, a red seaweed was collected from Njarakkal location. *G. corticata* was observed to be previously reported for its biodiesel productions (Sharmila et al., 2012), flavonoids, saponins, steroids and amino acids contents (Kumar & Kaladharan, 2007; Eahamban & Antonisamy, 2012; Balakrishnan et al., 2013), as trace metal bio indicator (Kalesh & Nair, 2006), as heavy metal bio indicator (Kalesh & Nair, 2005), for its commercially important polysaccharides (Andriamanantoanina et al., 2007), antioxidant (Sachindra et al., 2010) and anti-carcinogenic properties (Zandi et al., 2010). *Gracilaria corticata* var. *cylindrica*, a red seaweed was obtained from Njarakkal location. *G. corticata* var. *cylindrica* was observed to have appreciable protein, carbohydrates, amino acids and carotenoids contents (Thinakaran & Sivakumar, 2012). *G. corticata* var. *cylindrica* was also rich in agar contents (Kaliaperumal et al., 1992) and antimicrobial activities (Singh & Raadha, 2015). *Gracilaria foliifera*, a red seaweed was collected from Njarakkal location. *G. foliifera* was observed to be used as a bio indicator of toxic (Al-Shwafi & Rushdi, 2008) and heavy metals (Manivannan et al., 2008). Studies on biochemical composition were less reported.

1.7 Aim, Scope and Objectives of the Present Study

The present study aims for the characterization and quantification of major biochemical constituents and secondary metabolites of some green and red seaweeds from the Kerala coast, especially from the area where agricultural land is observed to decline. Detailed literature review encouraged

this attempt and was observed to be novel. In this context, the use of seaweeds as an alternative source of food and its contribution to the pharmaceutical industry requires more information on its biochemical composition. The general biochemical composition includes the quantification of total proteins, lipids, carbohydrates, ash content, crude fibre, iodine content and calorific value. The data could be used in determining the extent of the seaweed species to the nutritional index. Major secondary metabolites like the vitamins, phenolics, flavonoids, carotenoids, amino acids, saponins and polysaccharide contents were determined and these imparted the nutraceutical, nutritional and therapeutical qualities of the seaweeds. The therapeutical qualities were supported with their antioxidant and antimicrobial activities too. Volatile fractions of the seaweeds were also determined to identify the availability of commercially important organic molecules. Seaweeds generally exhibited species wise, class wise, seasonal wise and temporal wise variations in their overall bio-chemical compositions. Hence with, to cater the human needs, the investigation is essential for finding out the species that has to be promoted for cultivation. The quantification and characterization of the isolated novel secondary metabolites is a bench mark of this study which differentiates it from the classical research studies on seaweeds.

The objectives of the present study can be summarized as follows:

- Collection of the scientifically unattended seaweed samples from the central Kerala coast. Nine seaweeds samples were selectively collected.
- Evaluation of the general biochemical composition (total proteins, lipids, carbohydrates, ash content, crude fibre, iodine content and

calorific value) and the contribution of the seaweeds to the recommended daily intake (RDI) values.

- Estimation of the secondary metabolites such as the vitamins, phenolics, flavonoids and carotenoids of the seaweeds.
- Evaluation of amino acid compositions and the contribution of seaweeds to the RDI value.
- Screening of seaweeds for their saponins contents and their stage wise GC-MS analysis.
- Screening of seaweeds for their antioxidant and antimicrobial activities.
- Evaluation of the isolatable polysaccharide contents and speciation to monosaccharide units.
- Screening of the isolatable polysaccharides for their antioxidant and antimicrobial activities.
- Qualitative and quantitative evaluation of the isolated novel secondary metabolite from one of the abundant seaweed collected from the sampling site.

1.8 Scheme of the Thesis

The thesis is divided into eight chapters,

Chapter1: Introduction

Current chapter. This chapter gave a general introduction about seaweeds and their uses. The selected samples for this study and their peculiarities were discussed here. Aim, scope and objectives of this study were also discussed.

Chapter 2: Proximate composition

The general biochemical characterizations of the seaweeds starting with the analytical methodology are discussed here. The contribution to the nutritional RDI levels and statistical correlation of the biochemical compositions are also discussed.

Chapter 3: Vitamins, flavonoids and carotenoids

The selection of extraction solvent systems and quantification of vitamins, phenolics, flavonoids and carotenoids contents are dealt in this chapter. The chapter starts with the analytical methodologies. The antioxidant activity of the extract is discussed here along with its statistical correlations.

Chapter 4: Amino acids

This chapter deals with 16 amino acids and L-dopa contents. The body of the chapter discusses the contribution to nutritional RDI levels and statistical correlations.

Chapter 5: Biologically important metabolites

This chapter covers the quantification of saponins contents, characterization of the stage wise solvent extracts, its corresponding saponification and iodine values, antioxidant activity, antimicrobial activity, GC-MS profiling and statistical correlations. The chapter starts with the analytical methodologies.

Chapter 6: Polysaccharides

Total isolatable polysaccharides content, its saponification and iodine values, specific rotation, sulfate content, antioxidant and antimicrobial activity, its speciation into monosaccharide contents and statistical correlations were discussed in this chapter. The chapter begins with the methodology used.

Chapter 7: Isolation and characterization of a ketosteroid - *E*-guggulsterone

This chapter begins with the developed methodology for isolation of a ketosteroid from the seaweed. The isolation steps discusses the extraction, TLC screening, HPLC screening, column chromatography separation, TLC, GC, HPLC, HPLC-preparative, optical rotation, UV scan, FTIR scan, GC-MS, LC-MS/MS and NMR data of the isolated compound.

Chapter 8: Summary

Provides a brief summary on the overall outcome of the study and indicates the scope of future work.

All the chapters end with the respective references.

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Chapter 2

PROXIMATE COMPOSITION

Contents

2.1 Introduction

Seaweeds could be considered as a food source only if they are nutritive. Nutritive in this context are that part of substances which provide all the basic food necessities that are required for an organism to survive. It consists of water, carbohydrate, lipids, proteins, crude fibre, energy and minerals. Almost all seaweeds reported till date possesses these nutritive resources (Teas et al., 2004; Ortiz et al., 2006; Ahmad et al., 2012; Benjama & Masniyom, 2012). Chemical composition of seaweeds varies temporally, seasonally and spatially and so monitoring of the nutritional contents to select the better source is important. Seaweeds are used as a food item in many countries. The rich contribution of the seaweeds towards energy and vital biochemical requirements make them an excellent food source to the consumers.

Seaweeds are classified based on its chemical composition and nutrient content as Rhodophyta (red algae), Chlorophyta (green algae) and Phaeophyta (brown algae) (Dawczynski et al., 2007). The chemical compositions include water (moisture), protein, carbohydrate, lipids, iodine, ash, crude fibre, calorific value, vitamins, flavonoids, carotenoids, phenolics, amino acid compositions, saponins, polysaccharides etc. This chapter deal with the

screening of the seaweeds for the major biochemical compositions such as water (moisture), protein, carbohydrate, lipid, iodine, ash, crude fibre and calorific value. These beneficial components make the use of seaweeds in pharmaceutical activities (Lahaye, 1991). A brief introduction on some facts of these biochemical components is discussed ahead.

The water content of seaweeds depends on their physical structure. Water gets absorbed in seaweeds and gets interlocked in between its cell walls. It could act as a resource of water which could be used during the depletion in source of safe water availability. The water content in seaweeds reported in previous studies was ranging from 31 to 88 % dwt (Marsham et al., 2007).

All living organisms require proteins as an energy source and for the development of muscles. The nitrogen requirement of an organism is also supported by the proteins. Proteins are made up of amino acid units. One gram of protein is estimated to generate 5.65 kcal of energy. Several studies done on edible seaweeds have reported the quality and quantity of proteins (Ito & Hori, 1989; Fleurence, 1999; Wong & Cheung, 2001). Seaweeds were reported to have 6 to 44 % dwt of total proteins (Fleurence, 1999; Rupérez & Saura-Calixto, 2001; Marsham et al., 2007).

Carbohydrate consists of carbon, hydrogen and oxygen units combined in varying configurations. The change in configurations develops them into simple and complex, soluble and insoluble and digestible and indigestible carbohydrates. The extent of their complexity determines the extent of energy generation. On an average, one gram of carbohydrate is estimated to release 4.20 kcal of energy. The total carbohydrate in seaweeds was reported in previous studies as above 19 % dwt (Peña-Rodríguez et al., 2011; El-Said & El-Sikaily, 2013).

The indigestible carbohydrates are classified as crude fibres. Crude fibres have therapeutic effects like modulation of bowel movements, stabilization of gastro-intestinal tracts and removal of adsorbed chemicals adherents from the food tract which reduces the chances of heart diseases, obesity, cancer etc. The crude fibre contents are reported to vary seasonally (Indergaard & Knutsen, 1990). Seaweeds were reported to have 3 to 71 % dwt of crude fibre (Ortiz et al., 2006; Ratana-arporn & Chirapat, 2006; Dawczynski et al., 2007; Siddique et al., 2013; Hind et al., 2014).

Lipids are made of short or long, saturated or unsaturated, mono or poly unsaturated fatty acids and sterols. They consist of large amount of units and hence, estimated to release about 9.45 kcal of energy per gram. Lipid content or generally the fat content is also reported in seaweeds and variance on factors like seasonal, temporal and spatial have been established (Kolb et al., 1999). The lipid contents were accounted in the range of 1 - 6 % dwt (Jurković et al., 1995).

Sea which is a source of vital minerals and ions, also acts as a huge contributor to iodine (Apaydin et al., 2010). Iodine content in seaweeds is formed through the combination with proteins (Scott, 1954) and through accumulation along with the water from the sea. Iodine content in seaweeds has positive impact on its consumers as it functions as the major non metal responsible for thyroid functions. Seaweeds possess huge iodine content which could be consumed by animals as a potential source of iodine in thyroid malfunction. Iodine content was found in the range of 30 to 2984 $\mu g\ g^{-1}$ dwt in seaweeds (Teas et al., 2004).

Similar to all living organisms, seaweeds are a greater source of ions, minerals and metals. This could be absorbed or adsorbed during its dwelling on seashores. They consist of important nutritive minerals such as Na, Ca, K,

Fe, Zn etc. in free or combined forms. This content is collectively evidenced by its ash content. Ash content was reported in seaweeds from 9.3 to 77.8 % dwt in previous studies (Marsham et al., 2007; Peña-Rodríguez et al., 2011; Ahmad et al., 2012; Khairy & El-Shafay, 2013).

The calorific value is a combined input of all the major biochemical constituents. The energy that could be generated upon by the organisms through digestion is derived as the calorific value (Dare & Edwards, 1975). The calorific value in seaweeds varies from 0.64 to 4.37 kcal g^{-1} in previous studies (McDermid & Steurcke, 2003; Marsham et al., 2007; Kumar et al., 2009).

The major biochemical characterizations of seaweeds were reported in India and abroad. *Enteromorpha intestinalis, Ulva lactuca* and *Chaetomorpha linum* were studied for their biochemical compositions (Selvi et al., 1999). Seaweeds collected from Cape of Comorin were examined for its organic constituents such as the carbohydrates, lipids and proteins (Devi et al., 1996). Marine green algae collected from the Saurashtra coast was analysed for its amino acid contents (Dave & Parekh, 1997). Brown seaweeds *Sargassum wightii, Padina tetrastromatica, Chaetomorpha minima* and *Hormophysa triquetra* collected from the Mandapam region, Gulf of Mannar, India, was analysed for its total protein content, carbohydrate content and lipid content (Kokilam et al., 2013). *Sargassum wightii*, *Codium adhaerens* and *Acanthophora spicifera* collected from the Mandapam coast, Gulf of Mannar, India, were analysed for their total protein and lipid contents (Sreenivasan et al., 2012). The eleven seaweeds collected from the southeast coast of India, constituting of five green seaweeds - *Enteromorpha compressa, Ulva reticulata, Cladophora glomerata, Halimeda macroloba, Halimeda tuna,* three brown seaweeds - *Dictyota dichotoma, Padina pavonica* and *Turbinaria ornata* and three red seaweeds - *Gelidiella acerosa, Gracilaria crassa* and

Hypnea musciformis were reported with their total protein, carbohydrate and lipid contents (Manivannan et al., 2009). *Ulva rigida* collected from Chilka Lake was reported with total protein, carbohydrate, lipid and water contents (Satpati & Pal, 2011). *Dictyopteris repens* collected from the gangetic delta, India exhibited seasonal variation in total protein, lipids and carbohydrate contents (Banerjee et al., 2009). Ganesan & Kannan (1994) reported a seasonal variation in the biochemical constituents of some economically important seaweeds collected from Gulf of Mannar.

Edible marine alga collected from China was investigated for its nutrient compositions (Fan-Xiao et al., 1993). *Ulva lactuca* was screened for its chemical composition (Castro-Gonzalez et al., 1996). Seaweeds collected from Karachi coast was analysed for its biochemical compositions including crude fibre content (Qari & Qasim, 1993). Energy contribution of seaweeds was analysed by many researchers (Paine & Vadas, 1969; Sumitra et al., 1975; McQuaid, 1985; Qari & Qasim, 1993; Lamare & Wing, 2001).

The current search for commercial raw material to extract biologically important chemical constituents and as a highly nutritive food source has increased tremendously. Data on the biochemical compositions and cultivation of seaweeds all around the world are available. But the scope of its cultivation and utilization in India is not yet up to the required levels. Several Indian coasts are still unexplored. The growing demand for the non-conventional food sources and industrial raw materials highlights the importance of the exploration and evaluation of cheaply available seaweeds as an alternative. In this context, as an abandoned caution to the declination of cultivatable land in Kerala, it has been a noteworthy to study the biochemical composition and calorific values of seaweeds from the highly impacted Kerala coasts. The present study provides information on the available seaweeds from the two

Kerala coast, its major biochemical compositions, inter and intra species correlations and its contribution as a food source in comparison to the recommended daily intake values suggested by the Council for Responsible Nutrition (CRN, 2015), United States Food and Drug Administration (USFDA, 2011) and Electronic Code of Food Regulations (ECFR, 2015).

2.2 Materials and Methods

2.2.1 Algal Samples

Nine seaweeds with four green algae and five red algae collected from two locations - Njarakkal and Kayamkulam of the Kerala coast, India (Table 1.1; Fig. 1.2) were used for proximate composition analyses.

2.2.2 Proximate Composition Analyses

Fresh seaweeds upon removal of epiphytes and epifauna were analysed for its water content. Freeze dried samples were analysed for total proteins, carbohydrates, lipids, iodine, ash, crude fibre (dietary fibre) contents and calorific values. All the analyses were done in triplicates with results expressed with standard deviations (n=3) against dry weight and the algal materials were simultaneously dried at 100 ^{o}C in an oven for 24 h to determine the dry weight. The mean values of the observations were used for the discussions of this study.

2.2.2.1 Water Content

The moisture content (Karl Fischer moisture analysis (Bruttel & Schlink, 2003) was estimated potentiometrically, with the anode solution consisting of methanol, imidazole, SO_2 and iodine. The titration cell has a smaller compartment with a cathode immersed in the anode solution of the main compartment. The two compartments are separated by an ion-permeable

membrane, and the platinum anode generates I_2 when current is provided through the electric circuit. The net reaction commutes as one mole of I_2 is consumed for each mole of H_2O. In other words, 2 moles of electrons are consumed per mole of water. The end point is detected most commonly by a bipotentiometric method. A second pair of Pt electrodes is immersed in the anode solution known as the detector circuit which maintains a constant current between the two detector electrodes during titration. Prior to the equivalence point, the solution contains I^- and little I_2. At the equivalence point, excess of I_2 appears and an abrupt voltage drop denotes the end point. The amount of current needed to generate I_2 and reach the observed end point was used to calculate the amount of water in the original sample.

2.2.2.2 Protein Content

Total protein content was estimated by the UV-Vis spectrophotometric method (Lowry et al., 1951). UV-Vis Cary 60 spectrophotometer was used at 750 nm. 100 mg of the powdered sample was weighed into a 10 mL standard flask and made upto volume using double distilled water. 0.1 mL of this sample solution was mixed with 0.1 mL of 2N NaOH solution. The mixture was hydrolyzed at 100 °C for 10 min in a boiling water bath. The hydrolysate was cooled to room temperature and 1 mL of freshly mixed complex-forming reagent (containing 2 % Na_2CO_3, 2 % sodium potassium tartarate and 1 % $CuSO_4.5H_2O$) was added and the mixture was allowed to stand at room temperature for 10 min. To this mixture, 0.1 mL of Folin reagent was added, mixed using a vortex mixer and allowed to stand at room temperature for 30 - 60 min. The absorbance was measured. Bovine serum albumin was used as a standard with multi point calibration yielding the correlation factor r^2 as 0.999. Results are expressed as % to dry weight of the sample.

2.2.2.3 Carbohydrate Content

Total carbohydrates were estimated by the UV-Vis spectro-photometric method (Dubois et al., 1956). Estimation was done upon the dehydration reaction between the carbohydrates with con. sulphuric acid which produces furfural derivatives. The reaction between furfural derivatives and phenol develops detectable colour. 100 mg of the sample was measured into a 10 mL standard flask and made up with double distilled water. 2 mL aliquot of this sample solution was mixed with 1 mL of 5 % aqueous solution of phenol in a test tube. Subsequently, 5 mL of con. sulphuric acid was added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they were vortexed for 30 s and placed for 20 min in a water bath at room temperature for colour development. The absorbance was measured at 490 nm. The phenol used in this procedure was redistilled and 5 % phenol in water (w/w) was prepared immediately before the measurements. Glucose was used as standard with multi point calibration yielding the correlation factor r^2 as 0.999. Results are expressed as % to dry weight of the sample.

2.2.2.4 Lipid Content

Sulfophospho vanillin method (Barnes & Blackstock, 1973) was used to estimate the total lipids. 100 mg of the sample was weighed into a 10 mL standard flask and made up to the volume using HPLC grade methanol. 0.5 ml of this solution was taken into a clean test tube and dried under vacuum in a desiccator loaded with silica gel. The dried extract was dissolved in 0.5 ml of con. sulphuric acid and mixed well. The tube was plugged with non-absorbent cotton wool and placed in a boiling water bath for 10 min. The tubes were cooled to room temperature. 0.2 ml of this acid digest was mixed with 5 ml of vanillin reagent in another test tube, mixed well and allowed to stand for half an hour. The developed colour was measured at 520 nm. Cholesterol was used

as a standard with multi point calibration yielding the correlation factor r^2 as 0.999. Results are expressed as % to dry weight of the sample.

2.2.2.5 Iodine content

Total iodine content was determined spectrophotometrically (Saenko et al., 1978). 50 to 100 mg of the sample was taken in a crucible and moistened with 30 % K_2CO_3 solution. The crucible was kept in a muffle furnace for 4 - 5 h at 400 to 500 ^oC. Upon complete ash formation, the contents in crucible were transferred to a 100 mL standard flask. To this, 25 mL 20 % NaCl solution was added and mixed for 1 h. The mixture was filtered and the residue was placed in a calibrated test tube with a ground plug. The volume was increased to 10 mL with 20 % NaCl solution. To this mixture, a freshly prepared 0.5 % $NaNO_2$ solution (0.25 mL), 20 % NaBr solution (0.25 mL) and 0.5 % brilliant green solution (0.25 mL) were added. The mixture was then agitated for 2 min with 5 mL toluene and 1 mL 5N H_2SO_4. After 20 min, blue green toluene layer was taken off using a separating funnel into a cuvette and absorbance at 680 nm was measured. Potassium iodide was the standard with multi point calibrations yielding r^2 value 0.999. Results are expressed as $\mu g\ g^{-1}$ to dry weight of the sample.

2.2.2.6 Ash Content

Total ash content was estimated as per the method underlined in American Spice Trade Association manual (ASTA) (ASTA, 1999a). 2 to 3 g of the well mixed sample was placed in a pre-weighed crucible in the entrance of the open muffle furnace until the sample is well carbonized. The carbonized sample was placed in the furnace at 600 ^oC and incinerated for 2 h, until light gray ash was obtained to constant weight. The carbon remains were leached with hot water, filtered through an ash less filter paper. The filter paper was

washed thoroughly and the paper and contents were transferred to the original crucible. The crucible was dried and ignited in the muffle furnace at 600 ^{o}C until the ash was white and free from carbon. The dish was cooled and the weight of the ash was measured. The results are represented as % to dry weight of the sample.

2.2.2.7 Crude Fibre Content

Total crude fibre content was also estimated as per the method in ASTA (ASTA, 1999b). 2 g of sample was extracted three times with methylene chloride. The extract was discarded and the residue together with 0.5 g of ceramic fibre was added onto a digestion flask. 200 mL of 1N H_2SO_4 solution was added and refluxed for 30 min with frequent rotation of the flask to ensure thorough wetting and mixing of the sample. Upon completion of the boiling, the solution was filtered through a filter cloth under suction. The residue was washed with boiling water until washings are no longer acid. The residue was then transferred into another digestion flask containing 200 mL of 1N NaOH solution. The mixture was boiled for 30 min and upon completion; the mixture was filtered through a Gooch crucible. The residue was thoroughly cleaned with water and then with 15 mL of ethyl alcohol. The crucible was dried at 110 ^{o}C to a constant weight, cooled in a desiccator and weighed. The crucible was then ignited in an electric muffle furnace at 600 ^{o}C, for 20 min. The crucible was cooled in a desiccator and weighed to calculate the crude fibre content. The results are represented as % to dry weight of the sample.

2.2.2.8 Calorific Value

The calorific value of the sample was measured using a bomb calorimeter (Dare & Edwards, 1975). Small pellets of the dried sample (1 g) were placed in the bomb chamber, pressurized to 425 psi with pure oxygen, combusted and the

amount of difference of heat liberated was recorded. The bomb calorimeter was calibrated against benzoic acid standards prior to the analysis of samples. The results are expressed as kcal g^{-1} to dry weight of the sample.

2.2.3 Correlation Studies

The biochemical composition of the seaweeds was subjected to its inter-compositional Pearson correlation studies using the SPSS 16.0 software for windows. The species wise, division wise (Chlorophyta and Rhodophyta), and genera wise (genus *Gracilaria* including the *G. corticata, G. corticata* var. *cylindrica* and *G. foliifera*) correlations were attempted. The compositional ratios were determined to evaluate the biochemical distribution pattern.

2.2.4 Contribution to Recommended Daily Intake (RDI)

Recommended daily intake amount or the daily recommended intake values of protein, carbohydrate, lipid, iodine, crude fibre and calorific value stated by the CRN (CRN, 2015), USFDA (USFDA, 2011) and ECFR (ECFR, 2015) were compared with the corresponding values of the analysed seaweeds. The contribution of the seaweeds to the daily dietary requirement of an average person was evaluated for deriving the potential seaweed source that requires commercial attention. The results are reported as the amount of seaweeds to be consumed in gram per day. The seaweed with minimal intake quantity was concluded as the major contributor to the future dietary and commercial requirements.

2.3 Results and Discussions

2.3.1 Proximate Composition Analyses

Biochemical compositions of the nine seaweeds are illustrated in Table 2.1 and Fig. 2.1. In general, the red and green algae varieties showed large

differences in their chemical constituents. The contents varied considerably in division, family and species wise.

Table 2.1 Biochemical compositions of the selected seaweeds, (mean ± SD), (n=3).

Species	P	C	L	I
AS	9.37 ± 0.14	55.81 ± 0.86	2.49 ± 0.04	145.51 ± 2.23
CA	8.99 ± 0.18	48.44 ± 0.74	2.09 ± 0.03	57.81 ± 0.89
EPK	12.26 ± 0.19	50.33 ± 0.77	3.35 ± 0.05	52.83 ± 0.81
EPN	17.45 ± 0.27	50.83 ± 0.78	10.76 ± 0.17	41.46 ± 0.64
GC	6.18 ± 0.09	43.85 ± 0.67	1.89 ± 0.03	175.41 ± 2.69
GCC	5.98 ± 0.09	70.56 ± 1.08	1.49 ± 0.02	179.40 ± 2.75
GF	6.98 ± 0.11	31.02 ± 0.48	1.69 ± 0.03	185.38 ± 2.84
GP	5.98 ± 0.18	75.95 ± 1.16	2.65 ± 0.04	137.54 ± 2.11
UF	25.12 ± 0.39	33.17 ± 0.51	7.38 ± 0.11	62.49 ± 0.96
Species	**A**	**F**	**K**	**M**
AS	17.14 ± 0.26	61.89 ± 0.95	2.03 ± 0.03	83.65 ± 0.83
CA	21.03 ± 0.32	56.91 ± 0.87	1.39 ± 0.02	87.46 ± 1.34
EPK	27.71 ± 0.42	37.57 ± 0.58	3.01 ± 0.05	87.79 ± 0.14
EPN	24.12 ± 0.37	61.59 ± 0.94	3.49 ± 0.05	86.09 ± 0.72
GC	19.24 ± 0.29	45.58 ± 0.70	2.17 ± 0.03	86.69 ± 1.03
GCC	20.13 ± 0.31	40.07 ± 0.61	3.32 ± 0.05	86.06 ± 1.11
GF	23.12 ± 0.35	51.43 ± 0.79	1.67 ± 0.03	72.52 ± 0.80
GP	13.06 ± 0.20	63.39 ± 0.97	3.52 ± 0.05	86.44 ± 0.99
UF	20.43 ± 0.31	37.97 ± 0.58	2.99 ± 0.05	89.15 ± 0.27

AS - *A. spicifera*, CA - *C. antennina*, EPK - *E. prolifera* from Kayamkulam, EPN - *E. prolifera* from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera*, GP - *G. pusillum* and UF - *U. fasciata*.

P - Protein content (% dwt), C - Carbohydrate content (% dwt), L - Lipid content (% dwt), I - Iodine content (μg g^{-1} dwt), A - Ash content (% dwt), F - Crude fibre content (% dwt), K - Calorific value (kcal g^{-1}) and M - Water content (% dwt).

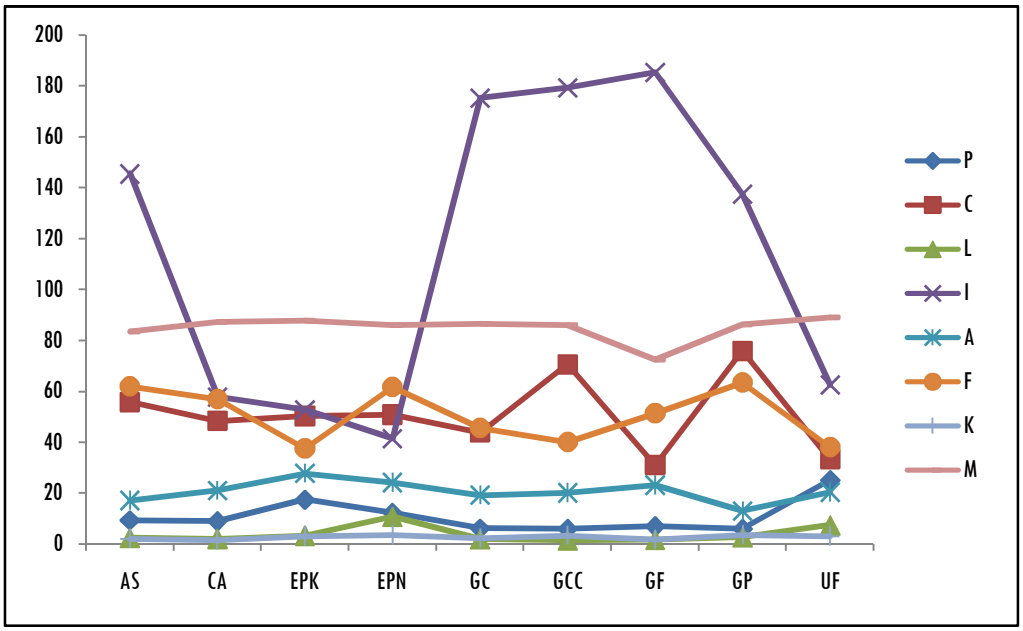

Fig. 2.1 Graphical cluster representation of biochemical composition of nine seaweeds.

P - Protein content (% dwt), C - Carbohydrate content (% dwt), L - Lipid content (% dwt), I - Iodine content (µg g^{-1} dwt), A - Ash content (% dwt), F - Crude fibre content (% dwt), K - Calorific value (kcal g^{-1}) and M - Water content (% dwt).

AS - *A. spicifera*, CA - *C. antennina*, EPK - *E. prolifera* from Kayamkulam, EPN - *E. prolifera* from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera*, GP - *G. pusillum* and UF - *U. fasciata*.

Water content in fresh raw seaweed exhibited a range of 72.52 to 86.69 % dwt in red seaweeds and 86.09 to 89.15 % dwt in green seaweeds (Table 2.1). The highest was observed in *G. corticata* (86.69 % dwt) among the red seaweed and *U. fasciata* (89.15 % dwt) among the green seaweeds. The least water clogging was observed inter variety in *G. foliifera* (72.52 % dwt). Almost similar water content was observed in previous studies done by Ahmad et al. (2012) and Satpati & Pal (2011) (Table 2.2).

Table 2.2 Literature review on biochemical composition studies done on seaweeds/seagrasses.

Species	Area	Concentration	Reference
Water content (% dwt)			
Ulva rigida	Chilka lake	76.00	Satpati & Pal (2011)
Various seaweeds	Malaysia	90.84 - 92.00	Ahmad et al. (2012)
Various seaweeds	Malaysia	75.95 - 96.03	Ahmad et al. (2012)
Various seaweeds	Malaysia	83.51 - 86.86	Ahmad et al. (2012)
Protein content (% dwt)			
Acanthophora spicifera	Mandapam coast, India	5.29	Sreenivasan et al. (2012)
Asparagopsis taxiformis	Brazil	11.70	Diniz et al. (2011)
Codium adhaerens	Mandapam coast, India	5.22	Sreenivasan et al. (2012)
Centroceras clavulatum	Brazil	11.30	Diniz et al. (2011)
Caulerpa lentillifera	Thailand	12.49	Ratana-arporn & Chirapat (2006)
Chnoospora minima	Gulf of Mannar, India	7.11	Kokilam et al. (2013)
Ceramium rubrum	Egypt	9.27	El-Shafay (2014)
Chaetomorpha aerea	Brazil	16.10	Diniz et al. (2011)
Durvillaea antarctica	Chile	10.40 - 11.60	Ortiz et al. (2006)
Dictyota repens	Gangetic delta, India	2.78 - 16.03	Banerjee et al. (2009)
Eucheuma cottoni	Malaysia	9.76	Matanjun et al. (2008)
Gracilaria changgi	Malaysia	6.90	Norziah & Ching (2000)
Gracilaria fisheri	Pattani Bay, Thailand	11.60	Benjama & Masniyom (2012)
Gelidium pusillum	St. Martin's Island, Bangladesh	11.31	Siddique et al. (2013)
Gracilaria tenuislipitata	Pattani Bay, Thailand	20.30 - 22.90	Benjama & Masniyom (2012)
Hypnea musciformis	St. Martin's Island, Bangladesh	18.64	Siddique et al. (2013)
Hypnea pannosa	St. Martin's Island, Bangladesh	16.31	Siddique et al. (2013)
Hormophysa triquetra	Gulf of Mannar, India	15.34	Kokilam et al. (2013)
Jania rubens	Abu Qir bay, Egypt	9.76-12.93	Khairy & El-Shafay (2013)
Pterocladia capillacea	Abu Qir bay, Egypt	17.35-23.72	Khairy & El-Shafay (2013)
Padina pavonica	Egypt	8.35	El-Shafay (2014)
Padina tetrastromatica	Gulf of Mannar, India	11.39	Kokilam et al. (2013)
Porphyra sp.	China, Japan and Korea	30.90 - 31.40	Dawczynski et al. (2007)
Sargassum filipendula	Brazil	8.72	Diniz et al. (2011)
Sargassum fusiforme	Egypt	8.85	El-Shafay (2014)
Spyridia hypnoides	Brazil	10.70	Diniz et al. (2011)
Sargassum vulgare	Egypt	5.85	El-Shafay (2014)
Sargassum wightii	Mandapam coast, India	6.40	Sreenivasan et al. (2012)
Sargassum wightii	Gulf of Mannar, India	15.10	Kokilam et al. (2013)
Ulva clathrata	Mexico	20.00 - 26.00	Peña-Rodríguez et al. (2011)
Ulva lactuca	Chile	27.20	Ortiz et al. (2006)
Ulva lactuca	Tunisia	7.90	Yaich et al. (2011)
Ulva lactuca	Abu Qir bay, Egypt	16.78-20.12	Khairy & El-Shafay (2013)
Ulva lactuca	Algeria	15.30	Hind et al. (2014)

Ulva reticulata	Thailand	21.06	Ratana-arporn & Chirapat (2006)
Ulva rigida	Chilka lake	6.64	Satpati & Pal (2011)
Various seaweeds	China, Japan and Korea	7.50 - 19.80	Dawczynski et al. (2007)
Various seaweeds	Southeast coast, India	9.65 - 31.07	Manivannan et al. (2009)
Various seaweeds	Malaysia	10.52 -13.24	Ahmad et al. (2012)
Various seaweeds	Malaysia	5.22 - 17.28	Ahmad et al. (2012)
Various seaweeds	Malaysia	5.93 - 7.78	Ahmad et al. (2012)
Carbohydrate content (% dwt)			
Asparagopsis taxiformis	Brazil	22.90	Diniz et al. (2011)
Centroceras clavulatum	Brazil	27.10	Diniz et al. (2011)
Caulerpa lentillifera	Thailand	59.27	Ratana-arporn & Chirapat (2006)
Chnoospora minima	Gulf of Mannar, India	42.98	Kokilam et al. (2013)
Ceramium rubrum	Egypt	88.76	El-Shafay (2014)
Chaetomorpha aerea	Brazil	29.40	Diniz et al. (2011)
Durvillaea antarctica	Chile	58.40 - 70.90	Ortiz et al. (2006)
Dictyota repens	Gangetic delta, India	21.52 - 35.74	Banerjee et al. (2009)
Eucheuma cottoni	Malaysia	26.49	Matanjun et al. (2008)
Gelidium pusillum	St. Martin's Island, Bangladesh	40.64	Siddique et al. (2013)
Hypnea musciformis	St. Martin's Island, Bangladesh	20.60	Siddique et al. (2013)
Hypnea pannosa	St. Martin's Island, Bangladesh	22.89	Siddique et al. (2013)
Hormophysa triquetra	Gulf of Mannar, India	49.06	Kokilam et al. (2013)
Jania rubens	Abu Qir bay, Egypt	34.57-42.18	Khairy & El-Shafay (2013)
Pterocladia capillacea	Abu Qir bay, Egypt	47.98-50.96	Khairy & El-Shafay (2013)
Padina pavonica	Egypt	90.50	El-Shafay (2014)
Padina tetrastromatica	Gulf of Mannar, India	59.30	Kokilam et al. (2013)
Sargassum filipendula	Brazil	16.80	Diniz et al. (2011)
Sargassum fusiforme	Egypt	90.71	El-Shafay (2014)
Spyridia hypnoides	Brazil	27.70	Diniz et al. (2011)
Sargassum vulgare	Egypt	93.34	El-Shafay (2014)
Sargassum wightii	Gulf of Mannar, India	40.21	Kokilam et al. (2013)
Ulva lactuca	Chile	61.50	Ortiz et al. (2006)
Ulva lactuca	Abu Qir bay, Egypt	42.09-46.42	Khairy & El-Shafay (2013)
Ulva reticulata	Thailand	55.77	Ratana-arporn & Chirapat (2006)
Ulva rigida	Chilka lake	22.00	Satpati & Pal (2011)
Various seaweeds	Southeast coast, India	14.73 - 17.49	Manivannan et al. (2009)
Various seaweeds	Malaysia	53.08 - 67.40	Ahmad et al. (2012)
Various seaweeds	Malaysia	57.79 - 74.11	Ahmad et al. (2012)
Various seaweeds	Malaysia	26.86 - 41.03	Ahmad et al. (2012)
Lipid content (% dwt)			
Acanthophora spicifera	Mandapam coast, India	1.11	Sreenivasan et al. (2012)
Asparagopsis taxiformis	Brazil	4.80	Diniz et al. (2011)
Codium adhaerens	Mandapam coast, India	1.21	Sreenivasan et al. (2012)
Centroceras clavulatum	Brazil	2.78	Diniz et al. (2011)

Caulerpa lentillifera	Thailand	0.86	Ratana-arporn & Chirapat (2006)
Chnoospora minima	Gulf of Mannar, India	0.45	Kokilam et al. (2013)
Ceramium rubrum	Egypt	0.01	El-Shafay (2014)
Chaetomorpha aerea	Brazil	5.49	Diniz et al. (2011)
Durvillaea antarctica	Chile	0.80 - 4.30	Ortiz et al. (2006)
Dictyota repens	Gangetic delta, India	0.17 - 0.24	Banerjee et al. (2009)
Eucheuma cottoni	Malaysia	1.10	Matanjun et al. (2008)
Gracilaria changgi	Malaysia	3.30	Norziah & Ching (2000)
Gracilaria fisheri	Pattani Bay, Thailand	1.70 - 2.70	Benjama & Masniyom (2012)
Gelidium pusillum	St. Martin's Island, Bangladesh	2.16	Siddique et al. (2013)
Gracilaria tenuislipitata	Pattani Bay, Thailand	1.90 - 3.60	Benjama & Masniyom (2012)
Hypnea musciformis	St. Martin's Island, Bangladesh	1.27	Siddique et al. (2013)
Hypnea pannosa	St. Martin's Island, Bangladesh	1.56	Siddique et al. (2013)
Hormophysa triquetra	Gulf of Mannar, India	0.11	Kokilam et al. (2013)
Jania rubens	Abu Qir bay, Egypt	1.47 - 2.39	Khairy & El-Shafay (2013)
Pterocladia capillacea	Abu Qir bay, Egypt	1.76 - 2.71	Khairy & El-Shafay (2013)
Padina pavonica	Egypt	0.01	El-Shafay (2014)
Padina tetrastromatica	Gulf of Mannar, India	0.55	Kokilam et al. (2013)
Porphyra sp.	China, Japan and Korea	1.00 - 2.80	Dawczynski et al. (2007)
Sargassum filipendula	Brazil	2.92	Diniz et al. (2011)
Sargassum fusiforme	Egypt	0.02	El-Shafay (2014)
Spyridia hypnoides	Brazil	4.20	Diniz et al. (2011)
Sargassum vulgare	Egypt	0.04	El-Shafay (2014)
Sargassum wightii	Mandapam coast, India	1.01	Sreenivasan et al. (2012)
Sargassum wightii	Gulf of Mannar, India	0.21	Kokilam et al. (2013)
Ulva clathrata	Mexico	2.50 - 4.00	Peña-Rodríguez et al. (2011)
Ulva lactuca	Chile	0.30	Ortiz et al. (2006)
Ulva lactuca	Tunisia	7.90	Yaich et al. (2011)
Ulva lactuca	Abu Qir bay, Egypt	3.14-4.09	Khairy & El-Shafay (2013)
Ulva reticulata	Thailand	0.75	Ratana-arporn & Chirapat (2006)
Ulva rigida	Chilka lake	12.00	Satpati & Pal (2011)
Various seaweeds	China, Japan and Korea	1.00 - 4.50	Dawczynski et al. (2007)
Various seaweeds	Southeast coast, India	0.26 - 3.58	Manivannan et al. (2009)
Various seaweeds	Thailand	7.30 - 8.70	Benjama & Masniyom (2011)
Various seaweeds	Malaysia	0.15 - 0.17	Ahmad et al. (2012)
Various seaweeds	Malaysia	0.18 - 0.54	Ahmad et al. (2012)
Various seaweeds	Malaysia	0.51 - 0.84	Ahmad et al. (2012)
Iodine content (μg g^{-1} dwt)			
Laminaria sp.	Taiwan, Japan, Thailand and Korea	241.00 - 4921.30	Yeh et al. (2014)
Postelsia palmaeformis	California	871.00	Teas et al. (2004)
Porphyra tenera	Taiwan, Japan, Thailand and Korea	29.30 - 45.80	Yeh et al. (2014)
Sargassum sp.	Washington	30.00	Teas et al. (2004)
Undaria pinnatifida	Taiwan, Japan, Thailand and Korea	93.90 - 185.10	Yeh et al. (2014)

Ash content (% dwt)			
Caulerpa lentillifera	Thailand	24.21	Ratana-arporn & Chirapat (2006)
Ceramium rubrum	Egypt	0.53	El-Shafay (2014)
Chaetomorpha sp.	Gulf of Kutch, India	23.00	Kumar et al. (2009)
Durvillaea antarctica	Chile	17.90 - 25.70	Ortiz et al. (2006)
Eucheuma cottoni	Malaysia	46.19	Matanjun et al. (2008)
Gracilaria changgi	Malaysia	22.70	Norziah & Ching (2000)
Gracilaria fisheri	Pattani Bay, Thailand	21.20 - 22.90	Benjama & Masniyom (2012)
Gelidium pusillum	St. Martin's Island, Bangladesh	21.15	Siddique et al. (2013)
Gracilaria tenuislipitata	Pattani Bay, Thailand	7.90 - 26.00	Benjama & Masniyom (2012)
Hypnea musciformis	St. Martin's Island, Bangladesh	21.57	Siddique et al. (2013)
Hypnea pannosa	St. Martin's Island, Bangladesh	18.65	Siddique et al. (2013)
Jania rubens	Abu Qir bay, Egypt	39.25 - 50.54	Khairy & El-Shafay (2013)
Pterocladia capillacea	Abu Qir bay, Egypt	13.02 - 23.68	Khairy & El-Shafay (2013)
Padina pavonica	Egypt	0.51	El-Shafay (2014)
Sargassum fusiforme	Egypt	0.33	El-Shafay (2014)
Sargassum vulgare	Egypt	0.19	El-Shafay (2014)
Sargassum sp.	Gulf of Kutch, India	41.60	Kumar et al. (2009)
Ulva clathrata	Mexico	28.00 - 50.00	Peña-Rodríguez et al. (2011)
Ulva lactuca	Chile	11.00	Ortiz et al. (2006)
Ulva lactuca	Tunisia	19.60	Yaich et al. (2011)
Ulva lactuca	Abu Qir bay, Egypt	17.56 - 23.19	Khairy & El-Shafay (2013)
Ulva lactuca	Algeria	39.10	Hind et al. (2014)
Ulva reticulata	Thailand	17.58	Ratana-arporn & Chirapat (2006)
Various seaweeds	Thailand	26.90 - 28.40	Benjama & Masniyom (2011)
Various seaweeds	Malaysia	10.64 - 14.10	Ahmad et al. (2012)
Various seaweeds	Malaysia	6.05 - 28.79	Ahmad et al. (2012)
Various seaweeds	Malaysia	21.37 - 45.04	Ahmad et al. (2012)
Crude fibre (% dwt)			
Caulerpa lentillifera	Thailand	3.17	Ratana-arporn & Chirapat (2006)
Durvillaea antarctica	Chile	56.40 - 71.40	Ortiz et al. (2006)
Eucheuma cottoni	Malaysia	5.91	Matanjun et al. (2008)
Gracilaria changgi	Malaysia	24.70	Norziah & Ching (2000)
Gracilaria fisheri	Pattani Bay, Thailand	57.50 - 64.00	Benjama & Masniyom (2012)
Gelidium pusillum	St. Martin's Island, Bangladesh	24.74	Siddique et al. (2013)
Gracilaria tenuislipitata	Pattani Bay, Thailand	56.60 - 60.20	Benjama & Masniyom (2012)
Hypnea musciformis	St. Martin's Island, Bangladesh	37.92	Siddique et al. (2013)
Hypnea pannosa	St. Martin's Island, Bangladesh	40.59	Siddique et al. (2013)
Porphyra sp.	China, Japan and Korea	45.70 - 49.80	Dawczynski et al. (2007)
Ulva clathrata	Mexico	24.00 - 27.00	Peña-Rodríguez et al. (2011)
Ulva lactuca	Chile	60.50	Ortiz et al. (2006)
Ulva lactuca	Algeria	22.80	Hind et al. (2014)

Ulva reticulata	Thailand	4.84	Ratana-arporn & Chirapat (2006)
Various seaweeds	China, Japan and Korea	36.00 - 62.30	Dawczynski et al. (2007)
Various seaweeds	Thailand	51.30 - 62.20	Benjama & Masniyom (2011)
Various seaweeds	Malaysia	11.29 - 19.40	Ahmad et al. (2012)
Various seaweeds	Malaysia	4.03 - 7.84	Ahmad et al. (2012)
Various seaweeds	Malaysia	21.66 - 34.71	Ahmad et al. (2012)
Calorific value (kcal g^{-1} dwt)			
Cymodocea rotundata	Palk Bay, India	60.62 - 63.68	Pradheeba et al. (2011)
Cymodocea serrulata	Palk Bay, India	37.33 - 43.40	Pradheeba et al. (2011)
Chaetomorpha sp.	Gulf of Kutch, India	2.70	Kumar et al. (2009)
Enhalus acoroides	Palk Bay, India	68.82 - 77.84	Pradheeba et al. (2011)
Gracilaria corticata	Palk Bay, India	18.69	Pradheeba et al. (2011)
Gracilaria edulis	Palk Bay, India	19.41	Pradheeba et al. (2011)
Gelidium pusillum	Palk Bay, India	27.46	Pradheeba et al. (2011)
Halophila beccarii	Palk Bay, India	29.16 - 38.84	Pradheeba et al. (2011)
Hypnea musciformis	Palk Bay, India	12.08	Pradheeba et al. (2011)
Halophila ovalis	Palk Bay, India	24.08 - 37.03	Pradheeba et al. (2011)
Halodule pinifoli	Palk Bay, India	28.23 - 28.39	Pradheeba et al. (2011)
Halodule uninervis	Palk Bay, India	37.16 - 40.15	Pradheeba et al. (2011)
Syringodium isoetifolium	Palk Bay, India	30.48 - 38.38	Pradheeba et al. (2011)
Sargassum wightii	Palk Bay, India	22.46	Pradheeba et al. (2011)
Sargassum sp.	Gulf of Kutch, India	5.40	Kumar et al. (2009)
Turbinaria conoides	Palk Bay, India	23.98	Pradheeba et al. (2011)
Various seaweeds	Hawai	> 3.00	McDermid & Steurcke (2003)

Protein content was observed in maxima for species grown at Kayamkulam location (Table 2.1). *U. fasciata* and *E. prolifera* exhibited the highest protein contents (25.12 and 12.26 % dwt respectively). With respect to division, the green seaweed had major concentration of protein contents (8.99 to 25.12 % dwt). In red seaweeds, *A. spicifera* (9.37 % dwt) had almost comparable protein content with the least protein containing green seaweed, *C. antennina* (8.99 % dwt). The least protein containing red seaweeds were *G. pusillum* (5.98 % dwt) and *G. corticata* var. *cylindrica* (5.98 % dwt) with almost similar protein content which shows the similarity in the point of sampling. *G. foliifera* and *G. corticata* also exhibited similar patterns (6.98 and 6.18 % dwt). Similar protein contents were observed in earlier studies too. Studies done by Manivannan et al. (2009), Satpati & Pal (2011), Sreenivasan et al. (2012) and Kokilam et al. (2013)

are some among the similar indigenous reports (Table 2.2). Recent studies reported by Ahmad et al. (2012), Benjama & Masniyom (2012), Khairy & El-Shafay (2013), Siddique et al. (2013), El-Shafay (2014) and Hind et al. (2014) (Table 2.2) had also similar protein contents.

The total carbohydrate contents which are the sources of agars, carrageenans and fucoidans were observed in high amounts in the red seaweeds collected from Njarakkal (Table 2.1). *G. pusillum* (75.95 % dwt) and *G. corticata* var. *cylindrica* (70.56 % dwt) showed similar carbohydrate contents which explain the spatial adherence of seaweed dwelling. The lowest carbohydrate content among the analysed seaweeds was in the red seaweed *G. foliifera* (31.02 % dwt). Among the green seaweeds, carbohydrate varied from 33.17 to 50.83 % dwt, with both *E. prolifera* possessing the highest contents and exhibiting almost similar carbohydrate concentrations which show their spatial relation. Carbohydrate pattern was almost evenly distributed, which indicates their dependence on the individual structural patterns and size. Carbohydrate contents were observed to be similar in between *A. spicifera* (55.81 % dwt) and both the *E. prolifera* (50.33 and 50.83 % dwt) collected. *C. antennina* (48.44 % dwt) was similar to *G. corticata* (43.85 % dwt) in reference to its carbohydrate contents. Indian seaweeds *U. rigida* (22 %) (Satpati & Pal, 2011), *S. wightii* (40.21 %), *Chnoospora minima* (42.98 %), *H. triquetra* (49.06 %) and *P. tetrastromatica* (59.30 %) (Kokilam et al., 2013) were reported to have comparable carbohydrate contents. Recent studies done on non indigenous seaweeds by Diniz et al. (2011), Ahmad et al. (2012), Khairy & El-Shafay (2013), Siddique et al. (2013) and El-Shafay (2014) were also observed to have similar carbohydrate contents (Table 2.2).

Lipid content exhibited a divisional variation with green seaweeds having the highest concentration in comparison with red (Table 2.1). Samples

from Kayamkulam area were rich in lipid contents where green seaweeds were found to grow in majority with *U. fasciata* and *E. prolifera* showing comparatively greater lipid content (7.38 and 3.35 % dwt respectively). The highest lipid containing seaweed was obtained from Njarakkal with *E. prolifera* possessing 10.76 % dwt. Green seaweeds varied from 2.09 to 10.76 % dwt in lipid content. Red seaweeds showed almost similar lipid contents ranging from 1.49 to 2.65 to dwt. The least was in *G. corticata* var. *cylindrica* (1.49 % dwt) and highest in *G. pusillum* (2.65 % dwt) which was inverse to the observation of total protein and carbohydrate contents. Lipid contents observed in this study was in relation with the previous reports on seaweeds (Table 2.2). Studies done by Sreenivasan et al. (2012) on indigenous seaweeds exhibited a lipid content of 1.01 to 1.21 % dwt. Satpati & Pal (2011) reported the presence of 12 % dwt of lipid content in *U. rigida* collected from Chilka Lake. Benjama & Masniyom (2011) reported 7.30 to 8.70 % dwt of lipid content in *Ulva intestinalis* collected from Thailand.

Iodine content exhibited the variety wise relation (Table 2.1). Chlorophyta ranged from 41.46 to 62.49 μg g^{-1} dwt in iodine content, whereas the red seaweeds had 137.54 to 185.38 μg g^{-1} dwt. *U. fasciata* (62.49 μg g^{-1} dwt) and *G. foliifera* (185.38 μg g^{-1} dwt) dominated in the green and red seaweed varieties respectively. Iodine content was observed to be in deviation from the spatial similarities. Similarity in iodine content was seen between *G. corticata* (175.41 μg g^{-1} dwt) and *G. corticata* var. *cylindrica* (179.40 μg g^{-1} dwt), *A. spicifera* (145.51 μg g^{-1} dwt) and *G. pusillum* (137.54 μg g^{-1} dwt), *U. fasciata* (62.49 μg g^{-1} dwt) and *C. antennina* (57.81 μg g^{-1} dwt) and both *E. prolifera* (52.83 and 41.46 μg g^{-1} dwt). *Sargassum* sp. (30 μg g^{-1} dwt) collected from Washington (Teas et al., 2004), *Porphyra tenera* (29.3 to 45.8 μg g^{-1} dwt) and *Undaria pinnatifida* (93.9 to 185.1 μg g^{-1} dwt) collected from Taiwan, Japan, Thailand and Korea were reported to have similar iodine contents (Yeh et al., 2014) (Table 2.2).

Total ash content which is the non combustible inorganic matter was observed to be in almost equal dispersion throughout the seaweeds (Table 2.1). The contents varied from 13.06 to 27.71 % dwt. In green seaweeds it ranged from 20.43 % dwt in *U. fasciata* to 27.71 % dwt in *E. prolifera* from the Kayamkulam location. In red seaweeds, it ranged from 13.06 % dwt in *G. pusillum* to 23.12 % dwt in *G. foliifera. C. antennina* (21.03 % dwt), *U. fasciata* (20.43 % dwt), *G. corticata* var. *cylindrica* (20.13 % dwt) and *G. corticata* (19.24 % dwt) had almost comparable ash contents. *A. spicifera* (17.14 % dwt) and *G. pusillum* (13.06 % dwt) had the least among the collected seaweeds. The trend was similar to the trends of carbohydrates, that it doesn't have any sort of relations with species or location and are seen to adhere to the structural patterns and size. Ash content observed was similar to the reports on the indigenous *Chaetomorpha* sp. collected from Gulf of Kutch (23 % dwt) (Kumar et al., 2009). Irrespective of sampling locations, similar ash contents were observed in recent studies reported by Ratana-arporn & Chirapat (2006), Ortiz et al. (2006), Yaich et al. (2011), Ahmad et al. (2012), Benjama & Masniyom (2012) and Siddique et al. (2013) (Table 2.2).

Crude fibre, also termed as dietary fibre demonstrated no similarities in variety wise or division wise (Table 2.1). It had a general pattern in the range of 37.57 to 61.59 % dwt in green seaweeds and 40.07 to 63.39 % dwt in red seaweeds. The highest was observed in *G. pusillum* (63.39 % dwt) and lowest in *E. prolifera* (37.57 % dwt) collected from the Kayamkulam location. The spatial similarity of *G. pusillum* (63.39 % dwt) and *G. corticata* var. *cylindrica* (40.07 % dwt) was also not observed in crude fibre content. *A. spicifera* (61.89 % dwt) and *E. prolifera* (61.59 % dwt) collected from the Njarakkal location showed comparable crude fibre contents. *C. antennina* was observed to have 56.91 % dwt and was comparable with the crude fibre contents of *G. foliifera* (51.43 % dwt).

G. corticata (45.58 % dwt) and *G. corticata* var. *cylindrica* (40.07 % dwt) had almost comparable crude fibre contents. The least was observed in *U. fasciata* (37.97 % dwt) and *E. prolifera* (37.57 % dwt) collected from the Kayamkulam location. Crude fibre contents were almost comparable to seaweeds reported from Bangladesh (Siddique et al., 2013) (Table 2.2). *Ulva lactuca* (60.50 % dwt) and *Durvillaea antarctica* (56.40 to 71.40 % dwt) obtained from Chile (Ortiz et al., 2006) and studies done by Benjama & Masniyom (2012) (56.60 to 64.00 % dwt) evidenced the presence of high crude fibre contents in seaweeds.

Calorific value, which is the energy released upon combustion of sample, exhibited a similarity in samples from Kayamkulam with 3.01 kcal g^{-1} dwt for *E. prolifera* and 2.99 kcal g^{-1} dwt for *U. fasciata* (Table 2.1). *C. antennina* exhibited the least calorific value with 1.39 kcal g^{-1} dwt which was collected from the Njarakkal location and belonging to the green seaweeds. The highest calorific value was for *G. pusillum* with 3.52 kcal g^{-1} dwt collected from the Njarakkal location. With respect to red seaweeds, *G. pusillum* and *G. corticata* var. *cylindrica* showed similarity in calorific value (3.52 and 3.32 kcal g^{-1} dwt respectively) which showed the adherence to spatial correlation. Least among the red seaweed was *G. foliifera* with 1.67 kcal g^{-1} dwt. The trends remark the contribution of proteins, carbohydrates and lipids to energy generation. Low calorific value reported in the current study was a remarkable observation which indicates the potential of seaweeds to be used as an alternative food source for diet conscious persons. Previous studies on the indigenous seaweeds were reported with relatively high calorific values (Table 2.2). Kumar et al. (2009) reported a comparable calorific value with 2.70 kcal g^{-1} for *Chaetomorpha* sp. and 5.40 kcal g^{-1} for *Sargassum* sp collected from Gulf of Kutch. Calorific value of *Monostroma oxyspermum* and *Dictyota sandvicensis* collected from Hawai was reported to be > 3 kcal g^{-1} (McDermid & Steurcke, 2003).

2.3.2 Correlation Studies among Biochemical Constituents

The correlation of the biochemical constituents to species determined the individual biochemical composition ratio (Protein content (P): Carbohydrate content (C): Lipid content (L): Ash content (A): Crude fibre content (F)) (Table 2.3). This study indicated the similarities in biochemical compositions between *A. spicifera* and *C. antennina*. A vague similarity was observed in between *G. corticata* var. *cylindrica* and *G. foliifera*. *A. spicifera*, *C. antennina*, *G. corticata* var. *cylindrica* and *G. foliifera* indicate similar P:L ratios. C:F ratio was almost similar throughout.

Table 2.3 Biochemical composition ratio of the nine seaweeds

Species	P:C:L:A:F Ratio
A. spicifera (N)	4:22:1:7:25
C. antennina (N)	4:23:1:10:27
E. prolifera (N)	1:5:1:2:6
G. pusillum (N)	2:29:1:5:24
G. corticata (N)	3:23:1:10:24
G. corticata var. *cylindrica* (N)	4:47:1:13:27
G. foliifera (N)	4:18:1:14:30
E. prolifera (K)	5:15:1:8:11
U. fasciata (K)	3:4:1:3:5

P - Total protein content (%), C - Total carbohydrate content (%), L - Total lipid content (%), A - Total ash content (%), F - Crude fibre content (%), N - Njarakkal and K - Kayamkulam.

Bivariate Pearson's correlation analysis indicated positive values, exhibiting direct progressive relation and negative values, exhibiting regressive relations. In the total biochemical compositional correlation analysis (Table 2.4 (a)), total protein content showed positive correlations with total lipid content (+ 0.586) and negative correlations with the iodine content (- 0.690), significant to < 0.001 level. Total carbohydrate content showed positive correlations with calorific

value (+ 0.528), and negative correlation with total ash content (- 0.513). Between total lipid content and iodine content, the correlation factor was - 0.682 i.e., significant to the level of < 0.001. No other correlations were observed in prominence between the biochemical compositions possessing significant relations. Negative correlation was reported between the total ash content and calorific value on the indigenous seaweeds viz., *Chaetomorpha* sp. and *Sargassum* sp. (Kumar et al., 2009).

Table 2.4 Pearson test bivariate correlation studies.

(a) Total biochemical compositional correlation analysis (n=27).

	P	C	L	I	A	F	K	M
P	1							
C	-0.470*	1						
L	0.586***	-0.222	1					
I	-0.690***	0.174	-0.682***	1				
A	0.410	-0.513**	0.296	-0.456*	1			
F	-0.484**	0.313	0.107	0.003	-0.476**	1		
K	0.241	0.528**	0.497**	-0.212	-0.059	-0.129	1	
M	0.407	0.345	0.300	-0.553**	-0.099	-0.208	0.449*	1

(b) Division wise biochemical compositional correlation analysis - Chlorophyta (n=12).

	P	C	L	I	A	F	K	M
P	1							
C	-0.822***	1						
L	0.220	-0.159	1					
I	0.503	-0.727***	-0.560	1				
A	-0.098	0.645*	-0.072	-0.535	1			
F	-0.830***	0.542	0.295	-0.614*	-0.209	1		
K	0.482	-0.075	0.780***	-0.514	0.452	-0.206	1	
M	0.794***	-0.841***	-0.335	0.921***	-0.360	-0.832***	-0.146	1

(c) Division wise biochemical compositional correlation analysis - Rhodophyta (n=15).

	P	C	L	I	A	F	K	M
P	1							
C	-0.238	1						
L	0.390	0.390	1					
I	-0.337	-0.605*	-0.965***	1				
A	0.001	-0.751***	-0.867***	0.933***	1			
F	0.505	0.193	0.934***	-0.863***	-0.675**	1		
K	-0.564	0.934***	0.179	-0.383	-0.620**	-0.010	1	
M	-0.227	0.738***	0.314	-0.458	-0.669**	-0.036	0.663**	1

(d) Family wise biochemical compositional correlation analysis - *Gracilaria* (n=9).

	P	C	L	I	A	F	K	M
P	1							
C	-0.861***	1						
L	0.189	-0.662	1					
I	0.826**	-0.424	-0.398	1				
A	0.917***	-0.586	-0.219	0.982***	1			
F	0.950***	-0.976***	0.485	0.610	0.747**	1		
K	-0.849***	0.999***	-0.680	-0.403	-0.567	-0.971***	1	
M	-0.974***	0.723*	0.039	-0.933***	-0.984***	-0.855***	0.706*	1

* - Correlation is significant at the 0.02 level (2-tailed).

** - Correlation is significant at the 0.01 level (2-tailed).

*** - Correlation is significant at the 0.001 level (2-tailed).

P - Total protein content (%), C - Total carbohydrate content (%), L - Total lipid content (%), I - Iodine content (μg g^{-1}), A - Total ash content (%), F - Crude fibre content (%), K - Calorific value (kcal g^{-1}) and M - Total moisture content.

The correlation studies upon extension to division wise analysis (Table 2.4 (b)) in Chlorophyta exhibited significant negative correlations between the total carbohydrate content and moisture content (- 0.841), crude fibre and moisture content (- 0.832), total protein content and carbohydrate content (- 0.822), total carbohydrate content and iodine content (- 0.727) and iodine

content and crude fibre content (- 0.614). Positive correlations were observed between iodine content and moisture content (+ 0.921), total protein content and moisture content (+ 0.794), total lipid content and calorific value (+ 0.780) and total carbohydrate content and ash content (+ 0.645).

Rhodophyta exhibited significant positive correlation (Table 2.4 (c)) between total carbohydrate content and calorific value (+ 0.934), total lipids content and crude fibre content (+ 0.934), iodine content and total ash content (+ 0.933), total carbohydrate content and moisture content (+ 0.738), and between calorific value and moisture content (+ 0.663). Significant negative correlations were observed between total lipids content and iodine content (- 0.965), total lipids content and ash content (- 0.867), iodine content and crude fibre content (- 0.863), total carbohydrate content and ash content (- 0.751), total ash content and crude fibre content (- 0.675), moisture content (- 0.669) and calorific value (- 0.620) and between the total carbohydrate content and iodine content (- 0.605).

Gracilaria genus on its intra species correlation (Table 2.4 (d)), exhibited significant positive correlation between total carbohydrate content and calorific value (+ 0.999), iodine content and ash content (+ 0.982), total protein content and crude fibre content (+ 0.950) and between total protein content and ash content (+ 0.917). Significant negative correlations were also observed between ash content and moisture content (- 0.984), total carbohydrate content and crude fibre content (- 0.976), total protein content and moisture content (- 0.974), crude fibre content and calorific value (- 0.971) and iodine content and moisture content (- 0.933).

2.3.3 Contribution of Seaweed Biochemical Constituents to RDI

Evaluation of seaweed biochemical constituents and their contribution to the RDI is important. RDI levels and the amount of seaweeds required to be

included in the daily food intake are described in Table 2.5. As per the stated standards, for an average person, a daily intake of 50 g protein, 300 g carbohydrate, 65 g lipid, 150 mg iodine, 25 g crude fibre and 2000 calories of energy is recommended. In order to meet the values, food has to be supplemented with other sources of nutritional inputs which are generally stated as the nutraceutical components. Among the sampled seaweeds, *E. prolifera* (Michalak & Chojnacka, 2009; Farasat et al., 2013), *U. fasciata* (Selvin & Lipton, 2004; Silva et al., 2013), *A. spicifera* (Devi et al., 2009; Flora & Rani, 2013; Guiry, 2015) and *Gracilaria corticata* (Zandi et al., 2010; Narasimman & Murugaiyan, 2012) were observed to be edible. In the present study, the seaweeds *U. fasciata* and *E. prolifera* were observed to be a potential source that could be suggested as a protein supplement. A daily intake of 199 g and 286 g would be enough to meet the required levels. *G. pusillum* and *G. corticata* var. *cylindrica* were observed to be a promising source of carbohydrates. *E. prolifera* and *U. fasciata* also proved to be an alternate source of lipids even though the consumption quantity was observed to be on a higher side. Hence with, these seaweeds could be utilized in the natural extraction sectors so as to develop an alternate mode of nutritional products in the form of value added food or health supplements. All the seaweeds were observed as a good source of iodine content and these could be taken along with food in the range of 0.81 to 3.62 g per day. The *Gracilaria* sp. was observed to be the major contributor to iodine. Similarly, all the seaweeds were observed as a rich source in dietary fibres and a daily consumption of 39 to 67 g per day could meet the daily requirements. Obesity is the current adverse impact on the changed food consumption patterns. Seaweeds, which provide the recommended energy levels with minimum

consumption, could result in the declination of the risk of obesity. All the analysed seaweeds were observed to be a good contributor to energy.

Table 2.5 Contribution of seaweed biochemical constituents to RDI.

Species	Dietary inputs					
	Protein	Carbohydrate	Lipid	Iodine	Crude fibre	Calorific value
RDI	50 g	300 g	65 g	150 µg	25 g	2 kcal
	Recommended daily intake of seaweed (g)					
A. spicifera (N)	533.62	537.54	2610.44	1.03	40.39	0.99
C. antennina (N)	556.17	619.32	3110.05	2.59	43.93	1.44
E. prolifera (N)	407.83	590.20	604.09	3.62	40.59	0.57
G. pusillum (N)	836.12	395.00	2452.83	1.09	39.44	0.57
G. corticata (N)	809.06	684.15	3439.15	0.86	54.85	0.92
G. corticata var. *cylindrica* (N)	836.12	425.17	4362.42	0.84	62.39	0.60
G. foliifera (N)	716.33	967.12	3846.15	0.81	48.61	1.20
E. prolifera (K)	286.53	596.07	1940.30	2.84	66.54	0.66
U. fasciata (K)	199.04	904.43	880.76	2.40	65.84	0.67

N- Njarakkal location and K- Kayamkulam location.

2.4 Conclusion

The chapter reported the determination of proximate composition of selected seaweeds from Kerala coast. Five among the nine seaweeds sampled were already reported to be edible. Other than the edible forms, *G. pusillum* and *Gracilaria* sp. were reported to be used for agar extractions. Green and red seaweeds examined in this study had almost comparable biochemical constituents. Green seaweeds were found to possess higher protein, lipid, calorific value and water holding capacity. Red seaweeds had higher carbohydrates, iodine content, crude fibre and calorific values. The correlation studies done were capable to explain how nutrition supplements can affect the concentrations of the corresponding counterparts. Compositions with positive correlation increases up with the supplement intakes and those with negative

correlation decreases up. The process is dynamic. In general, the protein content had significant correlations with lipid, ash and moisture contents. Carbohydrate had significant correlations with calorific value, crude fibre and moisture contents. High significance of correlations was observed when calorific value correlates with lipid and moisture contents. Water content which was considered upon in the study showed the promotional inputs on nutritional composition of the organism.

The comparative analysis to determine the acceptance in nutrient levels, as a source of food or on commercial aspects, screened out the analysed seaweeds and determined that the class of green seaweeds would be advisable for further exploitation. Edible seaweeds such as *E. prolifera* and *U. fasciata* contributed maximum to the RDI levels. The overall results under consideration with total ash content suggests *G. pusillum* as the best source for commercial exploitations, even though all the nine seaweeds studied could be considered as nutritional supplements. Energy contribution was found to be highest in *G. pusillum* and *E. prolifera* where the source could act as food and energy alternatives.

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Chapter 3

VITAMINS, FLAVONOIDS AND CAROTENOIDS

Contents

3.1 Introduction

The propagating world population has increased the demands for pharmaceutical products that are capable of treating illness. Illness in this context refers to those types of diseases which are caused due to the change in life style and those that couldn't be treated with the existing medicines. These diseases are formed due to the generation of free radicals as a result of oxidative stress. They are generally degenerative and results in chain reactions in our body. In order to protect our body from such damages, these free radicals have to be deactivated. This could be done by the administration of synthetic antioxidants such as butylatedhydroxytoluene (BHT), tertiary-butylatedhydroxyquinone (t-BHQ), resorcinol etc. But their prolonged usage reported to possess adverse actions such as tumours and carcinogenic growths. Hence, the search for an alternative and that too from the nature is inevitable. Phytochemicals with economic importance have attracted a vast attention, mainly on their therapeutic usage as a reduction of oxidative stress. Food with higher ends of nutritive values is still in its prime research. In the human health point of view, the vegetative metabolites are upcoming onto possess an

extreme importance by exhibiting anti-inflammatory, antimutagenic, antitumour, antidiabetic and antihypertensive actions (Andrade et al., 2013)

The extending consumer demands for bioactive food ingredients from natural sources have explored the possibilities of seaweeds in this sector. Seaweeds are generally exposed to extreme conditions like salt variations, nutrient variations, temperature fluctuations etc., and thus can result in the formation of free radicals and oxidising chemicals. The natural defence mechanism of the organism can thus create up an adaptive mechanism by the production of secondary metabolites which protects them from the oxidative stress (González et al., 2009). Seaweeds find its application in nutraceuticals as antioxidants, which are dietary in nature for human health in preventing ageing and degenerating diseases like the cardiovascular and cancerous growths through radical scavenging activities (Li et al., 2008a).

The extraction of these bioactive components and its quantifications from the algal source is generally complex in nature with the extractives compositing of various compounds. The contents generally vary temporally and seasonally. The effects of different extracting solvents have been reported from plant material (Pinelo et al., 2004). Most of the methods evaluated the extraction yields (Hayouni et al., 2007). López et al. (2011) established the mode of extraction which was observed to be effective when the water-methanol gradient system was used.

Algae/seaweeds which have the capability of photosynthesis led to the perception that they are autotrophic organism which only require light and a mixture of inorganic nutrients for their growth. The studies exhibited that Chlorophyta require thiamine as a growth factor (Lwoff & Dusi, 1937). The combination of cyanocobalamin (B12), thiamine (B1) and biotin (B7) was

found to be important for algal dwelling (Provasoli & Carlucci, 1974). Biotin acts as a growth promoting factor for yeast (Wildiers, 1901) and is also a cofactor for several essential caboxylase enzymes including acetyl coenzyme A (Acetyl-CoA) carboxylase (Streit & Entcheva, 2003). Thiamine also plays an important role in inter-mediatory carbon metabolism (Croft et al., 2006). It was the first vitamin that was found to be an algal growth factor (Lwoff & Dusi, 1937). Cyanocobalamin is a cobalt containing tetrapyrrole in relation to chlorophyll and heme. It was also reported to be the growth factor in algae (Robbins et al., 1950). Studies also showed that seaweeds have a B12 dependant methionine synthase which has the role in algal methionine biosynthesis (Met H). But higher plants don't require these due to the B12 independent methionine synthase (Met E) and not Met H. But the requirements for vitamins by seaweeds/algae for growth indicate that the source for it is from the environment through many mechanisms that aid in the uptake from its environment (Settembre et al., 2003). The uptake is not as simple, as the environmental concentration is too low (Provasoli & Carlucci, 1974) which could take place only through the uptake from the nearby dwelling bacteria. Algae grew rapidly in presence of bacteria where bacteria produce utilizable vitamins (Fogg, 1965; Kurata, 1986). Seaweeds are sources for water and fat soluble vitamins. Thus, seaweeds are finding application in nutraceuticals where vitamin C decreases blood pressure, betacarotene prevents cardiovascular diseases and vitamins E, C and carotenoids reduce risk for cancer (Škrovánková, 2011). Seaweeds from Hawaiian coast were already established for vitamin A and C (McDermid & Stuercke, 2003). Thus, human longetivity can be elaborated by the biochemical factors where vitamins also play a pivotal role (Kwak et al., 2010). Microalgae also contain water and fat soluble vitamins which are being used as food supplements. Therefore, both

micro and macro algae can be used as non conventional sources of vitamins or as vitamin supplements for human nutrition (Fabregas & Herrero, 1990).

The phenolic compounds are a general classification of compounds that contain a hydroxyl group attached to a benzene ring or its analogues. It constitutes of polyphenols and its monomers. They are generally observed in the terrestrial/aquatic flora and are beneficial to human health (Kuda et al., 2007). They are further classified as flavonoids and non-flavonoids. They are reported as non nutritive chemicals and do exhibits high biological effects. They are also capable of quenching free radicals. Artificial chemical antioxidants could work on these effects but, they show side effects like impairment to liver and carcinogenicity (Wichi, 1988). Therefore, the need to isolate and characterize natural antioxidants with less or no side effects was in greater considerations (Matsukawa et al., 1997). Seaweeds are known for its pharmacological and food additive effects (Lim et al., 2002; Athukurake et al., 2003). Naturally occurring phenolic substances are also attaining greater view as antioxidants and studies promote their pharmacological activities too (Bonilla et al., 1999; Kurihara et al., 1999; Konczak et al., 2010; Mudnic et al., 2010). Phenolics are used as natural antioxidants which are nowadays being consumed in larger quantities. The extending consumer demands for bioactive food ingredients of natural source explored the possibilities of seaweeds in matching the elongated needs (Kanazawa et al., 2008; Sangeetha et al., 2008; Granado-Lorencio et al., 2009; Miyashita, 2009). Polyphenolic compounds including flavonoids are found to be safe and non toxic antioxidants. The higher intake of polyphenols reduces the tendency of diseases like cancer, diabetics, obsesity etc. (Jonathan & Kevin, 2006; Halliwell, 2007; Yan & Asmah, 2010). Their presence in seaweeds is already established (Zhang et al., 2006) and its dissolution and diversity in water at coastal region of India are

known. They possess antioxidant properties too (Riso et al., 2004; Hu et al., 2008; Miyashita, 2009). Rhodophyceae are known to have higher concentrations of phenolic compounds (Zhao et al., 2004; Duan et al., 2007; Ma et al., 2007; Li et al., 2008b). The flavonoids act as potential antioxidants too (Pier-Giorgio-Pietta, 2000; Yan & Asmah, 2010; Lalopua et al., 2011).

Carotenoids constitute the hydrocarbon, carotene and their oxygenated derivatives, the xanthophylls. It is made of eight isoprenoid units. It has a characteristic linear C-40 molecular structure with 11 conjugated double bonds (Farré et al., 2010; Takaichi, 2011). They are generally produced by colourful organisms especially in red, yellow and orange coloured fruits, flowers and vegetables (Farré et al., 2010). Animals absorb the same and it acts as an antioxidant active component (Bartley & Scolinik, 1995; Vishnevetsky et al., 1999). Seaweeds being a marine photosynthetic organism are mainly classified on the presence of carotenoids as green, brown and red. Lutein and zeaxanthin contents in seaweeds express the two biosynthesis compounds of lycopene - α-carotene and β-carotene respectively (Mikami & Hosokawa, 2013). In animals, they are also required for normal growth and tissue repairs. Lutein and zeaxanthin have recently acquired a greater view in nutraceutical industries in paste and granular forms. The needs for natural colorants like carotenoids are now in greater considerations to replace toxic chemicals (Abd El-Baky et al., 2003).

This chapter deals with the evaluation of some economically important biochemical compounds from green and red seaweeds. The extracts of the algae were used to estimate the carotenoids -lutein and zeaxanthin, total phenolics, flavonoids - myricetin, quercetin, luteolin, kaempferol and apigenin and vitamins-thiamine, biotin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, ascorbic acid, ergocalciferol, tocopherol,

phylloquinone and retinol. The extract was also summarized for the antioxidant analysis. This study on the nine seaweeds will be a database for increasing the attention towards commercialized cultivation. The selectivity will be based upon the carotenoids and flavonoids contents, whereas the nutritive value would be promoted by the vitamins presence. The overall commercialization would depend on the activity potentials exhibited by their extracts (Farvin & Jacobsen, 2013). Moreover, the seaweeds were compared with the RDI values of vitamins as recommended by the Council of Responsible Nutrition (Council of Responsible Nutrition, 2015), in order to derive the minimum consumption quantities of seaweed extracts by a healthy individual so as to meet the vital requirements. Similar studies done in seaweeds on elsewhere are tabulated in the Table 3.1.

Table 3.1 Seaweed survey - biochemical compositions within the scope of this chapter.

Biochemical compound	Seaweed	Reference
Fucoxanthins	*Sargassum elegans*	Ayyad et al. (2011); Ragubeer et al. (2012)
	Undaria pinnatifida	Fung et al. (2013)
	Alaria crassiflora	Airanthi et al. (2011)
	Cladosiphon okamuranus	Mise et al. (2011)
	Cystoseira hakodatensis	Airanthi et al. (2011)
	Eisenia bicyclis	Airanthi et al. (2011)
	Fucus serratus	Strand et al. (1998)
	Fucus vesiculosus	Zaragoza et al. (2008)
	Hijika fusiformis	Yan et al. (1999)
	Ishige okamurae	Kim et al. (2010)
	Kjellmaniella crassifolia	Airanthi et al. (2011)
	Laminaria japonica	Das et al. (2008; Zhang et al. (2008; Miyata et al. (2009)
	Laminaria ochotensis	Miyata et al. (2009)
	Myagrophis myagroides	Heo et al. (2010)
	Padina tetrastromatica	Sangeetha et al. (2010)
	Petalonia binghamiae	Murakami et al. (2002)
	Sargassum fulvellum	Yan et al. (1999)
	Sargassum heterophyllum	Afolayan et al. (2008)
	Sargassum horneri	Airanthi et al. (2011)

	Sargassum siliquastrum	Heo et al. (2008)
	Undaria pinnatifida	Hosokawa et al. (1999)
Lutein, fucoxanthins, zeaxanthin	*Grateloupia asiatica*	Terasaki et al. (2012)
	Grateloupia livida	Terasaki et al. (2012)
Beta carotene	*Gracilaria changgi*	Norziah & Ching (2000)
Polyphenols	*Ulva clathrata*	Farasat et al. (2014)
	Ulva linza	Farasat et al. (2014)
	Ulva flexuosa	Farasat et al. (2014)
	Ulva intestinalis	Farasat et al. (2014)
	Stypocaulon scoparium	López et al. (2011)
	Fucus serratus	Farvin & Jacobsen (2013)
	Fucus vesiculosus	Farvin & Jacobsen (2013)
	Saccharina latissima	Farvin & Jacobsen (2013)
	Chnodrus crispus	Farvin & Jacobsen (2013)
	Gracilaria vermiculophyllum	Farvin & Jacobsen (2013)
	Sargassum muticum	Farvin & Jacobsen (2013)
	Dictyota dichotoma	Farvin & Jacobsen (2013)
	Enteromorpha intestinalis	Farvin & Jacobsen (2013)
	Ulva lactuca	Farvin & Jacobsen (2013)
	Palmaria plamata	Farvin & Jacobsen (2013)
	Porphyra purpurea	Farvin & Jacobsen (2013)
	Mastocarpus stellatus	Farvin & Jacobsen (2013)
	Polysiphonia fucoides	Farvin & Jacobsen (2013)
	Fucus distichus	Farvin & Jacobsen (2013)
	Fucus spiralis	Farvin & Jacobsen (2013)
	Chlorophyta, Rhodophyta, Phaeophyta	Andrade et al. (2013)
Flavanoids	Green, red and brown algae	Yoshie et al. (2002)
	Ulva clathrata	Farasat et al. (2014)
	Ulva linza	Farasat et al. (2014)
	Ulva flexuosa	Farasat et al. (2014)
	Ulva intestinalis	Farasat et al. (2014)
	Ulva lactuca	Meenakshi et al. (2009)
	Sargassum wightii	Meenakshi et al. (2009)
Myricetin	*Stypocaulon scoparium*	López et al. (2011)
Quercetin	*Stypocaulon scoparium*	López et al. (2011)
Bromophenols	*Rhodomela confervoides*	Li et al. (2012)
Ascorbic acid	*Gracilaria changgi*	Norziah & Ching (2000)
	Various seaweeds	Škrovánková (2011)
	Enteromorpha flexuosa	McDermid & Stuercke (2003)
	Tetraselmis suecica	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Biotin	*Tetraselmis suecica*	Fabregas & Herrero (1990)

	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Cyanocobalamin	*Tetraselmis suecica*	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
	Various seaweeds	Škrovánková (2011)
Folic acid	*Tetraselmis suecica*	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Nicotinic acid	*Tetraselmis suecica*	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Pantothenic acid	*Tetraselmis suecica*	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Pyridoxin	*Tetraselmis suecica*	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Retinol	Various seaweeds	Škrovánková (2011)
	Gracilaria changgi	Norziah & Ching (2000)
	Tetraselmis suecica	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Riboflavin	Various seaweeds	Škrovánková (2011)
	Tetraselmis suecica	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Thiamine	Various seaweeds	Škrovánková (2011)
	Tetraselmis suecica	Fabregas & Herrero (1990)
	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Tocopherol	*Durvillaea antarctica*	Ortiz et al. (2006)
	Ulva lactuca	Ortiz et al. (2006)
	Various seaweeds	Škrovánková (2011)
	Tetraselmis suecica	Fabregas & Herrero (1990)

	Isochrysis galbana	Fabregas & Herrero (1990)
	Dunaliella tertiolecta	Fabregas & Herrero (1990)
	Chlorella stigmatophora	Fabregas & Herrero (1990)
Tocotrienol	*Druvillaea antarctica*	Ortiz et al. (2006)
	Ulva lactuca	Ortiz et al. (2006)
Antioxidant Activity	*Undaria pinnatifida*	Fung et al. (2013)
	Stypocaulon scoparium	López et al. (2011)
	Rhodomela confervoides	Li et al. (2012)
	Ulva clathrata	Farasat et al. (2014)
	Ulva flexuosa	Farasat et al. (2014)
	Ulva intestinalis	Farasat et al. (2014)
	Ulva linza	Farasat et al. (2014)
	Sargassum elegans	Ragubeer et al. (2012)
	Sargassum wightii	Meenakshi et al. (2009)
	Ulva lactuca	Meenakshi et al. (2009)
	Chnodrus crispus	Farvin & Jacobsen (2013)
	Dictyota dichotoma	Farvin & Jacobsen (2013)
	Enteromorpha intestinalis	Farvin & Jacobsen (2013)
	Fucus distichus	Farvin & Jacobsen (2013)
	Fucus serratus	Farvin & Jacobsen (2013)
	Fucus spiralis	Farvin & Jacobsen (2013)
	Fucus vesiculosus	Farvin & Jacobsen (2013)
	Gracilaria vermiculophyllum	Farvin & Jacobsen (2013)
	Mastocarpus stellatus	Farvin & Jacobsen (2013)
	Palmaria plamata	Farvin & Jacobsen (2013)
	Polysiphonia fucoides	Farvin & Jacobsen (2013)
	Porphyra purpurea	Farvin & Jacobsen (2013)
	Saccharina latissima	Farvin & Jacobsen (2013)
	Sargassum muticum	Farvin & Jacobsen (2013)
	Ulva lactuca	Farvin & Jacobsen (2013)

3.2 Materials and Methods

3.2.1 Samples and Chemicals

All the nine seaweed samples (Table 1.1) were extracted and used for the estimations. All the determinations were done in triplicates against standards brought from Sigma (USA), Aldrich (USA) and Chromadex (USA). Chemicals used were from Merck (Germany) and all were in HPLC grade. Quantifications were done with calibrations equating to $r^2 > 0.950$ and all the

results were remarked with corresponding standard deviations. Purified Milli Q (Millipore- Bedford, MA, USA) water was used.

3.2.2 Preparation of Seaweed Extract

100 g of the dried algal powder was extracted for 2 h in 1:1 methanol-water solvent system consecutively for 5 times. Method suggested by López et al. (2011), had been adopted with slight modifications. The extract was filtered, centrifuged at 2000 rpm for 20 min to remove all the impurities, filtered through 0.45 µm filter paper and then dried under vacuum. The dried residues were weighed to obtain the yield on a dry weight basis of the algal material. This extract was found to be good enough for the total estimations. The extract was fool proofed by estimating the total phenolic contents in a specific seaweed *G. pusillum*, using solvents like water, methanol, isopropyl alcohol, dichloromethane, ammonia (24%), hexane, ethyl acetate, acetone, acetonitrile, butanol, a mixture of ethyl acetate with methanol (05:95, 10:90, 20:80, 25:75, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 95:05) and a mixture of water and methanol (05:95, 10:90, 20:80, 25:75, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 95:05) (Table 3.2).

3.2.3 Estimation of Vitamins

Vitamins (Fig 3.1) analyses and quantifications using HPLC techniques were done following the previously reported analytical procedures (Moreno & Salvado, 2000; Ekinci & Kadakal, 2005; Eriksen, 2008) with adequate modifications. Standards were procured from Chromadex (USA). Standards were prepared in pure methanol subjected to 10 min ultra-sonication. All the standards were soluble in methanol after ultra-sonication. The calibration was done with individual standards and mixtures (Table 3.3), and the correlation coefficients were obtained ($r^2 > 0.999$). The modified method was found to be

capable to detect vitamins at two wavelengths - 210 nm and 280 nm with excellent recovery factor and correlation.

Table 3.2 Comparative analysis of the extraction yields, total and composite phenolic contents, (mean ± SD), (n = 3).

Solvent system	Solvent ratio	Yield (% dwt)	Total phenolic contents (μg g^{-1} of extract)	Available content (in μg)
Hexane	100	11.06 ± 0.42	20.55 ± 0.79	226.07 ± 8.69
Dichloromethane	100	4.24 ± 0.16	9.60 ± 0.37	40.50 ± 1.56
Butanol	100	0.56 ± 0.02	11.40 ± 0.44	6.38 ± 0.25
Isopropyl alcohol	100	9.45 ± 0.36	48.83 ± 1.88	459.03 ± 17.64
Ehtyl acetate (EA)	100	17.89 ± 0.69	61.31 ± 2.36	1091.23 ± 41.94
Methanol (M)	100	14.43 ± 0.55	80.75 ± 3.10	1159.60 ± 44.56
Acetone	100	5.27 ± 0.20	77.04 ± 2.96	403.71 ± 15.51
Acetonitrile	100	3.54 ± 0.14	40.84 ± 1.57	143.77 ± 5.52
EA-M	95:05	17.52 ± 0.67	63.86 ± 2.45	1113.04 ± 42.77
EA-M	90:10	17.21 ± 0.54	65.81 ± 2.53	1126.62 ± 43.30
EA-M	80:20	17.02 ± 0.65	68.63 ± 2.36	1162.62 ± 44.68
EA-M	70:30	16.82 ± 0.72	69.89 ± 2.69	1169.92 ± 44.96
EA-M	60:40	16.41 ± 0.48	72.04 ± 2.47	1176.39 ± 45.21
EA-M	50:50	16.06 ± 0.62	74.01 ± 2.84	1182.65 ± 45.45
EA-M	40:60	15.57 ± 0.60	74.56 ± 2.62	1154.95 ± 44.38
EA-M	30:70	15.45 ± 0.59	75.23 ± 2.28	1156.35 ± 44.44
EA-M	25:75	15.34 ± 0.42	76.26 ± 2.34	1163.72 ± 44.72
EA-M	20:80	14.99 ± 0.58	78.30 ± 2.88	1168.23 ± 44.89
EA-M	10:90	14.92 ± 0.56	79.01 ± 2.04	1173.34 ± 45.09
EA-M	05:95	14.43 ± 0.55	80.75 ± 2.10	1159.60 ± 44.56
W-M	05:95	14.72 ± 0.57	78.75 ± 3.03	1153.71 ± 44.34
W-M	10:90	14.95 ± 0.46	77.57 ± 2.82	1154.18 ± 44.36
W-M	20:80	15.03 ± 0.36	74.02 ± 2.84	1107.31 ± 42.55
W-M	25:75	15.30 ± 0.62	72.62 ± 1.79	1105.30 ± 42.48
W-M	30:70	15.76 ± 0.60	72.30 ± 2.26	1133.66 ± 43.57
W-M	40:60	16.66 ± 0.63	71.95 ± 1.38	1192.90 ± 45.84
W-M	50:50	17.30 ± 0.37	71.06 ± 0.34	1229.06 ± 47.23
W-M	60:40	17.68 ± 0.69	68.56 ± 2.63	1205.99 ± 46.35
W-M	70:30	18.14 ± 0.76	64.70 ± 1.49	1167.87 ± 44.88
W-M	80:20	18.71 ± 0.72	56.94 ± 2.19	1060.28 ± 40.75
W-M	90:10	20.28 ± 0.82	53.52 ± 2.06	1080.04 ± 41.51
W-M	95:05	22.95 ± 0.88	48.93 ± 1.88	1117.46 ± 42.94
WATER (W)	100	23.66 ± 0.91	48.60 ± 1.87	1143.94 ± 43.96
AMMONIA	100	6.90 ± 0.27	18.09 ± 0.70	124.28 ± 4.78

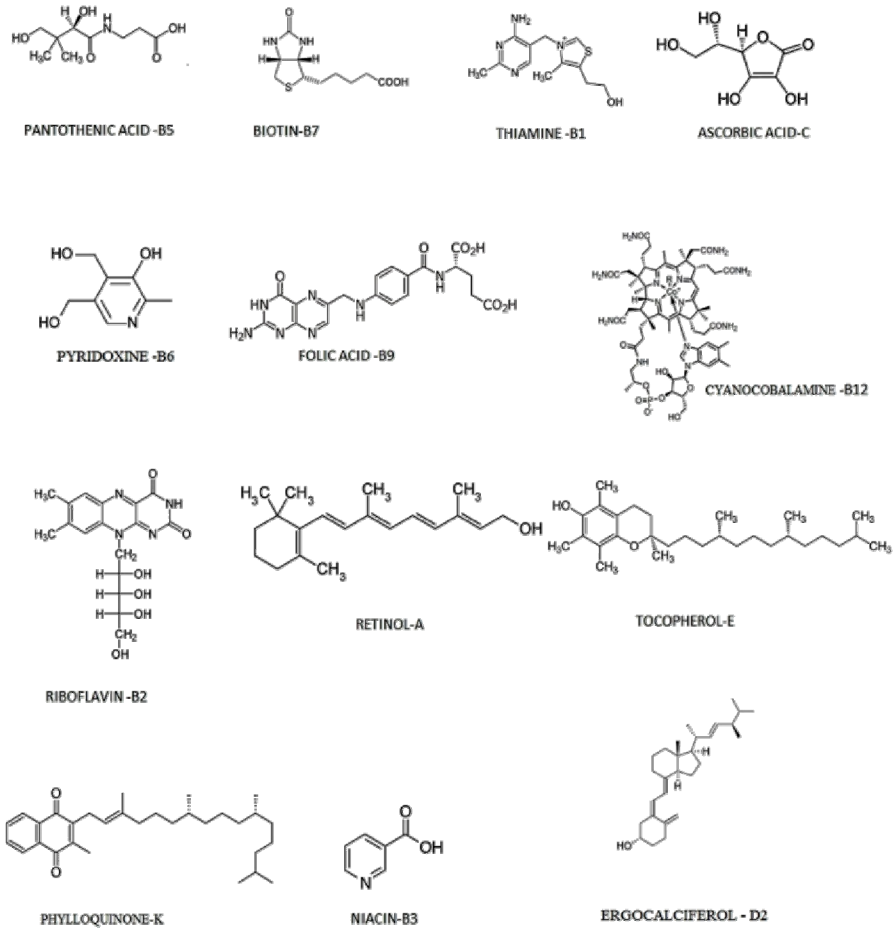


Fig. 3.1 13 vitamins quantified in the study.

Vitamin B7 (biotin) and vitamin B5 (pantothenic acid) were determined at 210 nm (Fig. 3.2), whereas vitamin B1 (thiamine), vitamin C (ascorbic acid), vitamin B6 (pyridoxine), vitamin B9 (folic acid), vitamin B12 (cyanocobalamin), vitamin B2 (riboflavin), vitamin A (retinol), vitamin E (α-tocopherol), vitamin K (phylloquinone), vitamin B3 (niacin) and vitamin D2 (ergocalciferol) were determined at 280 nm (Fig. 3.3). The recovery was determined as 99.88 % and 98.44 % with spiked concentration of ascorbic acid and α-tocopherol respectively in analytical samples which underlined the quantification of the vitamins in the analytes.

Table 3.3 Calibration data of vitamins, total phenolics content, flavonoids and carotenoids.

Standard	Calibration equations	r^2	Concentration (ppm)					Retention time
			1	2	3	4	5	
VITAMINS-HPLC								
B7	y = 0.0054x + .01	1.000	0.65	1.3	1.95	2.6	26	2.642
B5	y = 1.033x - 0.95	0.999	125	250	375	500	5000	3.165
B1	y = 0.2049x - 0.73	0.999	25	50	75	100	1000	3.562
C	y = 3.048x + 0.20	0.999	375	750	1125	1500	15000	5.188
B6	y = 0.0605x + 0.01	1.000	7.5	15	22.5	30	300	12.106
B9	y = 0.1301x + 0.80	0.999	16.25	32.5	48.75	65	650	15.928
B12	y = 2.9802e-05x - 0.33	0.999	0.0375	0.075	0.1125	0.15	1.5	16.519
B2	y = 0.1969x - 0.15	0.999	25	50	75	100	1000	17.658
A	y = 0.2929x + 0.80	1.000	37.5	75	112.5	150	1500	18.171
E	y = 3.8759x + 0.26	1.000	500	1000	1500	2000	20000	19.433
K	y = 1.077x + 0.42	1.000	14	28	42	56	560	19.645
B3	y = 1.4313x - 0.68	0.999	187.5	375	562.5	750	7500	20.826
D2	y = 1.8903e-05x - 0.50	0.999	0.025	0.05	0.075	0.1	1	20.927
AAC	y = 2.8219e-05 - 0.83	0.999	0.0375	0.075	0.1125	0.15	1.5	21.419
EAC	y = 2.799e-05 - 0.84	0.999	0.0375	0.075	0.1125	0.15	1.5	22.724
TOTAL PHENOLICS CONTENT - SPECTROPHOTOMETER								
GA	y = 0.0698x - 0.036	0.999	10000	20000	40000	60000	80000	-
FLAVONOIDS - HPLC								
M	y = 1.608e-0.5x - 0.37	0.999	5	7.5	10	12.5	15	5.481
Q	y = 1.3655e-0.5x + 0.07	0.998	5	7.5	10	12.5	15	8.971
L	y = 1.522e-0.5x - 0.02	0.997	5	7.5	10	12.5	15	10.355
KA	y = 1.45e-0.5x - 0.42	1.000	5	7.5	10	12.5	15	13.652
AP	y = 1.493e-0.5x - 0.03	0.995	5	7.5	10	12.5	15	14.986
CAROTENOIDS - HPLC								
LUT	y = 9.168e-05x + 0.45	1.000	9.84	19.68	29.52	39.36	49.2	3.567
ZEA	y = 4.696e-05x - 0.55	0.999	0.84	1.68	2.52	3.36	4.2	3.816

B7 - Biotin, B5 - Pantothenic acid, B1 - Thiamine, C - Ascorbic acid, B6 - Pyridoxine, B9 - Folic acid, B12 - Cyanocobalamin, B2 - Riboflavin, A - Retinol, E - Tocopherol, K - Phylloquinone, B3 - Niacin, D2 - Ergocalciferol, ACC - Retinol acetate, EAC - Tocopherol acetate, GA - Gallic acid, M - Myricetin, Q - Quercetin, L - Luteolin, KA - Kaempferol, AP - Apigenin, LUT - Lutein and ZEA - Zeaxanthin.

The instrument used was Shimadzu UFLC-LC-20AD equipped with UV-Vis SPD-20A detector, 20 µL loop, phenomenex luna 250 mm x 4.6 mm x 5µ C-18 column and LPGE pumping system. The column was maintained under oven temperature at 27 ± 0.1 ^{o}C. The solvent system was a gradient between acetonitrile-water (A) in the ratio 60:40 and KH_2PO_4 buffer (B) at 1 mL min^{-1} flow rate and 27 min as run time (Gradient system used was time : B concentration as 0:100, 4:100, 14:65, 14.5:20, 19:20, 19.5:100, 27:100). The buffer was made by dissolving 0.140 g KH_2PO_4 in 900 mL HPLC grade water followed with addition of 0.5 mL o-phosphoric acid and made up to 1 L with HPLC grade water.

1 g of the seaweed extracts was refluxed with 6 N HCl in water for 24 h at 120 ^{o}C. The extract was filtered and concentrated under vacuum to get free of HCl. It was diluted in methanol and made up to 25 mL in 75% methanol-water system, filtered through 0.45 µm nylon filter and injected. The results were reported as µg g^{-1} of the vitamins to the dry weight of the seaweed extract.

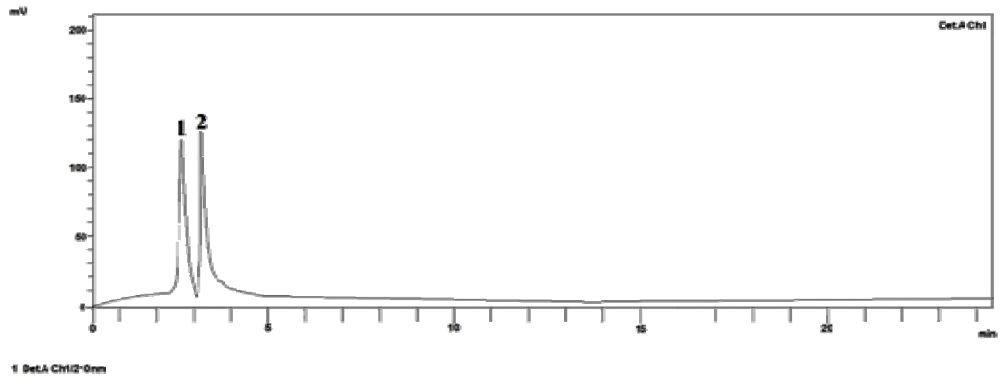


Fig. 3.2 HPLC chromatogram of the Vitamins B7 and B5 standards calibrated at 210 nm.
1 - B7 and 2 - B5.

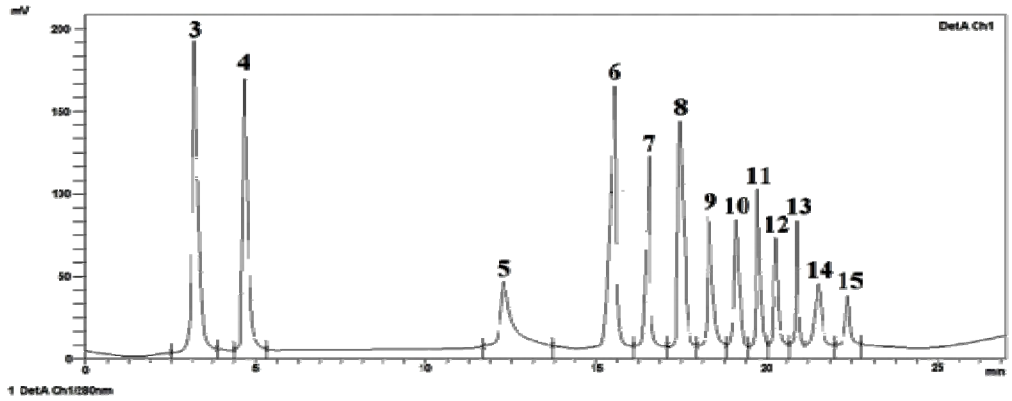


Fig. 3.3 HPLC chromatogram of the vitamins standards calibrated at 280 nm.

3 - B1, 4 - C, 5 - B6, 6 - B9, 7 - B12, 8 - B2, 9 - A, 10 - E, 11 - K2, 12 - B3, 13 - D2, 14 - AAC and 15 - EAC.

3.2.4 Estimation of Total Phenolics

The Folin-Ciocalteu reagent method (Blainski et al., 2013) was used to estimate the total phenolic contents of the seaweeds. 100 to 200 mg of the 1:1 methanol-water extract was dissolved in 100 mL of 80% methanol. 100 µL of the sample solution was dissolved in 500 µL of the Folin-Ciocalteu reagent and 1000 µL of distilled water. The solutions were then mixed and incubated at room temperature for 1 min. After 1 min, 1500 µL of 20 % sodium carbonate solution was added. The final mixture was shaken and then incubated for 2 h in dark at room temperature. The absorbance was measured at 760 nm using a Carry-60 UV-Vis spectrophotometer. A standard curve was plotted using gallic acid as the standard at different concentrations in 80% methanol (Table 3.3). The results were reported as µg g^{-1} of gallic acid equivalents to the dry weight of the seaweed extract.

3.2.5 Estimation of Flavonoids

Standards used were procured from Sigma (USA). Different concentrations (5.0 µg g^{-1} to 15.0 µg g^{-1}) of the standards were made in

methanol for analysis. Calibration was done based on this concentrations (Table 3.3) and the r^2 value was obtained as > 0.995 for all the standards - myrcetin, quercetin, luteolin, kaempferol and apigenin (Fig. 3.4) at the retention times 5.48, 8.97, 10.35, 13.65 and 14.98 min respectively (Fig. 3.5), which showed a good response to determinations with higher confidence. The instrument used was Shimadzu LC-20AD equipped with SPD-20A UV-Vis detector. The column used was phenomenex C-18, 250 mm x 4.6 mm x 5 µ column and detected at 370 nm. The run time was 30 min, at a flow rate of 1.2 mL min^{-1} with a solvent system of 1:1 acetonitrile-water.

The total flavonoid determination was done with the extraction procedures mentioned by Hertoj et al. (1992), with some modifications. 2 g of the extract was refluxed in 40 mL 50 % methanol-water containing 2 % t-BHQ and 60 mL 6N HCl in 50% methanol for 2 h. The extract was filtered and the filtrate was made up to 25 mL, and injected. The results were reported as µg g^{-1} of the flavonoid to the dry weight of the seaweed extract.

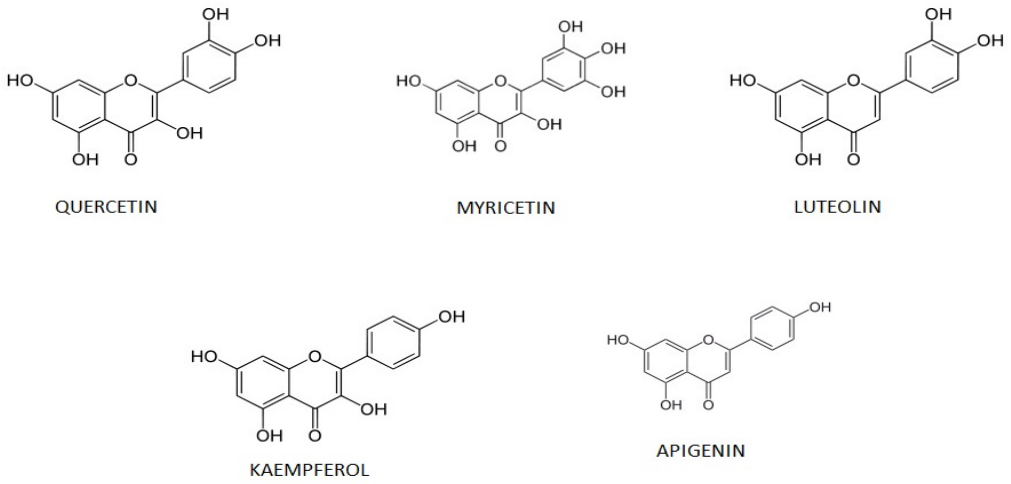


Fig. 3.4 Flavonoids quantified in the present study.

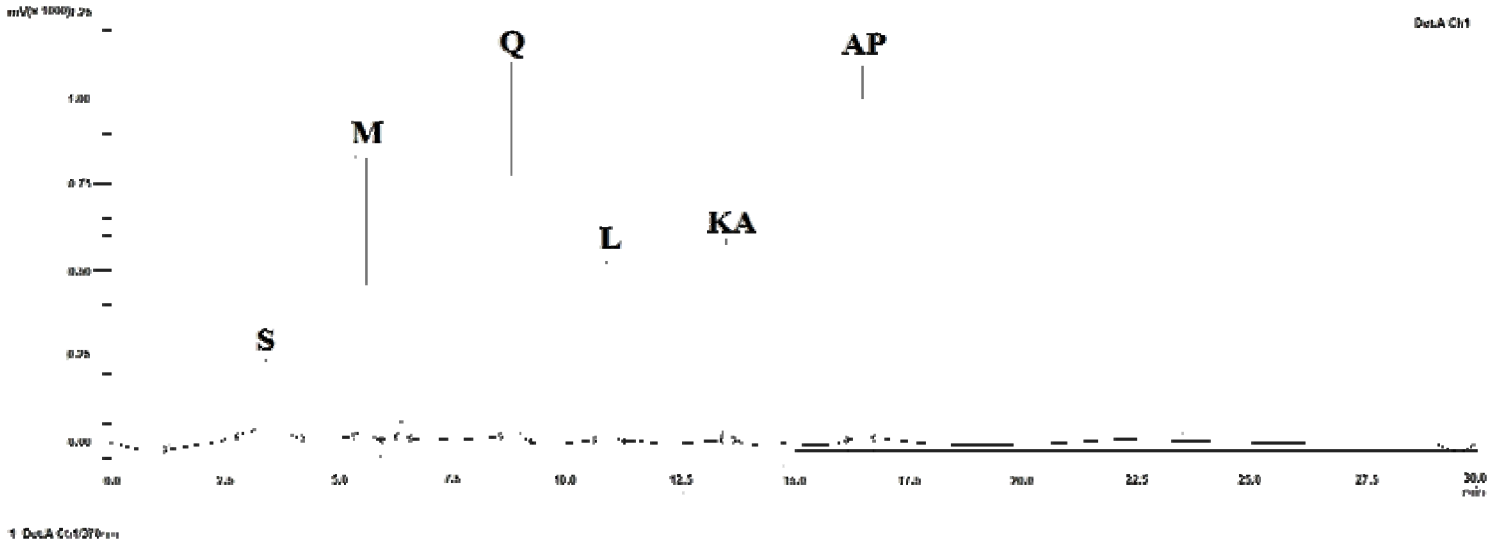


Fig. 3.5 HPLC chromatogram of the flavonoid standards calibrated at 370 nm.

S - Reagent, M - Myricetin, Q - Quercetin, L - Luteolin, KA - Kaempferol and AP - Apigenin.

3.2.6 Estimation of Carotenoids

The extraction of carotenoid pigments (Fig. 3.6) was taken up after reviewing the procedure of Hegazi (2009), where the methanolic extract was made up to 25 mL using hexane-ethyl acetate (70:30 with 1% DCM) and injected for analysis in HPLC. The instrument used was Shimadzu UFLC-LC-20AD equipped with UV-Vis SPD-20A detector, 20 µL loop, phenomenex luna-5 µ-250 mm x 4.6 mm x 5µ silica column and LPGE pumping system. The column was maintained under oven temperature at 27 ± 0.1 ^{o}C. The standards were procured from Aldrich (USA). The calibration was plotted for concentrations of 9.84 to 49.20 µg g^{-1} for lutein and 0.84 to 4.20 µg g^{-1} for zeaxanthin (Table 3.3, Fig. 3.7). The spike and recovery factor exhibited a value > 99% which well established the content in the seaweeds. The results were reported as µg g^{-1} of the corresponding carotenoids to the dry weight of the seaweed extract.

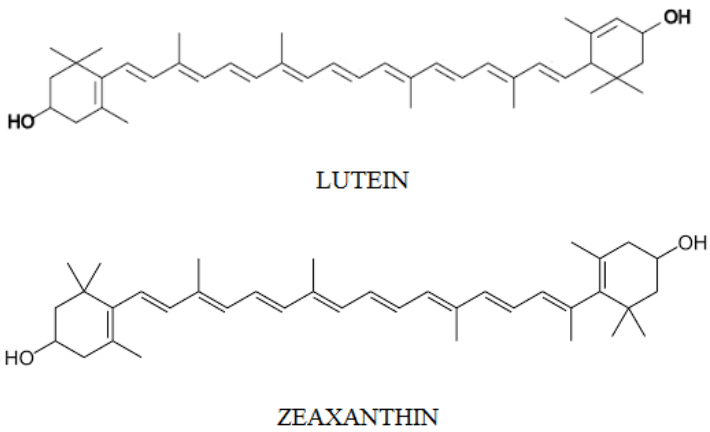


Fig. 3.6 Carotenoids quantified in the current study.

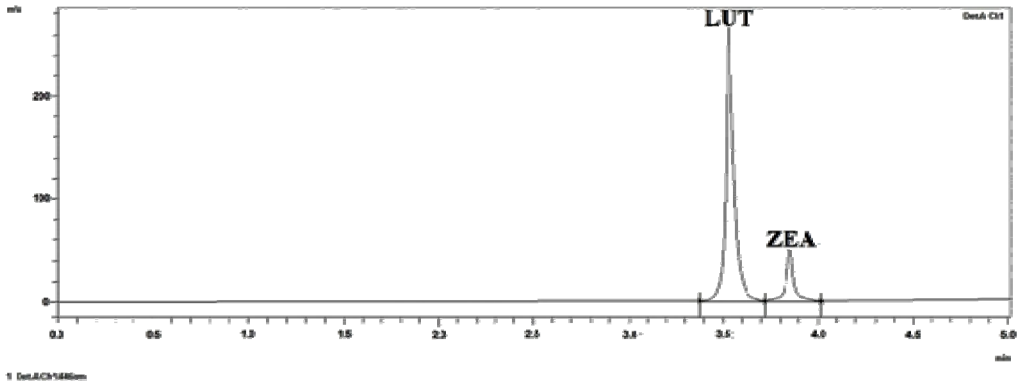


Fig.3.7 Carotenoids - lutein and zeaxanthin calibrated at 446 nm.

LUT - Lutein and ZEA - Zeaxanthin.

3.2.7 Antioxidant Activity

The antioxidant activities of the extracted fractions were surveyed using three prominent methods; 2, 2-diphenyl-1-picrylhydrazyl (DPPH), a modified UV-Vis ferrous tartarate and $KMnO_4$ titration methods against the four standards - ascorbic acid, BHT, α-tocopherol and resorcinol.

3.2.7.1 Antioxidant Assay - DPPH Method

The assay was done in accordance with the method given by Brand-Williams et al. (1995) with some modifications. DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 mL HPLC grade methanol and then stored at - 20 ^{o}C until needed. The working solution was obtained by mixing 10 mL stock solution with 45 mL HPLC grade methanol to obtain an absorbance of 1.170 ± 0.02 units at 515 nm using Cary-60 UV-Vis spectrophotometer. 1.5 mL of the sample solution (0.100 g of extract in 100 mL methanol) was allowed to react after mixing with 28.5 mL of DPPH solution for 24 h in dark. The absorbance was measured at 515 nm against reagent blank. The standard curve was linear between 403 to 1613 µg g^{-1} for ascorbic acid, 409 to 1637 µg g^{-1} for α-tocopherol, 405 to 1621 µg g^{-1} for BHT

and 406 to 1626 μg g^{-1} for resorcinol. Additional dilutions were done upon requirement when observed to be deviating from linear range. The r^2 values were > 0.995 as per the chart appended (Table 3.4). Results were expressed in percentage of equivalence to the corresponding standard to the dry weight of the seaweed extract.

Table 3.4 Calibration data of antioxidant assay.

Standard	Calibration equations	r^2	Concentration (ppm)			
			1	2	3	4
DPPH RADICAL SCAVENGING ACTIVITY-UV/VIS SPECTROPHOTOMETER						
ASC	y = -3.2176e-05x -0.081	0.995	403.36	806.72	1210.08	1613.44
RES	y = -3.1709e-05x - 0.088	0.999	406.56	813.12	1219.68	1626.24
TOC	y = -4.9594e-05x + 0.008	0.999	409.36	818.72	1228.08	1637.44
BHT	y = -2.9294e-05x - 0.064	0.996	405.32	810.64	1215.96	1621.28
FERROUS TARTARATE OXIDISING ACTIVITY-UV/VIS SPECTROPHOTOMETER						
ASC	y = 5.6376e-04x -0.013	0.978	403.36	806.72	1210.08	1613.44
RES	y = 7.2978e-06x - 0.012	0.992	405.60	811.20	1216.80	1622.40
TOC	y = 1.808e-03x + 0.556	0.995	409.36	818.72	1228.08	1637.44
BHT	y = 2.0351e-03x + 0.421	0.994	406.56	813.12	1219.68	1626.24
TOTAL ANTIOXIDANT POTENTIAL-TITRIMETRY						
Standard	**Weight of standards (g/50mL)**	**Blank readings**	**Titre Readings**			**Equivalence of 0.01N $KMnO_4$(g)**
			1mL	**2mL**	**3mL**	
ASC	0.504	0.9	23.0	41.0	61.3	0.00046
RES	0.507	0.9	1.1	1.3	1.4	0.00664
TOC	0.503	0.9	3.1	5.9	6.8	0.00457
BHT	0.507	0.9	2.4	4.0	6.0	0.05069

ASC- Ascorbic acid, RES- Resorcinol, TOC- Tocopherol and BHT- Butylatedhydroxytoluene.

3.2.7.2 Antioxidant Assay - UV-Vis Ferrous Tartarate Method

Antioxidant activity was determined by using the modified spectrophotometric methods (Liang et al., 2003; Li et al., 2005). Analysis was

carried out by accurately weighing 0.100 g of the extract into a 100 mL standard flask, made up to 100 mL using HPLC grade water. 2 mL of the solution was pipetted out into a 50 mL standard flask, to which 8 mL of HPLC grade water was added followed with 10 mL of tartaric acid ferrous sulfate solution and made up to the volume using phosphate buffer solution. The absorbance was measured at 540 nm against the reagent blank. The standard curve was linear between 403 to 1613 µg g^{-1} for ascorbic acid, 406 to 1626 µg g^{-1} for BHT, 409 to 1637 µg g^{-1} for α-tocopherol and 405 to 1622 µg g^{-1} for resorcinol. The r^2 values were > 0.950 (Table 3.4). Results were expressed in percentage of equivalence to the corresponding standard to the dry weight of the seaweed extract.

3.2.7.3 Antioxidant assay - $KMnO_4$ Method

Antioxidant assay was estimated using $KMnO_4$ and indigo carmine dye as an indicator (Ribereau-Gayon-Maurié titrimetric method) (Daničić, 1973; Radovanović, 1986). Indigo carmine dye was prepared by dissolving 1 g indigo carmine in 25 mL con. H_2SO_4, further diluted to 50 mL using con. H_2SO_4 and further diluted to 1 L with distilled water. 50-100 mg of the extract was transferred to 100 mL standard flask and made up to the mark with methanol. 3 mL was pipetted out into a conical flask; 15 mL methanol and 1 mL indigo carmine dye were added and titrated against 0.1N standard $KMnO_4$ solution against reagent blanks (Table 3.4). Results were expressed in percentage of equivalence to the corresponding standard to the dry weight of the seaweed extract.

3.2.8 Contribution to the Recommended Daily Intake (RDI) Levels

The recommended daily intake values for the carotenoids and the phenolics are not available. Council of responsible nutrition have defined the minimum requirements of vitamins for the vital functioning of a healthy individual. These values in comparison with the present values derived the minimum amounts of seaweed extracts to be administered to meet the daily requirements and in the cases of typical vitamin deficiencies.

3.2.9 Statistics

All the values of observations are means of three replicate determinations (± standard deviations). All the statistical correlation analyses were carried out using SPSS 16.0 for Windows. Pearson correlation test with bivariate significance was adopted and values of $p < 0.001$ were considered as significantly correlated (Li et al., 2012; Chakraborty et al., 2013; Imbs et al., 2014).

3.3 Results and Discussion

3.3.1 Seaweed Extraction

Aqueous methanolic extraction (1:1) of the seaweeds showed good extraction yields (Table 3.5). *G. pusillum* (17.21 %), *G. foliifera* (16.84 %) and *A. spicifera* (14.65 %) exhibited the highest extraction yields among the analysed seaweeds. Red seaweeds showed the highest yields with an exception in *G. corticata* (12.08 %) and *G. corticata* var. *cylindrica* (11.16 %). Green seaweeds had almost comparable yields. *C. antennina* (12.83 %), *E. prolifera* (12.04 %) from Njarakkal location and *U. fasciata* (12.18 %) exhibited

similarities in extraction yields. *E. prolifera* (13.14 %) from Kayamkulam location had the highest yield among the green seaweeds.

Table 3.5 Methanol - water extraction yield as % dwt, (mean ± SD), (n = 3).

Seaweed	1:1 Methanol-water Extraction Yield
C. antennina	12.83 ± 0.39
E. prolifera (K)	13.14 ± 0.40
E. prolifera (N)	12.04 ± 0.18
U. fasciata	12.18 ± 0.37
A. spicifera	14.65 ± 0.45
G. pusillum	17.21 ± 0.52
G. foliifera	16.84 ± 0.51
G. corticata	12.08 ± 0.37
G. corticata var. *cylindrica*	11.16 ± 0.34

K-Kayamkulam and N-Njarakkal

The extraction yields were seen in comparison with earlier reports carried out on various seaweed species. Seaweed *S. scoparium* collected from Spain was reported with 16.8% yield in methanol-water system (López et al., 2011). *G. corticata* collected from the Saurashtra coast, Gujarat, India, was observed to derive a methanolic extract yield of 6.19 % (Kumar et al., 2011).

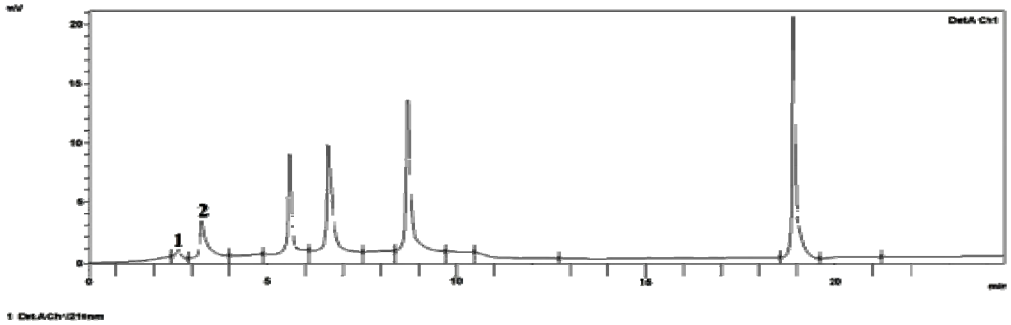
3.3.2 Vitamins

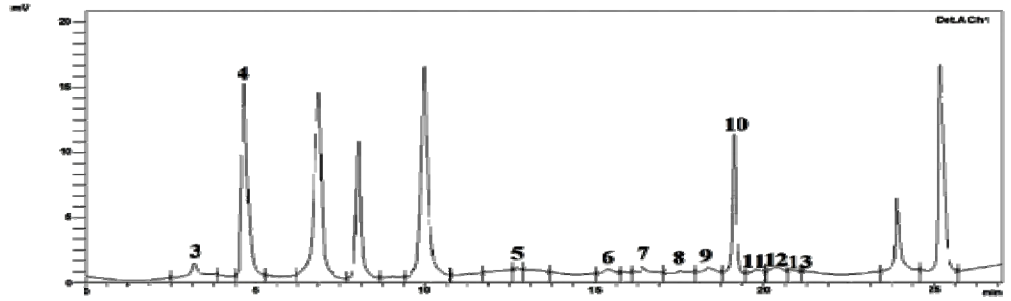
Concentrations of 13 vitamins detected from the nine seaweeds are given in the Table 3.6. Chromatograms of the nine seaweeds are given in Fig. 3.8. Analysis was done to estimate the concentrations of both water soluble and fat soluble vitamins. The detected vitamins were all of plant origins.

Table 3.6 Vitamin contents in the extracts of nine seaweeds analysed as μg g^{-1}, (mean ± SD), (n=3).

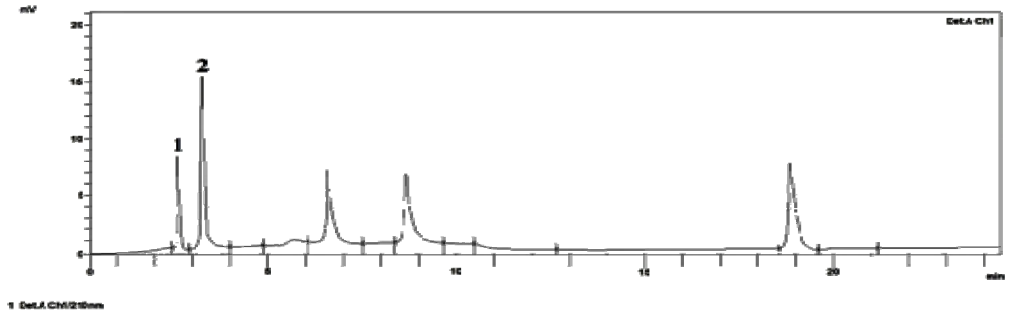
Seaweed	B7	B5	B1	C
CA	3.54 ± 0.055	769.22 ± 11.79	959.43 ± 14.71	17245.67 ± 264.31
EPK	6.06 ± 0.09	2334.18 ± 35.78	1725.74 ± 26.45	19098.45 ± 292.71
EPN	33.81 ± 0.52	3716.07 ± 56.95	1181.53 ± 18.11	12259.92 ± 187.90
UF	7.72 ± 0.12	3952.67 ± 60.58	2077.73 ± 31.84	23808.33 ± 364.90
AS	8.89 ± 0.14	10765.67 ± 164.99	1582.89 ± 24.26	32103.90 ± 492.04
GP	39.92 ± 0.62	13185.24 ± 202.08	5859.52 ± 89.81	22407.62 ± 343.43
GF	54.92 ± 0.84	9211.09 ± 141.17	3041.23 ± 46.62	17297.97 ± 265.15
GC	0.47 ± 0.01	25.84 ± 0.39	152.20 ± 2.33	332.76 ± 5.10
GCC	24.22 ± 0.37	4854.94 ± 74.41	1414.84 ± 21.68	14269.65 ± 218.70
Seaweed	**B6**	**B9**	**B12**	**B2**
CA	338.45 ± 5.19	872.33 ± 13.37	3.46 ± 0.05	209.25 ± 3.21
EPK	795.96 ± 12.19	967.10 ± 14.82	4.52 ± 0.07	346.97 ± 5.32
EPN	813.00 ± 12.46	1388.45 ± 21.28	4.93 ± 0.08	640.99 ± 9.82
UF	411.65 ± 6.31	476.93 ± 7.31	3.81 ± 0.06	836.27 ± 12.82
AS	3065.83 ± 46.99	3156.56 ± 48.38	12.31 ± 0.19	1520.61 ± 23.31
GP	1371.99 ± 21.03	2301.32 ± 35.27	21.37 ± 0.33	6754.29 ± 103.52
GF	742.61 ± 11.38	326.25 ± 5.00	8.61 ± 0.13	825.86 ± 12.66
GC	157.75 ± 2.42	1023.10 ± 15.68	1.54 ± 0.03	856.52 ± 13.13
GCC	222.10 ± 3.41	792.64 ± 12.15	2.23 ± 0.04	396.28 ± 6.07
Seaweed	**A**	**E**	**K**	**B3**
CA	529.37 ± 8.11	15834.59 ± 242.69	1872.95 ± 28.71	1717.05 ± 26.32
EPK	149.21 ± 2.29	4824.85 ± 73.95	1270.70 ± 19.48	1442.41 ± 22.11
EPN	436.29 ± 6.69	1544.68 ± 23.67	2290.29 ± 35.10	3497.51 ± 53.60
UF	337.38 ± 5.18	5311.82 ± 81.41	1070.96 ± 16.42	4821.06 ± 73.89
AS	2315.30 ± 35.49	7764.42 ± 119.00	4123.89 ± 63.20	7793.84 ± 119.45
GP	801.67 ± 12.29	23316.19 ± 357.36	7974.70 ± 122.22	7462.84 ± 114.38
GF	319.42 ± 4.89	4587.96 ± 70.32	521.74 ± 7.99	2403.09 ± 36.83
GC	383.98 ± 5.89	306.14 ± 4.69	1225.10 ± 18.78	573.50 ± 8.79
GCC	504.35 ± 7.73	2399.08 ± 36.77	891.08 ± 13.66	1357.36 ± 20.81
	Seaweed	**D2**	**Total**	
	CA	1.81 ± 0.03	4.04 ± 0.06	
	EPK	1.83 ± 0.03	3.30 ± 0.05	
	EPN	2.28 ± 0.04	2.78 ± 0.04	
	UF	2.38 ± 0.04	4.31 ± 0.07	
	AS	6.37 ± 0.09	7.42 ± 0.11	
	GP	22.26 ± 0.34	9.15 ± 0.14	
	GF	1.77 ± 0.03	3.94 ± 0.06	
	GC	3.71 ± 0.06	0.51 ± 0.01	
	GCC	1.51 ± 0.026	2.71 ± 0.04	

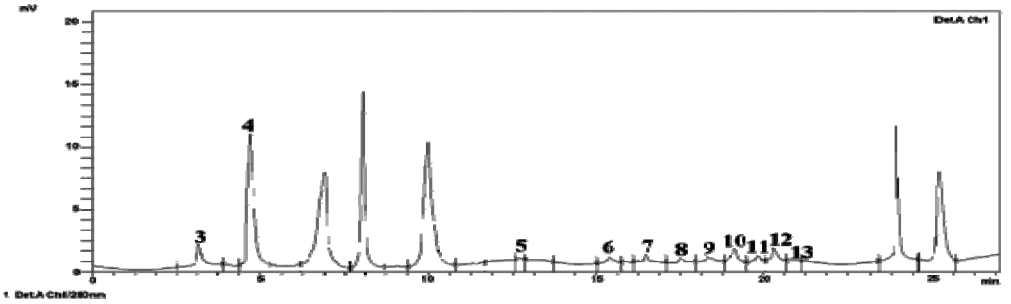
CA - *C. antennina*, EPK - *E. prolifera* from Kayamkulam, EPN - *E. prolifera* from Njarakkal, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, B7 - Biotin, B5 - Pantothenic acid, B1 - Thiamine, C - Ascorbic acid, B6 - Pyridoxine, B9 - Folic acid, B12 - Cyanocobalamin, B2 - Riboflavin, A - Retinol, E - Tocopherol, K - Phylloquinone, B3 - Niacin and D2 - Ergocalciferol.



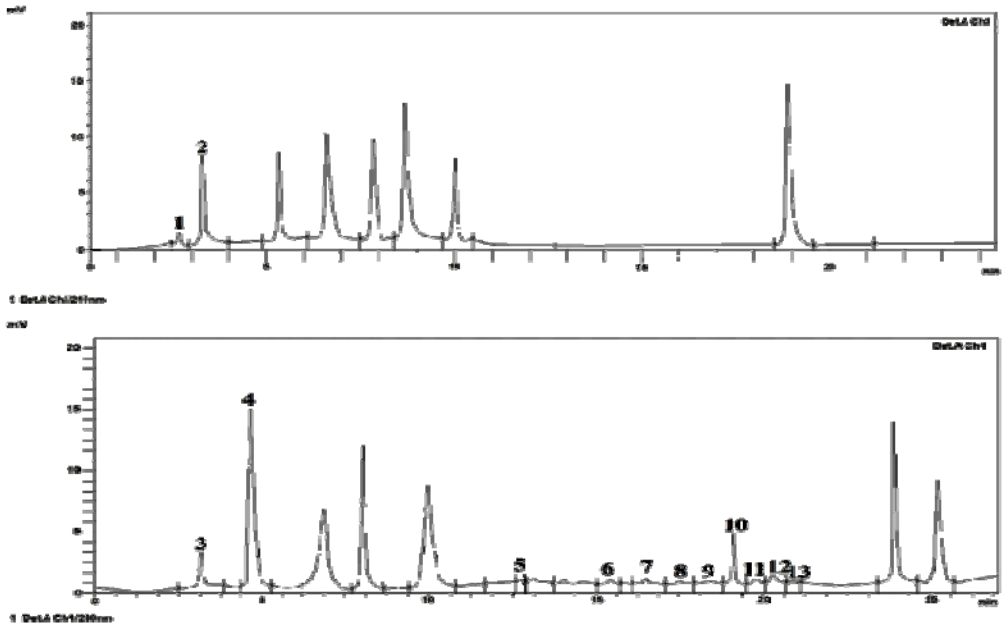


C. antennina

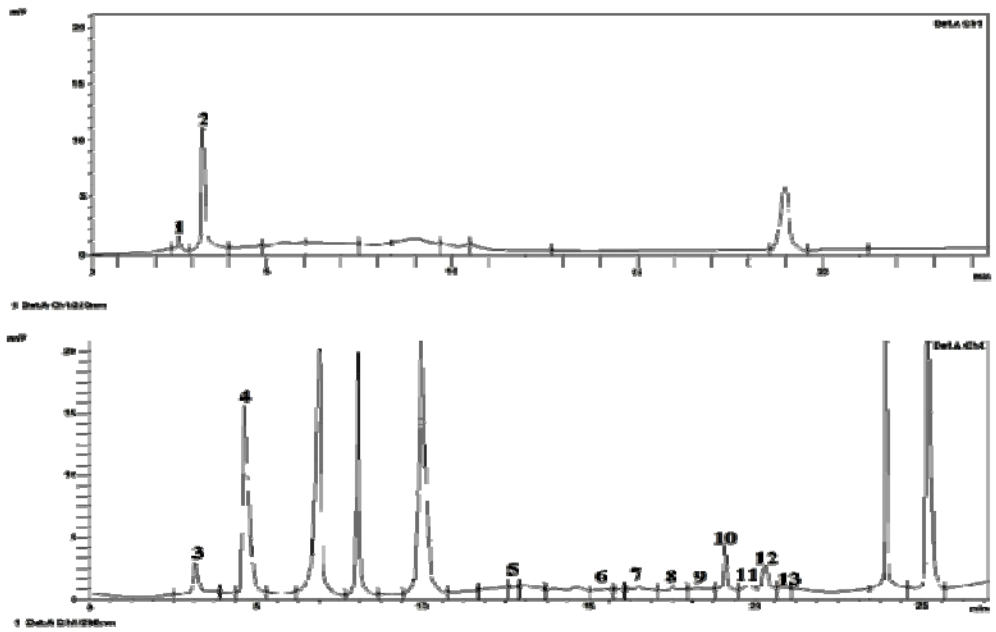




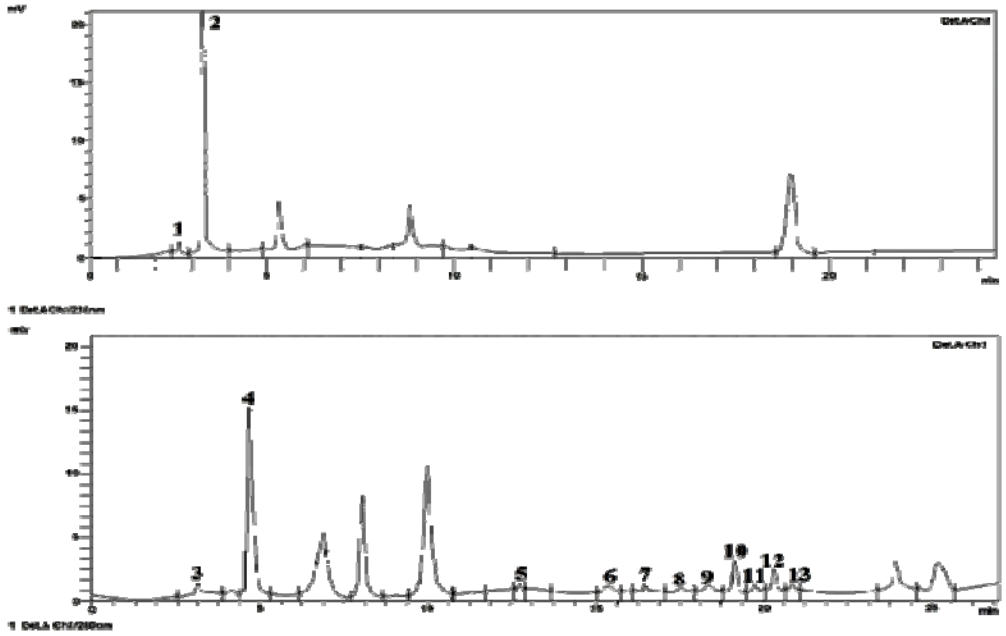
E. prolifera- Kayamkulam location



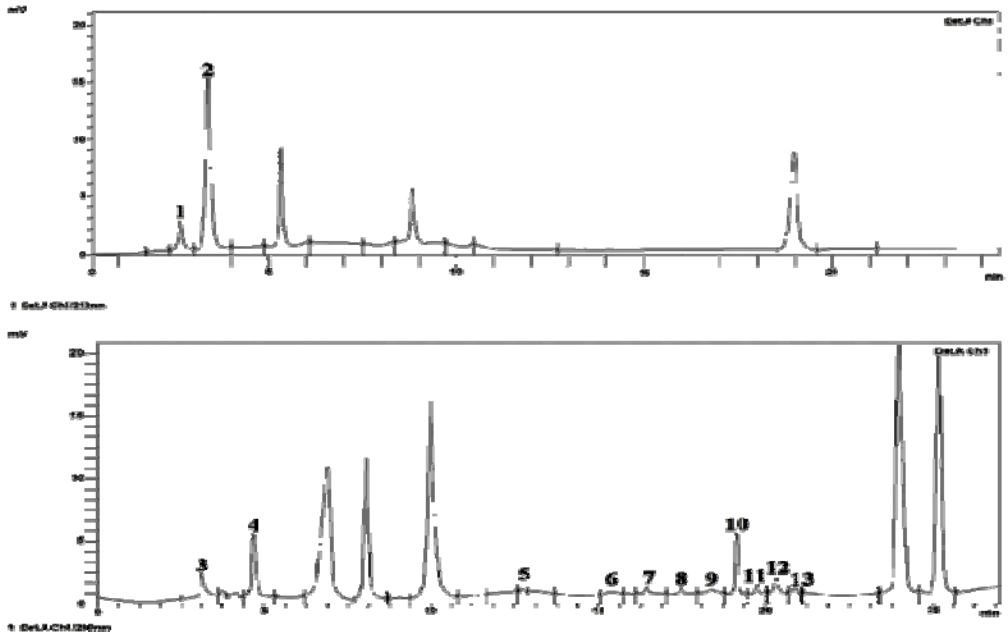
E. prolifera- Njarakkal location



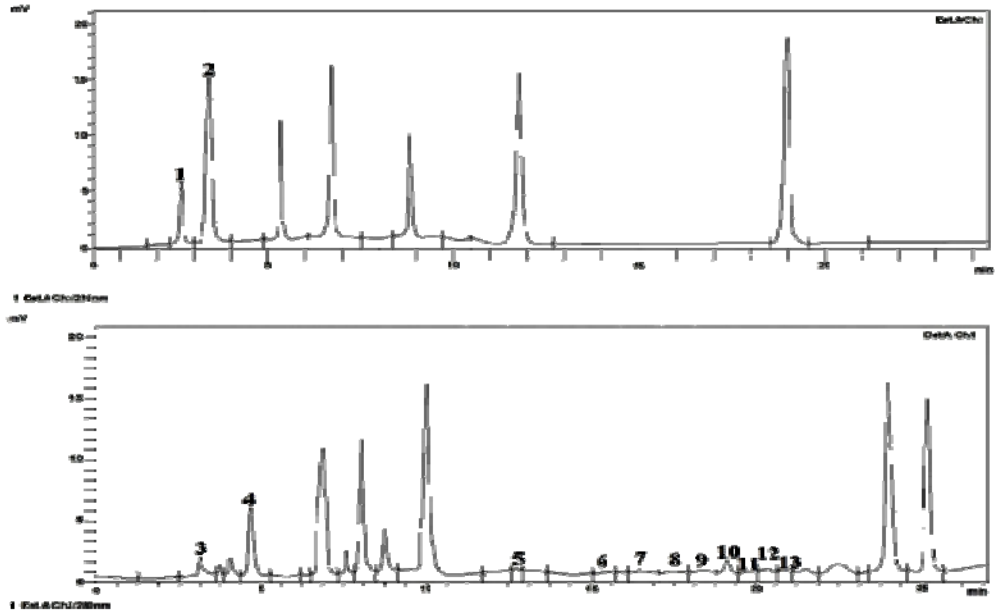
U. fasciata



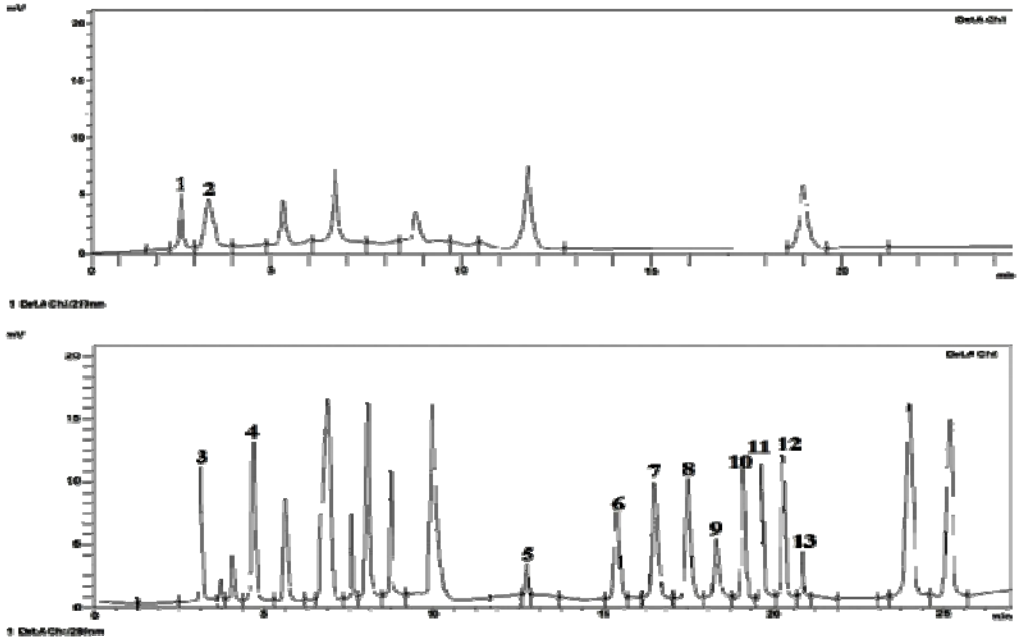
A. spicifera



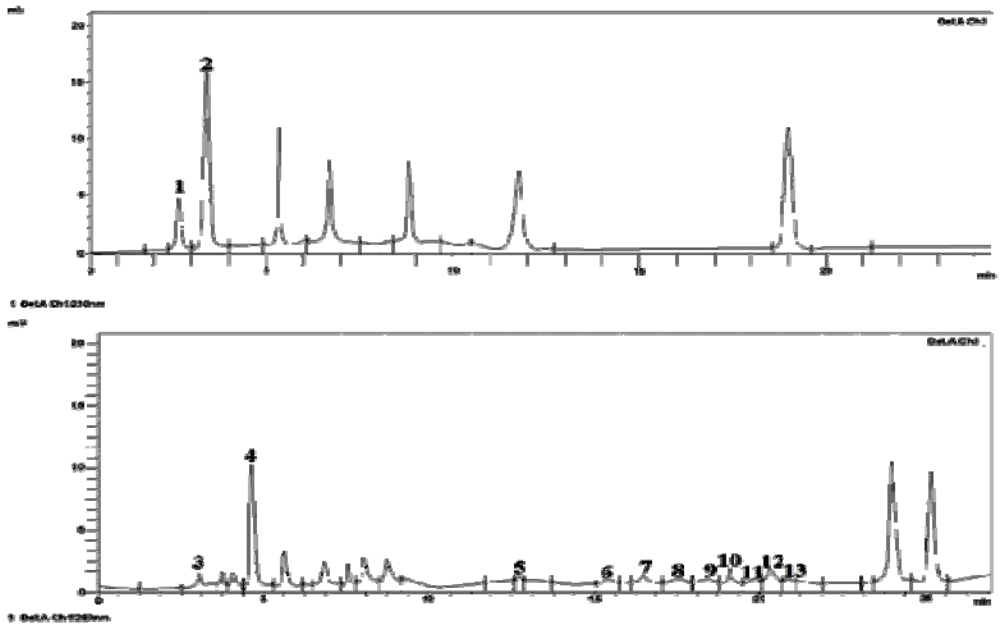
G. pusillum



G. foliifera



G. corticata



G. corticata var. *cylindrica*

Fig. 3.8 Chromatograms of the vitamin contents in the nine seaweed extracts.

1 - B7, 2 - B5, 3 - B1, 4 - C, 5 - B6, 6 - B9, 7 - B12, 8 - B2, 9 - A, 10 - E, 11 - K2, 12 - B3, 13 - D2, 14 - AAC and 15 - EAC.

All the analysed seaweeds are rich in ascorbic acid and α-tocopherol contents, with the exception of *G. corticata*. The highest biotin (54 µg g^{-1}) was reported in *G. foliifera*. Pantothenic acid (13185 µg g^{-1}), thiamine (5859 µg g^{-1}), cyanocobalamin (21 µg g^{-1}), riboflavin (6754 µg g^{-1}), α-tocopherol (23316 µg g^{-1}), ergocalciferol (22 µg g^{-1}) and phylloquinone (7974 µg g^{-1}) were observed to be the highest in *G. pusillum*, whereas retinol (2315 µg g^{-1}), ascorbic acid (32103 µg g^{-1}), folic acid (3156 µg g^{-1}), niacin (7793 µg g^{-1}) and pyridoxine (3065 µg g^{-1}) were highest in *A. spicifera*. Comparatively, lower concentrations of phylloquinone were observed in *G. foliifera* (521 µg g^{-1}) and *G. corticata* var. *cylindrica* (891 µg g^{-1}).

As a general discussion on the vitamin contents in the analysed seaweeds, the biotin contents of *G. pusillum* (39 µg g^{-1}) and *E. prolifera* (33

µg g^{-1}) from Njarakkal location were comparable. Low amounts were seen in *G. corticata* (0.47 µg g^{-1}). Pantothenic acid contents were comparable in between *A. spicifera* (10765 µg g^{-1}) and *G. foliifera* (9211 µg g^{-1}). Least was observed in *G. corticata* with 25 µg g^{-1}. Thiamine content was observed to be comparable in between *E. prolifera* (1725 µg g^{-1}) from Kayamkulam location, *A. spicifera* (1582 µg g^{-1}) and *G. corticata* var. *cylindrica* (1414 µg g^{-1}). Least was observed in *G. corticata* (152 µg g^{-1}). Ascorbic acid content was observed to be similar in between *C. antennina* (17245 µg g^{-1}) and *G. foliifera* (17297 µg g^{-1}). The same pattern was observed in between *U. fasciata* (23808 µg g^{-1}) and *G. pusillum* (22407 µg g^{-1}). The least content was seen in *G. corticata* with 332 µg g^{-1}. Pyridoxine content was comparable between the *E. prolifera* collected from Kayamkulam (795 µg g^{-1}) and Njarakkal (813 µg g^{-1}) locations and *G. foliifera* (742 µg g^{-1}). *C. antennina* (338 µg g^{-1}) and *U. fasciata* (411 µg g^{-1}) were also observed to be comparable. Least was observed in *G. corticata* (157 µg g^{-1}). Folic acid contents were seen comparable in *C. antennina* (872 µg g^{-1}) and *G. corticata* var. *cylindrica* (792 µg g^{-1}). Least was seen in *G. foliifera* (326 µg g^{-1}). Cyanocobalamin content was seen to be comparable in between the *E. prolifera* (4.52 µg g^{-1} and 4.93 µg g^{-1}) collected from both the sampling locations. *C. antennina* (3.46 µg g^{-1}) and *U. fasciata* (3.81 µg g^{-1}) also showed comparable B12 contents. Least was seen in *G. corticata* (1.54 µg g^{-1}). Riboflavin content was seen similar between *E. prolifera* (346 µg g^{-1}) collected from Kayamkulam location and *G. corticata* var. *cylindrica* (396 µg g^{-1}). *U. fasciata* (836 µg g^{-1}), *G. foliifera* (825 µg g^{-1}) and *G. corticata* (856 µg g^{-1}) also exhibited similar riboflavin contents. The least amount of riboflavin was seen in *C. antennina* (209 µg g^{-1}). Retinol content was observed to be comparable in between *E. prolifera* (436 µg g^{-1}) collected from Njarakkal location, *C. antennina* (529 µg g^{-1}) and *G. corticata* var. *cylindrica* (504 µg g^{-}

^{1}). Similarity was also observed in *U. fasicata* (337 µg g^{-1}), *G. foliifera* (319 µg g^{-1}) and *G. corticata* (383 µg g^{-1}). Least retinol content was seen in *E. prolifera* (149 µg g^{-1}) collected from the Njarakkal location.

α-tocopherol content was observed to have similarities between *E. prolifera* (4824 µg g^{-1}) collected from Kayamkulam location, *G. foliifera* (4587 µg g^{-1}) and *U. fasciata* (5311 µg g^{-1}). The least vitamin E content was seen in *G. corticata* (306 µg g^{-1}). Phylloquinone content was seen in similarities between *E. prolifera* collected from Kayamkulam location (1270 µg g^{-1}), *U. fasciata* (1070 µg g^{-1}) and *G. corticata* (1225 µg g^{-1}). Similarly between *E. prolifera* collected from the Njarakkal location (2290 µg g^{-1}) and *C. antennina* (1872 µg g^{-1}). The least content was observed in *G. foliifera* (521 µg g^{-1}). Niacin content was observed to be similar in between *C. antennina* (1717 µg g^{-1}), *E. prolifera* (1442 µg g^{-1}) collected from Kayamkulam location and *G. corticata* var. *cylindrica* (1357 µg g^{-1}). *A. spicifera* (7793 µg g^{-1}) and *G. pusillum* (7462 µg g^{-1}) also exhibited similar niacin contents. The least content was seen in *G. corticata* (573 µg g^{-1}). Ergocalciferol content was seen similar in between *C. antennina* (1.81 µg g^{-1}), *E. prolifera* (1.83 µg g^{-1}) collected from Kayamkulam location, *G. foliifera* (1.77 µg g^{-1}) and *G. corticata* var. *cylindrica* (1.51 µg g^{-1}), and *U. fasciata* (2.38 µg g^{-1}) and *E. prolifera* (2.28 µg g^{-1}) collected from the Njarakkal location.

Among the seaweeds studied, *G. pusillum* species of red seaweed is not only distinguished as affluent of several vitamins but maximised in total vitamin levels, whereas *G. corticata* (0.51 %) was impoverished in total vitamin contents (Fig. 3.9). Total vitamin content was seen similar in between *C. antennina* (4.04 %), *U. fasciata* (4.31 %) and *G. foliifera* (3.94 %). This pattern was also observed between *G. corticata* var. *cylindrica* (2.71 %) and *E. prolifera* (2.78 %) collected from the Njarakkal location.

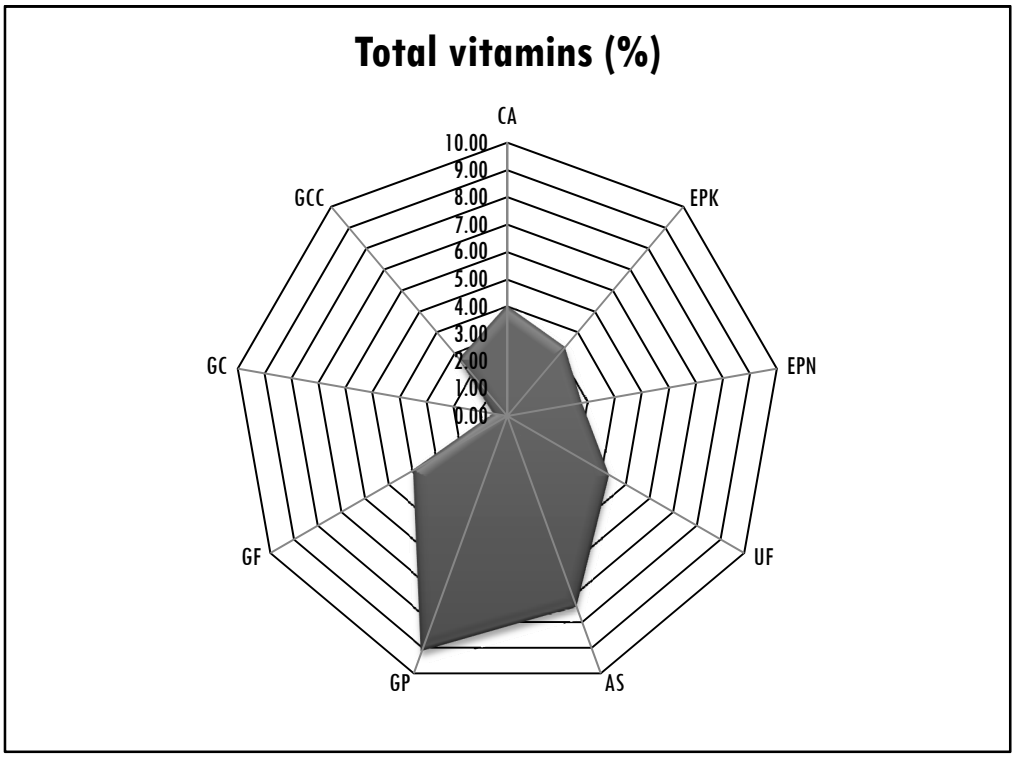


Fig. 3.9 The graphical representations of the total vitamin contents in nine seaweed extracts.

CA- *C. antennina*, EPK- *E. prolifera* -Kayamkulam location, EPN- *E. prolifera* - Njarakkal location, UF- *U. fasciata*, AS- *A. spicifera*, GP- *G. pusillum*, GF- *G. foliifera*, GC- *G. corticata* and GCC- *G. corticata* var. *cylindrica.*

Vitamin contents in seaweeds were already reported and known. *Porphyra yezoensis* was reported to possess vitamins C, A and B12 (McDermid & Stuercke, 2003). *Palmaria palmata* was reported to contain B2, B3, B6 and B12 (McDermid & Stuercke, 2003). *Eisenia bicyclis* contains B9, A and K, and *Undaria pinnatifida* contains B9 and beta-carotene (McDermid & Stuercke, 2003). *Gracilaria changgi*, an edible seaweed was already known to have vitamin A (Norziah & Ching, 2000). The edible seaweed *Durvillaea antarctica* and dried *Ulva lactuca* were also investigated for α-tocopherols and tocotrienols (provitamin E) (Ortiz et al., 2006). Seaweeds were already been

recognised as a natural source of vitamin E (Garrido et al., 2012) and was found to be better in comparison with artificial vitamin E. *G. changgi* collected from Malaysia had a level of 280 µg g^{-1} of vitamin C (Norziah & Ching, 2000). Vitamin A (104 µg g^{-1}), vitamin B2 (67 µg g^{-1}) and B6 (184 µg g^{-1}) were observed previously in *Halimeda opuntia* - a green seaweed as reported in the nutritional data published by USDA (2014). *U. lactuca* (Chlorophyta) and *Kappaphycus alvarezii* (Rhodophyta) collected from Tamil Nadu, India were reported to contain 42600 µg g^{-1} and 8700 µg g^{-1} respectively of ascorbic acid (Abirami & Kowsalya, 2011). Seaweed *U. pinnatifida* exhibited 30 µg g^{-1} of ascorbic acid, 0.6 µg g^{-1} of thiamine, 2.3 µg g^{-1} of riboflavin, 16 µg g^{-1} of niacin, 0.02 µg g^{-1} of pyridoxine, 1.96 µg g^{-1} of folic acid , 0.18 µg g^{-1} of retinol, 10 µg g^{-1} of tocopherol and 0.05 µg g^{-1} of phylloquinone (USDA, 2014). Seaweeds collected from the Bulgarian Black sea coast which include Chlorophyta *Ulva rigida* had 27.5 µg g^{-1} tocopherol and 0.29 µg g^{-1} ergocalciferol, *Cladophora vagabunda* had 16.8 µg g^{-1} tocopherol and Ochrophyta *Cystoseira barbata* and *Cystoseira crinita* were reported to contain 291 µg g^{-1} and 166 µg g^{-1} of tocopherols respectively (Panayotova et al., 2013). Seaweeds collected from Karachi coast which belong to Phaeophyta (*Dictyota dichotoma* var. *intricate* (52 µg g^{-1}), *Dictyota dichotoma* var. *indica* (32 µg g^{-1}), *Jolyna laminariodes* (27 µg g^{-1}), *Sargassum tenerrimum* (33 µg g^{-1}), *Sargassum ilicifolium* (32 µg g^{-1}), *Sargasssum swartzii* (52 µg g^{-1}), *Sargassum variegatum* (51 µg g^{-1}), *Spatoglossum asperum* (38 µg g^{-1}), *Cystoseira indica* (34 µg g^{-1}) and *Stoechospermum marginatum* (61 µg g^{-1})), Chlorophyta (*Halimeda tuna* (33 µg g^{-1}) and *Rhizoclonium implexum* (33 µg g^{-1})) and Rhodophyta (*Melanothamnus afaqhusainii* (34 µg g^{-1}) and *Solieria robusta* (27 µg g^{-1})) were also reported with ascorbic acid contents (Ambreen et al., 2012). Green

seaweed *H. opuntia* collected from Jeddah, Saudi Arabia was reported with vitamin A (104 µg g^{-1}), B1 (470 µg g^{-1}), B2 (67 µg g^{-1}), B6 (184 µg g^{-1}) and C (194 µg g^{-1}). Red seaweed, *G. corticata* collected from the same location was reported with vitamin A (74 µg g^{-1}), B1 (240 µg g^{-1}), B2 (32 µg g^{-1}), B6 (142 µg g^{-1}) and C (284 µg g^{-1}). Phaeophyta, *Turbinaria triquetra* was reported with vitamin A (45 µg g^{-1}), B1 (553 µg g^{-1}), B2 (54 µg g^{-1}), B6 (85 µg g^{-1}) and C (219 µg g^{-1}) (Omar et al., 2013).

Note-worthily, some of the vitamins detected in seaweeds were in higher concentrations on comparison to the conventional prominent food sources like the apple, banana, gooseberry and vegetables like cabbage, broccoli, French beans, spinach, Swiss chard etc. as evidenced with reported levels. Green peas, strawberry, potato, cauliflower, sweet potato, spinach etc. were reported to have vitamin C as its major vitamin (160 to 777 µg g^{-1}). B1 and B2 were observed only in certain vegetables (Watada & Iran, 1987). Gooseberries pronounced for its vitamin C (415 µg g^{-1}) and E (5.6 µg g^{-1}) contents, also did exhibit A (1.3 µg g^{-1}), B1 (0.6 µg g^{-1}), B2 (0.4 µg g^{-1}), niacin (4.5 µg g^{-1}), folate (0.1 µg g^{-1}), pantothenic acid (4.2 µg g^{-1}) and B6 (1.2 µg g^{-1}) (Watada & Iran, 1987).

3.3.3 Phenolics and Flavonoids

Remarkable variations were observed in total phenolic contents between the different species. Total phenolics were observed to be higher in the extracts of *A. spicifera* (red seaweed) having 91 µg g^{-1}, followed by *E. prolifera* (green seaweed) collected from the Kayamkulam location with 81 µg g^{-1} (Table 3.7). The lowest content was observed in *E. prolifera* collected from Njarakkal location (1.97 µg g^{-1}) and *G. corticata* (2.34 µg g^{-1}). Comparative polyphenolic contents were seen in *U. fasciata* (77 µg g^{-1}), *G.*

pusillum (71 μg g^{-1}) and *E. prolifera* (81 μg g^{-1}) from Kayamkulam location. Similarly, comparative polyphenolic contents were seen between *C. antennina* (17 μg g^{-1}) and *G. corticata* var. *cylindrica* (15 μg g^{-1}) and *G. foliifera* (21 μg g^{-1}). *E. prolifera* (1.97 μg g^{-1}) from Njarakkal location and *G. corticata* (2.34 μg g^{-1}) also exhibited similarities.

Table 3.7 Total phenolic and flavonoid contents in the extracts of nine seaweed samples as μg g^{-1}, (mean ± SD), (n=3).

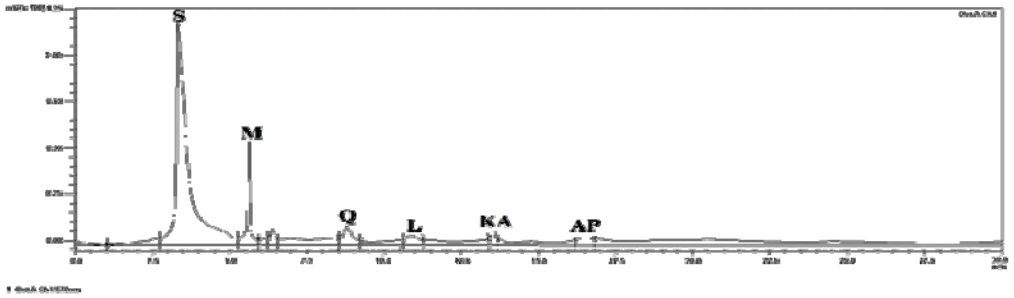
Seaweed	TPC	Myricetin	Quercetin	Luteolin	Kaempferol	Apigenin
CA	17.53 ± 0.15	7.97 ± 0.29	1.54 ± 0.06	0.99 ± 0.04	0.27 ± 0.01	0.67 ± 0.03
EPK	81.10 ± 0.36	57.88 ± 2.11	5.69 ± 0.21	0.60 ± 0.02	0.97 ± 0.04	0.25 ± 0.01
EPN	1.97 ± 0.13	0.02 ± 0.01	0.02 ± 0.03	0.05 ± 0.01	0.38 ± 0.02	0.02 ± 0.05
UF	77.14 ± 0.26	39.79 ± 1.45	5.47 ± 0.20	7.53 ± 0.28	1.80 ± 0.07	11.46 ± 0.42
AS	91.18 ± 0.40	31.85 ± 1.16	8.57 ± 0.31	6.65 ± 0.24	11.64 ± 0.43	13.38 ± 0.50
GP	71.06 ± 0.34	42.41 ± 1.55	0.62 ± 0.02	ND	2.36 ± 0.09	ND
GF	21.43 ± 0.36	7.78 ± 0.28	3.17 ± 0.12	0.07 ± 0.01	0.10 ± 0.01	2.42 ± 0.09
GC	2.34 ± 0.16	0.09 ± 0.01	0.14 ± 0.01	1.11 ± 0.04	0.11 ± 0.01	0.09 ± 0.01
GCC	15.15 ± 0.21	11.01 ± 0.40	1.97 ± 0.07	0.99 ± 0.04	0.09 ± 0.01	0.19 ± 0.01

TPC - Total Phenolics Content, CA - *C. antennina*, EPK - *E. prolifera* from Kayamkulam, EPN - *E. prolifera* from Njarakkal, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

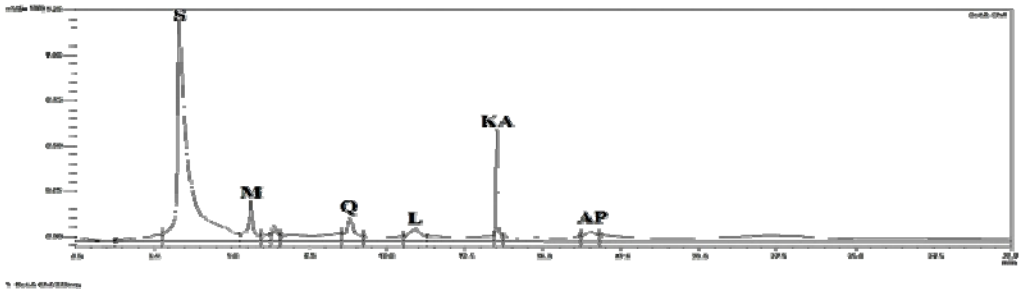
In general, total phenolic contents observed in the current study were considerably low. *G. corticata* (40 mg g^{-1}) and *U. fasciata* (41 mg g^{-1}) collected from the Saurashtra coast, Gujarat (India), were reported with much higher total phenolics contents (Kumar et al., 2011). *Himanthalia elongata*, a Phaeophyta obtained from Ireland was found to have 34 mg g^{-1} of total phenolic content (Cox et al., 2014). *A. spicifera* extracts showed 9.9 mg g^{-1} of total phenolic content and 5.8 mg g^{-1} in *G. corticata* as equivalents of catechol (Sachindra et al., 2010). The total phenolic contents in *A. spicifera* and *Gracilaria* sp. from Brazil, were also found to be 22 mg g^{-1} and 62 mg g^{-1} respectively (Martins et al., 2013). The low total phenolics contents in the present study could be the result of spatial

variations. Phenolic compounds which have been reported earlier for their antioxidant activities might have been used up to dwell through the adverse climatic conditions at the coastal area of Kerala.

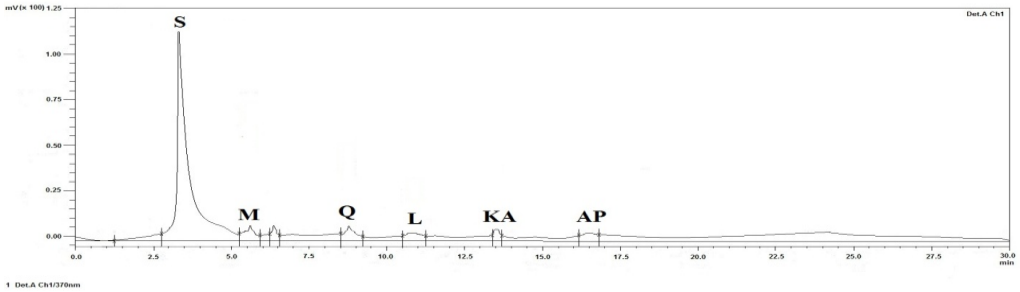
The result of the quantification of five types of flavonoids in nine seaweeds is given in Table 3.7. Chromatogram of 9 seaweeds is given in Fig. 3.10. The quantified flavonoids constituted of flavonols like quercetin, a major anticarcinogenic compound (Hollman et al., 1997; Vinson et al., 1995), myricetin, an active antioxidant, anticarcinogen and antimutagen compound (Ong & Khoo, 1997) and kaempferol, an antioxidant, antitumor and anti-inflammatory compound (Vinson et al., 1995), and flavones like apigenin and luteolin which possessed the least antioxidant activity due to the absence of o-dihydroxy groups (Asif & Khodadadi, 2013). The presently quantified flavonoids are prominent for their anti carcinogenic properties too as reported earlier (Hertog et al., 1992; Ohshima et al., 1998).



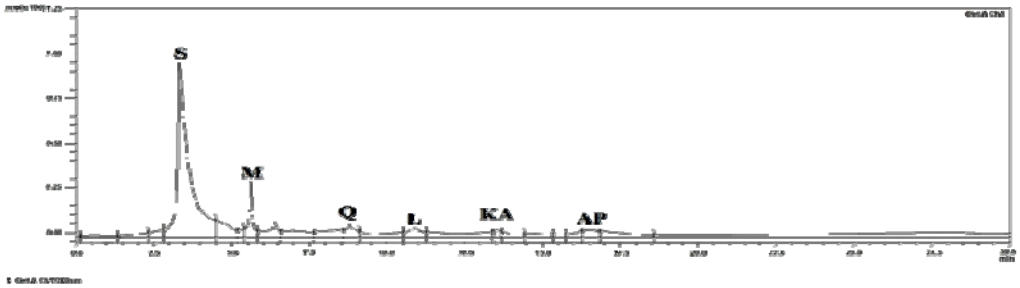
C. antennina



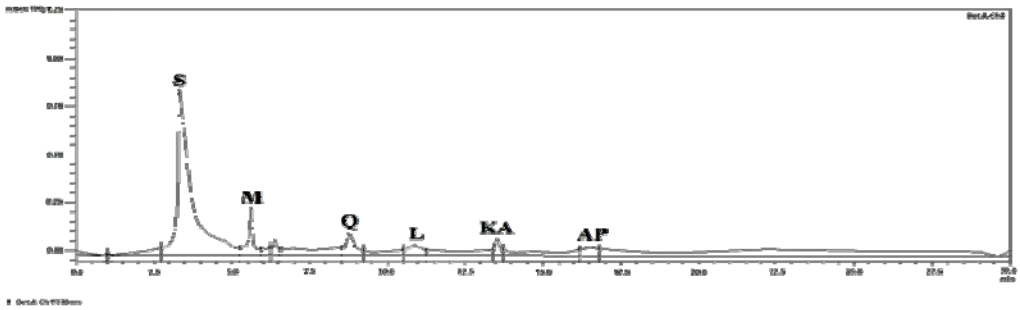
E. prolifera from Kayamkulam location



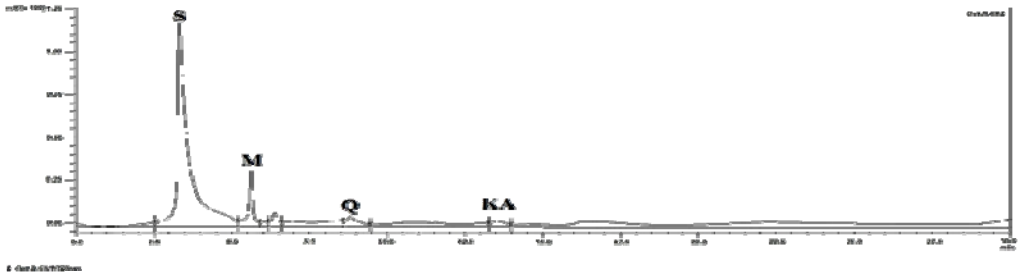
E. prolifera from Njarakkal location



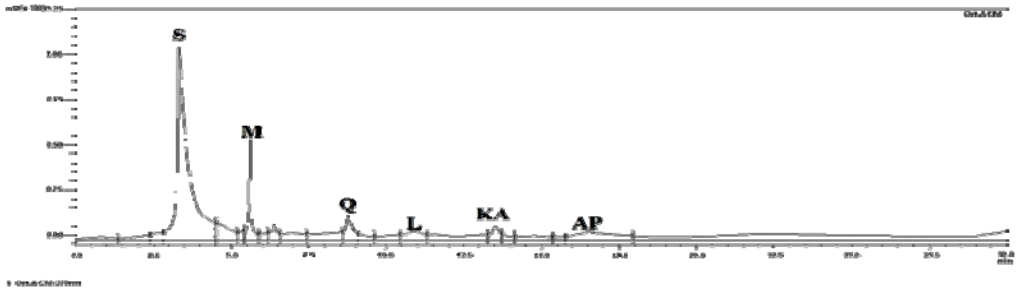
U. fasciata



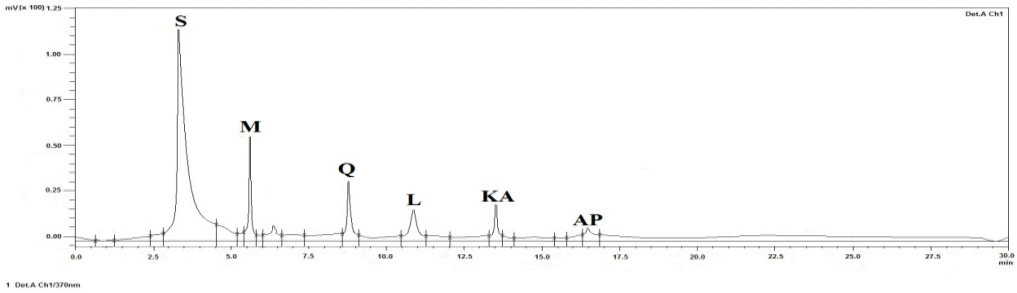
A. spicifera



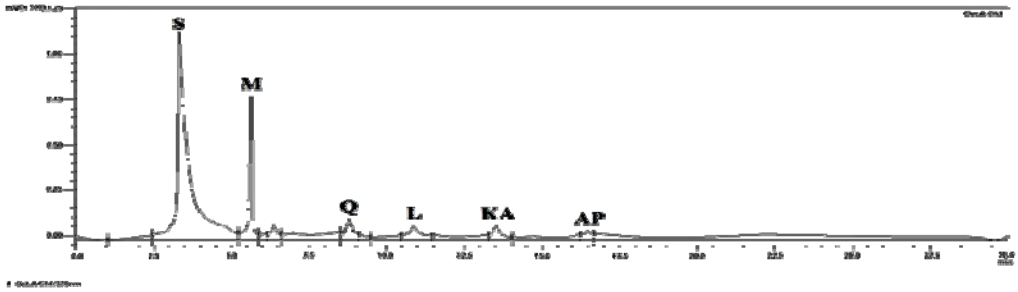
G. pusillum



G. foliifera



G. corticata



G. corticata var. *cylindrica*

Fig. 3.10 HPLC chromatogram of the flavonoids contents in the nine seaweed extracts.

S - Reagent, M - Myricetin, Q - Quercetin, L - Luteolin, KA - Kaempferol and AP - Apigenin.

Myricetin was observed to be the highly concentrated phenolic compound among the five flavonoids, followed by quercetin and apigenin. All the flavonoids standardized (except luteolin and apigenin in *G. pusillum*) were detected among the seaweeds collected from the Kerala coast. Noteworthily, it

is evidenced that the analysed flavonoids constitute more than 50% of the total phenolics in the seaweed, which was estimated on the basis of dry weight. *E. prolifera* collected from the Kayamkulam location (57 μg g^{-1}) and *G. pusillum* (42 μg g^{-1}) showed higher levels of myricetin. *U. fasciata* (39 μg g^{-1}), *A. spicifera* (31 μg g^{-1}) and *G. pusillum* (42 μg g^{-1}) showed comparative contents of myricetin. *C. antennina* (7.97 μg g^{-1}) and *G. foliifera* (7.78 μg g^{-1}) also exhibited similar contents of myricetin. Least myricetin content was observed in *E. prolifera* (0.02 μg g^{-1}) collected from the Njarakkal location.

Quercetin was seen in higher concentration in *A. spicifera* (8.57 μg g^{-1}) and *E. prolifera* collected from Kayamkulam location (5.69 μg g^{-1}). *E. prolifera* (5.69 μg g^{-1}) collected from the Kayamkulam location had similar quercetin content with *U. fasciata* (5.47 μg g^{-1}). *C. antennina* (1.54 μg g^{-1}) and *G. corticata* var. *cylindrica* (1.97 μg g^{-1}) also exhibited similarities between the quercetin contents. Least was observed in *E. prolifera* (0.02 μg g^{-1}) collected from the Njarakkal location.

Luteolin was found to be higher in *U. fasciata* (7.53 μg g^{-1}) and *A. spicifera* (6.65 μg g^{-1}). *U. fasciata* (7.53 μg g^{-1}) and *A. spicifera* (6.65 μg g^{-1}) had similar luteolin contents. Luteolin was not observed in *G. pusillum*. *C. antennina* (0.99 μg g^{-1}), *G. corticata* var. *cylindrica* (0.99 μg g^{-1}) and *G. corticata* (1.11 μg g^{-1}) exhibited similar luteolin contents.

A. spicifera (11.64 μg g^{-1}) and *G. pusillum* (2.36 μg g^{-1}) were noticed for their kaempferol level. *C. antennina* (0.27 μg g^{-1}) and *E. prolifera* (0.38 μg g^{-1}) collected from Njarakkal location, *U. fasciata* (1.80 μg g^{-1}) and *G. pusillum* (2.36 μg g^{-1}), *G. foliifera* (0.10 μg g^{-1}), *G. corticata* (0.11 μg g^{-1}) and *G. corticata* var. *cylindrica* (0.09 μg g^{-1}) exhibited comparable kaempferol contents.

Apigenin content recorded was comparatively higher in *A. spicifera* (13.38 μg g^{-1}) and *U. fasciata* (11.46 μg g^{-1}). Apigenin was absent in *G. pusillum*. This is further endorsed the fact that the nutraceutical potentiality of seaweeds have to be exploited as they are also a major source of flavonoids and to be considered as an alternate to the conventional food sources.

Few studies were available on the flavonoids content in seaweeds. These indicated the presence of myricetin, quercetin and kaempferol. Myricetin and quercetin were reported at 18.61 μg g^{-1} and 2.35 μg g^{-1} from *Stypocaulon scoparium* collected from Spain (López et al., 2011). *U. pinnatifida* and *Padina arborescens* belonging to Phaeophyta collected from Japan were reported to contain 202 μg g^{-1} and 466 μg g^{-1} of quercetin respectively. *Turbinaria ornata* (Phaeophyta) and *Chondrus verruscosus* (Rhodophyta) collected from Japan were reported to contain 346 μg g^{-1} and 270 μg g^{-1} respectively of myricetin (Yoshie et al., 2003). Seaweeds belonging to Chlorophyta such as *Halimeda macroloba* and *Halimeda opuntia* collected from Japan, were reported to contain 414 μg g^{-1} and 147 μg g^{-1} of myricetin respectively (Yoshie et al., 2002).

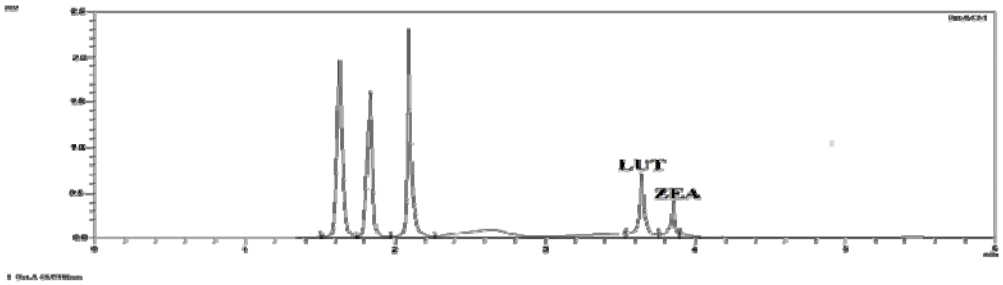
Tropical plants had major flavonols - kaempferol, quercetin, myricetin, and flavones - luteolin and apigenin, which were determined in guava, cabbage, broccoli, cauliflower, French beans, lady finger, green chilli, betel, onion, moringa oleifera, spinach, papaya etc. (Asif & Khodadadi, 2013). Studies on vegetables and fruits showed the presence of quercetin, kaempferol, apigenin and luteolin with almost 50 % of the analytes exhibiting either quercetin or kaempferol in majority (Yang & Zhang, 2008). However, no prior information was available on the exact concentration of the flavonoids in the seaweeds from Kerala coast which highlights the novelty of this study.

3.3.4 Carotenoids - Lutein and Zeaxanthin

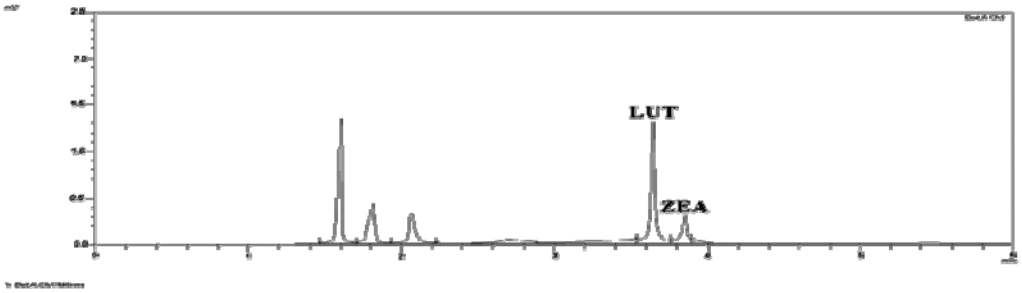
The major carotenoids contents of red and green seaweeds collected from the Kerala coast were examined and it was the first report of quantification from the site. The results indicate the beneficiary of those in dietary requirements. Both green and red seaweeds were examined and it was found that the red seaweeds possess the coin of importance (Table 3.8). Chromatograms of nine seaweeds are given in the Fig. 3.11.

Table 3.8 Lutein and zeaxanthin contents in the extracts of nine seaweeds analysed as $\mu g\ g^{-1}$, (mean $\pm$ SD), (n=3).

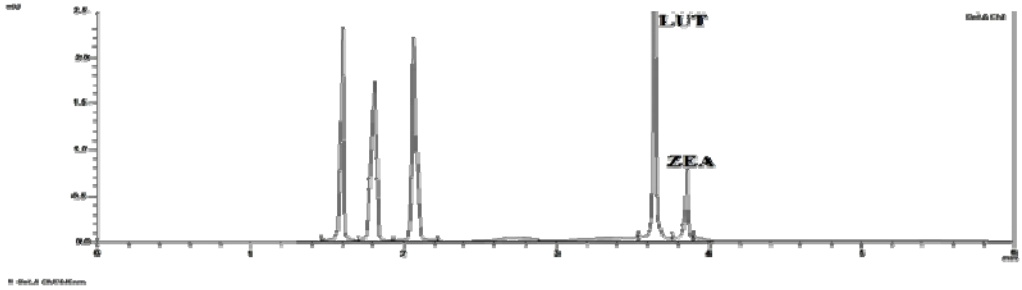
Seaweed	Lutein	Zeaxanthin
C. antennina	0.30 $\pm$ 0.01	0.10 $\pm$ 0.01
E. prolifera (K)	1.66 $\pm$ 0.03	0.20 $\pm$ 0.01
E. prolifera (N)	0.48 $\pm$ 0.01	0.09 $\pm$ 0.01
U. fasciata	0.73 $\pm$ 0.01	0.16 $\pm$ 0.01
A. spicifera	3.99 $\pm$ 0.06	0.30 $\pm$ 0.01
G. pusillum	4.91 $\pm$ 0.08	0.62 $\pm$ 0.01
G. foliifera	1.84 $\pm$ 0.03	0.29 $\pm$ 0.01
G. corticata	0.60 $\pm$ 0.01	0.14 $\pm$ 0.01
G. corticata var. *cylindrica*	0.43 $\pm$ 0.01	1.06 $\pm$ 0.02



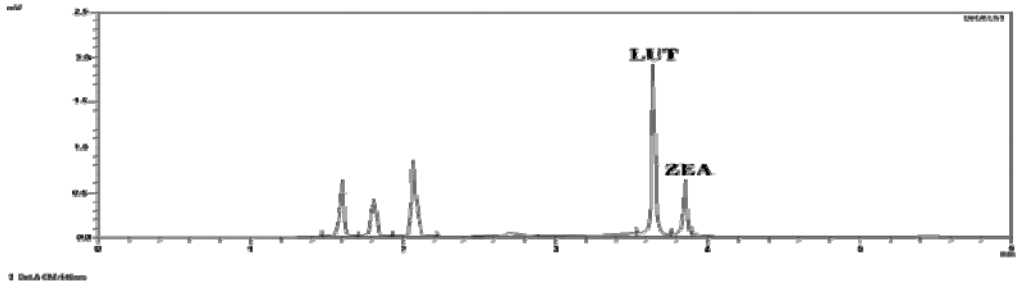
C. antennina



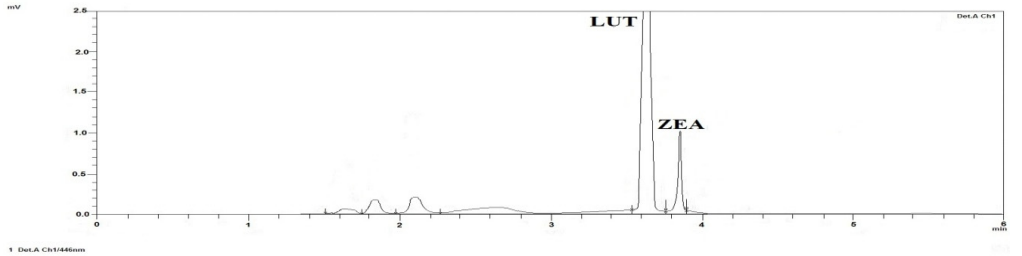
E. prolifera from Kayamkulam



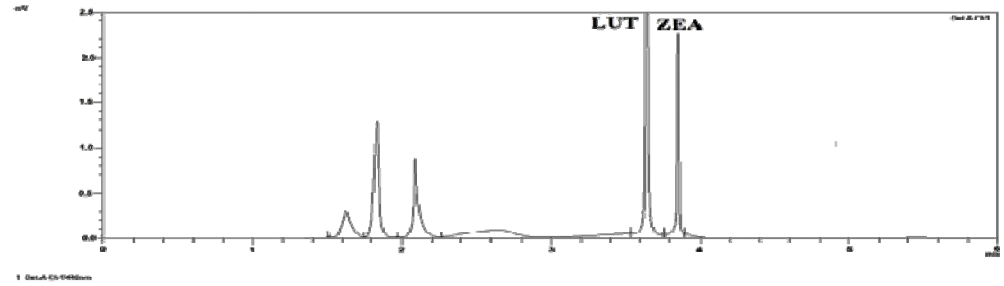
E. prolifera from Njarakkal



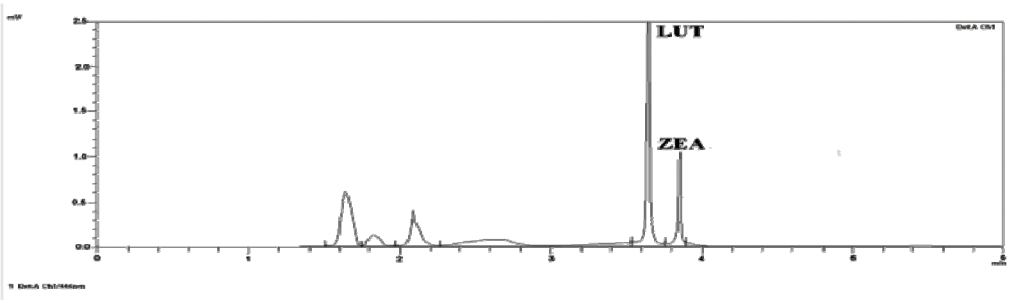
U. fasciata



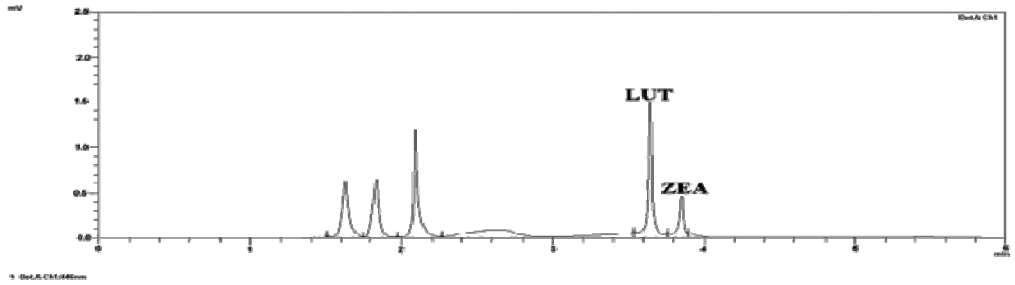
A. spicifera



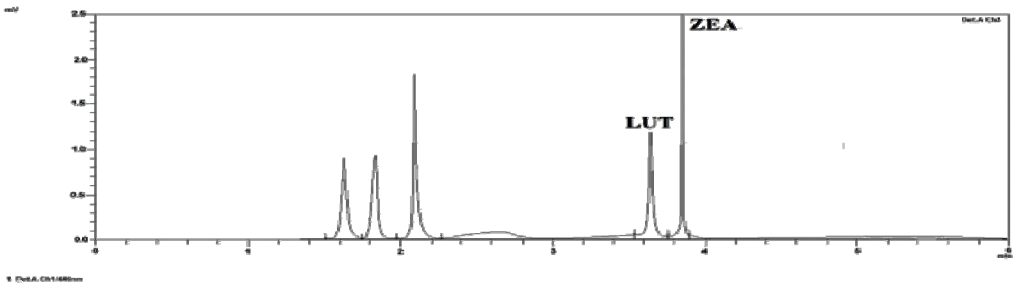
G. pusillum



G. foliifera



G. corticata



G. corticata var. *cylindrica*

Fig. 3.11 HPLC chromatograms of the lutein and zeaxanthin contents in the nine seaweed extracts.

LUT - Lutein and ZEA - Zeaxanthin

In Rhodophyceae, *G. pusillum* species showed the highest lutein content (4.91 μg g^{-1}) followed by *A. spicifera* (3.99 μg g^{-1}). Except *G. corticata* var. *cylindrica,* all other species have lutein as the major pigment than zeaxanthin. The contents of zeaxanthin pigments (1.06 μg g^{-1}) in the *G. corticata* var. *cylindrica* species are noticeable. Green seaweeds are generally observed to possess lower concentration of carotenoids due to the lack of colouring compounds in it. Apart from chlorophylls, it contains trace of carotenoids. *C. antennina* showed the least in lutein contents with 0.30 μg g^{-1}. The highest was for *E. prolifera* from Kayamkulam location with lutein concentration of 1.66 μg g^{-1}. *U. fasciata* had 0.73 μg g^{-1} lutein and 0.16 μg g^{-1} of zeaxanthin. Comparable lutein contents were observed in between *E. prolifera* (1.66 μg g^{-1}) collected from Kayamkulam location and *G. foliifera* (1.84 μg g^{-1}), *U. fasciata* (0.73 μg g^{-1}) and *G. corticata* (0.60 μg g^{-1}) and *G. corticata* var. *cylindrica* (0.43 μg g^{-1}) and *E. prolifera* (0.48 μg g^{-1}) collected from Njarakkal location. Zeaxanthin contents showed similarities in between *C. antennina* (0.10 μg g^{-1}) and *E. prolifera* (0.09 μg g^{-1}) collected from Njarakkal location, *U. fasciata* (0.16 μg g^{-1}) and *G. corticata* (0.14 μg g^{-1}) and *A. spicifera* (0.30 μg g^{-1}) and *G. foliifera* (0.29 μg g^{-1}).

Carotenoid contents as reported in the current study have been reported in many plants and seaweeds. The results of the current study showed that the lutein and zeaxanthin concentrations were appreciable enough to compare with the common sources of nutrition. Red seaweeds were found to be richer in carotenoids than green. The presence of lutein and zeaxanthin pigments had been reported in red algae from various locations (Marquardt & Hanelt, 2004; Schubert et al., 2006; Ortiz et al., 2009). The study also validated the observations. *U. pinnatifida* is the most investigated seaweed species for

carotenoids and are observed to possess lutein and zeaxanthin. Red seaweeds *Grateloupia asiatica* and *Grateloupia livida* from Japan coast were reported to contain 10.4 μg g^{-1} & 1.1 μg g^{-1} and 9.3 μg g^{-1} & 1.0 μg g^{-1} lutein and zeaxanthin respectively (Terasaki et al., 2012). Lutein and zeaxanthin have been observed in almost all vegetative sources (Sajilata et al., 2008). Green leafy vegetables, oranges, mangoes, raspberries, pink grapes, corns and even microbes have shown the carotenoid contents with lutein and zeaxanthin as the common carotenoids entity (Jin et al., 2003; Parry et al., 2005).

3.3.5 Antioxidant Activity

Compounds that are quantified in the current study are good antioxidant agents too. The composite antioxidant studies using 1:1 methanol water solvent system were carried out. DPPH radical scavenging antioxidant assay is the accepted methodology (Siriwardhana et al., 2003; Wang et al., 2009; Parys et al., 2010; Farvin & Jacobsen, 2013; Liu et al., 2013). But antioxidant properties are not purely based on free radical quenching (Balboa et al., 2013; Farvin & Jacobsen, 2013). Hence, the DPPH method was compared along with the ferrous tartarate and $KMnO_4$ titration methods, so as to confirm the antioxidant potential of seaweed extracts. In all the three methods used, the activity showed a concentration dependency which increased with concentrations. Each method was calibrated using ascorbic acid, α-tocopherol, BHT and resorcinol at various concentrations and the inhibition resulted in a linear slope. The antioxidant activities of the algal extracts were estimated in comparison with the corresponding standards and the results were reported in 4 equivalent values.

3.3.5.1 DPPH Antioxidant Activity

DPPH radical scavenging was observed highest in the extracts of *G. foliifera* in comparison to the activities of BHT (9.22 %) and resorcinol (10.12 %) (Table 3.9). *G. pusillum* and *G. corticata* var. *cylindrica* also exhibited greater amounts of inhibitory activities in comparison with ascorbic acid (10.11 %) and α-tocopherol (20.19 %) respectively. Since the vitamin C and E were exhibited by all seaweeds in appreciable levels, the antioxidant activity had to be relied upon the equivalence of BHT and resorcinol. In general, Rhodophyta showed greater equivalent activities with respect to standards, and among the class, *G. foliifera* (8.21 % ascorbic acid equivalence, 9.22 % BHT equivalence, 15.19 % tocopherol equivalence and 10.12 % resorcinol equivalence) showed comparatively higher activities and the results of total phenolics and carotenoids supported the results. Ascorbic acid antioxidant activity equivalence was observed to have similarities in between *A. spicifera* (8.62 %) and *G. foliifera* (8.21 %). The least activity was seen in *E. prolifera* (0.11 %) collected from Njarakkal location. BHT equivalence was observed to have similarities in the activities of the extracts of *A. spicifera* (7.22 %), *G. pusillum* (7.12 %) and *G. corticata* var. *cylindrica* (7.63 %). Least activity with respect to BHT equivalence was seen in the extracts of *E. prolifera* (0.08 %) collected from the Njarakkal location. Resorcinol equivalence exhibited activity similarities in between *A. spicifera* (8.02 %), *G. pusill*um (9.11 %) and *G. corticata* var. *cylindrica* (8.23 %). But in this study, Chlorophyta eventually showed lesser activities even though they possessed higher total phenolic contents. Hence, the present observations were revalidated to verify the oxidative studies using the ferrous tartarate oxidizing capability activities.

Table 3.9 Antioxidant potentials of the nine seaweed extracts (% of activity in comparison with the standards), (mean ±SD), (n=3).

Antioxidant activity (% of equivalence to standards)				
	Ascorbic acid	**BHT**	**Tocopherol**	**Resorcinol**
DPPH Method				
C. antennina	3.12 ± 0.03	3.38 ± 0.05	5.62 ± 0.08	3.61 ± 0.04
E. prolifera (K)	0.22 ± 0.01	0.18 ± 0.01	2.11 ± 0.04	0.12 ± 0.01
E. prolifera (N)	0.11 ± 0.03	0.08 ± 0.04	0.87 ± 0.22	0.05 ± 0.02
U. fasciata	0.83 ± 0.01	0.85 ± 0.01	2.92 ± 0.03	0.71 ± 0.01
A. spicifera	8.62 ± 0.49	7.22 ± 0.02	4.11 ± 0.03	8.02 ± 0.01
G. pusillum	10.11 ± 0.42	7.12 ± 0.08	8.01 ± 0.01	9.11 ± 0.01
G. foliifera	8.21 ± 0.12	9.22 ± 0.12	15.19 ± 0.22	10.12 ± 0.14
G. corticata	0.33 ± 0.01	0.38 ± 0.01	0.29 ± 0.01	0.29 ± 0.01
G. corticata var. *cylindrica*	7.22 ± 0.18	7.63 ± 0.01	20.19 ± 0.22	8.23 ± 0.11
Ferrous tartarate method				
C. antennina	0.94 ± 0.02	2.19 ± 0.02	1.99 ± 0.02	3.09 ± 0.03
E. prolifera (K)	0.97 ± 0.02	0.53 ± 0.01	0.52 ± 0.01	2.11 ± 0.01
E. prolifera (N)	0.54 ± 0.14	0.23 ± 0.16	0.34 ± 0.22	0.99 ± 0.03
U. fasciata	1.03 ± 0.01	1.08 ± 0.01	1.12 ± 0.01	0.68 ± 0.01
A. spicifera	0.69 ± 0.01	0.68 ± 0.01	0.63 ± 0.01	0.79 ± 0.01
G. pusillum	1.57 ± 0.02	1.38 ± 0.02	1.31 ± 0.02	1.85 ± 0.02
G. foliifera	0.68 ± 0.01	0.63 ± 0.01	0.62 ± 0.01	0.79 ± 0.19
G. corticata	1.20 ± 0.02	1.16 ± 0.01	1.17 ± 0.02	1.38 ± 0.02
G. corticata var. *cylindrica*	1.64 ± 0.02	1.52 ± 0.02	1.49 ± 0.02	1.79 ± 0.03
$KMnO_4$ method				
C. antennina	0.32 ± 0.01	3.44 ± 0.12	37.83 ± 0.31	4.96 ± 0.04
E. prolifera (K)	0.73 ± 0.01	6.25 ± 0.13	62.34 ± 0.32	9.13 ± 0.13
E. prolifera (N)	0.26 ± 0.11	3.11 ± 0.09	45.37 ± 0.68	5.37 ± 0.25
U. fasciata	0.29 ± 0.01	2.78 ± 0.01	30.11 ± 0.11	4.03 ± 0.06
A. spicifera	0.58 ± 0.34	5.12 ± 0.16	58.18 ± 0.64	6.42 ± 0.05
G. pusillum	0.69 ± 0.01	6.02 ± 0.28	59.81 ± 0.72	7.18 ± 0.08
G. foliifera	0.55 ± 0.01	5.02 ± 0.16	57.18 ± 0.73	7.40 ± 0.23
G. corticata	0.48 ± 0.01	4.81 ± 0.02	53.64 ± 0.18	7.19 ± 0.18
G. corticata var. *cylindrica*	0.61 ± 0.01	5.18 ± 0.07	56.83 ± 0.50	7.54 ± 0.13

K - Kayamkulam and N - Njarakkal location.

3.3.5.2 UV-Vis Ferrous Tartarate (Fe^{2+} to Fe^{3+}) Activity

The UV-Vis ferrous tartarate method where the oxidizing nature with respect to metal ions (Fe^{2+} to Fe^{3+}) was measured and the extracts of the

Chlorophyta species *C. antennina* showed a higher equivalence in comparison to BHT (2.19 %), α-tocopherol (1.99 %) and resorcinol (3.09 %) (Table 3.9). The antioxidant activity with respect to BHT and resorcinol was considered and the results showed the potential dependence on the total phenolic, carotenoid and vitamin contents in the analysed extracts. The least activity was exhibited by *A. spicifera* and *G. foliifera* (Rhodophyta). Ascorbic acid equivalence was seen to be the highest in *G. corticata* var. *cylindrica* (1.64 %) and *G. pusillum* (1.57 %). Similarity in activities was observed in between *C. antennina* (0.94 %), *E. prolifera* (0.97 %) from Kayamkulam location and *U. fasciata* (1.03 %). *E. prolifera* (0.54 %) collected from Njarakkal location, *A. spicifera* (0.69 %) and *G. foliifera* (0.68 %) also exhibited similarities in antioxidant activities. BHT equivalence also exhibited similarities in between the extracts of *U. fasciata* (1.08 %) and *G. corticata* (1.16 %), *A. spicifera* (0.68 %) and *G. foliifera* (0.63 %) and *G. pusillum* (1.38 %) and *G. corticata* var. *cylindrica* (1.52 %). Vitamin E equivalence was seen highest in *C. antennina* (1.99 %). Similarities in the vitamin E equivalence were exhibited by *U. fasciata* (1.12 %) and *G. corticata* (1.17 %) and in between *A. spicifera* (0.63 %) and *G. foliifera* (0.62 %). Least vitamin E equivalent activity was exhibited by the extracts of *E. prolifera* (0.34 %) collected from the Njarakkal location. Similarities in the resorcinol equivalence activity were also observed. *A. spicifera* (0.79 %) and *G. foliifera* (0.79 %), and *G. pusillum* (1.85 %) and *G. corticata* var. *cylindrica* (1.79 %) exhibited similarities in the resorcinol equivalent activity. Least activity was observed in *U. fasciata* (0.68 %).

3.3.5.3 $KMnO_4$ Total Oxidizing Activity

The variance in results using the two methods was then revalidated with the next method where total oxidizing capability/potency was analysed using $KMnO_4$ titrations (Table 3.9). $KMnO_4$ titration techniques showed greater

responses when tocopherol was used as a standard. Almost equal responses were seen throughout when ascorbic acid being a standard. Hence, the BHT and resorcinol equivalences were used and the extract of Chlorophyta, *E. prolifera* had the highest (0.73 % ascorbic acid equivalence, 6.25 % BHT equivalence, 62.34 % tocopherol equivalence and 9.13 % resorcinol equivalence) antioxidant activities. The results were advocated by the total phenolic contents which were 81 $\mu g\ g^{-1}$. Ascorbic acid equivalence was exhibited highest by the extracts of *E. prolifera* (0.73 %) collected from Kayamkulam location. The activity was almost comparable with the activities of *G. pusillum* (0.69 %). Comparable activities were exhibited by *C. antennina* (0.32 %), *E. prolifera* (0.26 %) collected from Njarakkal location and *U. fasciata* (0.29 %). Similar trend was exhibited by *A. spicifera* (0.58 %), *G. foliifera* (0.55 %) and *G. corticata* var. *cylindrica* (0.61 %). BHT equivalence was seen highest in *E. prolifera* (6.25 %) collected from the Kayamkulam location and comparable with *G. pusillum* (6.02 %). Comparable activities were also observed in between *C. antennina* (3.44 %) and *E. prolifera* (3.11 %) collected from the Njarakkal location and in between *A. spicifera* (5.12 %), *G. foliifera* (5.02 %) and *G. corticata* var. *cylindrica* (5.18 %). The activity was observed to be least in *U. fasciata* (2.78 %). Vitamin E equivalence was seen highest in the *E. prolifera* (62 %) collected from the Kayamkulam location and least in *U. fasciata* (30 %). Comparable activity was seen in between *A. spicifera* (58 %), *G. pusillum* (59 %), G. foliifera (57 %), *G. corticata* (53 %) and *G. corticata* var. *cylindrica* (56 %). Resorcinol equivalence was observed to have similarities in between the activities of *C. antennina* (4.96 %) and *U. fasciata* (4.03 %). Comparable activities were also exhibited by *G. pusillum* (7.18 %), *G. foliifera* (7.40 %), *G. corticata* (7.19 %) and *G. corticata* var. *cylindrica* (7.54 %).

The antioxidant activities observed in this study is highly appreciable. To add on, this is the first attempt to screen seaweeds collected from this

sampling location for such activities. The observed activity is a combined effect of the quantified compounds along with other compounds that are present in the analysed extracts (Imbs et al., 2014). Antioxidant properties are influenced by the mode of extraction too (Zhou & Yu, 2004) and the activity depends on the unique structure of compounds which varies with the compound combinations. The matrix in the extracts could highly influence the antioxidant activities (Kuda et al., 2006; Martins et al., 2013).

Antioxidant properties in food act as health protecting factor, and composes of vitamins C, E, carotenoids, phenolics, phytates and phytoestrogens (Asif & Khodadadi, 2013). Vitamin C, quercetin, myricetin, kaempferol, luteolin and apigenin were seen to be present in foods that exhibit antioxidant activities (Hollman et al., 1997). It may also chelate with metals and thus, generate the oxidising properties (Toyosaki & Iwabuchi, 2009). Vitamin C acts on the cytosol causing reactions with reactive oxygen species (ROS) and E act on the membranes to break the peroxidation chain of fatty acids (Weiss, 2005). Seaweeds which grow in extreme conditions like nutrient deficiency, varying salinity, environmental conditions, temperature, light, dissolved oxygen, carbon dioxide etc., (Langebartels et al., 2002) result in oxidative stress which produces free radicals (Matsukawa et al., 1997; Sharma & Davis, 1997). Despite of the dwelling conditions, the organisms seldom gets prone to such damages. They get protected due to the formations of strong protective defence systems that include an array of antioxidant compounds such as the secondary metabolites like vitamins, carotenoids, phenolics, phytates and phytoestrogens (Fung et al., 2013).

The antioxidant potential of carotenoids is mainly attributed to its redox properties which make them act as good reducing agents. The macro algal carotenoids induce cell cycle arrest and cell death via apoptosis, thereby reducing cancerous growth in liver, colon, white cells etc (Nishino et al., 1999;

Catherine-Murphy et al., 2014). The carotenoids antioxidant properties play a vital role in reducing oxidative stress (Yan et al., 1999) and ophthalmological diseases (Hosokawa et al., 1999).

Studies on *Ascophyllum nodosum* collected from Ireland were found to have antioxidant free radical inhibition for the extracts containing phenolic compounds (Jiménez et al., 2001). *U. pinnatifida* from New Zealand was also screened for the activities with DPPH-free radical scavenging and the results suggested contribution of phenolic compounds towards activity (Airanthi et al., 2011). The results also showed correlation between antioxidant activities and fucoxanthins. Antioxidants being considered as important nutraceuticals on health benefits were relied upon the DPPH assay with standards like the BHT and vitamin C (Sharma & Bhat, 2009). The methanol-water extracts of *Stypocaulon scoparium* collected from Spain showed 38.6 % radical scavenging activity (López et al., 2011). *H. elongata*, a Phaeophyta obtained from Ireland was found to have 13.98 % of antioxidant potential (Cox et al., 2014). *A. spicifera* from Brazil showed 50.86 % antioxidant activities, whereas *Gracilaria* sp. showed 11.46 % activities (Martins et al., 2013).

3.3.6 Contribution to the RDI Levels

The daily recommended intake of biochemical components for an adult is defined by the Council for Responsible Nutrition organization (Council for Responsible Nutrition, 2015). These values were compared with the quantification values of the present study. No recommended daily intake values were obtained for the carotenoids, phenolics and flavonoids. But data's were available for the vitamins and those values were used for the calculations to derive the minimum quantity of seaweeds that are suggestible for human consumption. Among the nine seaweeds, *E. prolifera* (Michalak & Chojnacka, 2009; Farasat et

al., 2013), *U. fasciata* (Selvin & Lipton, 2004; Silva et al., 2013), *A. spicifera* (Devi et al., 2009; Flora & Rani, 2013; Guiry, 2015) and *Gracilaria corticata* (Zandi et al., 2010; Narasimman & Murugaiyan, 2012) were observed to be edible. The data on comparison (Table 3.10) evidenced the usage of *G. pusillum* as a rich source of bioactive components for animal nutrition. The study recommends the usage of 7.52 g of seaweed extract per day. Similarly *G. foliifera* (8.32 g) and *E. prolifera* (9.71 g) from Njarakkal location also advocates their usage. *G. corticata* (634 g) and *C. antennina* (84 g) was not found to be suggestible due to their bulk mass required for consumption. In order to have the seaweed extracts such as to compensate individual vitamin deficiencies, the study suggest (Table 3.11) the use of 5.46 g of *G. foliifera* extract for B7, 0.76 g of *G. pusillum* extract for B5, B1, B12, B2, E, K and D2. 2.57 g of *A. spicifera* extract can full fill the requirements for C, B6, B9, A and B3.

Table 3.10 RDI values of the seaweed extracts in comparison with the standard RDI of vitamins.

Vitamins	RDI (µg of extract/day)	Weights in grams								
		CA	EPK	EPN	UF	AS	GP	GF	GC	GCC
B7	300	84.79	49.55	8.87	38.87	33.75	7.52	5.46	634.25	12.39
B5	10000	13.00	4.28	2.69	2.53	0.93	0.76	1.09	386.95	2.06
B1	1500	1.56	0.87	1.27	0.72	0.95	0.26	0.49	9.86	1.06
C	60000	3.48	3.14	4.89	2.52	1.87	2.68	3.47	180.31	4.20
B6	2000	5.91	2.51	2.46	4.86	0.65	1.46	2.69	12.68	9.01
B9	400	0.46	0.41	0.29	0.84	0.13	0.17	1.23	0.39	0.50
B12	6	1.73	1.33	1.22	1.58	0.49	0.28	0.70	3.90	2.70
B2	1700	8.12	4.90	2.65	2.03	1.12	0.25	2.06	1.98	4.29
A	900	1.70	6.03	2.06	2.67	0.39	1.12	2.82	2.34	1.78
E	15000	0.95	3.11	9.71	2.82	1.93	0.64	3.27	49.00	6.25
K	80	0.04	0.06	0.03	0.07	0.02	0.01	0.15	0.07	0.09
B3	20000	11.65	13.87	5.72	4.15	2.57	2.68	8.32	34.87	14.73
D2	10	5.55	5.48	4.38	4.21	1.57	0.45	5.64	2.69	6.62
Minimum intake quantity (g/day) to meet RDI		84.79	49.55	9.71	38.87	33.75	7.52	8.32	634.25	14.73

CA- *C. antennina*, EPK- *E. prolifera* -Kayamkulam location, EPN- *E. prolifera* - Njarakkal location, UF- *U. fasciata*, AS- *A. spicifera*, GP- *G. pusillum*, GF- *G. foliifera*, GC- *G. corticata*, GCC- *G. corticata* var. *cylindrica,* B7 - Biotin, B5 - Pantothenic acid, B1 - Thiamine, C - Ascorbic acid, B6 - Pyridoxine, B9 - Folic acid, B12 - Cyanocobalamin, B2 - Riboflavin, A - Retinol, E - Tocopherol, K - Phylloquinone, B3 - Niacin and D2 - Ergocalciferol.

Table 3.11 Suggestible seaweed extracts in treating particular vitamin deficiencies.

Suggestible seaweed extracts for specific vitamins		
Vitamin	**Genus**	**Required weight (g)**
Biotin-B7	*G. foliifera*	5.46
Pantothenic acid-B5	*G. pusillum*	0.76
Thiamine-B1	*G. pusillum*	0.26
Ascorbic acid-C	*A. spicifera*	1.87
Pyridoxine-B6	*A. spicifera*	0.65
Folic acid-B9	*A. spicifera*	0.13
Cyanocobalamin-B12	*G. pusillum*	0.28
Riboflavin-B2	*G. pusillum*	0.25
Retinol-A	*A. spicifera*	0.39
Tocopherol-E	*G. pusillum*	0.64
Phylloquinone-K	*G. pusillum*	0.01
Niacinaamide-B3	*A. spicifera*	2.57
Ergocalciferol-D2	*G. pusillum*	0.45

The recommended daily intake levels of the current study was observed to be very low than the conventional food sources. This property is attributed to the surplus biochemical compositions of the seaweed. The comparison of the current seaweed datas with conventional food sources are given in Table 3.12.

Table 3.12 Comparative data - seaweed samples vis-a-vis conventional food sources (μg g^{-1}).

Vegetative sources	LUT	ZEA	B7	B5	B1
B	25.6	17.36	0.01	4.8	0.9
R	2.7	6	0	5	1
O	11.8	0.67	0	3.28	1.14
CAR	2.71	0.36	0.01	2.73	5.1
LET	12.5	14.76	0.02	1.5	0.57
G	18.4	11.47	0	2.45	4.14
T	0.77	0.48	0.01	1.09	0.46
S	62.65	66.85	0.01	0.2	2
SC	5.2	4.4	0	9.35	1.1
CA	0.3	0.1	3.54	769	959
EPK	1.66	0.2	6.06	2334	1725
EPN	0.48	0.09	33.81	3716	1181
UF	0.73	0.16	7.72	3952	2077
AS	3.99	0.3	8.89	10765	1582

GP	4.91	0.62	39.92	13185	5859
GF	1.84	0.29	54.92	9211	3041
GC	0.59	0.14	0.47	25.84	152
GCC	0.43	1.06	24.22	4854	1414
Vegetative sources	**C**	**B6**	**B9**	**B12**	**B2**
B	506	1.56	0.84	-	0.96
R	1900	4	68.5	-	1
O	697	0.79	0.39	-	0.52
CAR	59	1.38	0.11	-	0.58
LET	37	0.82	0.73	-	0.62
G	227	3.46	1.01	-	2.38
T	156	0.98	0.18	-	0.23
S	84	0.59	0.58	-	0.57
SC	65	1.64	0.27	-	0.67
CA	17245	338	872	3.46	209
EPK	19098	795	967	4.52	346
EPN	12259	813	1388	4.93	641
UF	23808	411	476	3.81	836
AS	32103	3065	3156	12.31	1520
GP	22407	1371	2301	21.37	6754
GF	17298	742	326	8.61	825
GC	332	157	1023	1.54	856
GCC	14269	222	792	2.23	396
Vegetative sources	**A**	**E**	**K**	**B3**	**D2**
B	3.62	11.3	1.1	0.49	-
R	9.39	24	0.07	15	-
O	0.89	2.4	0	3.69	1.8
CAR	50.12	6.6	0.13	9.83	-
LET	0.83	1.8	1.02	3.75	0.8
G	3.85	2.2	0.41	32.34	-
T	3.08	6.6	0.1	7.31	-
S	28130	6.1	1.45	2.17	-
SC	0.93	1.1	0.01	19.86	-
CA	529	15834	1872	1717	1.81
EPK	149	4824	1270	1442	1.83
EPN	436	1544	2290	3497	2.28
UF	337	5311	1070	4821	2.38

AS	2315	7764	4123	7793	6.37
GP	801	23316	7974	7462	22.26
GF	319	4587	521	2403	1.77
GC	383	306	1225	573	3.71
GCC	504	2399	891	1357	1.51
Vegetative sources	**M**	**Q**	**L**	**KA**	**AP**
B	62.5	60	74.5	<1	<1
R	29.5	799.5	<1	<1	<1
O	<1	5.7	<1	<1	<1
CAR	<1	55	37.5	140	<1
LET	<1	14	<1	<2	<1
G	48.5	136.5	<1	<1	176
T	<1	8	<1	<1	<1
S	<1	<1	<1	<1	<1
SC	<1	<1	<1	<1	<1
CA	7.97	1.54	0.99	0.27	0.67
EPK	57.88	5.69	0.6	0.97	0.25
EPN	0.02	0.02	0.05	0.38	0.02
UF	39.79	5.47	7.53	1.8	11.46
AS	31.85	8.57	6.65	11.64	13.38
GP	42.41	0.62	0	2.36	0
GF	7.78	3.17	0.07	0.1	2.42
GC	0.09	0.14	1.11	0.11	0.09
GCC	11.01	1.97	0.99	0.09	0.19

Data taken from https://www.macular.org/lutein (Anonymous, 2015); United States Department of Agriculture (USDA, 2015) Food & Nutrition Center; http:// nutritiondata.self.com/ facts/vegetables-and-vegetable-products.

B - Brocolli, R- Red pepper, O - Orange, CAR - Carrot, LET - Lettuce, G - Green peas, T - Tomato, S - Spinach, SC - Sweet corn, CA - *C. antennina*, EPK - *E. prolifera* from Kayamkulam, EPN - *E. prolifera* from Njarakkal, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, LUT - Lutein, ZEA - Zeaxanthin, B7 - Biotin, B5 - Pantothenic acid, B1 - Thiamine, C - Ascorbic acid, B6 - Pyridoxine, B9 - Folic acid, B12 - Cyanocobalamin, B2 - Riboflavin, A - Retinol, E - Tocopherol, K - Phylloquinone, B3 - Niacin, D2 - Ergocalciferol, M - Myricetin, Q - Quercetin, L - Luteolin, KA - Kaempferol and AP - Apigenin.

3.3.7 Correlation Studies

An attempt was made to find out the Pearson type correlations among the different parameters (methanol extract yields, lutein and zeaxanthin contents, individual vitamins, total vitamins, total phenolic contents, individual flavonoids and antioxidant activities based on three methods and four standards) studied (Table 3.13). Correlation coefficients of $p < 0.001$ are considered as good correlation extend. To sum up the correlation matrixes, all the parameters, except luteolin, showed positive correlations with the observed antioxidant activity studies in one form or the other. Methanol yield correlated with cyanocobalamin (+ 0.853). Lutein correlated with pantothenic acid (+ 0.914), cyanocobalamin (+ 0.960), phylloquinone (+ 0.885), niacin (+ 0.885), ergocalciferol (+ 0.838) and total vitamin content (+ 0.892). Pantothenic acid correlated with cyanocobalamin (+ 0.900) and total vitamin contents (+ 0.851). Thiamine was observed to have correlations with cyanocobalamin (+ 0.878) and riboflavin (+ 0.874). Pyridoxine had positive correlations with folic acid (+ 0.890) and retinol (+ 0.920). Folic acid correlated positively with retinol (+ 0.887). Cyanocobalamin correlated with riboflavin (+ 0.905), phylloquinone (+ 0.914), ergocalciferol (+ 0.901) and total vitamin contents (+ 0.917). Riboflavin correlated with phylloquinone (+ 0.934) and ergocalciferol (+ 0.994) contents. Phylloquinone was observed to have correlations with ergocalciferol (+ 0.962) and total vitamin contents (+ 0.863). Niacin correlated with total vitamin contents (+ 0.922), total polyphenols with myricetin content (+ 0.907) and luteolin with apigenin (+ 0.958). The antioxidant activities also correlated each other with, DPPH ascorbic acid equivalence in correlation with DPPH BHT (+ 0.953) and resorcinol equivalence (+ 0.977). DPPH BHT equivalence correlated with DPPH resorcinol equivalence (+ 0.994). Ferrous tartarate BHT equivalence correlated with ferrous tartarate tocopherol

equivalence (+ 0.995). $KMnO_4$ ascorbic acid equivalence correlated with all the other three equivalances (BHT + 0.985, tocopherol + 0.946 and resorcinol + 0.903). BHT equivalence also correlated with tocopherol (+ 0.969) and resorcinol equivalences (+ 0.927) and tocopherol equivalence had correlations with resorcinol equivalences (+ 0.919) as well.

Table 3.13 Pearson test bivariate correlation studies, (n=27), (Split into 10 units for view ability).

(a)

	MEY	LUT	ZEA	BIO	PAN	THI	ASC	PYR	FOL	CYA
MEY	1									
LUT	0.784*	1								
ZEA	-0.020	0.217	1							
BIO	0.744*	0.445	0.450	1						
PAN	0.813*	0.914*	0.400	0.701*	1					
THI	0.798*	0.737*	0.317	0.722*	0.804*	1				
ASC	0.389	0.563	0.019	0.132	0.631*	0.416	1			
PYR	0.489	0.781*	-0.020	0.060	0.691*	0.266	0.747*	1		
FOL	0.377	0.816*	0.122	-0.080	0.605*	0.288	0.537	0.890*	1	
CYA	.853*	0.960*	0.215	0.542	0.900*	0.878*	0.541	0.640*	0.688*	1

(b)

	MEY	LUT	ZEA	BIO	PAN	THI	ASC	PYR	FOL	CYA
RIB	0.670*	0.829*	0.304	0.445	0.716*	0.874*	0.255	0.342	0.549	0.905*
RET	0.250	0.666*	0.092	-0.100	0.546	0.041	0.619*	0.920*	0.887*	0.467
TOC	0.564	0.609*	0.075	0.248	0.483	0.703*	0.404	0.269	0.439	0.744*
PHL	0.608*	0.885*	0.233	0.239	0.705*	0.742*	0.424	0.565	0.776*	0.914*
NIA	0.563	0.885*	0.110	0.207	0.812*	0.621*	0.795*	0.802*	0.777*	0.823*
ERG	0.637*	0.838*	0.282	0.366	0.689*	0.826*	0.259	0.384	0.615*	0.901*
TVT	0.698*	0.892*	0.184	0.365	0.851*	0.775*	0.797*	0.725*	0.722*	0.917*
TPP	0.281	0.515	-0.170	-0.110	0.465	0.394	0.821*	0.673*	0.551	0.503
MYR	0.193	0.317	-0.130	-0.090	0.288	0.437	0.611*	0.373	0.315	0.399
QUE	0.012	0.165	-0.270	-0.220	0.222	-0.130	0.748*	0.662*	0.349	0.044

(c)

	MEY	LUT	ZEA	BIO	PAN	THI	ASC	PYR	FOL	CYA
LTL	-0.240	0.106	-0.230	-0.420	0.076	-0.210	0.561	0.431	0.277	-0.060
KAE	0.237	0.634*	-0.050	-0.170	0.521	0.041	0.712*	0.953*	0.855*	0.441
API	0.001	0.277	-0.230	-0.220	0.282	-0.090	0.685*	0.607*	0.377	0.105
DAS	0.676*	0.764*	0.622*	0.736*	0.888*	0.637*	0.448	0.547	0.517	0.742*
DBH	0.585*	0.560	0.625*	0.787*	0.768*	0.483	0.349	0.406	0.297	0.534
DTO	0.127	0.004	0.801*	0.699*	0.335	0.244	0.001	-0.170	-0.240	0.047
DRE	0.639*	0.627*	0.629*	0.803*	0.813*	0.561	0.362	0.427	0.346	0.614*
FTA	-0.220	0.037	0.731*	0.096	0.025	0.287	-0.330	-0.370	-0.010	0.113
FTB	-0.310	-0.220	0.223	-0.190	-0.310	-0.090	-0.250	-0.420	-0.130	-0.140
FTT	-0.350	-0.250	0.249	-0.200	-0.330	-0.100	-0.290	-0.470	-0.180	-0.180

(d)

	MEY	LUT	ZEA	BIO	PAN	THI	ASC	PYR	FOL	CYA
FTR	-0.210	-0.29	0.003	-0.260	-0.410	-0.100	-0.240	-0.340	-0.100	-0.140
KAS	0.390	0.430	0.406	0.374	0.463	0.416	0.094	0.350	0.392	0.463
KBH	0.414	0.421	0.328	0.350	0.410	0.377	-0.010	0.322	0.393	0.453
KTO	0.408	0.414	0.351	0.383	0.426	0.266	-0.050	0.375	0.404	0.395
KRE	0.194	0.073	0.241	0.268	0.115	0.114	-0.250	0.056	0.079	0.112

(e)

	RIB	RET	TOC	PHL	NIA	ERG	TVT	TPP	MYR	QUE
RIB	1									
RET	0.221	1								
TOC	0.761*	0.198	1							
PHL	0.934*	0.484	0.823*	1						
NIA	0.679*	0.722*	0.562	0.784*	1					
ERG	0.994*	0.279	0.777*	0.962*	0.689*	1				
TVT	0.765*	0.595*	0.781*	0.863*	0.922*	0.771*	1			
TPP	0.340	0.452	0.281	0.464	0.736*	0.354	0.669*	1		
MYR	0.343	0.093	0.281	0.390	0.482	0.346	0.507	0.907*	1	
QUE	-0.280	0.547	-0.240	-0.090	0.431	-0.260	0.281	0.747*	0.536	1

(f)

	RIB	RET	TOC	PHL	NIA	ERG	TVT	TPP	MYR	QUE
LTL	-0.180	0.512	-0.190	-0.040	0.510	-0.160	0.208	0.557	0.270	0.716*
KAE	0.172	0.961*	0.119	0.428	0.756*	0.225	0.592*	0.643*	0.299	0.728*
API	-0.110	0.637*	-0.140	0.032	0.620	-0.110	0.347	0.606*	0.266	0.801*
DAS	0.571	0.529	0.456	0.585*	0.587*	0.553	0.696*	0.101	-0.070	0.001
DBH	0.330	0.414	0.261	0.318	0.379	0.298	0.494	-0.080	-0.250	-0.010
DTO	0.001	-0.110	-0.050	-0.120	-0.140	-0.060	0.018	-0.390	-0.350	-0.240
DRE	0.428	0.418	0.341	0.410	0.433	0.397	0.559	-0.040	-0.200	-0.040
FTA	0.452	-0.270	0.218	0.328	-0.060	0.448	0.014	-0.210	0.001	-0.620
FTB	0.065	-0.160	0.454	0.115	-0.180	0.092	-0.040	-0.490	-0.430	-0.620
FTT	0.059	-0.200	0.404	0.084	-0.200	0.081	-0.090	-0.510	-0.440	-0.640

(g)

	RIB	RET	TOC	PHL	NIA	ERG	TVT	TPP	MYR	QUE
FTR	-0.010	-0.290	0.448	0.092	-0.380	0.038	-0.090	-0.290	-0.060	-0.460
KAS	0.399	0.132	0.078	0.383	0.139	0.405	0.277	0.272	0.407	0.064
KBH	0.403	0.114	0.091	0.388	0.080	0.416	0.225	0.180	0.318	-0.030
KTO	0.296	0.214	-0.040	0.294	0.050	0.309	0.158	0.072	0.141	0.007
KRE	0.083	-0.140	-0.210	0.027	-0.270	0.087	-0.130	-0.030	0.174	-0.080

(h)

	LTL	KAE	API	DAS	DBH	DTO	DRE	FTA	FTB	FTT
LTL	1									
KAE	0.639*	1								
API	0.958*	0.757*	1							
DAS	-0.140	0.391	0.067	1						
DBH	-0.180	0.266	0.031	0.953*	1					
DTO	-0.360	-0.250	-0.280	0.622*	0.776*	1				
DRE	-0.210	0.270	0.006	0.977*	0.994*	0.742*	1			
FTA	-0.350	-0.380	-0.500	0.144	0.044	0.333	0.090	1		
FTB	-0.220	-0.350	-0.350	-0.010	-0.020	0.150	-0.010	0.452	1	
FTT	-0.200	-0.390	-0.350	-0.040	-0.050	0.156	-0.040	0.501	0.995*	1

(i)

	LTL	KAE	API	DAS	DBH	DTO	DRE	FTA	FTB	FTT
FTR	-0.570	-0.400	-0.660*	-0.180	-0.200	-0.020	-0.180	0.297	0.694*	0.650*
KAS	-0.460	0.148	-0.350	0.413	0.325	0.239	0.364	0.271	-0.470	-0.500
KBH	-0.540	0.110	-0.430	0.379	0.289	0.174	0.331	0.245	-0.440	-0.470
KTO	-0.510	0.184	-0.360	0.449	0.402	0.255	0.426	0.145	-0.480	-0.510
KRE	-0.630*	-0.120	-0.550	0.132	0.135	0.209	0.149	0.175	-0.470	-0.490

(j)

	FTR	KAS	KBH	KTO	KRE
FTR	1				
KAS	0.021	1			
KBH	0.075	0.985*	1		
KTO	-0.040	0.946*	0.969*	1	
KRE	0.102	0.903*	0.927*	0.919*	1

*. Correlation is significant at the 0.001 level (2-tailed).

MEY - Methanol extraction yield (% to dwt), LUT - Lutein content (μg g^{-1} to dwt), ZEA - Zeaxanthin content (μg g^{-1} to dwt), BIO -Biotin - B7 (μg g^{-1} to dwt), PAN - Pantothenic acid - B5(μg g^{-1} to dwt), THI - Thiamine - B1(μg g^{-1} to dwt), ASC - Ascorbic acid - C (μg g^{-1} to dwt), PYR - Pyridoxine - B6 (μg g^{-1} to dwt), FOL - Folic acid - B9 (μg g^{-1} to dwt), CYA - Cyanocobalamin - B12 (μg g^{-1} to dwt), RIB- Riboflavin - B2 (μg g^{-1} to dwt), RET - Retinol - A (μg g^{-1} to dwt), TOC - Tocopherol - E (μg g^{-1} to dwt), PHL - Phylloquinone - K (μg g^{-1} to dwt), NIA - Niacin - B3 (μg g^{-1} to dwt), ERG - Ergocalciferol - D2 (μg g^{-1} to dwt), TVT - Total vitamin content (% to dwt), TPP - Total polyphenols (% to dwt), MYR - Myricetin (μg g^{-1} to dwt), QUE - Quercetin (μg g^{-1} to dwt), LTL - Luteolin (μg g^{-1} to dwt), KAE - Kaempferol (μg g^{-1} to dwt), API - Apigenin (μg g^{-1} to dwt), DAS - DPPH - Ascorbic acid (% to dwt), DBH - DPPH - BHT (% to dwt), DTO - DPPH - Tocopherol (% to dwt), DRE - DPPH - Resorcinol (% to dwt), FTA - FT -Ascorbic acid (% to dwt), FTB - FT - BHT (% to dwt), FTT - FT - Tocopherol (% to dwt), FTR - FT - Resorcinol (% to dwt), KAS - $KMnO_4$ - Ascorbic acid (% to dwt), KBH - $KMnO_4$ - BHT (% to dwt), KTO - $KMnO_4$ - Tocopherol (% to dwt) and KRE - $KMnO_4$ - Resorcinol (% to dwt).

The correlation studies done on the quantified biochemical constituents in the current study highlighted the positive correlations between vitamins, carotenoids and phenolics. It indicates the relation between the concentrations and comments that, the decrease of one form will impact on the concentration of the other. Commendable correlations were reported between total phenolics, antioxidant activities, vitamins and carotenoids in the present study as reported in earlier works too (Matanjun et al., 2008; Kuda & Ikemori, 2009; FitzGerald et al., 2011).

3.4 Conclusion

The last two decades have exhibited a major drift in the interests of the scientific community. The search for better alternatives in dealing with health risks has increased. The old forms of medical treatments could never be stated as a complete success. There were many major drawbacks which includes side effects such as unseen permanent disorders. In this context, the concept of food stuffs as natural medications were considered. The consideration was based on the observations made on the Asian populations with vegetarian food habits. They were observed to have reduced risk of cancer, cardiovascular and degenerative diseases. The fruits, vegetables and spices that are included in the food habits of the Asian peoples were investigated in detail. Plant derived compounds were observed to be the main component responsible for this activity and thus, they were extracted and included in the human diets under the title nutraceuticals. The extracts derived from the seaweeds have proven antioxidant potentials. Some extracts are found to have great pharmaceutical applications, whereas others can be used as growth, feeding and reproduction inhibitors too. The bioactivities of seaweeds are related to the chemical composition and its reactions.

The present study reveals the potent antioxidant properties of nine seaweeds viz., *A. spicifera*, *C. antennina*, *E. prolifera* from the two sampling locations, *G. pusillum*, *G. corticata*, *G. corticata* var. *cylindrica*, *G. foliifera* and *U. fasciata*. 1:1 methanol-water extract showed highest yield than other solvents such as water, methanol, isopropyl alcohol, dichloromethane, ammonia (24%), hexane, ethyl acetate, acetone, acetonitrile, butanol , a mixture of ethyl acetate with methanol and other mixed forms of water and methanol. Among the analysed seaweeds, *A. spicifera*, *G. pusillum* and *G. foliifera* exhibited maximum extractives and vitamin contents. The major vitamins observed were C, E and B5. Phenolics and carotenoids followed the same pattern with myricetin and lutein being the major forms.

The *in vitro* activities of the 1:1 methanol-water extracts exhibited concentration depended activities which increases with increase in concentrations. The correlations of the biochemical components with the antioxidant activities indicate the importance of vitamins, carotenoids and phenolics in seaweeds. This study provides appreciable information on these seaweeds so as to get them developed as natural sources of antioxidants for food, nutraceuticals and pharmaceuticals.

This is the first detailed study of *in vitro* antioxidant activity of seaweeds from the Kerala coast, India, in which the 13 vitamins, two carotenoids and five monophenolic compounds along with total phenolic contents were identified and quantified from a range of seaweeds. The results of this study demonstrated that the extracts of the seaweeds possessed good antioxidant activities. The resultant of the three methods of antioxidant analyses was that, species (dominating in total phenolic contents, carotenoids and potential vitamins) like *G. pusillum, G. foliifera, G. corticata* var. *cylindrica, C. antennina* and *E. prolifera* exhibited commendable antioxidant

activities. The study also indicated good correlations between vitamins, carotenoids, phenolic compounds and antioxidant activities.

As this study indicates seaweeds as a source of antioxidant substances and other important bio molecules of health importance, attempts should be focused on their better commercial utilization. The untouched and unrevealed seaweed sources at Kerala coast are rich in most of the important biochemical resources which have to be considered for future exploitation. The free resource has to be utilized as they are classified as the renewable sources gifted to mankind.

3.5 References

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Chapter 4

AMINO ACIDS

Contents

4.1 Introduction

The demand for proteinaceous food intended for human consumption is increasing continuously. This growing demand led to the search for such foods from the marine environment. Marine protein resources were completely relied on fish. But, recently the various other forms of protein sources such as crustaceans, phytoplankton and algae have been identified. In this context, marine macro algae or seaweeds, which are the replenishable source are investigated much in order to fulfil these demands. Seaweeds proteins are reported to be better in quality and contents (McKeith, 1998). They contain both the essential and non essential amino acids, which are required by the human body to meet the daily requirements. Seaweeds face daily wear and tear which results in the degradation of proteins to their basic forms of amino acids. These amino acids are available in its free form and they play a potential role in the rejuvenation process of proteins in human body.

Amino acids are the basic units of proteins which play the important functions in the metabolism of living organisms. Their presence in physiological fluids has flashed greater attention to its determination due to its importance in the pharma sectors (Li et al., 2011). Amino acids are important for clinical diagnosis

and treatments of diseases related to the hereditary, liver, neuropathy, diabetics, atherothrombotic, etc. (Li et al., 2011) and so the accurate determination is mandatory. Scientists are studying the amino acid compositions in detail from decades and the observations showed pronounced difference between proteins and amino acid compositions. Differences were observed in between the brown, red and green seaweeds (Qasim, 1991). Nutritional qualities of the proteins are determined by the different amino acid contents and availability (Gressler et al., 2010). Animals are incapable to synthesize all amino acids of primary metabolism by themselves and get supplied through their diets. Grains lack in the threonine and methionine contents and so are supplied through legumes, meat or fish (Joshi et al., 2010). Aspartic and glutamic acids are the leading amino acid contents and contribute to approximately 22 to 44 % of the total amino acid contents (Gressler et al., 2010). Amino acids are naturally available and safe to be used in pharmaceuticals, which promote it in the biotechnological applications widely.

Amino acids are compounds with vast peculiarities and the formation of amino acids in living organisms is observed through many interconnected biochemical pathways. Isoleucine is synthesized from threonine and methionine which are obtained from the enzymatic actions on aspartate. This makes the concrete basement to the statement that amino acids are inter dependent and bio-important (Joshi et al., 2010). Alanine accumulation is found to increase when oxygen availability decreases. The alanine metabolism helps in ATP production by glycolysis (Rocha et al., 2010). Glycine which doesn't have D- or L- form, is seen in mammals and other animals in majority as an extracellular structural protein. It is seen to be synthesized from serine, threonine, choline and hydroxyproline (Wang et al., 2013). Biosynthesis of the proteinogenic L-amino acids of higher plants is observed to proceed through well established biochemical pathways. They are stereo specific which results

in the formation of L-amino acids. Exceptionally, D-amino acids are also observed, which are the mirror images of L-amino acids. D-alanine, D-aspartic acid and D-glutamic acid were seen in free and conjugated form in the pea seed (*Pisum sativum*) (Ogawa et al., 1977), barley (*Hordeum vulgare*) and hops blossom (*Humulus lupulus*) (Erbe & Brückner, 2000). Brückner & Westhauser (2003) reported the presence of D-amino acids in plants. Alanine as alanine amino transferase is a pyridoxal phosphate dependent enzyme found in leaves and roots of plants (Rocha et al., 2010). Glycine, alanine, proline etc., exhibits osmolytic properties and are known as osmolytes or compatible molecules which act by raising the osmotic pressure in high salt concentration conditions (Yancey et al., 1982; Somero, 1986). They also act as stabilisers of proteins in frozen state by increasing the melting temperature points of proteins (Chang et al., 2005; Tang & Pikal, 2005).

Seaweeds were reported earlier as an abundant source of free amino acids that represent their taste. As a vegetarian resource, its consumption as a food source was found to be highly beneficial (Wang et al., 1990). Thus, the free amino acid analysis was considered as very important in the nutritional studies. Fowden (1962) stated that, nearly all the amino acids present in protein would be observed as free amino acids. Including some specific amino acids, seaweeds are found to possess more than 20 amino acids (Arasaki & Arasaki, 1983). The concern on the taste and its nutritive contents made the consumer aware of its constitution in seaweeds (Santoso, 2004). Korean and Japanese red algae, *Porphyra yezoensis* was found to have glutamic acid, alanine, taurine and aspartic acid as the major free amino acids which correspond to its taste (Yoshie et al., 1993; Yoshie et al., 1994). *Ulva pertusa, Laminaria japonica*, *Iridaea cornucopiae* and *Porphyra tenera* were reported with high alanine, aspartic acid, glutamic acid, serine and threonine concentrations (Takagi & Kuriyama, 1959). Although the sources of all

amino acids determined are present in almost all living organisms, L-dopa source in marine system is from the mussels which produce L-dopa rich adhesive which is used by them to stick on to rocks and other surfaces (Waite & Tanzer, 1981). Tyrosine and L-dopa are phenolic amino acids with mono- and di- phenolic units respectively. They exhibit *in vitro* antioxidant properties. They find application in reducing the primary and secondary products of lipid per oxidation which are detrimental to health like DNA damaging, ageing, heart diseases and cancer (Gülçin, 2007). L-dopa, an immediate precursor of the natural neurotransmitter dopamine is used up widely in the Parkinson's disease treatments. H_2O_2 oxidation is also reduced by its oxidation products. L-dopa is a biosynthetic product from L-tyrosine. Henceforth, both could be used as a substitute to synthetic antioxidants (Schapira, 2005).

Amino acid determination is not an easy process as the naturally occurring derivatives are acid labile and thus become undetected after its conventional acid hydrolysis. As no other applicable methods are suggestible, the process is done with different modifications and inter-comparisons (Tsiboli et al., 1997). Amino acids have no UV or fluorescence absorption and hence, its determination at lower levels is difficult. Derivatization is necessary to improve its detection sensitivity, where the post and pre column derivatization with HPLC is found to be adoptable (Li et al., 2011). Amino acid analysis has achieved a suitable workplace in most of the chemical laboratories equipped with the latest analytical instrumentations (Sharma et al., 2014). Due to greater economy and sensitivity (Cohen, 2000), the use of pre-column against post-column methods has introduced a large variants of pre-column derivatization techniques including o-phthalaldehyde (OPA) (Soto-Otero et al., 1994), phenylisothiocyanate (PITC) (Hariharan et al., 1993), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Cohen, 2000), 9-flouroenylmethyl-chloroformate (FMOC-Cl) (Einarsson

et al., 1983), ninhydrin, 5-dimethylaminonaphthalene-1-sulfonyl chloride (DANSYL-Cl), 4-dimethylaminoazobenzene-4-sulfonyl chloride (DABSYL-Cl) (Callejón et al., 2010) etc. But ninhydrin showed post coloumn derivatization, DABSYL-Cl showed long derivatization and DANSYL-Cl showed highly photo sensitive derivatives which limited their usage (Kivrak et al., 2014). OPA and PITC are the most widely used derivatizing agent where OPA are useful for the faster derivatization and detection of primary amino acids and PITC are used for single and stable derivatives with low detection limits. However, OPA's failure in secondary amino acids detection and PITC's drawback of the removal of excess derivatization agent to avoid column contamination are the potential headache (Cohen, 2000). HPLC techniques using PITC as derivatization agent were reported on 17 amino acids which showed complete separation and elution (Okayasu et al., 1997). HPLC analysis using OPA derivatization was introduced as a rapid and robust assay for amino acid determinations which detected all amino acids except the proline and hydroxyproline. Newer methodologies with GC-MS, LC-MS/MS are used to separate and analyse, but the un-affordability of those equipments for the day to day application by developing countries is the corner stone for the classical HPLC analysis.

Up to the current date, researches on algal utilization are concentrated on the commercial exploitation such as its biochemical extractions. Seaweeds provide greater contribution to the amino acid pool. Proteins in algae possess essential amino acids (EAA) and non essential amino acids (NEAA) too (Galland-Irmouli et al., 1999). The alternative usage of seaweeds as a significant nutritional source is yet to develop to its full extend due to its scarce availability and hence, the cultivation of such important seaweeds is required. The comparison of amino acid composition of seaweeds with respect to other food sources is frequently determined where the *Gracilaria* sp. was the most

investigated one. Various methods were adopted to quantify the amino acids, but a reliable method to confirm the quantification is a limitation.

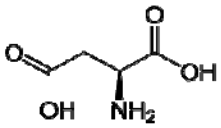
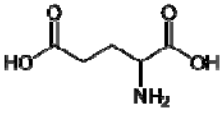
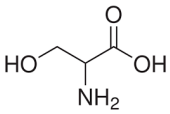
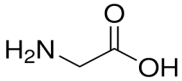
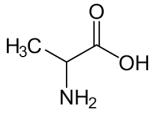
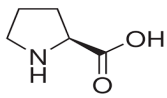
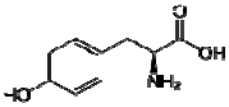
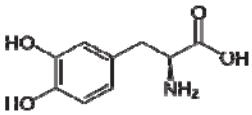
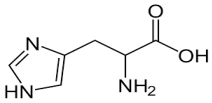
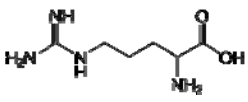
The current study uses two pre-column derivatization methods using PITC and OPA and compares the quantification in the nine seaweeds and recommends its suitability in routine analysis and as a useful data resource for the future exploitations. The energy and nutritional needs of the world population and the fundamental nutritional requirements were studied by the World Health Organisation (WHO), Food and Agriculture Organisation of the United Nations (FAO) and the United Nations University (UNU) and derived the recommended dietary requirements of EAA as the indispensable amino acids requirements in the reports of WHO/FAO/UNU (2007). The contribution of seaweeds in food with respect to the amino acids content was discussed in comparison with the above report for deriving the minimum levels of seaweed consumption to meet the daily requirements (RDI). The nutritive levels of the seaweeds were also determined from the average amino acid, free amino acid and L-dopa contents and advices the species which requires commercial attention for cultivation and exploitation using the RDI quantities. The correlation studies highlight the extent of adherence of amino acids to the total protein content.

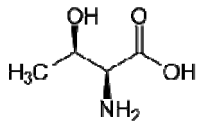
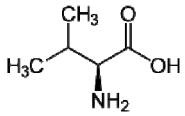
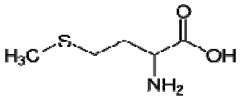
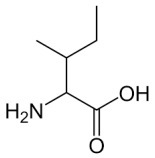
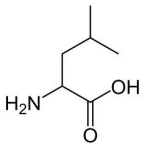
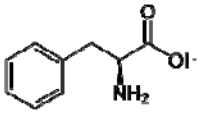
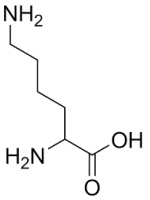
4.2 Materials and Methods

4.2.1 Standards, Chemicals and Samples

Amino acid standards were brought from Sigma (USA) and reagents were from Merck (Germany). All the analyses were done in triplicates and reported as with the corresponding standard deviations. The mean values of the observations were used for discussions. The analysed amino acids along with its structure and physiological importance are illustrated in Table 4.1. The nine seaweed samples (Table 1.1) in the form of dry powder were used in this study.

Table 4.1 Structure, abbreviated names, type and use of the analysed seventeen amino acids.

Amino acid	Structure	Abbreviation	Type	Important use	References
Aspartic acid		Asp	NEAA	As aspartate, the conjugate base stimulates the NMDA receptors eventhough not as strong as Glutamic acid as glutamate. It also has testosterone boosting capabilities.	Chen et al. (2005)
Glutamic acid		Glu	NEAA	As a neurotransmitter which improves learning skills and memory. Flavour enhancer, accelerates plant growth	Sapolsky (2005)
Serine		Ser	NEAA	As neurotransmitter and gilotransmitter	Mothet et al. (2000)
Glycine		Gly	NEAA	Biosynthetic nerotransmitter	Coyle & Tsai (2004)
Alanine		Ala	NEAA	Protein builing, Blood sugar level increaser in hypoglycemia	Muller et al. (1971)
Proline		Pro	NEAA	In brewing industries to complex polyphenols	Siebert (2010)
Tyrosine		Tyr	NEAA	Precursor to neurotransmitters and reducing stress	Rasmussen et al. (1983)
L-dopa		L-dopa	NEAA	Precursor to the neurotransmitter dopamine, norepinephrine, epinephrine. Treats parkinsons disease.	Waite &Tanzer (1981)
Histidine		His	EAA	Precursor to histamine	Fahey (2001)
Arginine		Arg	EAA	Cell division, healing wounds, de-ammonification, hormone release	Tapiero et al. (2002); Witte & Barbul (2003); Stechmiller et al. (2005)

Threonine		Thr	EAA	Used in treating nervous disorders, multiple sclerosis, myotrophic lateral sclerosis.	Growdon et al. (1991)
Valine		Val	EAA	Used in treating liver and gall bladder damages, tissue repairs	Mitrega et al. (2011)
Methionine		Meth	EAA	Prevents liver damage in acetaminophen poisoning, regulates urine pH	Bellone et al. (1997); Bellamy et al. (1998); Barshop (2000)
Isoleucine		Ileu	EAA	Muscular repairs,muscle rebuilding	Casperson et al. (2012)
Leucine		Leu	EAA	Maintain muscular tissue	Qin et al. (2010); Takeshita et al. (2012)
Phenylalanine		Phe	EAA	Treats depression, attention deficit hyperactivity disorder, parkinsons disease	Antoniou et al., (1989); Birkmayer et al. (1984)
Lysine		Lys	EAA	Treats cold stress caused by Herpes simplex	Griffith et al. (1981); DiGiovanna & Blank (1984); Flodin (1997)

4.2.2 Crude Protein Content

Total protein content estimated and discussed in Chapter 2 was compared with the amino acid contents.

4.2.3 Free Amino Acids

The free amino acids were estimated as per the UV-Vis method elaborated by Munda & Gubensek (1986). The standard used was leucine at 570 nm. The results were reported as percentage to dry weight of the sample.

4.2.4 Extraction of Amino Acids

Amino acids from the samples were extracted out following the method illustrated by Sánchez-Machado et al. (2003). 100 mg of sample was placed in 25 mm screw-cap tube and hydrochloric acid (6M, 10 mL) containing 1 % phenol was then added; the tubes were closed under nitrogen, placed in an electric oven at 110 ^{o}C for 24 h, cooled, and the contents were vacuum-filtered through Whatman no. 41 filter paper. The filtrate was diluted to 25 mL with HPLC water in a volumetric flask and 1 mL of the resulting liquid was membrane-filtered (Millipore 0.45 μm) to give the hydrolysate.

4.2.5 HPLC Amino Acid Analysis

Methods for separation and analysis of amino acids with GC and HPLC are used with high sensitivity for measuring picomole level of amino acids, but they consume a lot of time and money, along with the pre and post column derivatization. Hence, a more reliable and sensitive rapid method is required for the routine determination of amino acids in biological tissues (Fisher et al., 2001) which had to be derived upon responses and adaptability. This study used two pre column derivatization HPLC methods - OPA and PITC which are discussed here under.

4.2.5.1 Amino Acids Estimation using PITC

Amino acids were determined by the modified HPLC method-pre-column PITC derivatization (Sánchez-Machado et al., 2003). The technique was done with HPLC equipped with UV detection at 254 nm. The instrument used was Shimadzu UFLC LC-20AD with UV-Vis detector SPD-20AD equipped with phenomenex 250 mm x 1.6 mm x 5 μ C-18 column. The mobile phase was a gradient between solution A which is 0.14M ammonium acetate buffer containing 0.05 % (v/v) triethylamine (TEA) (pH adjusted to 6.4 with

glacial acetic acid) and solution B which is 60:40 (v/v) acetonitrile-water. The elution gradient (min : A %) was: 0:90, 8:90, 10:70, 12:70, 18:52, 20:0, 25:0, 28:90, 35:90 at 1.1 mL/min flow rate. Amino acid standard solution or sample hydrolysate (30 µL) was placed in a tube and dried in a vacuum oven for 20 min at 42 ^{o}C. Methanol-water-TEA (2:2:1, v/v; 50 µL) was then added to the residue and the resulting solution was vacuum dried for 20 min at ambient temperature. Methanol-water-TEA-PITC (7:1:1:1, v/v; 50 µL) was then added, and the tubes were vortex mixed for 15 s, then left for 20 min, at room temperature. The resulting solution was vacuum dried for 100 min, at ambient temperature (25 ^{o}C). After derivatization Na_2HPO_4 (5 mM containing 5 % acetonitrile; 100 µL) was added as diluents, with vortex mixing for 15 s. The results were reported as percentage on dry weight basis. Estimation was calibrated (Checa-Moreno et al., 2008) to correlation factor > 0.999 and spiked recovery > 99 % (Table 4.2; Fig. 4.1).

Table 4.2 HPLC calibration data of the 16 amino acids analysed using PITC derivatization.

Amino acid	Calibration equations	r^2	Concentration (ppm)					Retention time
			1	2	3	4	5	
Asp	y = 4.17 e-3x+ 0.088	0.999	33.28	83.19	166.38	249.56	332.75	4.576
Glu	y = 7.14 e-3x + 0.051	0.999	36.78	91.94	183.88	275.81	367.75	6.534
Ser	y = 5.56 e-3x + 0.066	0.999	26.28	65.69	131.38	197.06	262.75	12.857
Gly	y = 4.26 e-3x + 0.086	0.999	18.77	46.92	93.84	140.76	187.68	14.501
His	y = 3.33 e-2x + 0.109	0.998	38.80	97.00	194.00	291.00	388.00	17.858
Arg	y = 1.0 e-2x + 0.365	0.999	43.55	108.88	217.75	326.63	435.50	19.501
Thr	y = 1.25 e-2x + 0.292	0.999	29.78	74.44	148.88	223.31	297.75	20.002
Ala	y = 1.11 e-2x + 0.329	0.999	22.27	55.68	111.36	167.04	222.73	21.143
Pro	y = 1.29 e-2x - 0.028	0.999	28.78	71.94	143.88	215.81	287.75	22.625
Tyr	y = 5.56 e-3x - 0.065	0.999	45.30	113.25	226.50	339.75	453.00	29.504
Val	y = 7.14 e-3x + 0.052	0.999	29.30	73.25	146.50	219.75	293.00	32.375
Meth	y = 6.67 e-3x + 0.055	0.999	37.30	93.25	186.50	279.75	373.00	36.085
Leu	y = 6.25 e-3x + 0.058	0.999	32.80	82.00	164.00	246.00	328.00	39.562
Ileu	y = 8.33 e-3x - 0.044	0.999	32.80	82.00	164.00	246.00	328.00	44.121
Phe	y = 5.88 e-3x + 0.062	0.999	41.30	103.25	206.50	309.75	413.00	47.493
Lys	y = 2.03 e-2x - 0.183	0.999	36.55	91.38	182.75	274.13	365.50	49.945

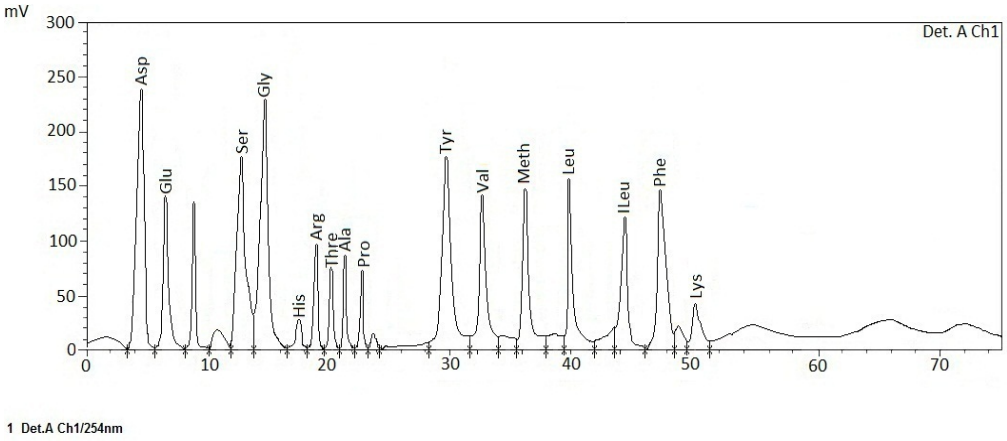


Fig. 4.1 HPLC chromatogram of 16 amino acid standards detected at 254 nm with PITC derivatization.

4.2.5.2 Amino Acids Estimation Using OPA

Amino acids were also determined by the modified HPLC method by pre-column derivatization with o-phthaladehyde (Bartolomeo & Maisano, 2006). The reagents used were o-phthalaldehyde, 2-mercaptoethanol, methanol, acetonitrile, water, sodium hydroxide, boric acid, sodium acetate and glacial acetic acid, all of HPLC grades. The OPA reagent was prepared by dissolving 5 mg of OPA in 0.05 mL methanol, added to 0.45 mL of 0.4M sodium borate buffer at pH 10.5, followed by 0.025 mL of 2-mercaptoethanol. 10 µL of sample/standard solution was mixed with 10 µL of above reagent, incubated at 25 ^{o}C for 2 min, and injected to Shimadzu UFLC LC-20AD equipped with UV-Vis SPD-20AD detector at 340 nm. The column used was phenomenex C-18 250mm x 1.6mm x 5 µ. The mobile phase used was between two solutions A and B. A is 40mM NaH_2PO_4/Na_2HPO_4 (1:1) buffer pH=7.8 and B is AcN/MeOH/H_2O (45/45/10 v/v/v). The flow rate was 1.0 mL/min. The elution gradient (min:A %) was: 0:100, 15:25, 17:0, 22:0, 25:100, 35:100. The estimation was done after due multi point calibration with r^2 value > 0.999 and spiked recovery > 99 % (Table 4.3; Fig. 4.2).

Table 4.3 HPLC calibration data of the 16 amino acids analysed using OPA derivatization.

Amino acid	Caliberation equations	r^2	Concentration (ppm)					Retention time
			1	2	3	4	5	
Asp	y = 3.33e-3x + 0.011	0.999	33.28	83.19	166.38	249.56	332.75	5.628
Glu	y = 9.09e-3x - 0.004	0.999	36.78	91.94	183.88	275.81	367.75	7.826
Ser	y = 7.69e-3x + 0.004	0.999	26.28	65.69	131.38	197.06	262.75	10.578
Gly	y = 2.52e-3x + 0.012	0.999	18.77	46.92	93.84	140.76	187.68	11.322
His	y = 7.14e -3x -0.003	0.999	38.80	97.00	194.00	291.00	388.00	12.003
Arg	y = 5.55e-3x - 0.005	0.999	43.55	108.88	217.75	326.63	435.50	12.911
Thr	y = 7.14e-3x + 0.004	0.999	29.78	74.44	148.88	223.31	297.75	13.502
Ala	y = 6.25e-3x -0.005	0.999	22.27	55.68	111.36	167.04	222.73	14.204
Tyr	y = 5.26e-3x + 0.006	0.999	45.30	113.25	226.50	339.75	453.00	14.942
Val	y = 4.54e-3x + 0.007	0.999	29.30	73.25	146.50	219.75	293.00	15.607
Meth	y = 4.082e-3x - 0.009	0.999	37.30	93.25	186.50	279.75	373.00	16.506
L-dopa	y = 6.06e-3x + 0.006	0.999	28.00	70.00	140.00	210.00	280.00	17.624
Leu	y = 5.03e-3x - 0.008	0.999	32.80	82.00	164.00	246.00	328.00	19.213
Ileu	y = 4.26e-3x - 0.010	0.999	32.80	82.00	164.00	246.00	328.00	20.732
Phe	y = 8.69e-3x + 0.006	0.999	41.30	103.25	206.50	309.75	413.00	21.322
Lys	y = 2.44e-3x + 0.021	0.999	36.55	91.38	182.75	274.13	365.50	21.958

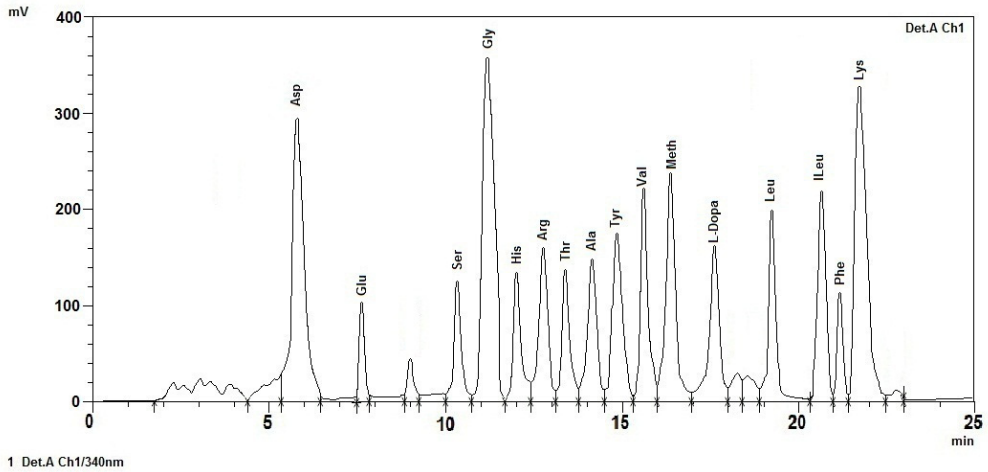


Fig. 4.2 HPLC chromatogram of 16 amino acid standards detected at 340 nm with OPA derivatization.

L-Dopa - L-dopa.

4.2.6 Estimation of L-dopa

L-dopa could be easily detectable without pre-column derivatization, but to get better response, it was pre-column derivatized using OPA as per the methods in 4.2.5.2. The multi point calibration was done to a correlation factor > 0.999 and a spike recovery of > 99 % was obtained (Table 4.3; Fig. 4.2).

4.2.7 Contribution of EAA to the RDI

The revised WHO/FAO/UNU (2007) report with the latest recommended daily intake amounts of amino acids was compared with the average EAA contents determined by both the methods. This evaluation was done in consideration with an average human body weighing 70 kgs. The comparison results were expressed as grams of dried seaweed required for consumption to meet the RDI.

4.2.8 Correlation Studies

The correlation studies were done on the average amino acid contents derived from the analysed methods, free amino acid contents, L-dopa and total protein contents upon subjecting the observations to the inter compositional correlation studies using the SPSS (16.0) for windows by utilizing the bivariate Pearson correlation.

4.3 Results and Discussions

4.3.1 Crude Protein Content

The green seaweeds analysed in the current study (Table 4.4) showed a higher content of protein in the range of 8.99 to 25.12 %. The species from Kayamkulam exhibited the higher concentration with *U. fasciata* having 25.12 %. *E. prolifera* obtained from Njarakkal location (17.45 %) and Kayamkulam location (12.26 %) also exhibited appreciable concentrations of proteins. In the

red seaweeds, *A. spicifera* showed the maximum concentration (9.37 %) which was at par with the content of the lowest concentrated green species *C. antennina* (8.99 %). The lowest among the analysed seaweeds was the *G. pusillum* (5.98 %) and *G. corticata* var. *cylindrica* (5.98 %) with the comparable concentrations which remark the correlation of seaweeds from the same point of growth at the sampling location.

Table 4.4 Total protein content (%) and EAA/Protein content calculated with both the methods, (mean ± SD), (n=3).

Particulars	CHLOROPHYTA			
	CA	EPK	EPN	UF
TPC	8.99 ± 0.18	12.26 ± 0.19	17.45 ± 0.27	25.12 ± 0.39
EAA/P-PITC	0.39 ± 0.01	0.49 ± 0.01	0.42 ± 0.01	0.42 ± 0.01
EAA/P- OPA	0.42 ± 0.01	0.41 ± 0.01	0.42 ± 0.01	0.48 ± 0.01

Particulars	RHODOPHYTA				
	AS	GP	GF	GC	GCC
TPC	9.37 ± 0.14	5.98 ± 0.09	6.98 ± 0.11	6.18 ± 0.09	5.98 ± 0.16
EAA/P-PITC	0.39 ± 0.01	0.35 ± 0.01	0.36 ± 0.01	0.39 ± 0.01	0.39 ± 0.01
EAA/P- OPA	0.43 ± 0.01	0.35 ± 0.01	0.41 ± 0.01	0.46 ± 0.01	0.44 ± 0.01

TPC - Total protein content, EAA - Essential amino acids, P - Protein, PITC - Phenylisothiocyanate method, OPA - o-phthalaldehyde method, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

Traditionally green leafy vegetables were advised by medical practitioners and dieticians as a nutritive source. The reported levels of protein content in this study were found to be suitable to fulfil those advices. Green seaweeds were observed to have appreciable levels of protein contents and the results were observed in comparison with the earlier studies. *Eisenia arborea* collected from Mexico showed 9.44 % protein (Hernández-Carmona et al., 2009). Five macroalgae such as the *Chaetomorpha aerea*, *Asparagopsis*

taxiformis, *Spyridia hypnoides*, *Centroceras clavulatum* and *Sargassum filipendula* collected from Brazil showed 8.72 to 16.1 % of protein content (Diniz et al., 2011). *Ulva rigida* collected from the Chilka Lake, were reported with 6.64 % protein content (Satpati & Pal, 2011).

4.3.2 Free Amino Acids

The free amino acids in a living organism are the basic building blocks of protein in their muscular developments. In plants, they are the base units of nucleic acid formation and protein developments. A certain level of free amino acid contents is present due to the degradation of proteins as a result of adverse dwelling conditions. The estimation of free amino acids helps in correlating the concentrations of total amino acids with crude protein content.

In the current study (Table 4.5) the free amino acids were found to be higher in *U. fasciata* (2.66 %) from Kayamkulam location and *E. prolifera* obtained from Njarakkal location (2.20 %). *E. prolifera* (1.61 %) obtained from Kayamkulam location also exhibited appreciable free amino acid contents. The lowest was in *G. pusillum* (0.58 %) among the analysed species. Rhodophyta showed free amino acids to the lower extend.

Table 4.5 Free amino acid contents in nine seaweeds, (% to dwt), (mean ± SD), (n=3).

Species	Free amino acids
C. antennina	0.96 ± 0.01
E. prolifera (K)	1.61 ± 0.02
E. prolifera (N)	2.20 ± 0.02
U. fasciata	2.66 ± 0.03
A. spicifera	1.02 ± 0.01
G. pusillum	0.58 ± 0.01
G. foliifera	1.39 ± 0.01
G. corticata	0.89 ± 0.01
G. corticata var. *cylindrica*	0.78 ± 0.01

K - Kayamkulam location and N - Njarakkal location.

The observations of the current study were similar to the previous studies done in this field. Indonesian seaweeds like *Caulerpa sertularoides*, *Caulerpa racemosa*, *Cladophoropsis vauchaeriaeformis*, *Halimeda macroloba* and *Ulva reticulata* belonging to green seaweeds, *Padina australis*, *Sargassum polycystum* and *Turbinaria conoides* belonging to brown seaweeds and *Kappaphycus alvarezii* belonging to red seaweeds showed low free amino acid contents with 0.00029 to 0.05189 % (Santoso, 2004). 14.47 % glycine and 7.37 % threonine were the major amino acids among the reported free amino acids in *C. vauchaeriaeformis*. 2.65 % and 5.72 % of aspartic acid were reported in *S. polycytum* and *T. conoides* respectively (Santoso, 2004). Free amino acid contents in Japanese green algae *Ulva pertusa* and *Enteromorpha linza* were reported as 0.37 and 0.35 % respectively (Takagi et al., 1967). *U. pinnatifida* (1.70 %), *Sargassum confusum* (0.54 %) (Takagi et al., 1967) and *Laminaria japonica* (5.97 to 6.62 %) were also reported with free amino acid contents (Noda, 1987). *Porphyra yezoensis* were reported with free amino acids, which consisted of taurine (0.63 to 1.64 %), alanine (0.31 to 1.60 %), glutamic acid (0.33 to 1.84 %) and aspartic acid (0.07 to 0.28 %) in majority (Yoshie et al., 1993). *Ulva rigida* collected from the Chilka Lake, were reported with 8.9 % free amino acid content (Satpati & Pal, 2011).

4.3.3 Quantification of Amino Acids with PITC Derivatization Technique

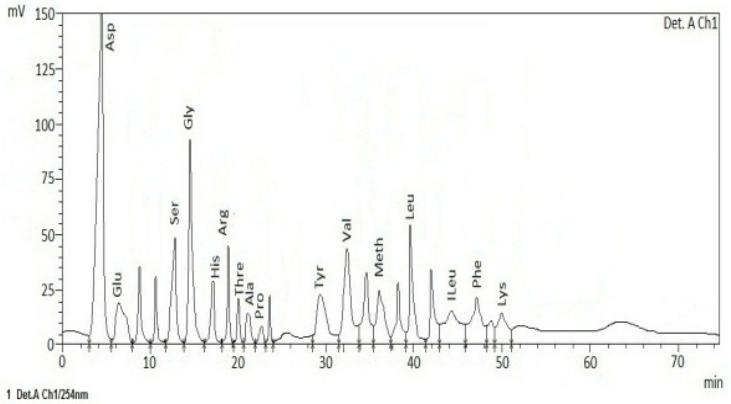
PITC derivatization in presence of TEA was done in the current study to obtain the stable derivatives of amino acids. High amounts of amino acids (Table 4.6), were observed in *U. fasciata* (EAA - 10.46 % and NEAA - 8.57 %). HPLC chromatogram of the nine seaweeds is given in Fig. 4.3.

Table 4.6 Amino acid contents in nine seaweeds (PITC derivatization), (% to dwt), (mean ± SD), (n=3).

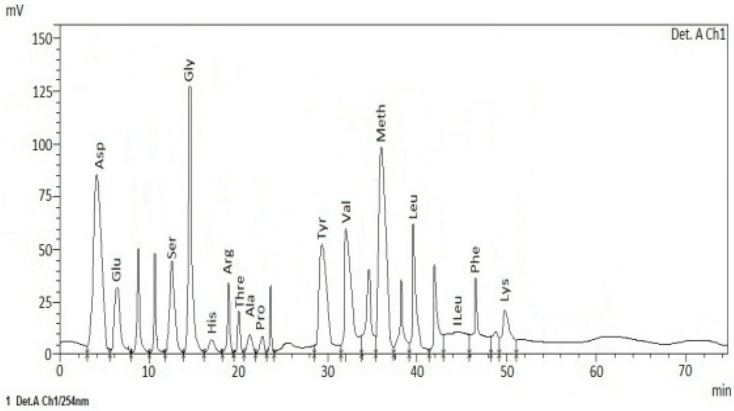
Amino acids	CHLOROPHYTA			
	CA	EPK	EPN	UF
His	0.35 ± 0.01	0.56 ± 0.01	0.53 ± 0.01	1.24 ± 0.02
Arg	0.61 ± 0.01	0.46 ± 0.01	1.08 ± 0.02	1.32 ± 0.02
Thr	0.64 ± 0.01	0.63 ± 0.01	0.83 ± 0.01	1.32 ± 0.02
Val	0.36 ± 0.01	0.57 ± 0.01	1.23 ± 0.02	1.18 ± 0.02
Meth	0.21 ± 0.00	0.63 ± 0.01	1.10 ± 0.02	0.82 ± 0.01
Ileu	0.27 ± 0.00	0.49 ± 0.01	0.57 ± 0.01	1.11 ± 0.02
Leu	0.38 ± 0.01	0.49 ± 0.01	0.58 ± 0.01	0.97 ± 0.02
Phe	0.44 ± 0.01	0.66 ± 0.01	0.55 ± 0.01	1.22 ± 0.02
Lys	0.29 ± 0.00	0.58 ± 0.01	0.91 ± 0.01	1.31 ± 0.02
Asp	0.85 ± 0.01	0.47 ± 0.01	0.92 ± 0.01	1.83 ± 0.02
Glu	0.45 ± 0.01	0.71 ± 0.01	0.99 ± 0.01	1.65 ± 0.02
Ser	0.64 ± 0.01	0.52 ± 0.01	0.87 ± 0.01	1.04 ± 0.02
Gly	0.47 ± 0.01	0.66 ± 0.01	1.25 ± 0.02	0.89 ± 0.01
Ala	0.67 ± 0.01	0.72 ± 0.01	1.17 ± 0.02	1.01 ± 0.02
Pro	0.44 ± 0.01	0.81 ± 0.01	1.32 ± 0.02	0.85 ± 0.01
Tyr	0.28 ± 0.00	0.58 ± 0.01	0.89 ± 0.01	1.33 ± 0.02
Total EAA	3.54 ± 0.05	5.02 ± 0.08	7.38 ± 0.11	10.46 ± 0.16
Total NEAA	3.79 ± 0.06	4.43 ± 0.07	7.41 ± 0.11	8.57 ± 0.13
EAA/NEAA	0.93 ± 0.01	1.13 ± 0.02	0.99 ± 0.02	1.22 ± 0.02

Amino acids	RHODOPHYTA				
	AS	GP	GF	GC	GCC
His	0.49 ± 0.01	0.30 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.33 ± 0.01
Arg	0.48 ± 0.01	0.18 ± 0.00	0.22 ± 0.00	0.12 ± 0.00	0.10 ± 0.00
Thr	0.36 ± 0.01	0.28 ± 0.00	0.33 ± 0.01	0.43 ± 0.01	0.33 ± 0.01
Val	0.32 ± 0.01	0.18 ± 0.00	0.22 ± 0.00	0.17 ± 0.00	0.21 ± 0.00
Meth	0.17 ± 0.01	0.11 ± 0.00	0.14 ± 0.00	0.12 ± 0.00	0.16 ± 0.00
Ileu	0.29 ± 0.01	0.29 ± 0.01	0.36 ± 0.01	0.44 ± 0.01	0.43 ± 0.01
Leu	0.45 ± 0.01	0.21 ± 0.00	0.25 ± 0.00	0.15 ± 0.00	0.19 ± 0.00
Phe	0.61 ± 0.01	0.35 ± 0.01	0.39 ± 0.01	0.42 ± 0.01	0.38 ± 0.01
Lys	0.48 ± 0.01	0.23 ± 0.00	0.25 ± 0.00	0.18 ± 0.00	0.24 ± 0.00
Asp	1.26 ± 0.01	0.89 ± 0.01	1.71 ± 0.02	0.16 ± 0.00	0.13 ± 0.00
Glu	0.98 ± 0.01	0.49 ± 0.01	0.59 ± 0.01	0.42 ± 0.00	0.54 ± 0.01
Ser	0.29 ± 0.00	0.21 ± 0.00	0.25 ± 0.00	0.27 ± 0.00	0.25 ± 0.00
Gly	0.37 ± 0.01	0.17 ± 0.00	0.21 ± 0.00	0.14 ± 0.00	0.16 ± 0.00
Ala	0.32 ± 0.01	0.27 ± 0.00	0.34 ± 0.01	0.47 ± 0.01	0.29 ± 0.00
Pro	0.58 ± 0.01	0.38 ± 0.01	0.47 ± 0.01	0.46 ± 0.01	0.47 ± 0.01
Tyr	0.39 ± 0.01	0.24 ± 0.00	0.28 ± 0.00	0.26 ± 0.00	0.32 ± 0.00
Total EAA	3.64 ± 0.06	2.10 ± 0.03	2.52 ± 0.04	2.40 ± 0.04	2.36 ± 0.04
Total NEAA	4.18 ± 0.06	2.62 ± 0.04	3.83 ± 0.06	2.17 ± 0.03	2.15 ± 0.03
EAA/NEAA	0.89 ± 0.01	0.79 ± 0.01	0.65 ± 0.01	1.10 ± 0.02	1.09 ± 0.02

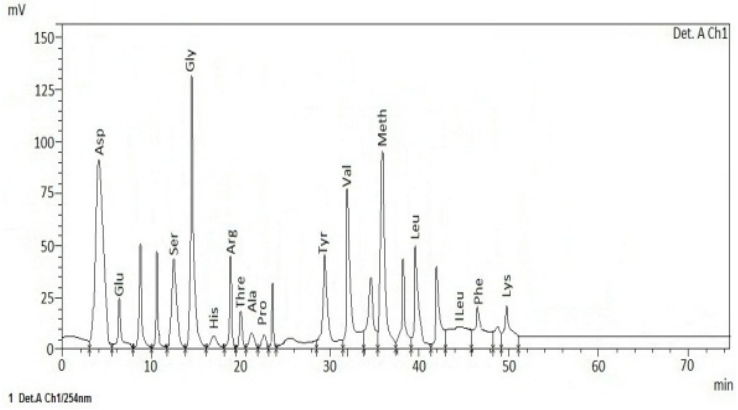
EAA - Essential Amino Acids, NEAA - Non Essential Amino Acids, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.



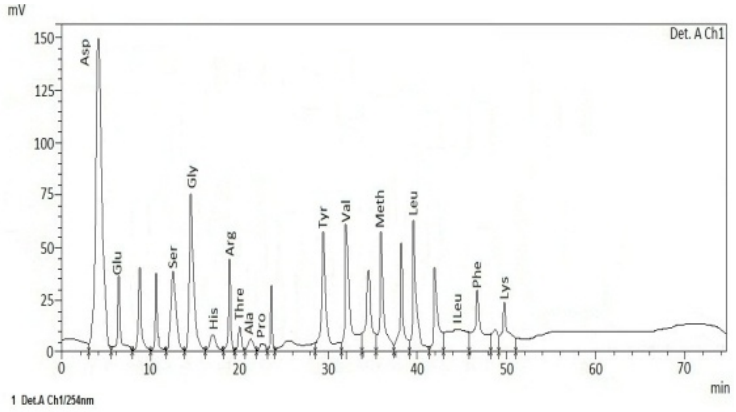
C. antennina



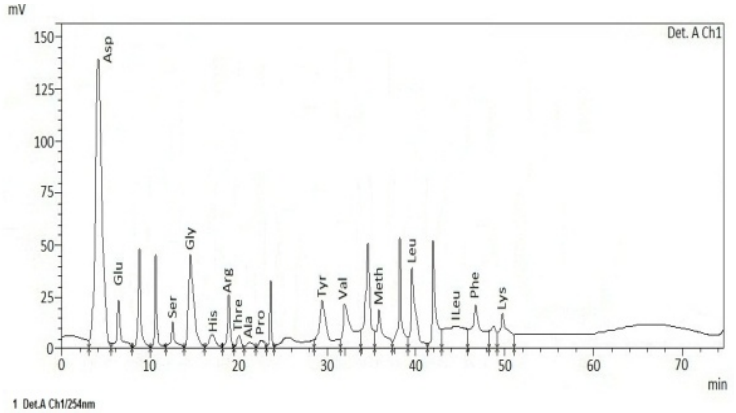
E. prolifera-Kayamkulam



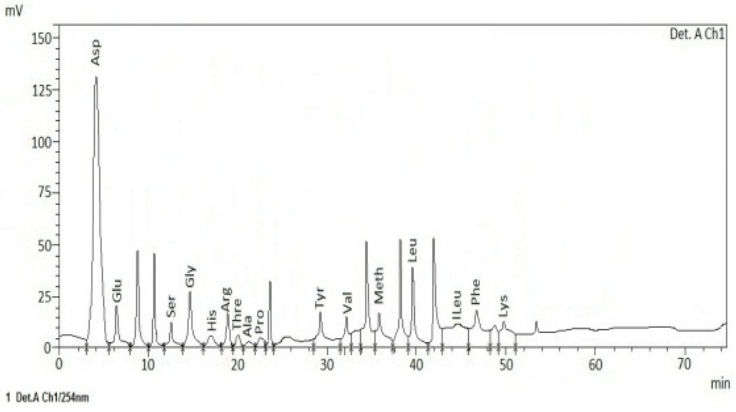
E. prolifera-Njarakkal



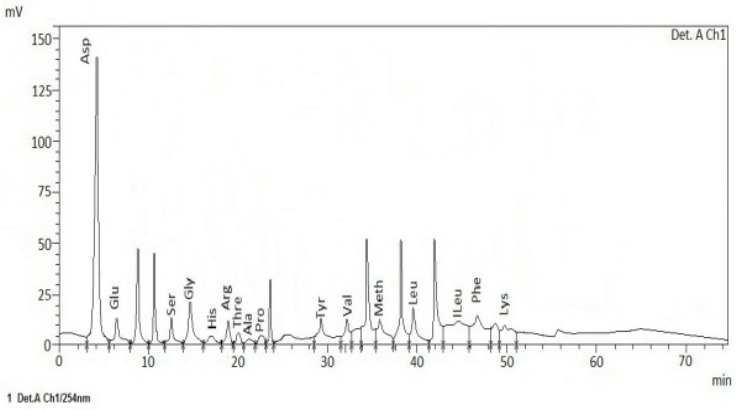
U. fasciata



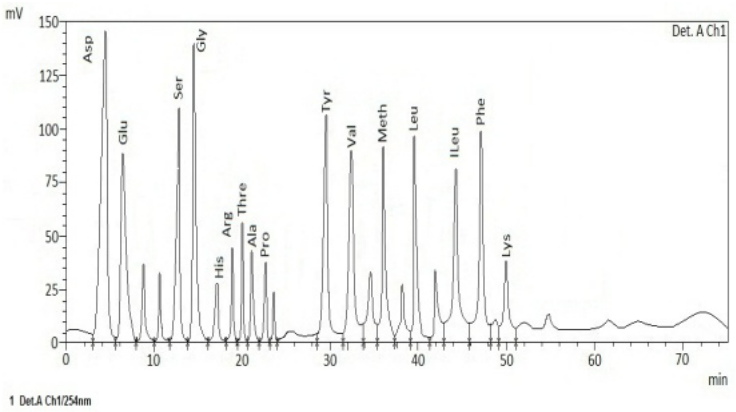
A. spicifera



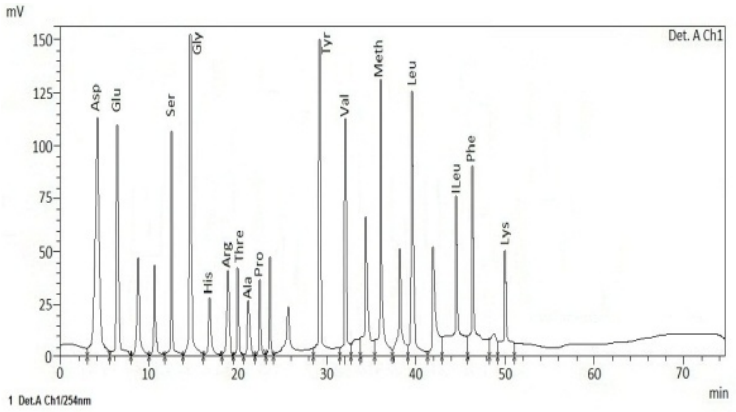
G. pusillum



G. foliifera



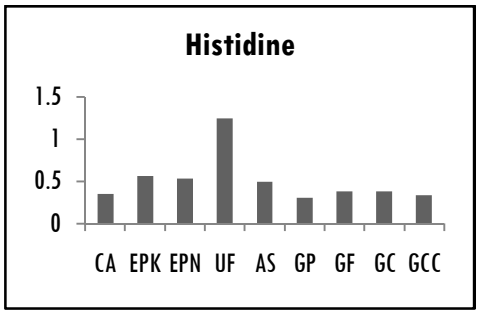
G. corticata



G. corticata var. *cylindrica*

Fig. 4.3 HPLC chromatogram of nine seaweeds using PITC derivatization method.

Histidine content was seen highest in *U. fasciata* (1.24 %) (Fig. 4.4). Comparable histidine content was observed in between *E. prolifera* obtained from the Kayamkulam location (0.56 %) and Njarakkal location (0.53 %). Comparable histidine content was observed between *G. pusillum* (0.30 %) and *G. corticata* var. *cylindrica* (0.33 %) and in between *C. antennina* (0.35 %), *G. foliifera* (0.38 %) and *G. corticata* (0.38 %). Least content was reported in *G. pusillum*. Arginine content was observed highest in *U. fasciata* (1.32 %). Comparable arginine content was seen between *E. prolifera* obtained from Kayamkulam location (0.46 %) and *A. spicifera* (0.48 %), *G. corticata* (0.12 %) and *G. corticata* var. *cylindrica* (0.10 %) and *G. pusillum* (0.18 %) and *G. foliifera* (0.22 %). The least content was reported in *G. corticata* var. *cylindrica*. Threonine content was seen highest in *U. fasciata* (1.32 %) and least in *G. pusillum* (0.28 %). Comparable threonine concentrations were seen between *C. antennina* (0.64 %) and *E. prolifera* obtained from Kayamkulam location (0.63 %) and *A. spicifera* (0.36 %), *G. foliifera* (0.33 %) and *G. corticata* var. *cylindrica* (0.33 %). Valine was observed in higher levels in *E. prolifera* obtained from the Njarakkal location (1.23 %). The contents were similar with *U. fasciata* (1.18 %). Least was observed in *G. corticata* (0.17 %). *C. antennina* (0.36 %) and *A. spicifera* (0.32 %), *G. pusillum* (0.18 %) and *G. corticata* (0.17 %) and *G. foliifera* (0.22 %) and *G. corticata* var. *cylindrica* (0.21 %) exhibited comparable valine contents.


Histidine
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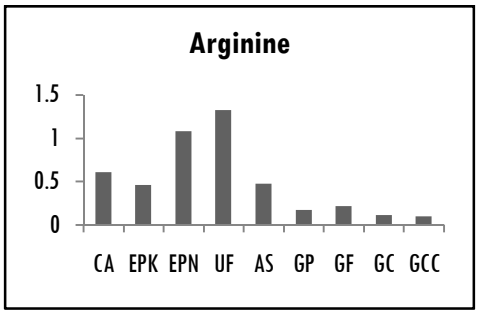
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC


Arginine
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1
0.5
0
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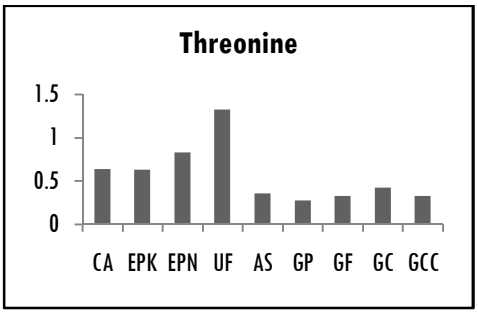
Threonine
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1
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0
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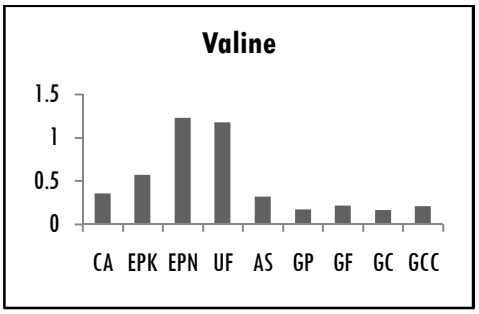
Valine
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0.5
0
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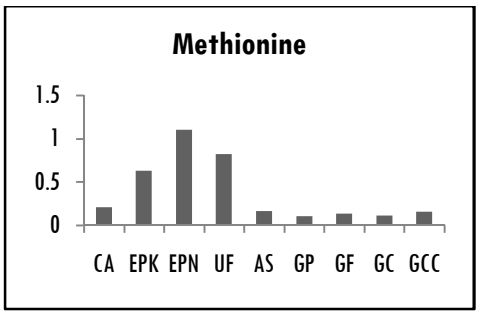
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0.5
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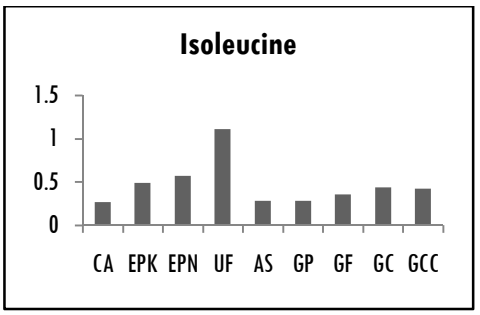
Isoleucine
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1
0.5
0
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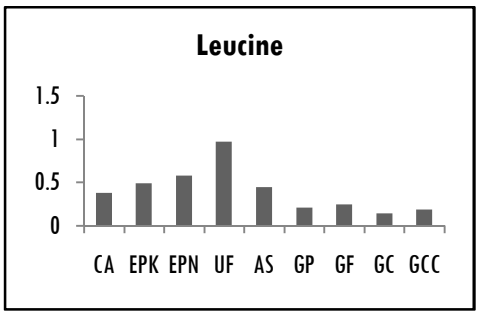
Leucine
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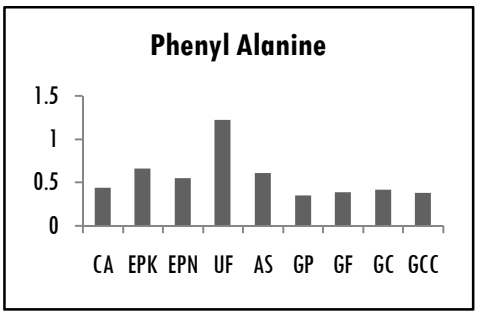
0.5
0
CA EPK EPN UF AS GP GF GC GCC


Phenyl Alanine
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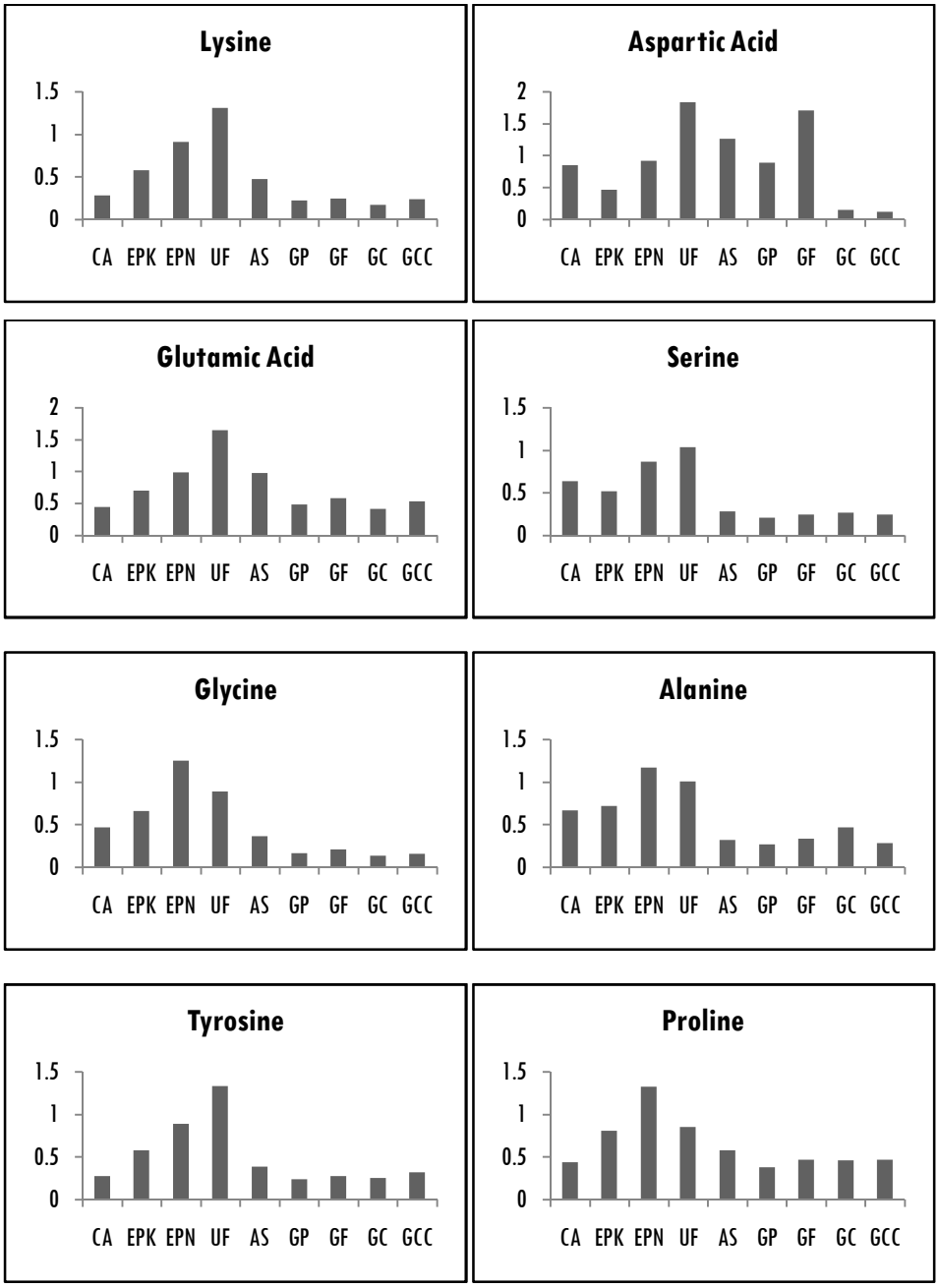
0.5
0
CA EPK EPN UF AS GP GF GC GCC


Lysine
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0.5
0
CA EPK EPN UF AS GP GF GC GCC
Aspartic Acid
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1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC
Glutamic Acid
2
1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC
Serine
1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC
Glycine
1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC
Alanine
1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC
Tyrosine
1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC
Proline
1.5
1
0.5
0
CA EPK EPN UF AS GP GF GC GCC

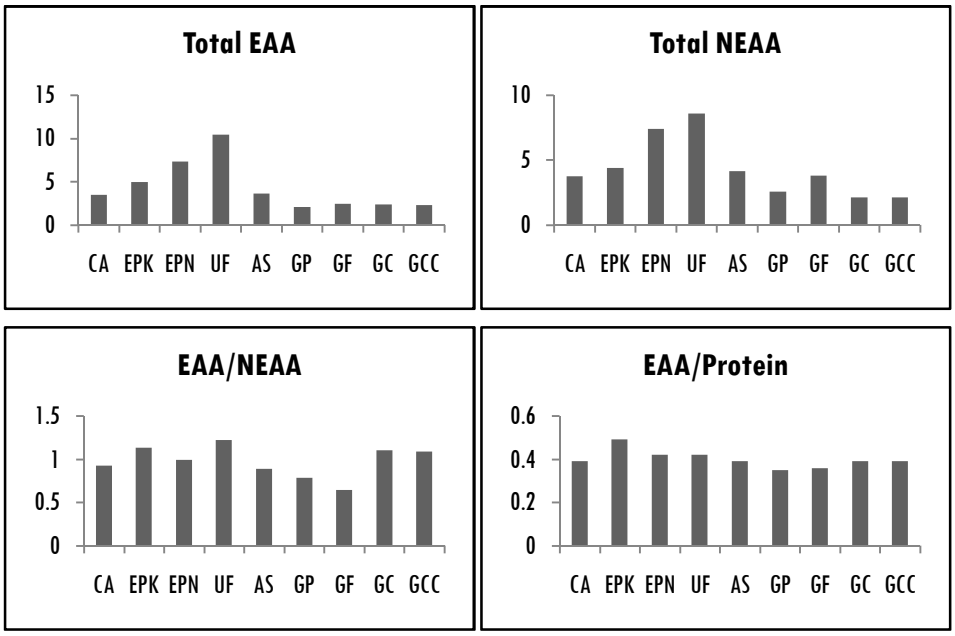


Fig. 4.4 Graphical representation of the amino acid contents in nine seaweeds (PITC derivatization).

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

Methionine content was observed to be high in *E. prolifera* obtained from Njarakkal location (1.10 %) and low in *G. pusillum* (0.11 %). Comparable methionine content was seen in between *A. spicifera* (0.17 %) and *G. corticata* var. *cylindrica* (0.16 %) and *G. pusillum* (0.11 %) and *G. corticata* (0.12 %). Isoleucine was reported to be high in *U. fasciata* (1.11 %) and low in *C. antennina* (0.27 %). Comparable isoleucine content was seen in between *A. spicifera* (0.29 %) and *G. pusillum* (0.29 %) and in between *G. corticata* (0.44 %) and *G. corticata* var. *cylindrica* (0.43 %). Leucine was high in *U. fasciata* (0.97 %) and low in *G. corticata* (0.15 %). Phenylalanine was observed to be high in *U. fasciata* (1.22 %) and low in *G. pusillum* (0.35 %). Comparable content was seen in between *C. antennina* (0.44 %) and *G.*

corticata (0.42 %) and in between *G. foliifera* (0.39 %) and *G. corticata* var. *cylindrica* (0.38 %). High contents of lysine were seen in *U. fasciata* (1.31 %) and low amounts in *G. corticata* (0.18 %). Comparable lysine contents were observed in between *G. pusillum* (0.23 %), *G. corticata* var. *cylindrica* (0.24 %) and *G. foliifera* (0.25 %).

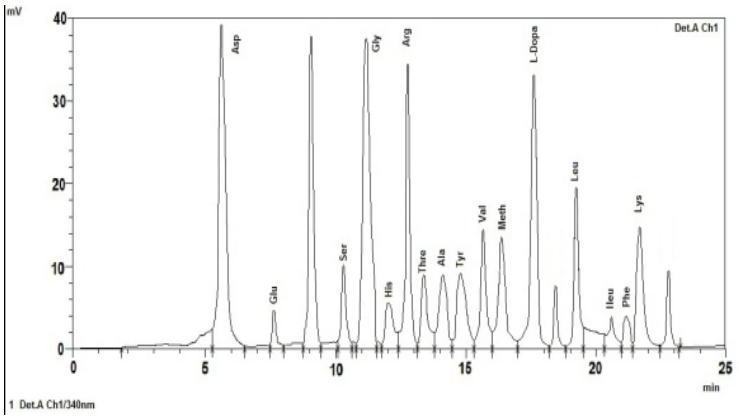
Aspartic acid which is one of the major amino acids, was observed to be high in *U. fasciata* (1.83 %) and *G. foliifera* (1.71 %). Least was observed in *G. corticata* var. *cylindrica* (0.13 %). Similarly glutamic acid was seen high in *U. fasciata* (1.65 %) and least in *G. corticata* (0.42 %). *G. corticata* showed comparable contents with *C. antennina* (0.45 %). *E. prolifera* (0.99 %) collected from Njarakkal location exhibited comparable concentrations with *A. spicifera* (0.98 %). Serine content was seen highest in *U. fasciata* (1.04 %) and least in *G. pusillum* (0.21 %). Comparable serine contents were seen in between *A. spicifera* (0.29 %) and *G. corticata* (0.27 %), and in between *G. foliifera* (0.25 %) and *G. corticata* var. *cylindrica* (0.25 %). Glycine, a sweet amino acid was seen in higher levels in *E. prolifera* (1.25 %) obtained from the Njarakkal location and lower levels in *G. corticata* (0.14 %). Comparable contents were seen in between *G. pusillum* (0.17 %) and *G. corticata* var. *cylindrica* (0.16 %). Alanine was seen in higher concentrations in *E. prolifera* (1.17 %) obtained from Njarakkal and in *U. fasciata* (1.01 %). Low concentration of alanine was seen in *G. pusillum* (0.27 %) and *G. corticata* var. *cylindrica* (0.29 %). Comparable alanine contents were seen in between *A. spicifera* (0.32 %) and *G. foliifera* (0.34 %). Proline content was seen high in *E. prolifera* (1.32 %) obtained from Njarakkal location and low in *G. pusillum* (0.38 %). *C. antennina* (0.44 %), *G. foliifera* (0.47 %), *G. corticata* (0.46 %) and *G. corticata* var. *cylindrica* (0.47 %) showed comparable proline concentrations. Tyrosine was seen in higher levels in *U. fasciata* (1.33 %).

Comparable, but low contents of tyrosine was seen in between *G. pusillum* (0.24 %) and *G. corticata* (0.26 %). Comparable tyrosine contents were also observed in between *C. antennina* (0.28 %) and *G. foliifera* (0.28 %). The total amount of essential amino acids (EAAs) was seen to be comparable in between *C. antennina* (3.54 %) and *A. spicifera* (3.64 %). Low EAA contents were observed in *G. pusillum* (2.10 %) which was comparable with *G. foliifera* (2.52 %), *G. corticata* (2.40 %) and *G. corticata* var. *cylindrica* (2.36 %).

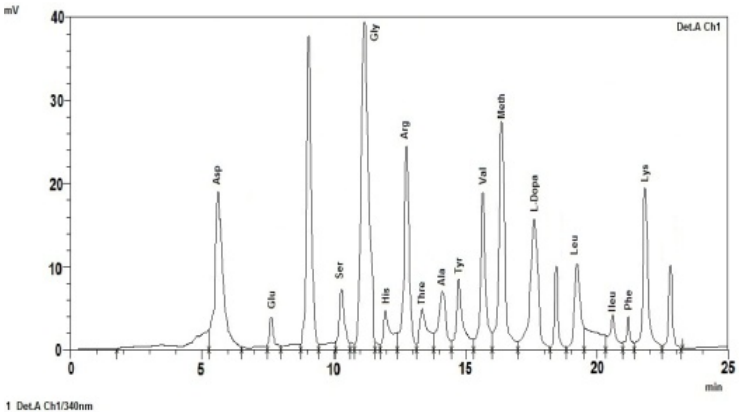
Comparable non essential amino acids content were observed in between *E. prolifera* (4.43 %) obtained from Kayamkulam location and *A. spicifera* (4.18 %), and in between *G. corticata* (2.17 %) and *G. corticata* var. *cylindrica* (2.15 %). The essential amino acids to non essential amino acids ratio was seen high in *U. fasciata* (1.22). Comparative ratio was seen in *E. prolifera* obtained from Kayamkulam location (1.13), *U. fasciata* (1.22), *G. corticata* (1.10) and *G. corticata* var. *cylindrica* (1.09). *C. antennina* (0.93) and *E. prolifera* (0.99) obtained from Njarakkal location also exhibited comparable EAA/NEAA ratio.

4.3.4 Quantification of Amino Acids with OPA Derivatization Technique

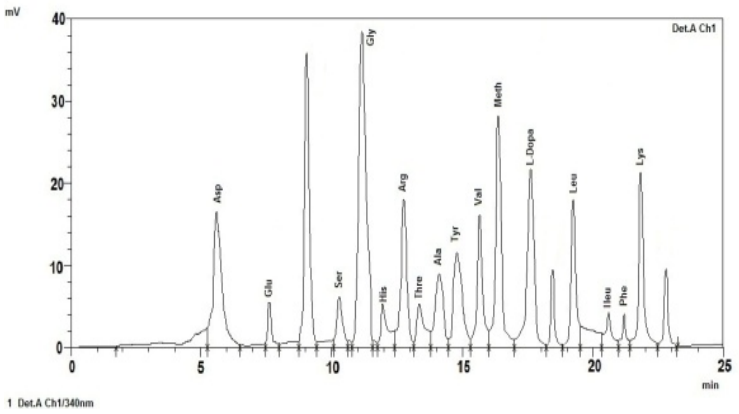
OPA technique with fluorescence detection was the commonly adopted methodology. The procedure used here is based on the reaction between amino acids with OPA in presence of β-mercaptoethanol and measuring the UV active complex at 340 nm. It's a very rapid method and is 500 times more sensitive than other conventional methods (Fisher et al., 2001). HPLC chromatogram of the nine seaweeds analysed using the OPA method is depicted in Fig. 4.5.



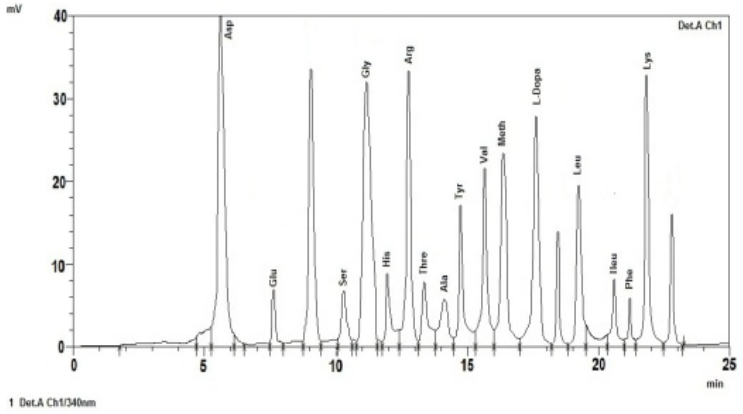
C. antennina



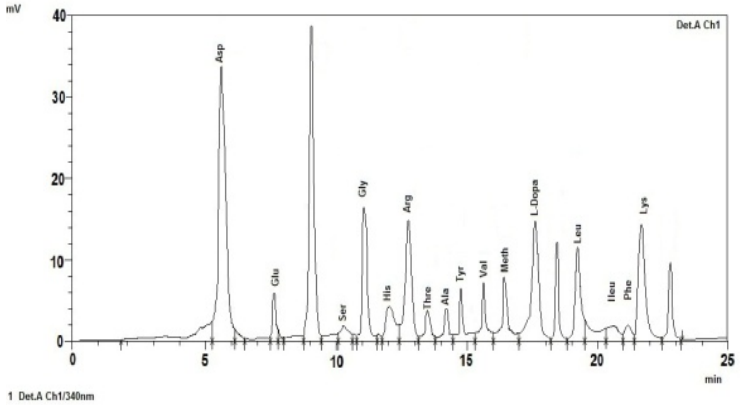
E. prolifera-Kayamkulam



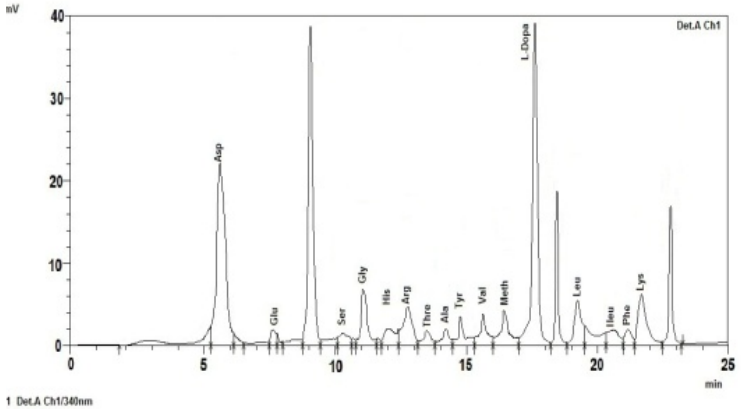
E. prolifera-Njarakkal



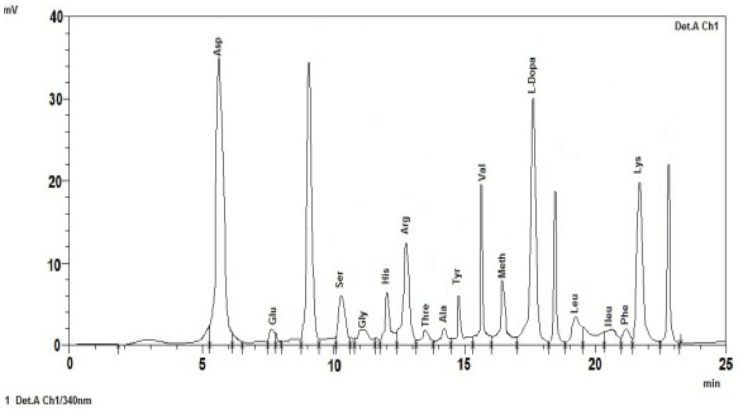
U. fasciata



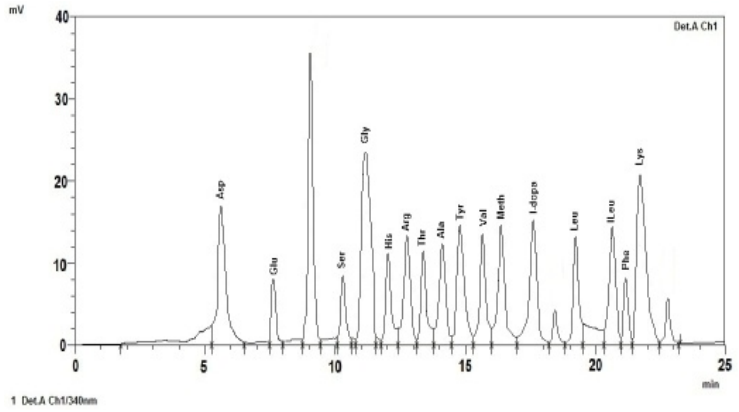
A. spicifera



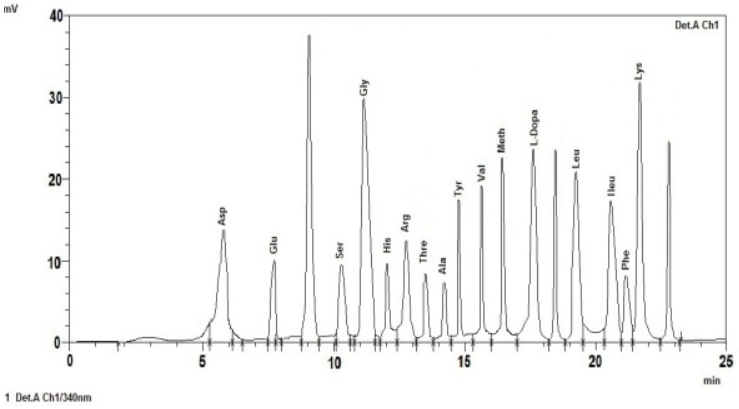
G. pusillum



G. foliifera



G. corticata



G. corticata var. *cylindrica*

Fig. 4.5 HPLC chromatogram of nine seaweeds using OPA derivatization method.

In this study (Table 4.7; Fig. 4.6), the essential amino acids and non essential amino acids were seen in higher concentrations in *U. fasciata* (12.19 % and 9.01 % respectively). Histidine content was seen high in *U. fasciata* (1.44 %) and low in *G. pusillum* (0.29 %). *A. spicifera* (0.54 %) and *G. foliifera* (0.51 %), and *C. antennina* (0.38 %) and *G. corticata* var. *cylindrica* (0.37 %) showed comparable histidine contents. Arginine content was seen high in *U. fasciata* (1.54 %). Least level of arginine was seen in *G. corticata* var. *cylindrica* (0.11 %) and *G. corticata* (0.14 %). Comparable arginine contents were seen in between *E. prolifera* (0.52 %) obtained from Kayamkulam location and *A. spicifera* (0.53 %). Threonine contents were seen high in *U. fasciata* (1.53 %) and low in *G. foliifera* (0.13 %). Comparable threonine contents were seen in between *A. spicifera* (0.39 %) and *G. corticata* var. *cylindrica* (0.37 %). Valine contents were seen high in *U. fasciata* (1.37 %), which was comparable with *E. prolifera* obtained from Njarakkal location (1.31 %). Low valine contents were seen in *G. pusillum* (0.17 %). Comparable valine contents were seen in between *G. corticata* (0.20 %) and *G. corticata* var. *cylindrica* (0.23 %). Methionine content was seen high in *E. prolifera* obtained from Njarakkal location (1.18 %). Comparable methionine contents were seen in between *A. spicifera* (0.19 %), *G. foliifera* (0.19 %) and *G. corticata* var. *cylindrica* (0.18 %). Least levels of methionine contents were seen in *G. pusillum* (0.11 %) and *G. corticata* (0.14 %). High content of isoleucine was seen in *U. fasciata* (1.29 %). Comparable isoleucine contents were seen in between *E. prolifera* obtained from Njarakkal (0.61 %) and Kayamkulam (0.61 %) locations. *A. spicifera* (0.32 %), *G. pusillum* (0.29 %) and *C. antennina* (0.29 %) exhibited comparable isoleucine contents. Low content of isoleucine was seen in *G. foliifera* (0.06 %).

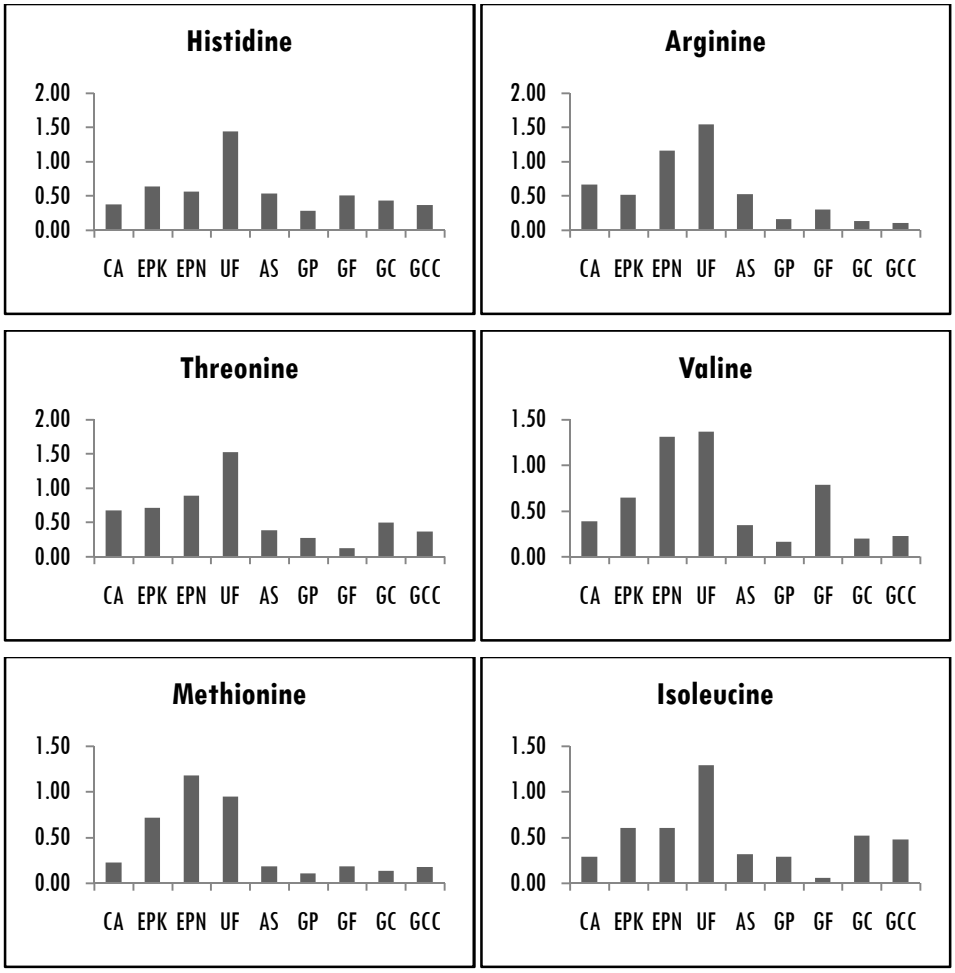
Table 4.7 Amino acids content in nine seaweeds (OPA derivatization), (% to dwt), (mean ± SD), (n=3).

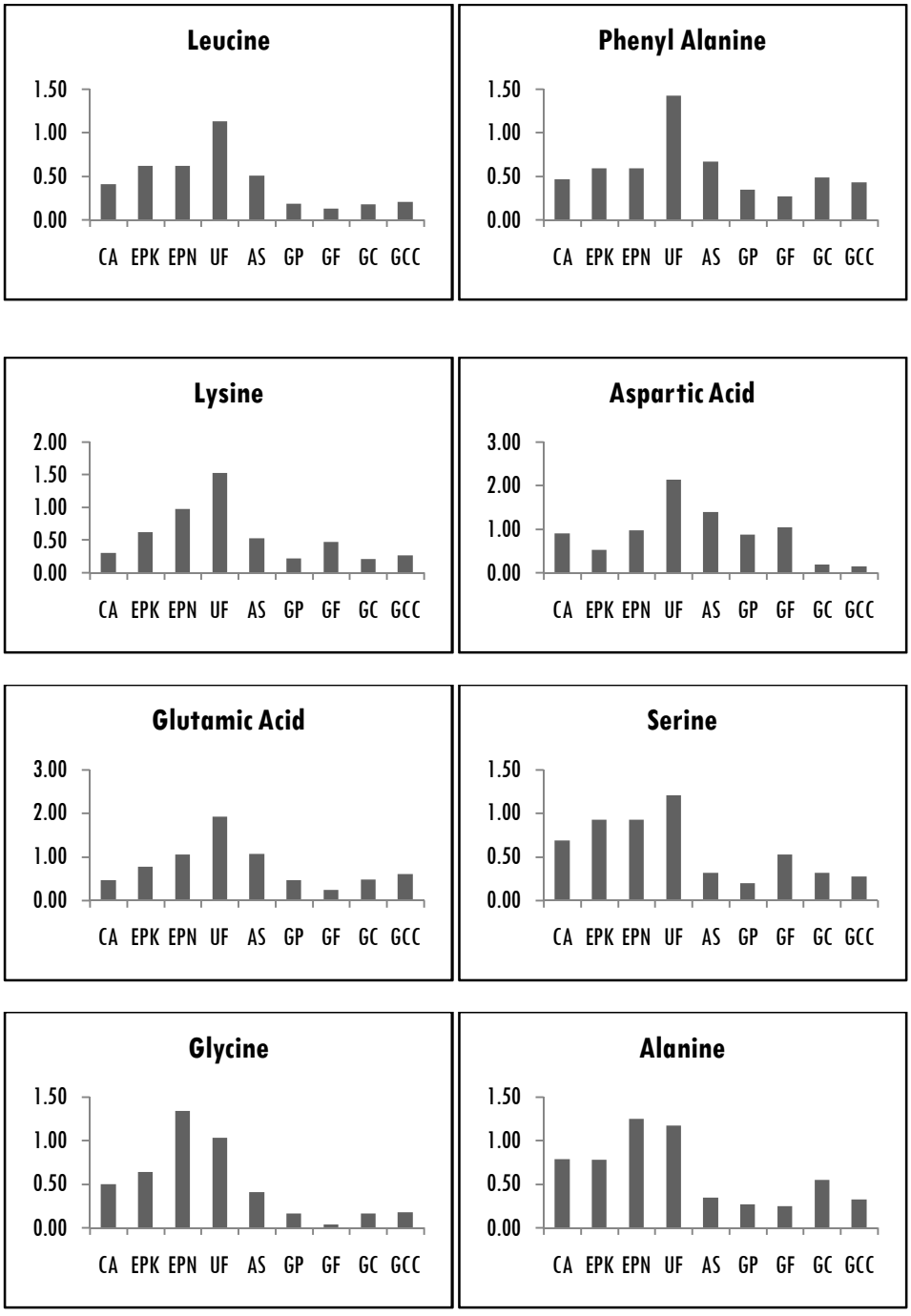
	CHLOROPHYTA			
Amino acids	**CA**	**EPK**	**EPN**	**UF**
His	0.38 ± 0.01	0.64 ± 0.01	0.57 ± 0.01	1.44 ± 0.02
Arg	0.67 ± 0.01	0.52 ± 0.01	1.16 ± 0.02	1.54 ± 0.02
Thr	0.68 ± 0.01	0.72 ± 0.01	0.89 ± 0.01	1.53 ± 0.02
Val	0.39 ± 0.01	0.65 ± 0.01	1.31 ± 0.02	1.37 ± 0.02
Meth	0.23 ± 0.00	0.72 ± 0.01	1.18 ± 0.02	0.95 ± 0.02
Ileu	0.29 ± 0.00	0.61 ± 0.01	0.61 ± 0.01	1.29 ± 0.02
Leu	0.41 ± 0.01	0.62 ± 0.01	0.62 ± 0.01	1.13 ± 0.02
Phe	0.47 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	1.42 ± 0.02
Lys	0.31 ± 0.01	0.62 ± 0.02	0.98 ± 0.02	1.53 ± 0.02
Asp	0.91 ± 0.01	0.53 ± 0.02	0.98 ± 0.02	2.13 ± 0.03
Glu	0.48 ± 0.01	0.78 ± 0.02	1.06 ± 0.02	1.92 ± 0.03
Ser	0.69 ± 0.01	0.93 ± 0.00	0.93 ± 0.01	1.21 ± 0.02
Gly	0.50 ± 0.01	0.64 ± 0.02	1.34 ± 0.02	1.03 ± 0.02
Ala	0.79 ± 0.01	0.78 ± 0.02	1.25 ± 0.02	1.17 ± 0.02
Tyr	0.30 ± 0.01	0.63 ± 0.02	0.95 ± 0.02	1.55 ± 0.02
Total EAA	3.81 ± 0.06	5.02 ± 0.08	7.38 ± 0.11	12.19 ± 0.19
Total NEAA	3.60 ± 0.06	4.43 ± 0.07	7.41 ± 0.11	9.01 ± 0.14
EAA/NEAA	1.05 ± 0.02	1.13 ± 0.02	0.99 ± 0.02	1.35 ± 0.02

	RHODOPHYTA				
Amino acids	**AS**	**GP**	**GF**	**GC**	**GCC**
His	0.54 ± 0.01	0.29 ± 0.01	0.51 ± 0.01	0.44 ± 0.01	0.37 ± 0.01
Arg	0.53 ± 0.01	0.17 ± 0.00	0.31 ± 0.01	0.14 ± 0.00	0.11 ± 0.00
Thr	0.39 ± 0.01	0.28 ± 0.00	0.13 ± 0.00	0.50 ± 0.01	0.37 ± 0.01
Val	0.35 ± 0.01	0.17 ± 0.00	0.79 ± 0.01	0.20 ± 0.00	0.23 ± 0.00
Meth	0.19 ± 0.00	0.11 ± 0.00	0.19 ± 0.00	0.14 ± 0.00	0.18 ± 0.00
Ileu	0.32 ± 0.01	0.29 ± 0.00	0.06 ± 0.00	0.52 ± 0.01	0.48 ± 0.01
Leu	0.51 ± 0.01	0.19 ± 0.00	0.13 ± 0.00	0.18 ± 0.00	0.21 ± 0.00
Phe	0.67 ± 0.01	0.35 ± 0.01	0.27 ± 0.00	0.49 ± 0.01	0.43 ± 0.01
Lys	0.53 ± 0.01	0.22 ± 0.00	0.47 ± 0.01	0.21 ± 0.00	0.27 ± 0.00
Asp	1.39 ± 0.02	0.88 ± 0.01	1.04 ± 0.02	0.19 ± 0.00	0.15 ± 0.00
Glu	1.08 ± 0.02	0.48 ± 0.01	0.25 ± 0.00	0.49 ± 0.01	0.61 ± 0.01
Ser	0.32 ± 0.01	0.20 ± 0.00	0.53 ± 0.01	0.32 ± 0.01	0.28 ± 0.00
Gly	0.41 ± 0.01	0.17 ± 0.00	0.04 ± 0.00	0.17 ± 0.00	0.18 ± 0.00
Ala	0.35 ± 0.01	0.27 ± 0.00	0.25 ± 0.00	0.55 ± 0.01	0.33 ± 0.01
Tyr	0.43 ± 0.01	0.24 ± 0.00	0.27 ± 0.00	0.31 ± 0.01	0.36 ± 0.01
Total EAA	4.03 ± 0.06	2.08 ± 0.03	2.85 ± 0.04	2.83 ± 0.04	2.66 ± 0.04
Total NEAA	3.98 ± 0.06	2.22 ± 0.03	2.37 ± 0.04	2.02 ± 0.03	1.89 ± 0.03
EAA/NEAA	1.01 ± 0.02	0.93 ± 0.01	1.19 ± 0.02	1.39 ± 0.02	1.39 ± 0.02

EAA - Essential Amino Acids, NEAA - Non Essential Amino Acids, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

Leucine content was observed high in *U. fasciata* (1.13 %) and low in *G. foliifera* (0.13 %). Comparable leucine content was seen in between *E. prolifera* obtained from Njarakkal (0.62 %) and Kayamkulam (0.62 %) locations. *G. pusillum* (0.19 %), *G. corticata* (0.18 %) and *G. corticata* var. *cylindrica* (0.21 %) had comparable concentrations. Phenylalanine was observed to be high in *U. fasciata* (1.42 %) and least in *G. foliifera* (0.27 %). Comparable phenylalanine contents were observed in between both the *E. prolifera* (0.59 %) and in between *C. antennina* (0.47 %) and *G. corticata* (0.49 %). Lysine content was observed high in *U. fasciata* (1.53 %). Low levels of lysine were seen in *G. corticata* (0.21 %) and *G. pusillum* (0.22 %).

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0.50
0.00
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Phenyl Alanine
1.50
1.00
0.50
0.00
CA EPK EPN UF AS GP GF GC GCC
Lysine
2.00
1.50
1.00
0.50
0.00
CA EPK EPN UF AS GP GF GC GCC
Aspartic Acid
3.00
2.00
1.00
0.00
CA EPK EPN UF AS GP GF GC GCC
Glutamic Acid
3.00
2.00
1.00
0.00
CA EPK EPN UF AS GP GF GC GCC
Serine
1.50
1.00
0.50
0.00
CA EPK EPN UF AS GP GF GC GCC
Glycine
1.50
1.00
0.50
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Alanine
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1.00
0.50
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CA EPK EPN UF AS GP GF GC GCC

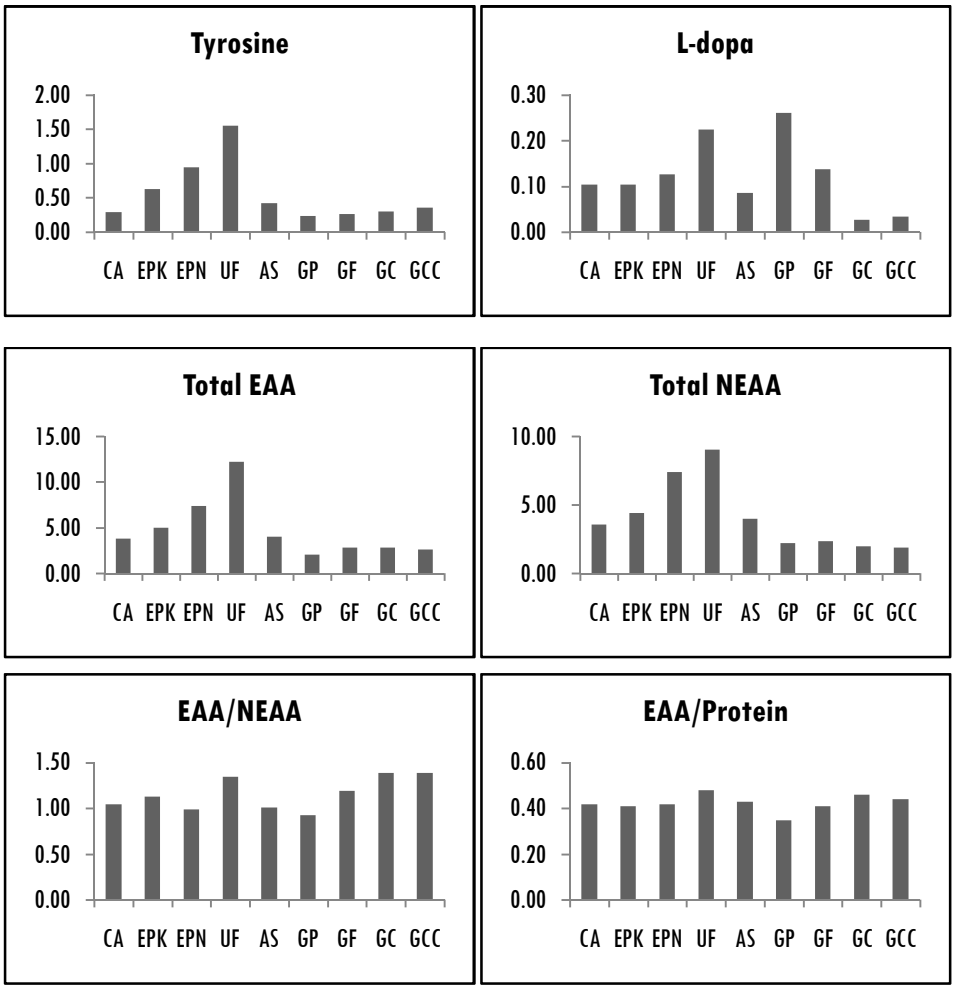


Fig. 4.6 Graphical representation of the amino acid contents in nine seaweeds (OPA derivatization).

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

Aspartic acid was seen high in *U. fasciata* (2.13 %) and low in *G. corticata* var. *cylindrica* (0.15 %). Glutamic acid was seen high in *U. fasciata* (1.92 %). Comparable glutamic acid contents were seen in between *A. spicifera* (1.08 %) and *E. prolifera* obtained from Njarakkal location (1.06 %). *C. antennina* (0.48 %), *G. pusillum* (0.48 %) and *G. corticata* (0.49 %)

exhibited similar levels of glutamic acid contents. A low level of glutamic acid content was observed in *G. foliifera* (0.25 %). Serine content was seen high in *U. fasciata* (1.21 %) and low in *G. pusillum* (0.20 %). Comparable serine contents were seen in between *E. prolifera* obtained from Njarakkal (0.93 %) and Kayamkulam (0.93 %) locations, and in between *A. spicifera* (0.32 %) and *G. corticata* (0.32 %). Glycine was seen high in *E. prolifera* (1.34 %) obtained from Njarakkal location and low in *G. foliifera* (0.04 %). *G. pusillum* (0.17 %), *G. corticata* (0.17 %) and *G. corticata* var. *cylindrica* (0.18 %) exhibited comparable glycine contents. Alanine was seen in higher concentration in *E. prolifera* (1.25 %) obtained from Njarakkal location and *U. fasciata* (1.17 %). Low alanine contents were seen in *G. foliifera* (0.25 %) and *G. pusillum* (0.27 %). *G. corticata* var. *cylindrica* (0.33 %) and *A. spicifera* (0.35 %), and *C. antennina* (0.79 %) and *E. prolifera* (0.78 %) obtained from Kayamkulam location showed comparable alanine concentrations. Tyrosine content was observed to be high in *U. fasciata* (1.55 %). Comparable tyrosine content was observed in between *C. antennina* (0.30 %) and *G. corticata* (0.31 %). Low tyrosine content was seen in *G. pusillum* (0.24 %) and *G. foliifera* (0.27 %).

Essential amino acid contents were comparable in between *C. antennina* (3.81 %) and *A. spicifera* (4.03 %). *G. pusillum* (2.08 %), *G. corticata* (2.83 %), *G. corticata* var. *cylindrica* (2.66 %) and *G. foliifera* (2.85 %) exhibited comparable EAA concentrations. Non essential amino acid contents were comparable in between *C. antennina* (3.60 %) and *A. spicifera* (3.98 %). Low amounts of NEAA were seen in *G. pusillum* (2.22 %) which was comparable with *G. foliifera* (2.37 %). Essential amino acids to non essential amino acids ratio was seen comparable in between *G. corticata* (1.39) and *G. corticata* var. *cylindrica*

(1.39). Low ratio was seen in *G. pusillum* (0.93). Comparable ratio was seen in between *C. antennina* (1.05) and *A. spicifera* (1.01).

4.3.5 HPLC Method Comparison and Data Analysis

Overall, the reported amino acids pattern was almost similar, irrespective of the derivatization methods used, except that proline which is a secondary amino acid couldn't be quantified with OPA. The derivatives of the total amino acid content, total EAA content, total EAA to protein content and total amino acid content to protein content were used in the method comparison studies. When PITC derivatization method was adopted, the total amino acid content, total EAA content, total EAA to protein content and total amino acid content to protein content were 7.35%, 3.55 %, 39.49 % and 81.76 % in *C. antennina* , 9.54 %, 5.07 %, 49.20 % and 77.08 % in *E. prolifera* obtained from Kayamkulam location, 14.92 %, 7.39 %, 42.35 % and 85.49 % in *E. prolifera* obtained from Njarakkal location, 19.09 %, 10.49 %, 41.76 % and 75.99 % in *U. fasciata,* 7.84 %, 3.65 %, 38.95 % and 83.67 % in *A. spicifera,* 4.78 %, 2.13 %, 35.62 % and 79.93 % in *G. pusillum,* 6.39 %, 2.54 %, 36.39 % and 91.55 % in *G. foliifera,* 4.59 %, 2.41 %, 38.99 % and 74.27 % in *G. coritcata* and 4.53 %, 2.37 %, 39.63 % and 75.75 % in *G. corticata* var. *cylindrica* respectively. Essential amino acids to protein content (Table 4.4) were seen high in *E. prolifera* (0.49 %) obtained from Kayamkulam location. Similarities were observed in between *E. prolifera* (0.42 %) obtained from Njarakkal location and *U. fasciata* (0.42 %), *C. antennina* (0.39 %), *A. spicifera* (0.39 %), *G. corticata* (0.39 %) and *G. corticata* var. *cylindrica* (0.39 %), and in between *G. pusillum* (0.35 %) and *G. foliifera* (0.36 %).

Considering the OPA derivatization method, the total amino acid content, total EAA content, total EAA to protein content and total amino acid content to protein content were 7.60 %, 3.83 %, 42.60 % and 84.54 % in *C. antennina*, 9.45 %, 5.02 %, 40.91 % and 77.09 % in *E. prolifera* obtained from Kayamkulam location, 14.91 %, 7.38 %, 42.30 % and 85.43 % in *E. prolifera* obtained from Njarakkal location, 21.44 %, 12.2 %, 48.57 % and 85.35 % in *U. fasciata*, 8.10 %, 4.03 %, 43.01 % and 86.45 % in *A. spicifera*, 4.57 %, 2.07 %, 34.62 % and 76.42 % in *G. pusillum*, 5.38 %, 2.86 %, 40.97 % and 77.08 % in *G. foliifera*, 4.88 %, 2.82 %, 45.63 % and 78.96 % in *G. corticata* and 4.6 %, 2.65 %, 44.31 % and 76.92 % in *G. corticata* var. *cylindrica* respectively. OPA derivatization analysis (Table 4.4) exhibited high essential amino acids to protein content in *U. fasciata* (0.48 %) and *G. corticata* (0.46 %). The low contents were seen in *G. pusillum* (0.35 %). Comparable EAA to protein content was seen in between *A. spicifera* (0.43 %), *G. corticata* var. *cylindrica* (0.44 %), *C. antennina* (0.42 %), *E. prolifera* obtained from Njarakkal location (0.42 %), *E. prolifera* obtained from Kayamkulam location (0.41 %) and *G. foliifera* (0.41 %).

Both the methods proved equally good in analysis. But the ease of analysis, response and result calculations were found better when OPA was used, even though PITC had a broad spectrum of analysis. Both methods could be used, but the selection depends on the amino acid to be analysed. From the results of the current study upon considerations of the total amino acid to protein content, it's evident that the OPA derivatization methodology promotes greater acceptance and responses.

4.3.5.1 Average Amino Acid Contents (PITC & OPA)

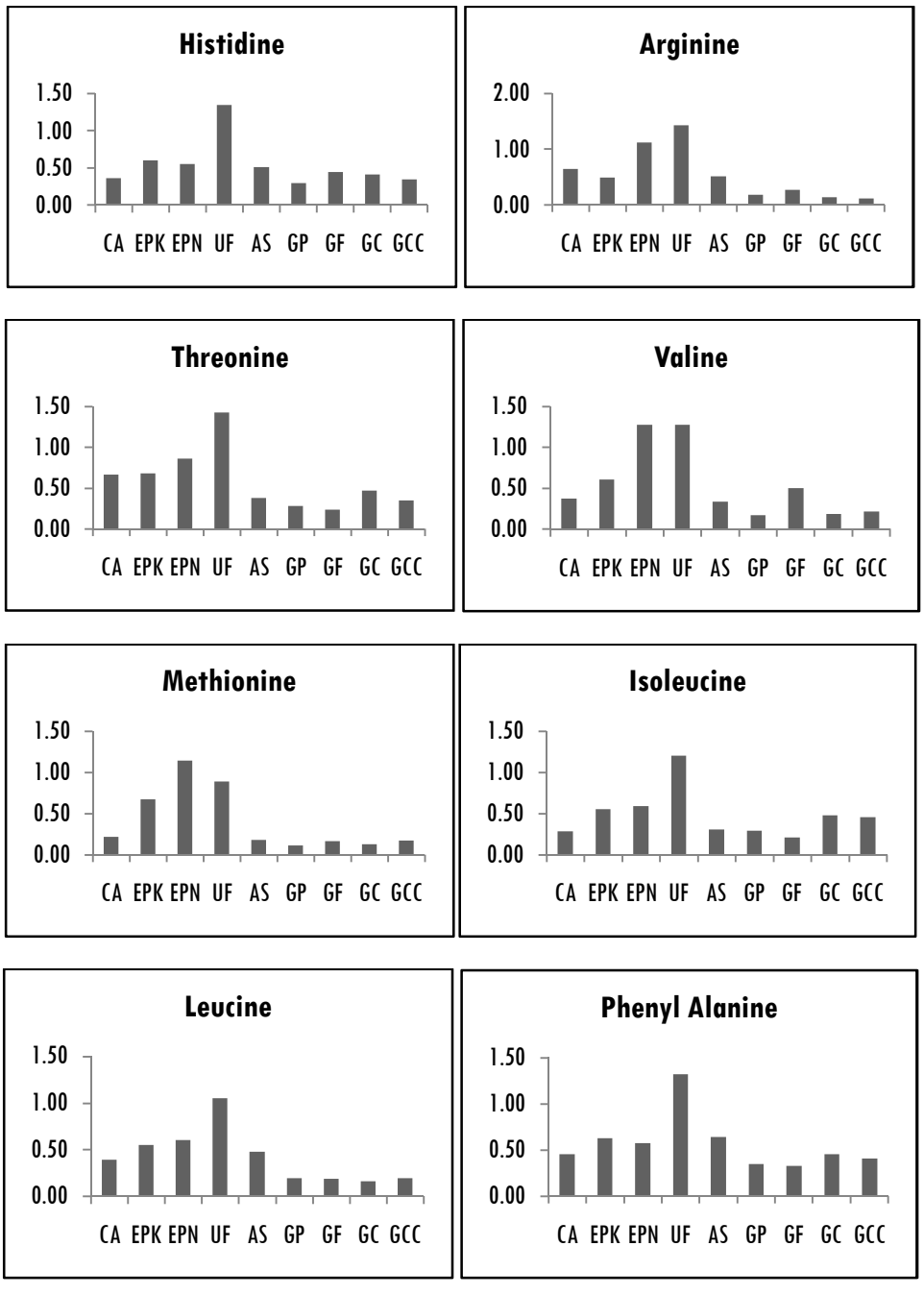
The greater acceptance of PITC derivatization method among the various earlier reports on amino acid determinations and the exceptions in observations between both the methods regarding the variability in the least contents of threonine, valine, isoleucine, leucine, phenylalanine, glutamic acid, glycine and alanine, concluded the current study to move forward with the amino acid content discussions on the nine seaweeds after deriving the average of the results deduced by both the methods (Table 4.8; Fig. 4.7).

Among the green seaweeds, *U. fasciata* (1.34 %) had the highest contents of histidine. Least was observed in *C. antennina* (0.37 %). Arginine (1.43 %), threonine (1.43 %), valine (1.28 %), isoleucine (1.20 %), leucine (1.05 %), phenylalanine (1.32 %), lysine (1.42 %), aspartic acid (1.98 %), glutamic acid (1.79 %), serine (1.13 %), tyrosine (1.44 %) and L-dopa (0.23 %) contents were seen to be in high in *U. fasciata*. *E. prolifera* obtained from Njarakkal location had the highest contents of methionine (1.14 %), glycine (1.29 %), alanine (1.21 %) and proline (1.32 %). Least contents of arginine (0.49 %) and aspartic acid (0.50 %) were observed in *E. prolifera* obtained from Kayamkulam location. *C. antennina* had the least threonine (0.66 %), valine (0.38 %), methionine (0.22 %), isoleucine (0.28 %), leucine (0.40 %), phenylalanine (0.46 %), lysine (0.30 %), glutamic acid (0.47 %), serine (0.67 %), glycine (0.49 %), alanine (0.73 %), proline (0.44 %), tyrosine (0.29 %) and L-dopa (0.10 %) contents. Total essential amino acids, non essential amino acids, EAA/NEAA ratio and EAA/protein ratio were high in *U. fasciata*.

Table 4.8 Average amino acid contents (%) estimated by both the methods (PITC and OPA).

Amino acid	CA	EPK	EPN	UF	AS	GP	GF	GC	GCC
His	0.37	0.60	0.55	1.34	0.52	0.30	0.45	0.41	0.35
Arg	0.64	0.49	1.12	1.43	0.51	0.18	0.27	0.13	0.11
Thr	0.66	0.68	0.86	1.43	0.38	0.28	0.23	0.47	0.35
Val	0.38	0.61	1.27	1.28	0.34	0.18	0.51	0.19	0.22
Meth	0.22	0.68	1.14	0.89	0.18	0.11	0.17	0.13	0.17
Ileu	0.28	0.55	0.59	1.20	0.31	0.29	0.21	0.48	0.46
Leu	0.40	0.56	0.60	1.05	0.48	0.20	0.19	0.17	0.20
Phe	0.46	0.63	0.57	1.32	0.64	0.35	0.33	0.46	0.41
Lys	0.30	0.60	0.94	1.42	0.51	0.23	0.36	0.20	0.26
Asp	0.88	0.50	0.95	1.98	1.33	0.89	1.38	0.18	0.14
Glu	0.47	0.75	1.03	1.79	1.03	0.49	0.42	0.46	0.58
Ser	0.67	0.73	0.90	1.13	0.31	0.21	0.39	0.30	0.27
Gly	0.49	0.65	1.29	0.96	0.39	0.17	0.13	0.16	0.17
Ala	0.73	0.75	1.21	1.09	0.34	0.27	0.30	0.51	0.31
Pro	0.44	0.81	1.32	0.85	0.58	0.38	0.47	0.46	0.47
Tyr	0.29	0.61	0.92	1.44	0.41	0.24	0.28	0.29	0.34
L-dopa	0.10	0.11	0.13	0.23	0.09	0.26	0.14	0.03	0.04
Protein	8.99	12.26	17.45	25.12	9.37	5.98	6.98	6.18	5.98
Total EAA	3.69	5.38	7.65	11.35	3.84	2.10	2.70	2.62	2.51
Total NEAA	4.06	4.90	7.76	9.46	4.47	2.90	3.49	2.37	2.31
EAA/NEAA	0.91	1.10	0.99	1.20	0.86	0.73	0.77	1.11	1.09
EAA/Protein	0.41	0.44	0.44	0.45	0.41	0.35	0.39	0.42	0.42

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.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CA EPK EPN UF AS GP GF GC GCC
Threonine
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0.50
0.00
CA EPK EPN UF AS GP GF GC GCC
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1.00
0.50
0.00
CA EPK EPN UF AS GP GF GC GCC
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0.00
CA EPK EPN UF AS GP GF GC GCC
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0.50
0.00
CA EPK EPN UF AS GP GF GC GCC
Leucine
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1.00
0.50
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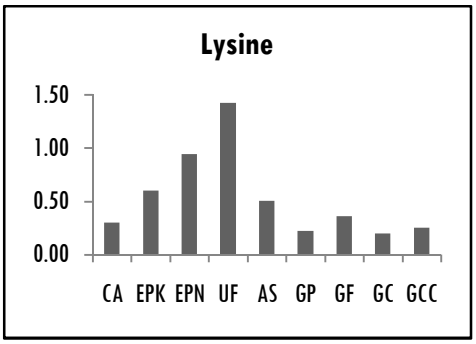
CA EPK EPN UF AS GP GF GC GCC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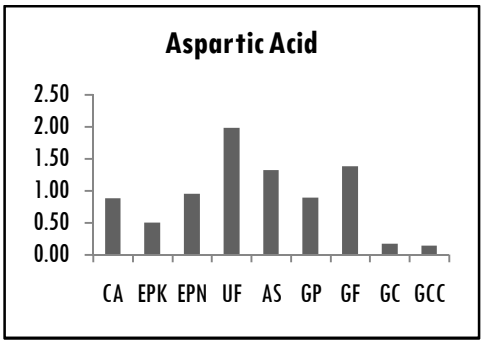
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1.50
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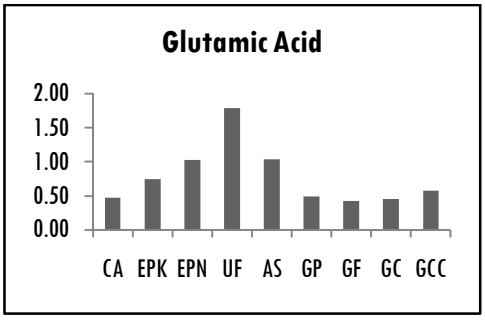
0.50
0.00
CA EPK EPN UF AS GP GF GC GCC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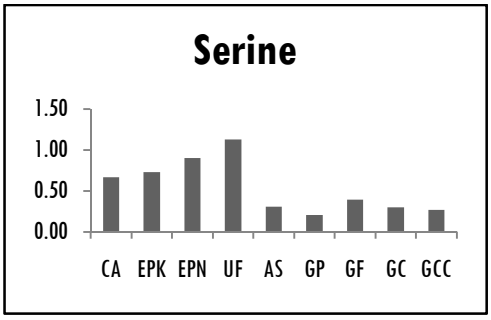
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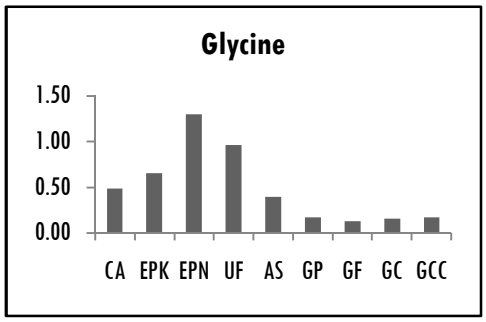
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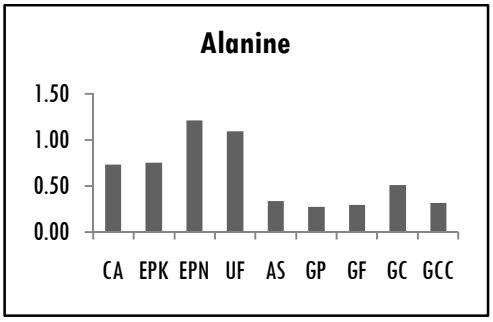
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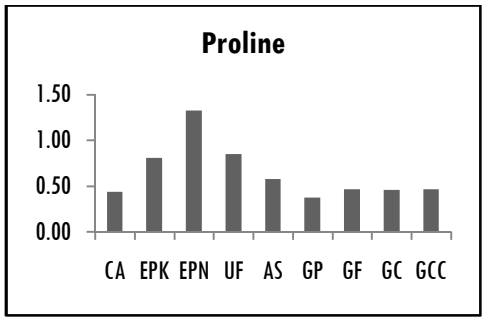
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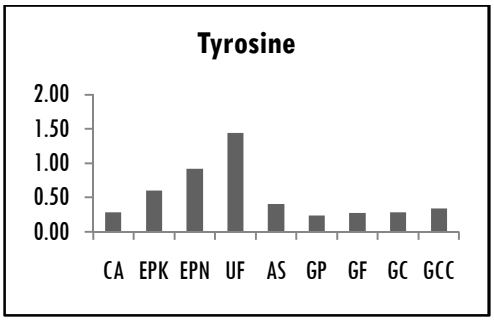
0.00
CA EPK EPN UF AS GP GF GC GCC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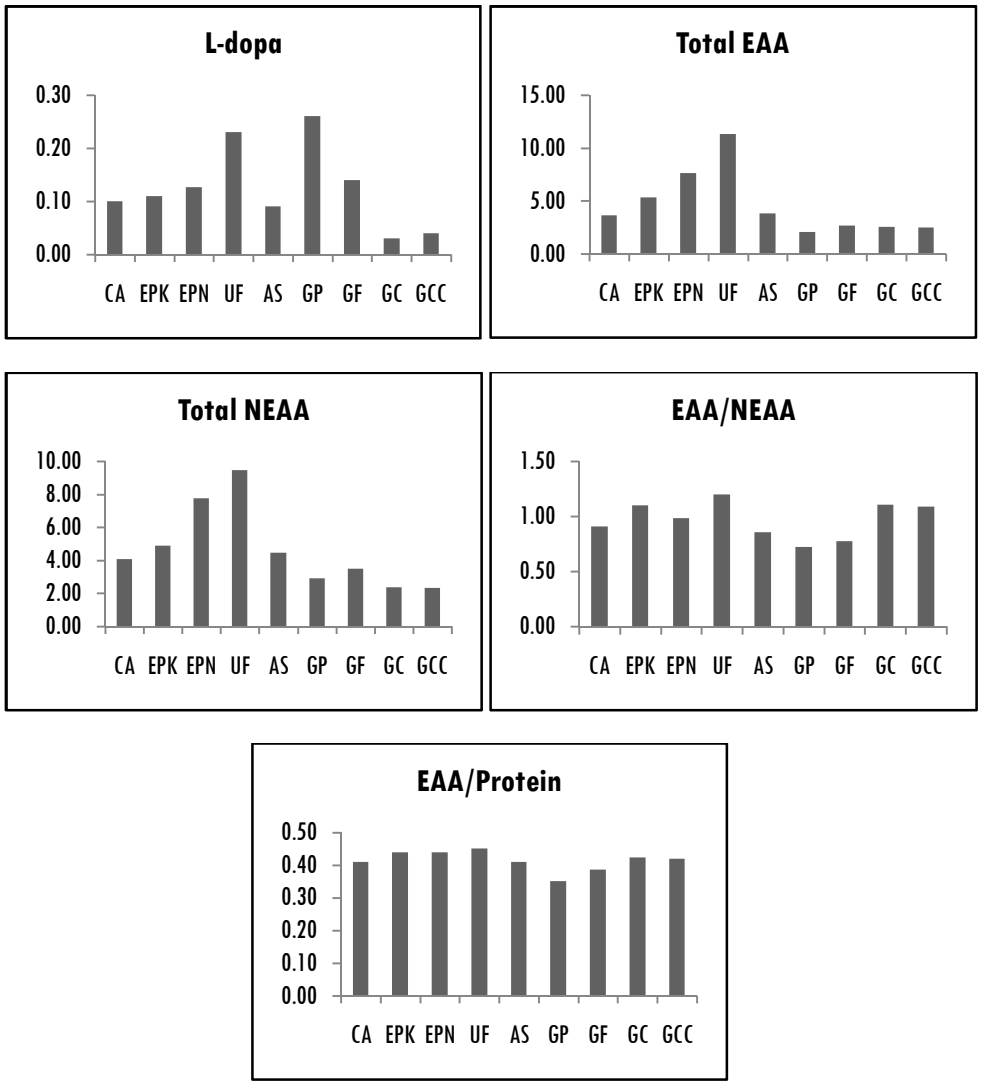


Fig. 4.7 Graphical representation of the average amino acid contents in the nine seaweeds.

CA- *C. antennina*, EPK- *E. prolifera* obtained from Kayamkulam location, EPN- *E. prolifera* obtained from Njarakkal location, UF- *U. fasciata*, AS- *A. spicifera*, GP- *G. pusillum*, GF- *G. foliifera*, GC- *G. corticata* and GCC- *G. corticata* var. *cylindrica*.

Among the red seaweeds, *A. spicifera* had high contents of histidine (0.52 %), arginine (0.51 %), methionine (0.18 %), leucine (0.48 %), phenylalanine (0.64 %), lysine (0.51 %), glutamic acid (1.03 %), glycine (0.39 %), proline (0.58 %) and tyrosine (0.41 %). Threonine (0.47 %), isoleucine

(0.48 %) and alanine (0.51 %) were observed in higher concentrations in *G. corticata*. Valine (0.51 %), aspartic acid (1.38 %) and serine (0.39 %) were observed in higher concentrations in *G. foliifera*. L-dopa was seen in higher concentrations in *G. pusillum* (0.26 %). Least content of histidine (0.30%), valine (0.18 %), methionine (0.11 %), serine (0.21 %), alanine (0.27 %), proline (0.38 %) and tyrosine (0.24 %) was observed in *G. pusillum*. Threonine (0.23 %), isoleucine (0.21 %), phenylalanine (0.33 %), glutamic acid (0.42 %) and glycine (0.13 %) were observed in the lowest levels in *G. foliifera*. Low levels of leucine (0.17 %), lysine (0.20 %) and L-dopa (0.03 %) contents were observed in *G. corticata*, and arginine (0.11 %) and aspartic acid (0.14 %) in *G. corticata* var. *cylindrica*. Total essential amino acids and non essential amino acids were observed in *A. spicifera*. EAA/NEAA ratio and EAA/protein ratio was high in both *G. corticata* and *G. corticata* var. *cylindrica*.

4.3.5.2 Average Amino Acids to Protein Contents

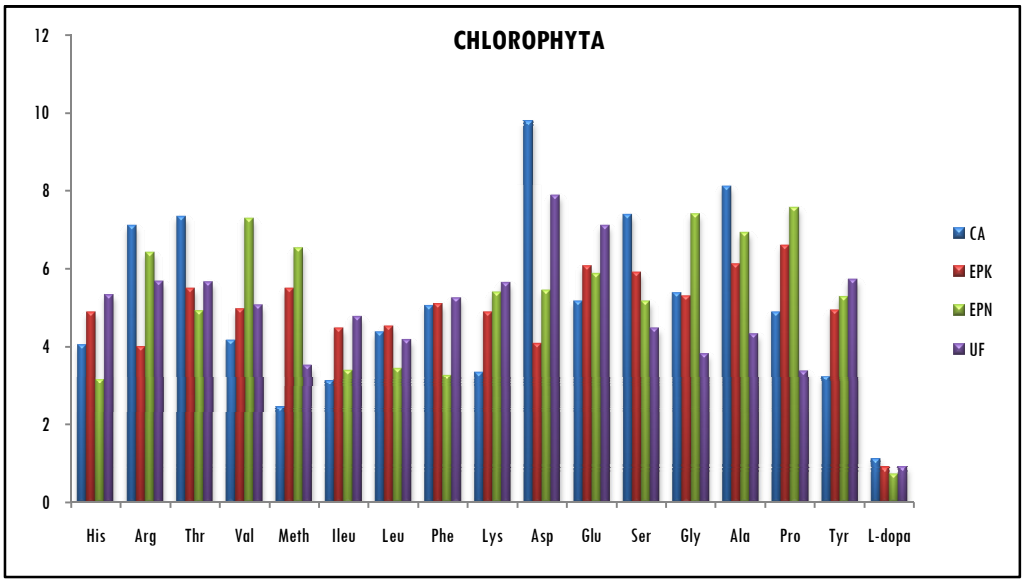
The results in comparison with the protein contents (Table 4.9; Fig. 4.8) were entirely different from the average amino acid contents. Among the green seaweeds, a high content of histidine was observed in *U. fasciata* (5.33 %), whereas the least was in *E. prolifera* obtained from Njarakkal location (3.15 %). Arginine was high in *C. antennina* (7.12 %) and least in *E. prolifera* obtained from Kayamkulam location (4.00 %). Threonine was high in *C. antennina* (7.34 %) and low in *E. prolifera* obtained from Njarakkal location (4.93 %). Valine was high in *E. prolifera* obtained from Njarakkal location (7.30 %) and least in *C. antennina* (4.17 %). Methionine was high in *E. prolifera* obtained from Njarakkal location (6.53 %) and least in *C. antennina* (2.45 %). Isoleucine was high in *U. fasciata* (4.78 %) and least in *C. antennina* (3.11 %). Leucine was high in *E. prolifera* obtained from Kayamkulam location (4.53 %) and least in *E. prolifera* obtained from Njarakkal location (3.45 %). Phenylalanine was high

in *U. fasciata* (5.25 %) and least in *E. prolifera* obtained from Njarakkal location (3.27 %). Lysine was high in *U. fasciata* (5.65 %) and least in *C. antennina* (3.34 %). Aspartic acid was highest in *C. antennina* (9.79 %) and least in *E. prolifera* obtained from Kayamkulam location (4.08 %). Glutamic acid was high in *U. fasciata* (7.11 %) and least in *C. antennina* (5.17 %). Serine was high in *C. antennina* (7.40 %) and least in *U. fasciata* (4.48 %). Glycine was observed to be high in *E. prolifera* obtained from Njarakkal location (7.41 %) and least in *U. fasciata* (3.82 %). Alanine was seen to be high in *C. antennina* (8.12 %) and low in *U. fasciata* (4.34 %). Proline was high in *E. prolifera* obtained from Njarakkal location (7.58 %) and least in *U. fasciata* (3.38 %). Tyrosine was observed to be high in *U. fasciata* (5.73 %) and least in *C. antennina* (3.23 %). L-dopa was high in *C. antennina* (1.11 %) and least in *E. prolifera* obtained from Njarakkal location (0.73 %).

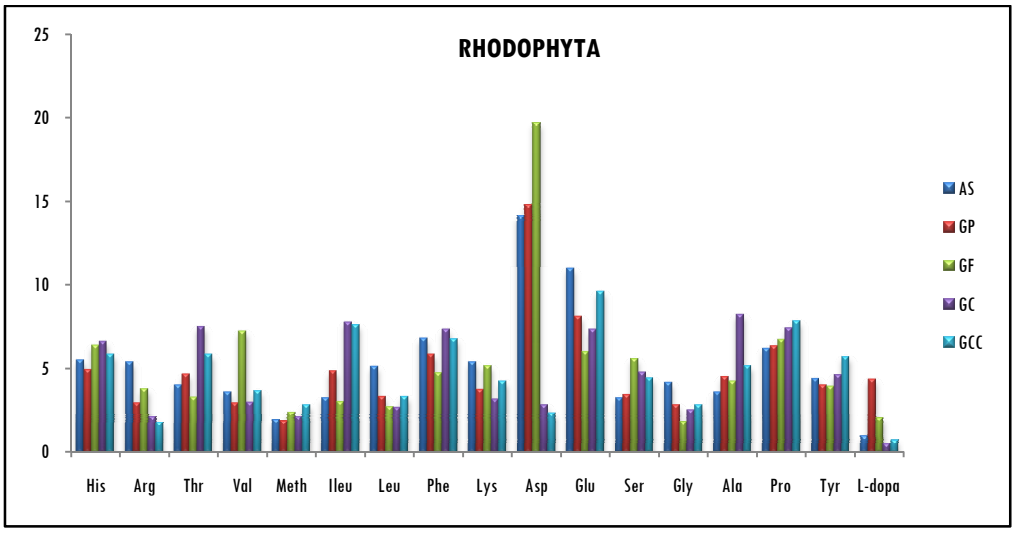
Table 4.9 Amino acid contents in 100 g of protein (average of PITC and OPA methods).

Amino acid	CA	EPK	EPN	UF	AS	GP	GF	GC	GCC
His	4.06	4.89	3.15	5.33	5.50	4.93	6.38	6.63	5.85
Arg	7.12	4.00	6.42	5.69	5.39	2.93	3.80	2.10	1.76
Thr	7.34	5.51	4.93	5.67	4.00	4.68	3.30	7.52	5.85
Val	4.17	4.98	7.30	5.08	3.58	2.93	7.23	2.99	3.68
Meth	2.45	5.51	6.53	3.52	1.92	1.84	2.36	2.10	2.84
Ileu	3.11	4.49	3.39	4.78	3.26	4.85	3.01	7.77	7.61
Leu	4.39	4.53	3.45	4.18	5.12	3.34	2.72	2.67	3.34
Phe	5.06	5.10	3.27	5.25	6.83	5.85	4.73	7.36	6.77
Lys	3.34	4.89	5.41	5.65	5.39	3.76	5.16	3.16	4.26
Asp	9.79	4.08	5.46	7.88	14.14	14.80	19.70	2.83	2.34
Glu	5.17	6.08	5.88	7.11	10.99	8.11	6.02	7.36	9.62
Ser	7.40	5.91	5.17	4.48	3.26	3.43	5.59	4.77	4.43
Gly	5.39	5.30	7.41	3.82	4.16	2.84	1.79	2.51	2.84
Ala	8.12	6.12	6.94	4.34	3.58	4.52	4.23	8.25	5.18
Pro	4.89	6.61	7.58	3.38	6.19	6.35	6.73	7.44	7.86
Tyr	3.23	4.94	5.29	5.73	4.38	4.01	3.94	4.61	5.69
L-dopa	1.11	0.90	0.73	0.92	0.96	4.35	2.01	0.49	0.67

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.



A



B

Fig. 4.8 Graphical representation of the amino acid contents in 100g of proteins.

A represents Chlorophyta and B represents Rhodophyta.

CA- *C. antennina*, EPK- *E. prolifera* obtained from Kayamkulam location, EPN- *E. prolifera* obtained from Njarakkal location, UF- *U. fasciata*, AS- *A. spicifera*, GP- *G. pusillum*, GF- *G. foliifera*, GC- *G. corticata* and GCC- *G. corticata* var. *cylindrica*.

Among the red seaweeds, high content of histidine was observed in *G. corticata* (6.63 %) and least in *G. pusillum* (4.93 %). Arginine content was high in *A. spicifera* (5.39 %) and low in *G. corticata* var. *cylindrica* (1.76 %). Threonine was high in *G. corticata* (7.52 %) and low in *G. foliifera* (3.30 %). Valine was observed to be high in *G. foliifera* (7.23 %) and least in *G. pusillum* (2.93 %). Methionine was observed to be high in *G. corticata* var. *cylindrica* (2.84 %) and least in *G. pusillum* (1.84 %). Isoleucine content was high in *G. corticata* (7.77 %) and low in *G. foliifera* (3.01 %). Leucine content was observed to be high in both *G. pusillum* (3.34 %) and *G. corticata* var. *cylindrica* (3.34 %). Low amounts of leucine were seen in *G. corticata* (2.67 %). Phenylalanine is high in *G. corticata* (7.36 %) and low in *G. foliifera* (4.73 %). Lysine was observed to be high in *A. spicifera* (5.39 %) and least in *G. corticata* (3.16 %). Aspartic acid was seen to be in higher concentrations in *G. foliifera* (19.70 %) and least in *G. corticata* var. *cylindrica* (2.34 %). Glutamic acid was observed to be high in *A. spicifera* (10.99 %) and low in *G. foliifera* (6.02 %). Serine content was high in *G. foliifera* (5.59 %) and low in *A. spicifera* (3.26 %). Glycine content was high in *A. spicifera* (4.16 %) and low in *G. foliifera* (1.79 %). Alanine was observed to be high in *G. corticata* (8.25 %) and least in *A. spicifera* (3.58 %). Proline content was high in *G. corticata* var. *cylindrica* (7.86 %) and low in *A. spicifera* (6.19 %). Tyrosine content was high in *G. corticata* var. *cylindrica* (5.69 %) and low in *G. foliifera* (3.94 %). L-dopa content was high in *G. pusillum* (4.35 %) and low in *G. corticata* (0.49 %).

4.3.5.3 Dispersion Extent of Amino Acids

The extent of distributions of amino acids in total amino acid pool of nine seaweeds individually were also derived (Table 4.10; Fig. 4.9). *C. antennina* possessed low L-dopa (1.29 %) and comparable isoleucine (3.60 %), tyrosine (3.73 %) and lysine (3.86 %), histidine (4.76 %) and valine (4.88 %), and arginine (8.23 %), threonine (8.48 %) and serine (8.61 %) dispersion

pattern. Aspartic acid (11.31 %) was the major contributor to amino acid pool in *C. antennina*. *E. prolifera* obtained from Kayamkulam location had L-dopa (1.07 %) as the least contributor to the pool. Arginine (4.75 %) and aspartic acid (4.85 %), isoleucine (5.33 %) and leucine (5.43 %), lysine (5.82 %) and histidine (5.82 %), tyrosine (5.92 %) and valine (5.92 %), methionine (6.60 %) and threonine (6.60 %), and glutamic acid (7.27 %) and alanine (7.27 %) exhibited comparable contributory patterns. *E. prolifera* obtained from Njarakkal location had low L-dopa (0.84 %) contributions. Comparable contributing patterns were observed in between isoleucine (3.83 %) and leucine (3.90 %), serine (5.85 %) and tyrosine (5.98 %) and lysine (6.11 %) and aspartic acid (6.17 %).

Table 4.10 Dispersion of amino acids in nine seaweeds (%).

Amino acid	CA	EPK	EPN	UF	AS	GP	GF	GC	GCC
His	4.76	5.82	3.57	6.43	6.23	5.96	7.22	8.15	7.22
Arg	8.23	4.75	7.28	6.87	6.11	3.58	4.33	2.58	2.27
Thr	8.48	6.60	5.59	6.87	4.55	5.57	3.69	9.34	7.22
Val	4.88	5.92	8.25	6.14	4.07	3.58	8.19	3.78	4.54
Meth	2.83	6.60	7.41	4.27	2.16	2.19	2.73	2.58	3.51
Ileu	3.60	5.33	3.83	5.76	3.71	5.77	3.37	9.54	9.48
Leu	5.14	5.43	3.90	5.04	5.75	3.98	3.05	3.38	4.12
Phe	5.91	6.11	3.70	6.34	7.66	6.96	5.30	9.15	8.45
Lys	3.86	5.82	6.11	6.82	6.11	4.57	5.78	3.98	5.36
Asp	11.31	4.85	6.17	9.51	15.93	17.69	22.15	3.58	2.89
Glu	6.04	7.27	6.69	8.59	12.34	9.74	6.74	9.15	11.96
Ser	8.61	7.08	5.85	5.42	3.71	4.17	6.26	5.96	5.57
Gly	6.30	6.30	8.38	4.61	4.67	3.38	2.09	3.18	3.51
Ala	9.38	7.27	7.86	5.23	4.07	5.37	4.82	10.14	6.39
Pro	5.66	7.86	8.58	4.08	6.95	7.55	7.54	9.15	9.69
Tyr	3.73	5.92	5.98	6.91	4.91	4.77	4.49	5.77	7.01
L-dopa	1.29	1.07	0.84	1.10	1.08	5.17	2.25	0.60	0.82
Total EAA	47.43	52.18	49.71	54.49	45.99	41.75	43.34	52.09	51.75
Total NEAA	52.19	47.53	50.42	45.42	53.53	57.65	56.02	47.12	47.63

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

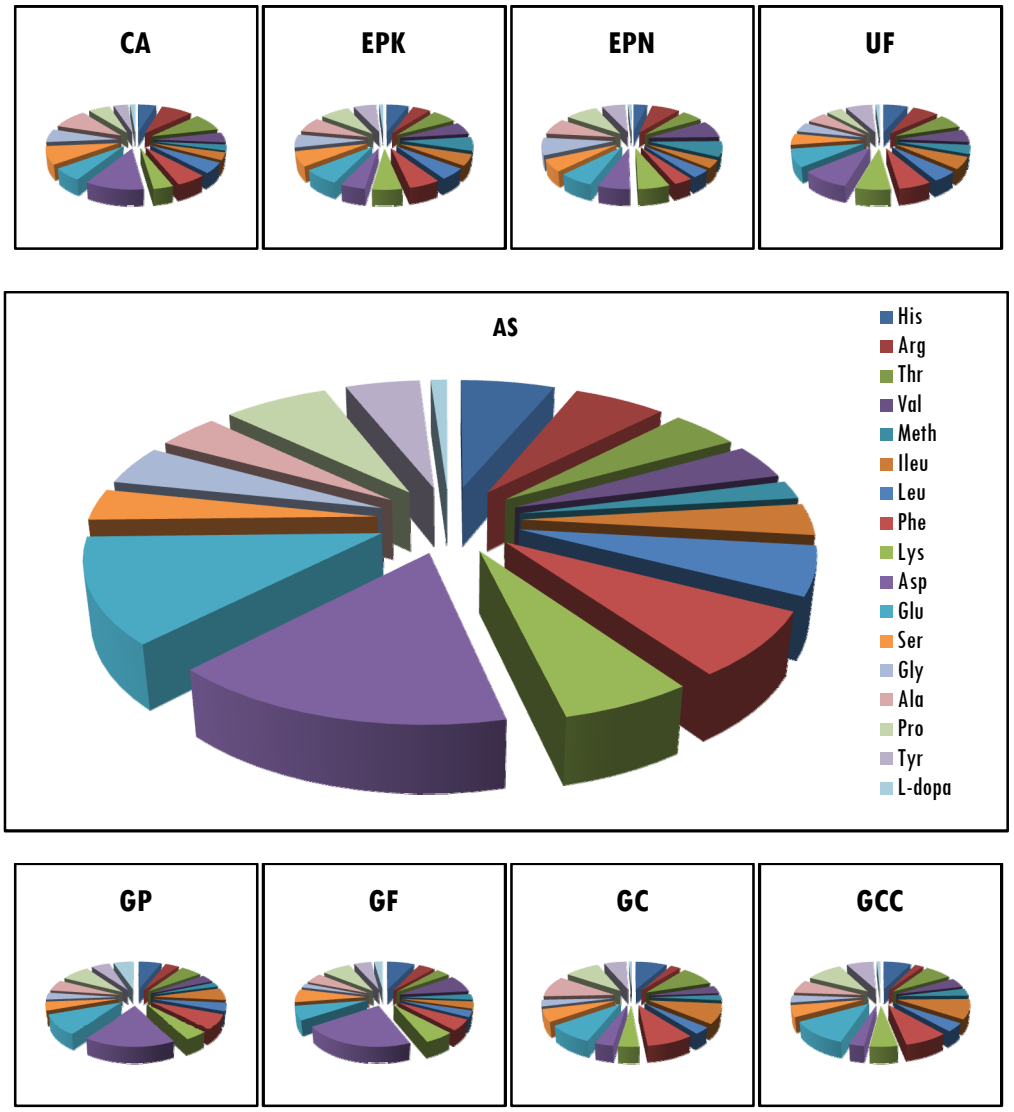


Fig. 4.9 Graphical representation of the contributions of amino acids to total amino acid pool in each seaweed.

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

L-dopa (1.10 %) was the least contributor to the amino acid pool of *U. fasciata*. Phenylalanine (6.34 %) and histidine (6.43 %) and threonine (6.87 %) and arginine (6.87 %) had comparable distribution pattern in the pool.

Aspartic acid (9.51 %) possessed the major contribution in *U. fasciata*. *A. spicifera* had low L-dopa (1.08 %) and comparable serine (3.71 %) and isoleucine (3.71 %), alanine (4.07 %) and valine (4.07 %), and lysine (6.11 %) and arginine (6.11 %) contribution patterns. Aspartic acid (15.93 %) possessed the major area of contribution in *A. spicifera*. *G. pusillum* had low methionine (2.19 %) contribution. Valine (3.58 %) and arginine (3.58 %) had comparable area in the pool. Aspartic acid (17.69 %) possessed the major part.

G. foliifera had low glycine (2.09 %) and high aspartic acid (22.15 %) contributions. *G. corticata* had low L-dopa (0.60 %) and comparable methionine (2.58 %) and arginine (2.58 %), phenylalanine (9.15 %), glutamic acid (9.15 %) and proline (9.15 %) contributions in the pool. Alanine (10.14 %) had the major area in the pool. *G. corticata* var. *cylindrica* had low L-dopa (0.82 %) and comparable methionine (3.51 %) and glycine (3.51 %), and histidine (7.22 %) and threonine (7.22 %) contribution patterns. Major area of contribution was exhibited by glutamic acid (11.96 %).

Above observations were aligning with the previous reports on amino acids from various sources. The seaweeds proteins generally contain 4.4 to 9.9 % alanine, 6.5 to 24 % glutamic acid, 6.1 to 12 % aspartic acid (Hernández-Carmona et al., 2009). *Solanum macrocarpon* and *Solanum aethiopicum* collected from Nigeria showed 3.78 and 3.35 % lysine, 2.18 and 2.01 % histidine, 5.01 and 4.75 % arginine, 6.95 and 7.23 % aspartic acid, 2.21 and 1.85 % threonine, 2.14 and 1.85 % serine, 9.70 and 10.6 % glutamic acid, 1.63 and 1.32 % proline, 3.70 and 2.91 % glycine, 4.10 and 3.23 % alanine, 4.16 and 3.90 % valine, 0.86 and 0.70 % methionine, 3.17 and 3.01 % isoleucine, 6.64 and 5.70 % leucine, 2.38 and 2.11 % tyrosine and 3.77 and 3.53 % phenylalanine to its total protein content

respectively (Adeyeye & Adanlawo, 2011). EAA was 50.3% and 48.9 % and NEAA was 49.8 % and 51.1 % respectively (Adeyeye & Adanlawo, 2011). *Kappaphycus alvarezii* collected from Tuticorin, Tamil Nadu, India was observed to have high amounts of tyrosine (2.6 %) and arginine (1.6 %). Histidine, glutamic acid and aspartic acid ranged from 0.4 to 0.5 %. Methionine was reported with 0.2 % and proline was reported as undetected (Raman & Doble, 2014). *Eisenia arborea* collected from Mexico showed seventeen amino acids with 9.88 to 10.52 % glutamic acid, 5.85 to 7.88 % aspartic acid, 5.19 to 5.89 % leucine, 4.75 to 5.38 % arginine, 3.95 to 4.88 % alanine, 2.88 to 4.28 % valine, 3.58 to 3.97 % lysine, 3.04 to 3.43 % isoleucine, 2.56 to 3.45 % threonine, 2.28 to 3.26 % glycine, 2.41 to 2.99 % proline, 2.47 to 2.96 % tyrosine, 2.41 to 2.99 % serine, 2.30 to 2.67 % phenylalanine, 1.40 to 1.73 % methionine and 1.00 to 1.46 % histidine (Hernández-Carmona et al., 2009). *Chlorella* sp. was reported earlier with 12.42 % aspartic acid, 18.61 % glutamic acid, 13.41 % arginine and 25.45 % lysine (Sorimachi, 2002). *Chlorella* sp. also exhibited 9.73 % aspartic acid, 9.97 % glutamic acid, 11.38 % alanine and 12.03 % lysine (Sorimachi, 1999). *Padina tetrastomatica* collected from Kanyakumari, Tamil Nadu, India was reported with isoleucine and asparagines as the major amino acids followed by phenylalanine and leucine. Lysine was the least observed (Sethi, 2012). *G. corticata*, *Hypnea musciformis*, *A. spicifera*, *Sargassum wightii*, *Ulva lactuca* and *K. alvarezii* collected from the Kerala coast, India were reported with 5 to 20 % amino acid content (Kumar & Kaladharan, 2007). The EAA to protein ratio was between 45 to 49 % (Kumar & Kaladharan, 2007). Among the above, the aspartic acid (0.66 to 2.46 %) and glutamic acid (0.73 to 2.67 %) were seen highest. Threonine was observed in the range of 0.50 to 1.78 %. The lowest among the reported amino

acids was tyrosine (0.13 to 0.61 %) followed with cystine (0.06 to 0.25 %) (Kumar & Kaladharan, 2007). EAA to protein ratio was observed in the range of 37 to 38 % in *Porphyra tenera*, *Grateloupia turuturu*, *Ulva pertusa* and *Codium fragile* (Fujiwara-Arasaki et al., 1984), 37 - 42 % in *Ulva lactuca* and *Gelidium amansii* (Ochiai et al.,1987), 36.5 to 38.6 % in *Ulva rigida* and *Ulva rotundata* (Fleurence et al., 1995). *Palmaria palmata* reported to contain 8 to 35 % of amino acids with aspartic acid and glutamic acid as the major forms and histidine as the minor form. In *Ulva pertusa*, 20 to 26 % of amino acids were reported with arginine, glutamic acid and leucine as the major forms and tryptophan as the minor form. In *P. tenera*, 33 - 47 % of amino acids were reported with arginine and leucine as the major amino acids, and methionine as the minor amino acid (Fleurence, 1999). 3.21 % of taurine and 2.86 % of glutamic acid were reported in *K. alvarezii* (Santoso, 2004). The nutraceutical importance of seaweed protein was demonstrated with glycine, alanine, proline, glutamic acid and aspartic acids in major concentrations. Their protein profile was comparable with the egg protein where the NEAA were almost comparable with the green, brown and red algae and the EAA was almost half of the protein weights (Černá, 2011).

The six seaweeds collected from the Galcian coast, Spain and the north Portuguese coast constituting of the *Saccorhiza polyschides, Himanthalia elongata, Laminaria ochroleuca, Undaria pinnatifida, Palmaria* sp. and *Porphyra* sp. exhibited 0.71, 0.16, 0.38, 0.6, 0.87 and 1.15 % aspartic acid, 0.87, 0.33, 0.47, 1.15, 1.02 and 1.27 % glutamic acid, 0.30, 0.10, 0.20, 0.29, 0.60 and 0.67 % serine, 0.43, 0.06, 0.21, 0.39, 0.77 and 0.94 % glycine, 0.77, 0.17, 0.41, 0.53, 1.01 and 0.83 % histidine, 0.79, 0.15, 0.50, 1.39, 1.32, 0.76 % arginine, 0.15, 0.08, 0.16, 0.08, 0.34 and 0.97 % threonine, 1.03, 0.36, 0.49, 0.78, 1.54 and 1.45 %

alanine, 0.48, 0.20, 0.25, 0.48, 0.80 and 0.84 % proline, 0.18, 0.06, 0.13, 0.20. 0.37 and 0.44 % tyrosine, 0.49, 0.07, 0.19, 0.35, 0.66 and 0.72 % valine, 0.19, 0.01, 0.07, 0.19, 0.17 and 0.16 % methionine, 0.41, 0.08, 0.16, 0.30, 0.47 and 0.46 % isoleucine, 0.65, 0.06, 0.18, 0.48, 0.67 and 0.71 % leucine, 1.50, 0.24, 0.57, 1.66, 1.89 and 1.66 % phenylalanine and 0.43, 0.11, 0.22, 0.57, 0.68 and 0.77 % lysine contents respectively. The total amino acids and EAA were reported as 9.38 % and 40.7 %, 2.24 % and 29 %, 4.59 % and 33.8, 9.44 % and 38.5 %, 13.18 % and 37 and 13.80 % and 39.5 % in the *S. polychides, H. elongata, L. ochroleuca, U. pinnatifida, Palmaria* sp. and *Porphyra* sp. respectively (Sánchez-Machado et al., 2003).

Conventional food sources like the golden apple, red apple, broccoli, purple onion, white onion, peach, red bell pepper, potato, tomato, buffalo gourd, cucumber, pumpkin and watermelon showed aspartic acid and methionine, aspartic acid and methionine, aspartic acid and glutamic acid, arginine and glutamic acid, arginine and methionine, methionine and serine, proline and aspartic acid, proline and valine, glutamic acid and proline, aspartic acid and proline, alanine and glycine, aspartic acid and arginine and aspartic acid and methionine as the major amino acids respectively (Fish, 2012). Conventional food source, cooked *Oryza sativa* exhibited 0.151 % alanine, 0.196 % arginine, 0.242 % aspartic acid, 0.526 % glutamic acid, 0.127 % glycine, 0.066 % histidine, 0.109 % isoleucine, 0.214 % leucine, 0.099 % lysine, 0.058 % methionine, 0.133 % phenylalanine, 0.121 % proline, 0.134 % serine, 0.095 % threonine, 0.097 % tyrosine and 0.151 % valine (Kalman, 2014). Non conventional leafy vegetables like the *Hibiscus cannabinus* and *Haematostaphis barteri* collected from Nigeria showed 3.96 and 3.02 % lysine, 3.25 and 2.26 % threonine, 3.85 and 3.25 %

valine, 0.91 and 0.86 % methionine, 2.81 and 3.03 % isoleucine, 7.05 and 5.66 % leucine, 3.06 and 2.86 % tyrosine, 4.55 and 3.86 % phenylalanine, 2.41 and 2.01 % histidine, 5.02 and 5.11 % arginine, 7.02 and 6.69 % aspartic acid, 1.45 and 1.72 % serine, 11.11 and 9.52 % glutamic acid, 2.50 and 2.06 % proline, 0.72 and 1.02 % glycine and 1.65 and 2.25 % alanine to the total protein contents respectively (Kubmarawa, 2009).

Conventional and non conventional food sources as discussed above indicate similar amino acid patterns as observed in this study. This highlights the importance of the seaweeds in nutrition. Detailed investigation on this part is required and hence with, the amino acid content in comparison with the recommended daily intake is discussed ahead (Section 4.3.7).

4.3.6 L-dopa Content with OPA Technique

Most of the marine organisms which stick on the surface of rocks, seabeds or other substrates use a particular adhesive for its strong anchoring to the surfaces inorder to overcome the adverse climatic conditions. For the production of such a strong adhesives, they modify their amino acids to certain high surface affinity compounds that could form coordinate surface complexes upon substrate surface delivery. These adhesives are generally generated in a fluid form which solidifies upon, to form a permanent natural adhesive. L-dopa is one of the typically modified amino acids, reported earlier in mussels and barnacles which aid in their strong adherence to surfaces (Stewart et al., 2011). These are observed to be emitted to the seawater for aiding the purpose. Seaweeds which are one among the marine organisms clinging onto strong surfaces, could either be using the same protocols adopted by similar organisms. L-dopa content in the seaweeds suggests the adoption of the same adherence protocol of barnacles or its

absorbance from the seawater at its dwelling environment. This study investigated the levels of L-dopa content in the nine seaweeds.

L-dopa derivatization was found to respond better with OPA than PITC. *G. pusillum* of the Rhodophyta family (Table 4.11) collected from Njarakkal location showed better concentration of L-dopa (0.26 %). *U. fasciata* of Chlorophyta species of Kayamkulam location showed 0.23 % of L-dopa. Comparable L-dopa contents were seen in between *E. prolifera* obtained from Njarakkal (0.13 %) and Kayamkulam (0.11 %) location. *C. antennina* (0.10 %), *A. spicifera* (0.09 %) and *G. foliifera* (0.14 %) exhibited comparable L-dopa contents. Low content of L-dopa was observed in *G. corticata* (0.03 %) and *G. corticata* var. *cylindrica* (0.04 %). Presence of L-dopa in seaweeds was observed to be novel. But L-dopa was observed to be reported in various terrestrial plants along with their therapeutical importance. Herbs such as *Mucuna pruriens, Vicia faba, Ginkgo biloba, Plumbago zeylanica, Vigna aconitifolia*, *Vigna unguiculata, Vigna vexillata, Prosopis chilensis, Pileostigma malabarica, Phanera vahlis, Parkinsonia acculeata, Macuna urens, Canvavalia glandiata, Cassia floribanda, Casia hirsute and Dalbergia retusa* were reported with L-dopa contents with *M. pruriens* alone being used for the treatment of Parkinsons disease (Ramya & Thakkur, 2007). Since almost all the terrestrial plants exhibited L-dopa greater than the results reported in current study, the L-dopa content in the analysed seaweeds could be the leftover of the adherence processes of the well rooted seaweeds or the accumulation from the seawater. The correlation analysis discussed ahead supports the observations.

Table 4.11 L-dopa content in nine seaweeds (OPA derivatization), (% to dwt), (mean ± SD), (n=3).

Amino acids	L-dopa
C. antennina	0.10 ± 0.00
E. prolifera (K)	0.11 ± 0.00
E. prolifera (N)	0.13 ± 0.00
U. fasciata	0.23 ± 0.00
A. spicifera	0.09 ± 0.00
G. pusillum	0.26 ± 0.00
G. foliifera	0.14 ± 0.00
G. corticata	0.03 ± 0.00
G. corticata var. *cylindrica*	0.04 ± 0.00

K-Kayamkulam location and N- Njarakkal location

4.3.7 Contribution of Seaweeds EAA to RDI

The recommended daily amino acid intake for an average man weighing 70 kg stated by WHO/FAO/UNU (2007) along with the amount of analysed seaweeds required to meet the stated requirements is illustrated in the Table 4.12. *E. prolifera* (Michalak & Chojnacka, 2009; Farasat et al., 2013), *U. fasciata* (Selvin & Lipton, 2004; Silva et al., 2013), *A. spicifera* (Devi et al., 2009; Flora & Rani, 2013; Guiry, 2015) and *G. corticata* (Zandi et al., 2010; Narasimman & Murugaiyan, 2012) were reported to be edible which enhances the importance of this study. For meeting the RDI, 260 g of the *U. fasciata*, 453.49 g of *E. prolifera* or 583.33 g of *A. spicifera* could be suggested as an appreciable food source. The RDI comparison could also advice a consumption of *C. antennina* at 700 g/day even though its edibility is still unknown. All the other seaweeds were seen to be inappropriate for meeting the RDI, but could be considered in contributing as compensation to the deficiency of selected EAAs. *G. pusillum* could be consumed upto 500 g/day for meeting the RDI values of histidine, threonine, isoleucine and phenylalanine. *G. foliifera* upto 666.67 g/day would meet the RDI for all the

EAAs except leucine. *G. corticata* upto 384.62 g/day would compensate the RDI for histidine, threonine, isoleucine and phenylalanine. *G. corticata* var. *cylindrica* upto 617.65 g/day was enough to meet the RDI for histidine, threonine, isoleucine, methionine and phenylalanine. Since the consumption of seaweed greater than 1 kg/day couldn't be imagined, *U. fasciata* and *E. prolifera* could be considered as an alternate food source which this study recommends for further considerations.

Table 4.12 Recommended daily intake (RDI) values of nine seaweeds in comparison with its amino acid contents.

Amino acids	RDI (mg/kg/day)	RDI (mg/70kg/day)	CHLOROPHYTA (g)			
			CA	EPK	EPN	UF
His	10	700	191.78	116.67	127.16	52.24
Thr	15	1050	159.09	155.56	122.02	73.68
Val	26	1820	485.33	298.36	142.91	142.75
Meth	15	1050	477.27	155.56	92.19	118.64
Ileu	20	1400	500.00	254.55	236.49	116.67
Leu	39	2730	691.14	491.89	453.49	260.00
Phe	25	1750	384.62	280.00	306.48	132.58
Lys	30	2100	700.00	350.00	222.34	147.89
Minimum intake quantity (g/day)			700.00	491.89	453.49	260.00

Amino acids	RDI (mg/kg/day)	RDI (mg/70kg/day)	RHODOPHYTA (g)				
			AS	GP	GF	GC	GCC
His	10	700	135.92	237.29	157.30	170.73	200.00
Thr	15	1050	280.00	375.00	456.52	225.81	300.00
Val	26	1820	543.28	1040.00	360.40	983.78	827.27
Meth	15	1050	583.33	954.55	636.36	807.69	617.65
Ileu	20	1400	459.02	482.76	666.67	291.67	307.69
Leu	39	2730	568.75	1365.00	1436.84	1654.55	1365.00
Phe	25	1750	273.44	500.00	530.30	384.62	432.10
Lys	30	2100	415.84	933.33	583.33	1076.92	823.53
Minimum intake quantity (g/day)			583.33	1365.00	1436.84	1654.55	1365.00

CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam location, EPN - *E. prolifera* obtained from Njarakkal location, UF - *U. fasciata*, AS - *A. spicifera*, GP - *G. pusillum*, GF - *G. foliifera*, GC - *G. corticata* and GCC - *G. corticata* var. *cylindrica*.

4.3.8 Correlation Studies

All the amino acid content on its average, free amino acids, L-dopa and total protein content were subjected to inter compositional correlation study (Table 4.13). The results were highly promising with the significance of the relation ($p < 0.001$) between amino acids and total protein contents. Free amino acids also showed significant correlations with total protein contents which show the relation that they are outputs from protein. L-dopa had relatively low correlations with serine (0.343), glycine (0.351), alanine (0.230), proline (0.200), tyrosine (0.432), free amino acids (0.315) and protein contents (0.460). Low level of correlations shows the non-dependence of amino acids to the free amino acids and proteins. Aspartic acid which is the dominant amino acid (Dave & Lewis, 1976) exhibited independent existence. The total amino acid content of algae to total protein contents generally varies (Dave et al., 1988; Dave & Parekh, 1994). It has been observed that in certain cases the total amino acids are higher than protein contents and in certain other cases, its lower. This could be due to the degradation of proteins and amino acids during the preparation of acid hydrolysates. The low correlation of L-dopa underlined the earlier stated observation that the presence in seaweeds is due to the absorbance from its environment or its utilization in rooting which has to be proved by more in depth studies.

Table 4.13 Pearson's bivariate correlation analysis data of amino acids, (n=27), (Split into 10 sections for enhanced readability).

(a)

	His	Arg	Thre	Val	Meth
His	1				
Arg	0.919*	1			
Thre	0.918*	0.933*	1		
Val	0.962*	0.923*	0.892*	1	
Meth	0.885*	0.826*	0.888*	0.911*	1

(b)

	His	Arg	Thre	Val	Meth
Ille	0.928*	0.798*	0.922*	0.846*	0.861*
Leu	0.938*	0.964*	0.930*	0.919*	0.910*
Phe	0.976*	0.931*	0.938*	0.904*	0.868*
Lys	0.990*	0.936*	0.905*	0.967*	0.901*
Asp	0.688*	0.758*	0.510	0.727*	0.452

(c)

	His	Arg	Thre	Val	Meth
Glu	0.942*	0.889*	0.843*	0.855*	0.794*
Ser	0.860*	0.920*	0.942*	0.922*	0.918*
Gly	0.860*	0.931*	0.930*	0.873*	0.928*
Ala	0.806*	0.866*	0.956*	0.824*	0.873*
Pro	0.835*	0.743*	0.779*	0.835*	0.951*

(d)

	His	Arg	Thre	Val	Meth
Tyr	0.989*	0.910*	0.938*	0.949*	0.915*
L-dopa	0.426	0.460	0.343	0.471	0.364
FA	0.935*	0.897*	0.963*	0.888*	0.944*
TPC	0.982*	0.962*	0.957*	0.966*	0.920*

(e)

	Ille	Leu	Phe	Lys	Asp
Ille	1				
Leu	0.849*	1			
Phe	0.931*	0.966*	1		
Lys	0.898*	0.964*	0.973*	1	
Asp	0.403	0.666*	0.631*	0.723*	1

(f)

	Ille	Leu	Phe	Lys	Asp
Glu	0.869*	0.937*	0.974*	0.957*	0.688*
Ser	0.794*	0.906*	0.844*	0.868*	0.546
Gly	0.796*	0.971*	0.899*	0.892*	0.543
Ala	0.808*	0.860*	0.824*	0.792*	0.384
Pro	0.782*	0.875*	0.835*	0.858*	0.409

(g)

	Ille	Leu	Phe	Lys	Asp
Tyr	0.957*	0.947*	0.979*	0.986*	0.618*
L-dopa	0.314	0.413	0.370	0.473	0.662*
FA	0.938*	0.961*	0.966*	0.936*	0.478
TPC	0.911*	0.978*	0.977*	0.988*	0.681*

(h)

	Glu	Ser	Gly	Ala	Pro
Glu	1				
Ser	0.736*	1			
Gly	0.834*	0.946*	1		
Ala	0.685*	0.962*	0.923*	1	
Pro	0.796*	0.810*	0.878*	0.764*	1

(i)

	Glu	Ser	Gly	Ala	Pro
Tyr	0.942*	0.871*	0.885*	0.825*	0.852*
L-dopa	0.401	0.343	0.351	0.230	0.200
FA	0.902*	0.900*	0.950*	0.905*	0.900*
TPC	0.932*	0.921*	0.933*	0.871*	0.850*

(j)

	Tyr	L-dopa	FA	TPC
Tyr	1			
L-dopa	0.432	1		
FA	0.962*	0.315	1	
TPC	0.986*	0.460	0.963*	1

*. Correlation is significant at the 0.001 level (2-tailed).

FA- Free amino acids and TPC- Total Protein Content.

4.4 Conclusion

The present study done on the nine seaweeds collected from Kerala coast showed that the OPA derivatization is the best method for amino acid analysis. Free amino acids in seaweeds were from the degradation of proteins. Free amino acids were seen high in Chlorophyta especially in *U. fasciata*. Similarly essential amino acids (EAA) to protein contents were observed high in Chlorophyta. Generally, essential amino acids concentrations were found to be high which are being discussed in other studies too (Kumar & Kaladharan,

2007). Seaweeds are capable of absorbing biochemical compounds from its dwelling surroundings and in rooting to surface using similar adhesives of that of barnacles and mussels. L-dopa which is an enzymatic derivative of phenylalanine and seen to have the ability to treat parkinsons disease by crossing the blood brain barrier and increasing the dopamine concentration, was seen higher in *G. pusilium*. *U. fasciata* also had high L-dopa contents.

Among the 16 amino acids, threonine, histidine and alanine were the major EAA forms, and aspartic acid and proline were the major NEAA forms. *U. fasciata* obtained from Kayamkulam location and *E. prolifera* obtained from Njarakkal location were observed to be rich in amino acids. Since *E. prolifera*, *U. fasciata*, *A. spicifera* and *G. corticata* were already reported to be edible, the amino acids content observed in this study supports their enhanced utilization in food. Comparison to the RDI values of amino acids suggests the utilization of *U. fasciata*, *E. prolifera* and *A. spicifera* as a food source. Correlation studies evidenced that, all the 16 amino acids had significant and appreciable inter correlation coefficients. This study also highlighted the presence of L-dopa in seaweeds. L-dopa whose inter correlation values were not appreciable indicated that, it is either getting accumulated in seaweeds from the environment and or being formed temporarly *in vivo*.

The current study concludes that green seaweeds especially *U. fasciata* and *E. prolifera* represent an important source of protein with higher EAA must be extended for cultivation and considered as a food source. The seaweed consumption has an inclination in European countries (Dawczynski et al., 2007) which has to be brought to the Indian coast too. The cultivation of all the studied species for food may not be advisable as it may not necessarily be edible. But the cultivation would be viable where the species could be considered as a nutraceutical extractive source.

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Chapter 5

BIOLOGICALLY IMPORTANT METABOLITES

Contents

5.1 Introduction

Seaweeds are photosynthetic organisms that flourish well in adverse environmental conditions such as high sunlight, varying temperature and pH, alternating salinity, inconsistent tides, etc. These conditions generate degenerative toxic chemicals inside the organisms which are harmful in nature (Amsler & Fairhead, 2006; Paul et al., 2006; Matanjun et al., 2008). In-depth analysis exhibited the presence of secondary metabolites in seaweeds that are capable of neutralizing these adverse chemicals. Secondary metabolites were observed to vary with the intrinsic and extrinsic factors such as genetic aspects, age, heat, light, air, mode of extraction, extraction solvent medium, extraction duration, drying procedure and storage (Cavalcanti et al., 2008). Traditionally, seaweeds are observed to be used in medicinal applications (Holdt & Kraan, 2011) which are due to the presence of many bioactive components including the carbohydrates, proteins, lipids, crude fibers, vitamins, carotenoids, amino acids, phenolics, flavonoids etc. These components are discussed in detail in the previous chapters. But, seaweeds produce many more multifaceted compounds which include the saponins, terpenoids, saturated fatty acids (SFAs),

monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), etc., (Blunt et al., 2006; Matanjun et al., 2008) whose assessment is an essential element to evaluate its economical, ecological and biological roles.

Saponins are a class of compounds which consist of a steroid moiety with a glycoside linkage. They are considered as a key ingredient in the traditional Indian and Chinese medicine and are observed for most of the biological effects. This group of compounds are known to produce healing effects on inflammations and so is used up commercially by the nutraceutical industries (Mandal et al., 2005; Manjunatha, 2006). Saponins also exhibit beneficial effects on the lowering of blood cholesterol, and in acting against cancer along with antiviral and antimicrobial properties (Daniel et al., 2011). They also include the cardiac glycosides which are used in the treatment of cardiac disorders and cardiac arrhythmias (Krishnamurthy & Asha, 2011). Saponins content in seaweeds is a less studied area. Saponins were observed in the extracts of *Chaetomorpha crassa, Caulerpa scalpelliformis, Caulerpa veravalensis, Ulva fasciata, Ulva lactuca, Padina pavonica, Padina tetrastomatica, Sargassum marginatum, Sargassum tennerimum* and *Sargassum wightii* collected from the four coastal districts of southern Tamil Nadu (Sahayaraj et al., 2014). Saponins were observed in ethanol, chloroform and petroleum ether extracts of *Acanthophora nayadiformis* and *Chondracanthus teedei* (Jeeva et al., 2013), in acetic acid, dimethyl sulfoxide and chloroform extracts of *Sargassum longifolium* (Kumar et al., 2014) and aqueous, chloroform, ethanol and acetone fractions of *U. fasciata, U. lactuca* and *Ulva intestinalis* collected from Kanyakumari, Tamil Nadu (Manchu et al., 2014).

Selection of extracting solvents plays a great role in the determination of antioxidant and antimicrobial activities. Studies promote the use of methanolic extract for higher antimicrobial activity than n-hexane and ethyl acetate (Sastry

& Rao, 1994; Paul & Puglisi, 2004). Organic solvents were observed to yield high activities in comparison to water based extractions (Lima-Filho et al., 2002). Mixture of dichloromethane, methanol and acetone was reported to extract diterpenes from *Dictyota menstrualis* (Cavalcanti et al., 2008).

Seaweeds (red, green and brown) with high contents of bioactive composition exhibit strong bactericidal and antioxidant activities (Hosokawa et al., 2006; Zaragoza et al., 2008; Cox et al., 2009). Reactive oxygen species (ROS) and oxidative stress that are generated in human beings and classified as the root cause of life style diseases, have led to the occurrence of a variety of chronic diseases, including coronary heart disease, diabetics etc. (Chauhan & Chauhan, 2006). ROS are highly toxic resulting in cell death and degradation of tissues. These highly reactive species need to be destroyed or neutralised by antioxidants which are either artificial or natural (Matanjun et al., 2008). Artificial antioxidants like butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA), resorcinol etc., were found to be highly effective, but the concerns on its toxicity and carcinogenicity, led to the search for natural sources of antioxidants (Kranl et al., 2005).

The changing life style has resulted in the upcoming trend for the consumption of advanced food resources that requires low processing time. Fresh and processed food products are hence preserved in order to maintain its quality and to extend the shelf life. Chemical preservatives and antioxidants are generally used, which have negative reactions on body that results in the formation of new diseases such as cancer, tumor and cell degenerative diseases. Even though, these chemicals and its usage were known to be harmful, the current consumer preferences lead to the development of foods that contain minimal antioxidant chemicals and are microbiologically safe. The use of natural antioxidant and antimicrobial agents that inhibit pathogen growth and

deterioration has gained recent attractions. Seaweeds were reported to have high antioxidant activities. Many related works suggested the need to carry out more than one type of antioxidant activity measurement, in order to determine the various mechanisms of antioxidative activities. No particular antioxidation study would reflect the various mechanisms of antioxidants. The methods for antioxidant capacity determination differ in terms of their assay principles and experimental conditions (Cao & Prior, 1998). In general, the 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) are used. *Turbinaria ornata* and *Sargassum polycystum* collected from Thailand were reported to have high free radical scavenging properties and reducing properties (Rattaya et al., 2015). Methanolic extracts of *Sargassum tenerrimum, Chaetomorpha indica, Ulva rigida, Gracilaria acerosa* and *Sargassum filiforme* exhibited high ascorbic acid equivalent antioxidant activity (Kumar et al., 2011). Treatment of sliced vegetables with edible seaweed juice containing PUFAs and volatile compounds were reported for the prevention of vegetable browning and poisoning (Kajiwara et al., 2006).

Requirements for antimicrobial derivatives that are isolated from floral sources are increasing tremendously. It finds application in food and pharma sectors. In food, many sterilization methods are adopted to maintain the microbiological load to minimum level, with non-thermal processes gaining higher attractions. These processes include the addition of natural antibacterial extracts (Hayes et al., 2010), high pressure CO_2 (Garcia-Gonzalez et al., 2009), high intensity pulsed electric field (Mosqueda-Melgar et al., 2008), irradiation (Alighourchi et al., 2008), ultrasound (Schenk et al., 2008) etc., which could preserve food without degradation of its organoleptic quality. In the pharma sector, the bacterial diseases are usually arrested using drugs or

chemicals, which results in the mutation of bacteria due to their indiscriminate uses. It becomes a greater problem of giving treatments to those infected with such mutated microorganisms. Advanced antibiotics are being developed whose cost is high and efficacy are still interrogative. The decreased efficacy of antibiotics to drug resistance bacteria has made the search for alternatives from natural sources. Marine biota has proved to be worth in this search which constituted of diverse secondary metabolites with the seaweeds showing bactericidal and bacteriostatic properties (Vedhagiri et al., 2009).

In addition to the food and pharma sectors, antimicrobial compounds find application in commercial sectors. Bio fouling is a natural process observed in marine ecosystem which is due to the excess growth of micro and macro fouler and results in huge economic and environmental losses. This effect is observed on natural and man-made marine structures. Marine organisms including sponges, seaweeds, sea grass etc., develop secondary metabolites which have antifouling activity. Since ancient times, the antimicrobial properties of the seaweeds were recognized, even though the in-depth exploration has not been done so far. Algal species are good sources of active principles that have bactericidal effects. Many studies on marine algal extracts support these observations. Brown alga was reported to be active against both Gram - positive and Gram - negative organisms (Ara et al., 2002). *Ecklonia kurome* extracts also showed high bactericidal activities (Nagayama et al., 2002). *T. ornata* and *S. polycystum* collected from Thailand had antimicrobial activities against *Staphylococcus aureus* (Rattaya et al., 2015). The extracts of the three seaweeds *Caulerpa lentillifera, Caulerpa racemosa* var. *clavifera* f. *microphysa* and *Caulerpa racemosa* var. *laetevirens* were observed to have bactericidal activities against *Escherichia coli, S. aureus, Streptococcus* sp. and *Salmonella* sp. (Nagappan & Vairappan, 2014). Methanolic extracts of *Gracilaria edulis*, *Gracilaria verrucosa*, *Ulva fasciata*,

Ulva lactuca, *Kappaphycus spicifera*, *Sargassum ilicifolium, Sargassum wightii, Padina tetramatica* and *Padina gymnospora* were observed to have antimicrobial activities against *Vibrio alginolyticus*. The acetone fractions of *G. edulis, G. verrucosa, A. spicifera, U. fasciata, U. lactuca, S. ilicifolium* and *S.wightii* and the chloroform fractions of *A. spicifera, U. fasciata, U. lactuca, S. wightii, P. tetramatica* and *P. gymnospora* also exhibited bactericidal activities against *V. alginolyticus* (Thirunavukkarasu et al., 2013).

Commendable antimicrobial activity was exhibited by the alcoholic extracts of *S. wightii, Ulva reticulata and Halimeda macroloba* against the biofilm bacterial strains viz., *Pseudomonas* sp., *Flavobacterium* sp., *Cytophaga* sp. and *Bascillus* sp. (Prabhakaran et al., 2012). Methanol extracts of *Cladophora glomerata* exhibited bactericidal activity against *Vibrio parahaemolyticus, Vibrio anguillarum, Vibrio fischeri, Vibrio vulnificus, Escherichia coli, Bacillus cereus* and *Acinetobacter baumannii* (Yuvaraj et al., 2011). Turkish seaweed *Chaetomorpha prolifera* and *Codium fragile* exhibited high bactericidal activities against *Enterococcus faecalis* and *E. coli* (Taşkin et al., 2011). Marine seaweed *Asparagopsis taxiformis* was observed to possess antimicrobial activity against *Leptospira javanica* (Vedhagiri et al., 2009). Methanolic extracts of *A. taxiformis* exhibited commendable inhibitory effects on the Gram - negative bacteria *E. coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* and Gram - positive bacteria non-haemolytic *Streptococcus*, *S. aureus, Staphylococcus epidermidis*, *E. faecalis*, *Micrococcus luteus* and *Bacillus subtilis*. Similarly, inhibitory effects were exhibited by the methanolic extracts of *Laurencia brandenii*, *Laurencia ceylanica* and *Hypnea valentiae* (Manilal et al., 2009).

Chemical morphology of the bioactive extracts obtained from seaweeds with respect to GC-MS analysis was reported with an array of different organic

compounds. They constituted of the alkanes, alkenes, alcohols, steroids etc. The petroleum ether fraction of *Laminaria japonica* was observed to have two alcohols, two alkanes, one phenol etc. (Cai et al., 2014). GC-MS analysis of the essential oils of brown, red and green seaweeds, extracted using dichloromethane and pentane mixture showed the presence of flavour compounds such as hexanal, hexenal, nonanal and nonenal with antimicrobial activities against *E. coli* and *Erwinia carotovora* (Kajiwara et al., 2006). Alcoholic extracts of *S. wightii* exhibited the presence of alcohols, phenols and carboxylic acids. Similarly, the alcoholic extracts of *U. reticulata* exhibited the presence of alkenes, alcohols, phenols, carboxylic acids and its esters. In the alcoholic extracts of *H. macroloba*, presence of alcohols and phenols were observed (Prabhakaran et al., 2012). Steroids were observed in the petroleum ether and ethanol fractions of *Acanthophora nayadiformis,* aqueous, chloroform, ethanol and petroleum ether fractions of *Gracilaria idinhtakaraiensis*, chloroform extract of *Padina tetrastomatica* and ethanol extract of *Ulva lactuca* (Jeeva et al., 2013) and in the extracts obtained from the *Chaetomorpha crassa, Caulerpa scalpelliformis, Caulerpa veravalensis, U. fasciata, U. lactuca, Padina pavonica, Padina tetrastomatica, Sargassum marginatum, Sargassum tennerimum* and *Sargassum wightii* collected from the four coastal districts of southern Tamil Nadu, India (Sahayaraj et al., 2014). Steroids were observed in the petroleum ether, chloroform, ethanol and acetone fractions, and carboxylic acid content was seen in chloroform, ethanol and acetone fractions of *U. fasciata, U. lactuca* and *U. intestinalis* collected from Kanyakumari, Tamil Nadu (Manchu et al., 2014).

Seaweeds are sources of important fatty acids. They constitute of SFAs, MUFAs and PUFAs. Unsaturated fatty acids are generally classified as omega fatty acids on the basis of their double bond position. Omega fatty acids are found to be good for health, brain development, cholesterol level regulations etc. Marine

algae have been reported as good sources of omega fatty acids (Ragonese et al., 2014). Generally C-7 to C-24 SFAs, C-14 to C-22 MUFAs (omega (ω) 5, 7, 9 and 11) and C16 to C-22 PUFAs (ω-1, 3, 4, 6, 7 and 9) were observed (Ragonese et al., 2014). Earlier studies on seaweeds were reported to have the above fatty acids in varying concentrations. Seaweeds were reported to have low lipid contents with high PUFA contents in comparison to terrestrial plants (Ragonese et al., 2014). The ω-3 PUFAs including the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found to have beneficial clinical and nutraceutical applications (Ginzberg et al., 2000). ω-3 fatty acids are essential PUFAs which have to be provided through diet for human and the primary sources are represented by marine fishes and fish oils (Saito & Aono, 2014).

As an alternative source, marine macro algae have been studied as potential resources of PUFAs which could be cultivated on large scale (Bhosale et al., 2008; Kumari et al., 2010). Fatty acids like the linolenic acid (Ohta et al., 1994), palmitoleic acid, hexadecatrienoic acid (HTA), EPA, other SFAs, ω-3 and ω-6 PUFAs were reported with antimicrobial activities which acts through their detergent activity by destroying the bacterial membranes through auto oxidation (Park et al., 2013). Many topical applications containing these fatty acids have seen to be highly effective against bacterial infections (Naqvi et al., 2010).

Green seaweeds were observed to have SFAs ranging from C-8 to C-24, MUFAs from C-16 to C-22 (ω-5,7,9 and 11) and PUFAs from C-16 to C-22 (ω-1,3,4,6,7 and 9) (Ragonese et al., 2014). Red seaweeds had SFAs ranging from C-6 to C-24, MUFAs from C-16 to C-22 (ω-5,7,9 and 11) and PUFAs from C-16 to C-22 (ω-1,3,4,6,7 and 9) (Ragonese et al., 2014). Brown seaweeds had SFAs ranging from C-7 to C-24, MUFAs from C-16 to C-22 (ω-5,7,9 and 11) and PUFAs from C-16 to C-22 (ω-1,3,4,6,7 and 9) (Ragonese et

al., 2014). SFAs such as C-14 to C-18, MUFAs such as C-16 to C-22 and PUFAs such as the C-18 to C-22 were observed in the petroleum ether fractions of *Turbo cornutus* (Saito & Aono, 2014). The petroleum ether fraction of *Laminaria japonica* had rich contents of fatty acids such as the tetradecanoic acid, linoleic acid, palmitic acid, oleic acid etc. (Cai et al., 2014).

Ulva lactuca, Chondrus crispus, Laminaria hyperborea, Fucus serrata, Undaria pinnatifida, Palmaria palmata, Caulerpa taxifolia, Ascophyllum nodosum and *Sargassum natans* collected from the north Atlantic and tropical seas were reported to have appreciable concentrations of C-14 to C-24 SFAs, ω-9 MUFAs and ω-3, 6 and 9 PUFAs (van Ginneken et al., 2011). *U. rigida* collected from Chilka Lake exhibited C-14 to C-22 SFAs, C14 to C-18 MUFAs and C-16 to C-22 PUFAs (Satpati & Pal, 2011). *Gracilaria domingensis*, *Gracilaria birdiae*, *Laurencia filiformis* and *Laurencia intricata* collected from Brazil was reported with the presence of C-12, C-14, C-16 and C-18 as the major SFAs, C-16 (ω-7) and C-18 (ω-9) as the major MUFAs and C-18 (ω-6) and C-20 (ω-6) as the major PUFAs (Gressler et al., 2010). Bactericidal active fractions of *Asparagopsis taxiformis* exhibited the presence of saturated and unsaturated fatty acids (Manilal et al., 2009). Marine seaweed *A. taxiformis* had both SFAs and MUFAs (Vedhagiri et al., 2009). *Gracilaria changgi* collected from Malaysia, had EPA (ω-3), palmitic acid, oleic acid and DHA (ω-3) as the main free fatty acid contents (Norziah & Ching, 2000). Red algae observed to have high contents of ω-fatty acids (Fleurence et al., 1994).

This study deals with the quantification and bioactivity properties of the total saponins isolated from the seven seaweeds. The antimicrobial and antioxidant activities of the isolated saponins and the fractions collected using petroleum ether, chloroform and ethyl acetate were estimated. Antioxidant activity studies were done as per the methods discussed in the previous chapters.

Antimicrobial study was done as per the Kirby-Bauer disc diffusion method against four pathogenic bacteria - *E. coli, S. abony, B. cereus* and *S. aureus* with respect to two positive controls - chloramphenicol and tetracycline. Iodine and saponification values of the fractions and the isolated saponins were estimated to get an idea regarding the saponifiable matter and extend of their unsaturation. Bioactive extracts were further investigated for the chemical speciation using GC-MS in comparison with the NIST MS search, version 2.0. All the quantified bioactive contents and their bioactivity results were subjected to Pearson bivariate correlation analysis. Correlation studies indicated the cross dependence of the biochemical constituents to activity.

5.2 Materials and Methods

5.2.1 Chemicals Used

All the standards were brought from Sigma (USA), Aldrich (USA) and Chromadex (USA). Chemicals used were of analytical grade and solvents were purchased from Merck (Germany) in HPLC grade. Water used was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Quantifications were done in triplicates against standards and results were reported with standard deviations. Chloramphenicol and tetracycline were purchased from Sigma (USA). Mannitol salt agar (MSA), nutrient agar, Levin eosin methylene blue agar (L-EMBA), xylose lysine deoxycholate agar (XLDA) and soyabean casein digest agar were purchased from Difco, USA.

5.2.2 Pathogen Culture Used

The lyophilised cultures (live strains) of *Escherichia coli* ATCC 25922 - Gram - negative rod, *Salmonella abony* NCTC 6017 - Gram - negative rod, *Bacillus cereus* ATCC 10876 - Gram - positive rod and *Staphylococcus aureus* ATCC 6538 - Gram - positive cocci were used.

5.2.3 Samples

Ground samples of the seven seaweeds, comprising of Chlorophyta (*Chaetomorpha antennina, Enteromorpha prolifera* and *Ulva fasciata*) and Rhodophyta (*Gracilaria corticata, Gracilaria corticata* var. *cylindrica* and *Gracilaria foliifera)* were studied (Table 5.1).

Table 5.1 Taxonomic classification of seaweeds collected from the Kerala coast and the four solvent extraction yields, (% to dwt), (mean ± SD), (n = 3).

Seaweed	Extraction yields (%)			
	PEF	EAF	CF	SF
Chaetomorpha antennina (Bory de Saint-Vincent) Kützing	0.98 ± 0.04	0.02 ± 0.00	1.98 ± 0.09	1.72 ± 0.16
Enteromorpha prolifera (O.F.Müller) J.Agardh (K)	0.57 ± 0.03	0.13 ± 0.01	0.98 ± 0.05	1.31 ± 0.12
Enteromorpha prolifera (O.F.Müller) J.Agardh (N)	1.00 ± 0.05	0.07 ± 0.01	1.18 ± 0.05	1.19 ± 0.11
Gracilaria corticata (J.Agardh) J.Agardh	0.48 ± 0.02	0.16 ± 0.01	1.10 ± 0.05	0.56 ± 0.05
Gracilaria corticata var. *cylindrica* Umamaheswara Rao	0.54 ± 0.02	0.17 ± 0.01	2.46 ± 0.11	0.27 ± 0.02
Gracilaria foliifera (Forsskål) Børgesen	0.43 ± 0.02	0.34 ± 0.02	1.01 ± 0.05	1.21 ± 0.11
Ulva fasciata Delile	1.14 ± 0.05	1.50 ± 0.07	0.63 ± 0.03	1.54 ± 0.14

PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction and SF - Saponins fraction. K - Kayamkulam and N - Njarakkal.

5.2.4 Estimation of Saponins

Saponins content of the seaweed sample was estimated by the double solvent gravimetric method (Harbone, 1973). 2 g of the ground seaweed sample was mixed with 50 mL of 20 % aqueous ethanol solution and heated at 50 - 60 ^{o}C in a water bath for 90 min. The supernatant solution was filtered off through a Whatman no. 40 filter paper. The residue was re-extracted twice and

the supernatants were combined. The combined supernatant was reduced to 40 mL at 90 ^{o}C and transferred to a separating funnel. 40 mL of diethyl ether was added and vigorously shaken. The ether layer was separated off. The aqueous layer was re-extracted twice with 40 mL of diethyl ether. The diethyl ether layer was discarded. The aqueous layer was extracted further with 60 mL of n-butanol thrice (Fig. 5.1). The combined n-butanol layer was dried in a pre weighed evaporating dish at 60 ^{o}C, and the weight of residue was estimated and reported as the % of saponins to dry weight of the seaweeds (SF).

5.2.5 Preparation of Fractions

Saponins fraction from the above was identified as SF. The residue of the above saponins extractions including the seaweed residue, diethyl ether layer and aqueous layer were combined together, desolventized and then repeatedly extracted using 50 mL each of 90 % methanol-water until the supernatant was clear (Fig. 5.1). The aqueous methanolic solution was then reduced to half the extract volume. 100 mL of 6N methanolic KOH was added and refluxed for 3 h. The extract volume was again reduced to one fourth under vacuum at 50 ^{o}C and transferred to a separating funnel. 25 mL of petroleum ether was added and shaken vigorously. The petroleum ether layer was separated. The aqueous layer was re-extracted twice with 25 mL of petroleum ether. The petroleum ether layer was concentrated under vacuum at 40 ^{o}C and stored in glass vials at < 4 ^{o}C. This layer was identified as petroleum ether fraction (PEF). The aqueous layer was then kept in a water bath to remove the traces of petroleum ether. The aqueous layer was then transferred into a separating funnel and extracted thrice with ethyl acetate. The ethyl acetate layer was collected, concentrated under vacuum at 40 ^{o}C and stored in glass vials at < 4 ^{o}C. This layer was identified as ethyl acetate fraction (EAF). The aqueous layer was then kept in boiling water bath for 5 min, cooled and

the pH of the solution was adjusted to 2 - 2.5 using drops of concentrated hydrochloric acid. The aqueous layer was again transferred to a separating funnel and extracted thrice with chloroform. The chloroform layer was concentrated at 40 ^{o}C under vacuum, transferred to a glass vial and stored at < 4 ^{o}C. The chloroform layer was identified as chloroform fraction (CF). All the above fractions were further subjected to iodine value, saponification value, antioxidant activity, antibacterial activity and GC-MS profiling analysis.

5.2.6 Determination of Iodine and Saponification Values

Iodine and saponification values provide an information regarding the extend of unsaturation and availability of saponifiable matters. Estimations were done based on the analytical procedures evidenced in the Indian Pharmacopoeia (Indian Pharmacopoeia, 1996a; Indian Pharmacopoeia, 1996b). Iodine value was reported as the grams of iodine absorbed per 100 gram of the sample and saponification value was reported as the mg of KOH required per gram of the sample.

5.2.7 Antioxidant Activity

DPPH free radical scavenging antioxidant assays are the generally accepted methodology (Farvin & Jacobsen, 2013; Xin et al., 2013). But the antioxidant properties are not purely based on the free radical quenching (Balboa, et al., 2013; Farvin, & Jacobsen, 2013). Henceforth, the antioxidant activity of the extracted fractions was surveyed using three prominent methods. - DPPH, a modified UV-Vis ferrous tartarate method and $KMnO_4$ titration method.

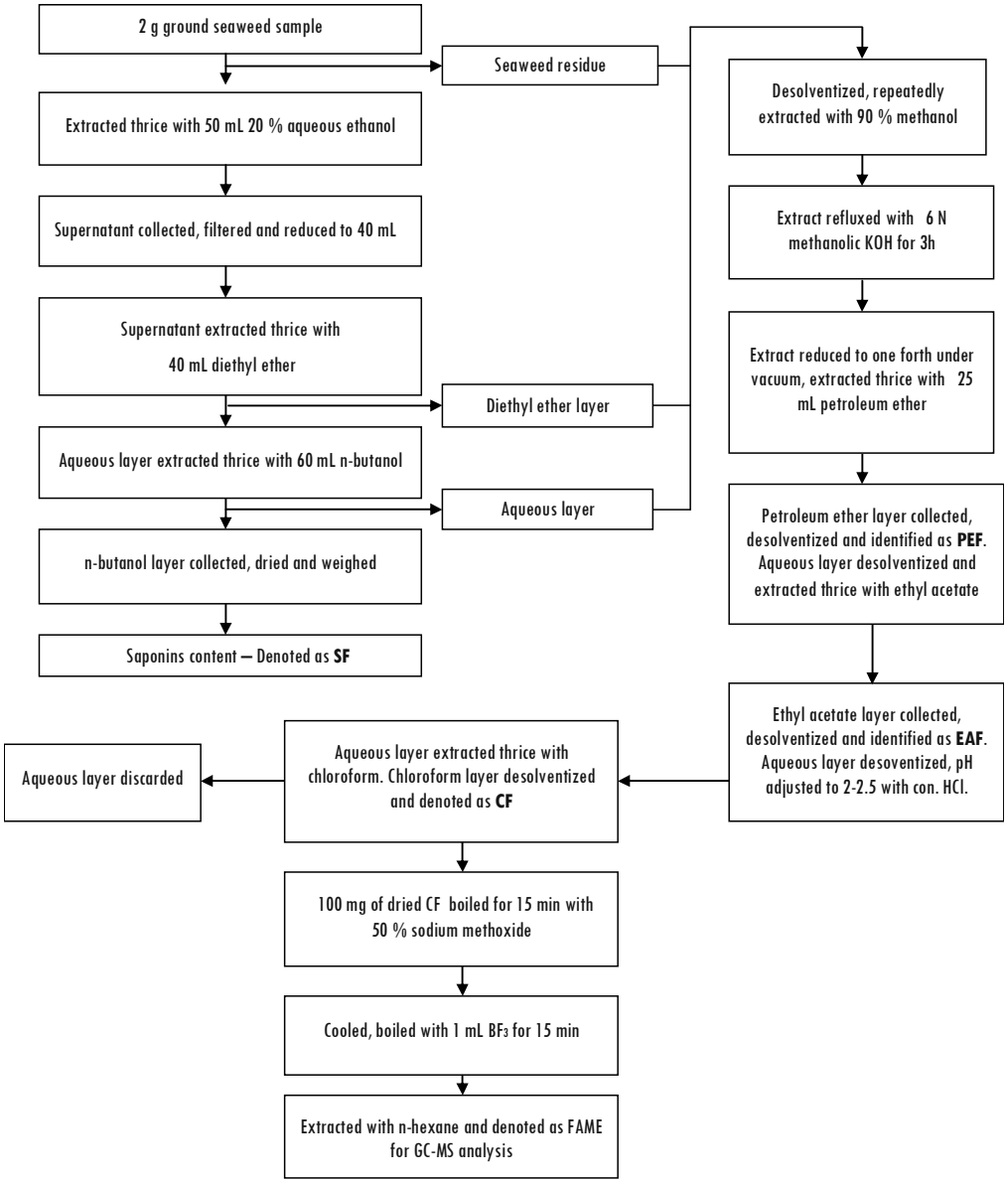


Fig. 5.1 Flow chart of the extraction process of the saponins content (Harbone, 1973) and the four solvent fractions (Rajpal, 2005). The fatty acid methyl esters (FAMEs) synthesis for GC - MS analysis is also shown. (PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction and SF - Saponins fraction).

5.2.7.1 Antioxidant assay - DPPH Method

The assay was done as per the method given by Brand-Williams et al. (1995) with some modifications. DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 mL HPLC grade methanol and stored at - 20 ^{o}C until needed. The working solution was obtained by mixing a 10 mL stock solution with 45 mL HPLC grade methanol to obtain an absorbance of 1.170 ± 0.02 units at 515 nm using Cary-60 UV-Vis spectrophotometer. 1.5 mL of the sample solution (0.100 g of extract in 100 mL methanol) was allowed to react upon mixing with 28.5 mL of DPPH solution for 24 h in dark. The absorbance was measured at 515 nm against the reagent blank. The standard curve was observed to be linear between 403 to 1613 µg g^{-1} for ascorbic acid, 409 to 1637 µg g^{-1} for α-tocopherol, 405 to 1621 µg g^{-1} of BHT and 406 to 1626 µg g^{-1} of resorcinol. Results were expressed as the percentage of equivalence to the corresponding standard concentration to the dry weight of the sample. Additional dilutions were done upon the requirement, when observed to be deviating from linear range. The r^2 values were > 0.995 (Table 5.2).

5.2.7.2 Antioxidant Assay - UV-Vis Ferrous Tartarate Method

Antioxidant activity was determined by using the modified UV-visible spectrophotometric methods (Liang et al., 2003; Li et al., 2005). Analysis was carried out by accurately weighing 0.100 g of the extract into a 100 mL standard flask, made up to the mark, using HPLC grade water. 2 mL of the solution was pipetted into a 50 mL standard flask, to which 8 mL of HPLC grade water was added followed with 10 mL of tartaric acid ferrous sulfate solution and made up to the volume using phosphate buffer solution. The absorbance was measured at

540 nm nullifying the reagent blank. The standard curve was observed to be linear between 403 to 1613 μg g^{-1} for ascorbic acid, 406 to 1626 μg g^{-1} for BHT, 409 to 1637 μg g^{-1} for α-tocopherol and 405 to 1622 μg g^{-1} for resorcinol. Results were expressed as the percentage of equivalence to the corresponding standard concentration to the dry weight of the sample. Additional dilutions were done upon the requirement, when observed to be deviating from linear range. The r^2 values were > 0.950 (Table 5.2).

Table 5.2 Calibration data for the antioxidant activities of standards using DPPH, ferrous tartarate and $KMnO_4$ titration methods.

Standard	Calibration equations	r^2	Concentration (ppm)			
			1	2	3	4
DPPH RADICAL SCAVENGING ACTIVITY						
ASC	y = -3.2176e-05x -0.081	0.995	403.36	806.72	1210.08	1613.44
RES	y = -3.1709e-05x - 0.088	0.999	406.56	813.12	1219.68	1626.24
TOC	y = -4.9594e-05x + 0.008	0.999	409.36	818.72	1228.08	1637.44
BHT	y = -2.9294e-05x - 0.064	0.996	405.32	810.64	1215.96	1621.28
FERROUS TARTARATE OXIDISING ACTIVITY						
ASC	y = 5.6376e-04x -0.013	0.978	403.36	806.72	1210.08	1613.44
RES	y = 7.2978e-06x - 0.012	0.992	405.60	811.20	1216.80	1622.40
TOC	y = 1.808e-03x + 0.556	0.995	409.36	818.72	1228.08	1637.44
BHT	y = 2.0351e-03x + 0.421	0.994	406.56	813.12	1219.68	1626.24

$KMnO_4$ TITRIMETRY						
Standard	Weight of standards (g/50mL)	Blank readings	Titre readings			Equivalence of 0.01N $KMnO_4$(g)
			1mL	2mL	3mL	
ASC	0.504	0.9	23.0	41.0	61.3	0.00046
RES	0.507	0.9	2.4	4.0	6.0	0.05069
TOC	0.507	0.9	1.1	1.3	1.4	0.00664
BHT	0.503	0.9	3.1	5.9	6.8	0.00457

ASC- Ascorbic acid, RES- Resorcinol, TOC- Tocopherol and BHT- Butylatedhydroxytoluene.

5.2.7.3 Antioxidant Assay - $KMnO_4$ Method

The antioxidant assay was estimated adopting the Ribereau-Gayon-Maurié titrimetric method with $KMnO_4$ and indigo carmine dye as an indicator (Daničić, 1973; Radovanović, 1986). 50 - 100 mg of the extract of seaweed was transferred to 100 mL standard flask and made up to the mark with methanol. 3 mL was pipette out into a conical flask, 15 mL methanol and 1 mL indigo carmine dye (prepared by dissolving 1 g indigo carmine in 25 mL con. H_2SO_4, further diluted to 50 mL using con. H_2SO_4 and further diluted to 1 L with distilled water), were added and titrated against 0.1N standard $KMnO_4$ solution against reagent blanks. The results were calculated with the estimated observations against the standards such as ascorbic acid, α-tocopherol, BHT and resorcinol and were expressed in percentage of equivalence to the corresponding standard concentration to the dry weight of the sample (Table 5.2).

5.2.8 Antibacterial Study

Positive cultures purchased were initially cultured in the respective selective culture medium. The live *S. aureus* was streaked on mannitol salt agar plates adjusted to pH 6.8 to 7.2. *B. cereus* was streaked on nutrient agar, *E. coli* on eosin methylene blue agar and *S. abony* on the xylose lysine deoxycholate agar plates at pH 6.8 to 7.2. The grown colonies were carefully collected using a sterile platinum loop in the presence of the flame of a spirit lamp inside a bio safety cabinet pre-sterilised using UV rays, and inoculated in to the soyabean casein digest medium. The log phase culture was swabbed into soyabean casein digest agar plates. pH was maintained at 6.8 to 7.20. The antibacterial activity study was done based on the Kirby-Bauer disc diffusion method (Bauer et al., 1966). This test is done in order to find whether the extract - impregnated discs made of Whatman no. 40 filter paper, is active

against a known strain of bacteria which are grown on the agar plates. If the extracts are active, an area of clarity is surrounded the disc which is the zone of inhibition and the observations are reported in comparison to the inhibition area of the standard. All the fractions were screened for its activity which is stated as the biological activity with pathogenic two Gram - positive (*S. aureus* and *B. cereus*) and two Gram - negative forms (*E. coli* and *S. abony*). The 4 fractions, namely PEF, EAF, CF and SF were dissolved in methanol at a concentration level of 100 mg L^{-1}. Chloramphenicol and tetracycline was used as the positive controls, dissolved in methanol at a level of 100 mg L^{-1}. Methanol was used as the analytical blank whose activity observations were deducted during the results calculations. The methanolic solution of the positive controls, samples and methanol was impregnated in the paper discs made of Whatman no. 40 filter paper, dried at 40 ^{o}C under vacuum and refrigerated at a temperature of < 4 ^{o}C until analysis. The filter paper discs were placed onto the streaked up plates. The activity was measured as in mm of the clearance area around the disc, discarding the diameter of the disc and reported as % of inhibition against the positive controls (Table 5.3).

Table 5.3 Standardisation of antimicrobial methods using three positive controls, (mean ± SD), (n = 3).

Positive control (100 mg L^{-1})	Inhibition level (mm)			
	Gram +ve		Gram -ve	
	S. aureus	*B. cereus*	*E. Coli*	*S. abony*
Solvent	2.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.2	2.0 ± 0.1
Tetracycline	9.0 ± 0.4	10.0 ± 0.5	8.0 ± 0.4	9.0 ± 0.4
Chloramphenicol	8.0 ± 0.4	12.0 ± 0.6	10.0 ± 0.4	12.0 ± 0.4

5.2.9 Preparation of Fatty acid Methyl Esters (FAMEs) for GC Analysis

The fatty acid methyl esters (FAMEs) from the algal extracts were obtained as follows; 100 mg of the dried chloroform extract was taken in a pyrex tube and dried under nitrogen. 1 mL of 50 % sodium methoxide (NaOH/Methanol) in methanol was added and heated in a boiling water bath

for 15 min. After cooling, 1 mL of BF_3 was added and the solution was kept in boiling water bath for 15 min. After cooling, 1 mL of HPLC grade n-hexane (Fig. 5.1) was added followed by 1 mL of saturated NaCl solution and swirled for 10 min. The upper layer was collected, moisture removed by addition of Na_2SO_4 and injected to GC-MS.

5.2.10 GC-MS Analysis

The different fractions were chromatographically analysed on Perkin Elmer Clarus 680 gas chromatograph equipped with mass spectrometer with electron ionization as mass identification source. Dimethyl polysiloxane with 5 % diphenyl (Elite 5MS) column was used for the separation having 30 m-length and 250 μm internal diameters. High pure helium was used as the carrier gas. Pneumatic split/splitless injection was used to inject the sample having a split ratio 50:1. The oven temperature was initially kept at 35 ^{o}C and increased up to 280 ^{o}C at the rate of 10 ^{o}C per min keeping hold 10 min in the maximum temperature. The injector temperature was kept at 280 ^{o}C. Inlet and source temperature of the mass spectrometer was kept at 200 ^{o}C, scan range 4 - 500 a.m.u. and solvent delay was at four min. The extract was diluted with corresponding solvent, filtered through 0.2 μ sterile syringe filters and 0.5 μL of each fraction was injected into the gas chromatograph and analysed with single quadruple mass spectrometric detector. Structural assignments were done based on analysis of fragmentation pattern of mass spectra and direct comparison of mass spectral data with profiles in the National Institute of Standards and Technology (NIST MS search, version 2.0). Results were reported with a hit value range greater than 85 % and the quantified values in % were calculated upon the relative concentration of the compounds in the

injected volume of sample to the dry weight of the seaweed. Fatty acids contents were reported as its actual concentration and relative concentrations.

5.2.11 Statistics

All the analytical observations are reported as the mean of triplicate determinations (± standard deviations). All the statistical correlation analyses were carried out using SPSS 16.0 for Windows. The Pearson correlation test with bivariate significance was adopted and the positive r^2 values explained the relation between the chemical constituents and activities.

5.3 Results and Discussions

5.3.1 Saponins Content

Saponins are steroids or similar compounds with glycosidic linkages. Saponins contents were observed and quantified in all the analysed samples (Table 5.1). Chlorophyta exhibited the maximum concentrations (1.19 to 1.72 %). Among Rhodophyta, *G. foliifera* alone exhibited similar concentration patterns of Chlorophyta (1.21 %). The saponin fractions (SF) of *G. foliifera* were observed to be comparable with *E. prolifera* obtained from Njarakkal location (1.19 %). The least saponin content was observed in *G. corticata* var. *cylindrica* (0.27 %) (Fig. 5.2). The results obtained were aligning with the previous studies done on seaweeds. Saponins were observed in extracts of seaweeds collected from the Indian coast (Jeeva et al., 2013). 0.35 to 1.08 % of saponin contents were reported in the five Chlorophyta and five Phaeophyta seaweeds collected from the southwest coast of India (Sahayaraj et al., 2014).

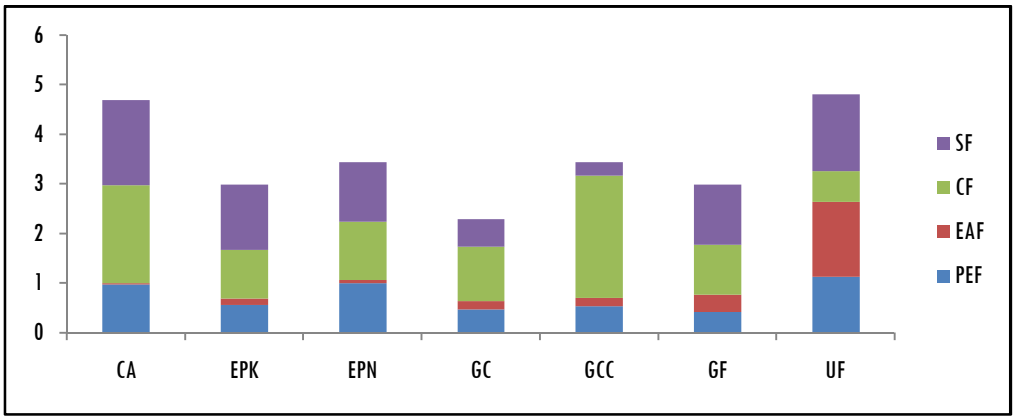


Fig. 5.2 Graphical representation of the total saponin fraction (SF), chloroform fraction (CF), ethyl acetate fraction (EAF) and petroleum ether fraction (PEF).

CA- *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN- *E. prolifera* obtained from Njarakkal, GC- *G. corticata*, GCC- *G. corticata* var. *cylindrica*, GF- *G. foliifera* and UF- *U. fasciata*.

5.3.2 Preparation of Seaweed Fractions for Bioactivity and GC-MS Profiling

The fractions collected after sequential solvent extractions were dried and yield was estimated and reported as the % to dry weight of the sample (Table 5.1). High concentrations of the non polar fractions (PEF) were obtained from Chlorophyta (0.57 to 1.14 %). All the Rhodophyta species had almost comparable non polar contents (0.43 to 0.54 %). It was observed that, the PEF was comparable in between *C. antennina* (0.98 %) and *E. prolifera* obtained from Njarakkal location (1.00 %) and in between *E. prolifera* obtained from Kayamkulam location (0.57 %) and *G. corticata* var. *cylindrica* (0.54 %). The least PEF was observed in the Rhodophyta, *G. foliifera*. Ethyl acetate fraction (slightly polar) (EAF) was seen highest in *U. fasciata* (1.50 %) but generally in Rhodophyta (0.16 to 0.34 %). Comparable EAF was observed in between *G. corticata* (0.16 %) and *G. corticata* var. *cylindrica* (0.17 %). Least amount of EAF was observed in *C. antennina* (0.02 %). Chloroform

fraction (CF) was seen highest in the Rhodophyta (1.01 to 2.46 %) eventhough, Chlorophyta such as the *C. antennina* (1.98 %) and *E. prolifera* from Njarakkal location (1.18 %) yielded appreciable concentrations. Comparable CF was observed in between *E. prolifera* obtained from Kayamkulam location (0.98 %) and *G. foliifera* (1.01 %). Least CF was observed in *U. fasciata* (0.63 %) (Fig. 5.2).

5.3.3 Iodine and Saponification Values

Iodine value which denotes the extent of unstaturation, exhibits relatively low values for SF and EAF (Table 5.4). PEF of *G. foliifera* (102.0), *E. prolifera* (Kayamkulam) (97.66) and *E. prolifera* (Njarakkal) (87.32) had relatively high iodine values which indicate the presence of unsaturated compounds. Least iodine value was observed in *G. corticata* (3.80). EAF obtained from *E. prolifera* obtained from Kayamkulam location (79.44) had the highest iodine value. Comparable iodine values were observed in between *C. antennina* (13.87) and *U. fasciata* (14.22), *E. prolifera* obtained from Njarakkal (18.54) and *G. corticata* var. *cylindrica* (19.33) and *G. corticata* (11.25) and *G. foliifera* (12.00). The chloroform fraction had high iodine values which highlighted the content of unsaturated fatty acids. CF of *E. prolifera* obtained from Njarakkal location (131.66) and Kayamkulam location (113.53) exhibited high iodine values. Comparable iodine value was observed in between *G. corticata* var. *cylindrica* (27.54) and *G. foliifera* (24.35). Least iodine value was observed in *G. foliifera*. SF obtained from *U. fasciata* had the highest iodine value (22.76). Least was observed in *G. foliifera* (9.65). Comparable iodine value was observed in between *C. antennina* (12.64) and *G. corticata* var. *cylindrica* (13.22) and in between *E. prolifera* obtained from Njarakkal (11.65) and *G. corticata* (10.87).

Table 5.4 Saponification and iodine values of the four extract fractions of seaweeds, (mean ± SD), (n = 3).

Seaweed	Fractions	Saponification value	Iodine value
CA	PEF	7.56 ± 0.69	7.55 ± 0.69
	EAF	34.36 ± 3.14	13.87 ± 1.27
	CF	335.31 ± 30.61	59.48 ± 5.43
	SF	90.81 ± 8.29	12.64 ± 1.15
EPK	PEF	34.33 ± 3.13	97.66 ± 8.92
	EAF	76.43 ± 6.98	79.44 ± 7.25
	CF	356.09 ± 32.51	113.53 ± 10.36
	SF	43.87 ± 4.00	17.65 ± 1.61
EPN	PEF	23.95 ± 2.19	87.32 ± 7.97
	EAF	10.77 ± 0.98	18.54 ± 1.69
	CF	413.66 ± 37.76	131.66 ± 12.02
	SF	43.30 ± 3.95	11.65 ± 1.06
GC	PEF	5.49 ± 0.50	3.80 ± 0.35
	EAF	7.45 ± 0.68	11.25 ± 1.03
	CF	322.45 ± 29.44	33.64 ± 3.07
	SF	41.44 ± 3.78	10.87 ± 0.99
GCC	PEF	1.56 ± 0.14	4.97 ± 0.45
	EAF	22.65 ± 2.07	19.33 ± 1.76
	CF	332.60 ± 30.36	27.54 ± 2.51
	SF	53.98 ± 4.93	13.22 ± 1.21
GF	PEF	3.13 ± 0.29	102.00 ± 9.31
	EAF	12.33 ± 1.26	12.00 ± 1.09
	CF	315.95 ± 28.84	24.35 ± 2.22
	SF	92.63 ± 8.46	9.65 ± 0.88
UF	PEF	65.23 ± 5.95	29.43 ± 2.69
	EAF	44.30 ± 4.04	14.22 ± 1.30
	CF	311.07 ± 28.40	67.09 ± 6.12
	SF	95.64 ± 8.73	22.76 ± 2.08

PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction and SF - Saponins fraction. CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

Determination of the saponification value, results in the interpretation of the total saponifiable matter and acidic matter in the sample. In the current samples, the saponification values (Table 5.4) were seen > 300 in the

chloroform fractions which indicate the contribution of fatty acids in the fraction. *C. antennina* (335) and *G. corticata* var. *cylindrica* (332) exhibited comparable saponification value. *G. foliifera* (315) and *U. fasciata* (311) also exhibited comparable saponification value. High saponification value was observed in *E. prolifera* obtained from Njrakkal location (413) and least was observed in *U. fasciata*. PEF had low saponification value with an exception in the fractions obtained from *U. fasciata* (65) and *E. prolifera* from both locations (34 and 23). The exceptional high saponification value of PEF in *U. fasciata* and *E. prolifera*, remarks the availability of fatty acids which weren't saponified during the extraction procedures. Least saponification value in PEF was observed in *G. corticata* var. *cylindrica* (1.56 ± 0.14). EAF fractions of *E. prolifera* obtained from Kayamkulam location had the highest saponification value (76). Chlorophyta had high saponification value of EAF (10 to 76). Least saponification value of EAF was observed in *G. corticata* (7.45). SF also exhibited a moderate saponification value indicating the presence of acidic functions in the glycosidic linkages. SF obtained from *C. antennina* (90), *G. foliifera* (92) and *U. fasciata* (95) had comparable saponifcation values. *E. prolifera* obtained from Kayamkulam location (43), Njarakkal location (43) and *G. corticata* (41) exhibited comparable saponification values.

5.3.4 Antioxidant Activity

With the three methods, the activity showed a concentration dependency which increased with concentrations. The antioxidant activities of the algal extracts were estimated in comparison with the corresponding standards and the results were reported as the % of its equivalence.

DPPH free radical scavenging was seen (Table 5.5) highest in the CF of *G. corticata* var. *cylindrica* with 3.97 %, 4.63 %, 12.77 % and 5.06 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences respectively (Fig. 5.3). SF of *G. foliifera* also exhibited similar patterns with 3.87 %, 4.34 %, 8.03 % and 4.64 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences respectively. Highest antioxidant PEF and EAF was exhibited by the *G. foliifera* (0.12 %) and *G. corticata* var. *cylindrica* (2.37 %) with respect to ascorbic acid equivalence respectively. Least antioxidant potential was indicated by the PEF of *E. prolifera* from Kayamkulam location (0.01 %) and *G. corticata* (0.01 %) with respect to ascorbic acid equivalence. Based on the standards, highest free radical quenching was shown by PEF of *G. foliifera* which exhibited 0.31 % of resorcinol equivalence, 0.11 % of BHT equivalence and 0.12 % ascorbic acid equivalence. PEF of *G. corticata* var. *cylindrica* exhibited 0.06 % of α-tocopherol equivalence. EAF of *G. corticata* var. *cylindrica* exhibited the highest activity based on the standards with 2.37 %, 2.70 %, 6.09 % and 2.91 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. CF of *G. corticata* var. *cylindrica* exhibited the highest activity based on the standards with 3.97 %, 4.63 %, 12.77 % and 5.06 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. SF of *G. foliifera* exhibited the highest activity based on the standards with 3.87 %, 4.34 %, 8.03 % and 4.64 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. In general seaweeds with all the fractions exhibiting high antioxidant potential were the *G. foliifera* and *G. corticata* var. *cylindrica*. Least antioxidant potential was observed in *G. corticata.*

Table 5.5 Antioxidant activity study of the fractions of seaweed extracts based on three methods, (mean ± SD), (n = 3).

Seaweed	Fraction	DPPH Method-% of Inhibition			
		AAE	BHTE	α-TE	RE
CA	PEF	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.04 ± 0.01
	EAF	1.23 ± 0.06	1.37 ± 0.07	2.45 ± 0.12	1.46 ± 0.07
	CF	0.97 ± 0.05	1.07 ± 0.05	1.63 ± 0.08	1.13 ± 0.05
	SF	0.89 ± 0.04	0.98 ± 0.05	1.40 ± 0.07	1.03 ± 0.05
EPK	PEF	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	EAF	0.08 ± 0.01	0.06 ± 0.01	0.39 ± 0.02	0.05 ± 0.01
	CF	0.08 ± 0.01	0.07 ± 0.01	0.36 ± 0.02	0.06 ± 0.01
	SF	0.08 ± 0.01	0.04 ± 0.01	1.24 ± 0.06	0.01 ± 0.01
EPN	PEF	0.10 ± 0.01	0.09 ± 0.01	0.04 ± 0.01	0.25 ± 0.01
	EAF	1.98 ± 0.09	2.16 ± 0.10	2.43 ± 0.12	2.26 ± 0.11
	CF	2.33 ± 0.11	2.55 ± 0.12	3.20 ± 0.15	2.68 ± 0.13
	SF	2.41 ± 0.11	2.65 ± 0.13	3.48 ± 0.17	2.79 ± 0.13
GC	PEF	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	EAF	0.08 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.07 ± 0.01
	CF	0.14 ± 0.01	0.14 ± 0.01	0.24 ± 0.01	0.14 ± 0.01
	SF	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.07 ± 0.01
GCC	PEF	0.10 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.26 ± 0.01
	EAF	2.37 ± 0.11	2.70 ± 0.13	6.09 ± 0.29	2.91 ± 0.14
	CF	3.97 ± 0.19	4.63 ± 0.22	12.77 ± 0.61	5.06 ± 0.24
	SF	0.28 ± 0.01	0.29 ± 0.01	0.05 ± 0.01	0.30 ± 0.01
GF	PEF	0.12 ± 0.01	0.11 ± 0.01	0.06 ± 0.01	0.31 ± 0.01
	EAF	1.14 ± 0.05	1.22 ± 0.06	0.71 ± 0.03	1.25 ± 0.06
	CF	3.25 ± 0.15	3.62 ± 0.17	6.03 ± 0.29	3.85 ± 0.18
	SF	3.87 ± 0.18	4.34 ± 0.21	8.03 ± 0.38	4.64 ± 0.22
UF	PEF	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	EAF	0.30 ± 0.01	0.26 ± 0.01	1.28 ± 0.06	0.23 ± 0.01
	CF	0.22 ± 0.01	0.21 ± 0.01	0.61 ± 0.03	0.19 ± 0.01
	SF	0.26 ± 0.01	0.24 ± 0.01	0.92 ± 0.04	0.22 ± 0.01

Seaweed	Fraction	Ferrous Tartrate Method-% of Activity			
		AAE	BHTE	α-TE	RE
CA	PEF	0.04 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.12 ± 0.01
	EAF	0.14 ± 0.01	0.76 ± 0.04	0.75 ± 0.04	1.28 ± 0.06
	CF	0.09 ± 0.01	0.64 ± 0.03	0.63 ± 0.03	1.18 ± 0.06
	SF	0.63 ± 0.03	0.59 ± 0.03	0.59 ± 0.03	0.70 ± 0.03
EPK	PEF	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.01
	EAF	0.22 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.46 ± 0.02
	CF	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
	SF	0.57 ± 0.03	0.23 ± 0.01	0.23 ± 0.01	1.35 ± 0.06
EPN	PEF	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ±0.01
	EAF	0.48 ± 0.02	0.43 ± 0.02	0.43 ± 0.02	0.57 ± 0.03
	CF	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
	SF	0.52 ± 0.02	0.47 ± 0.02	0.47 ± 0.02	0.63 ± 0.03
GC	PEF	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
	EAF	0.45 ± 0.02	0.43 ± 0.02	0.43 ± 0.02	0.47 ± 0.02
	CF	0.20 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.28 ± 0.01
	SF	0.55 ± 0.03	0.55 ± 0.03	0.55 ± 0.03	0.54 ± 0.03
GCC	PEF	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
	EAF	0.47 ± 0.02	0.47 ± 0.02	0.47 ± 0.02	0.45 ± 0.02
	CF	0.40 ± 0.02	0.39 ± 0.02	0.39 ± 0.02	0.39 ± 0.02
	SF	0.68 ± 0.03	0.59 ± 0.03	0.59 ± 0.03	0.88 ± 0.04
GF	PEF	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
	EAF	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.19 ± 0.01
	CF	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
	SF	0.40 ± 0.02	0.35 ± 0.02	0.35 ± 0.02	0.50 ± 0.02
UF	PEF	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
	EAF	0.33 ± 0.02	0.44 ± 0.02	0.43 ± 0.02	0.10 ± 0.01
	CF	0.28 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.29 ± 0.01
	SF	0.35 ± 0.02	0.36 ± 0.02	0.35 ± 0.02	0.35 ± 0.02

Seaweed	Fraction	$KMnO_4$ method-% of Activity			
		AAE	BHTE	α-TE	RE
CA	PEF	0.02 ± 0.01	0.18 ± 0.01	2.26 ± 0.11	0.21 ± 0.01
	EAF	0.09 ± 0.01	0.87 ± 0.04	9.64 ± 0.46	1.26 ± 0.06
	CF	0.08 ± 0.01	0.76 ± 0.04	8.46 ± 0.40	1.11 ± 0.05
	SF	0.17 ± 0.01	1.73 ± 0.08	19.14 ± 0.91	2.51 ± 0.12
EPK	PEF	0.02 ± 0.01	0.28 ± 0.01	3.49 ± 0.17	0.33 ± 0.02
	EAF	0.18 ± 0.01	1.79 ± 0.09	19.80 ± 0.94	2.59 ± 0.12
	CF	0.08 ± 0.01	0.79 ± 0.04	8.70 ± 0.41	1.14 ± 0.05
	SF	0.37 ± 0.02	3.65 ± 0.17	40.43 ± 1.93	5.30 ± 0.25
EPN	PEF	0.02 ± 0.01	0.22 ± 0.01	2.71 ± 0.13	0.26 ± 0.01
	EAF	0.07 ± 0.01	0.69 ± 0.03	7.61 ± 0.36	1.00 ± 0.05
	CF	0.04 ± 0.01	0.40± 0.02	4.47 ± 0.21	0.59 ± 0.03
	SF	0.32 ± 0.02	3.19 ± 0.15	35.33 ± 1.68	4.63 ± 0.22
GC	PEF	0.02 ± 0.01	0.26 ± 0.01	3.22 ± 0.15	0.31 ± 0.01
	EAF	0.10 ± 0.01	0.95 ± 0.05	10.53 ± 0.50	1.38 ± 0.07
	CF	0.04 ± 0.01	0.42 ± 0.02	4.63 ± 0.22	0.61 ± 0.03
	SF	0.34 ± 0.02	3.39 ± 0.16	37.64 ± 1.79	4.93 ± 0.23
GCC	PEF	0.02 ± 0.01	0.24 ± 0.01	3.01 ± 0.14	0.29 ± 0.01
	EAF	0.14 ± 0.01	1.40 ± 0.07	15.53 ± 0.74	2.04 ± 0.10
	CF	0.12 ± 0.01	1.23 ± 0.06	13.67 ± 0.65	1.79 ± 0.09
	SF	0.24 ±0.01	2.44 ± 0.12	27.03 ± 1.29	3.54 ± 0.17
GF	PEF	0.02 ± 0.01	0.26 ± 0.01	3.24 ± 0.15	0.31 ± 0.01
	EAF	0.16 ± 0.01	1.58 ± 0.08	17.53 ± 0.83	2.30 ± 0.11
	CF	0.08 ± 0.01	0.83 ± 0.04	9.23 ± 0.44	1.21 ± 0.06
	SF	0.26 ± 0.01	2.63 ± 0.13	29.17 ± 1.39	3.82 ± 0.18
UF	PEF	0.01 ± 0.01	0.16 ± 0.01	2.02 ± 0.10	0.19 ± 0.01
	EAF	0.12 ± 0.01	1.20 ± 0.06	13.28 ± 0.63	1.74 ± 0.08
	CF	0.03 ± 0.01	0.28 ± 0.01	3.11 ± 0.15	0.41 ± 0.02
	SF	0.12 ± 0.01	1.18 ± 0.06	13.10 ± 0.62	1.72 ± 0.08

AAE - Ascorbic acid equivalence, BHTE - Butylatedhydroxy toluene equivalence, α-TE - α-tocopherol equivalence, RE - Resorcinol equivalence, PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction, SF - Saponins fraction. CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

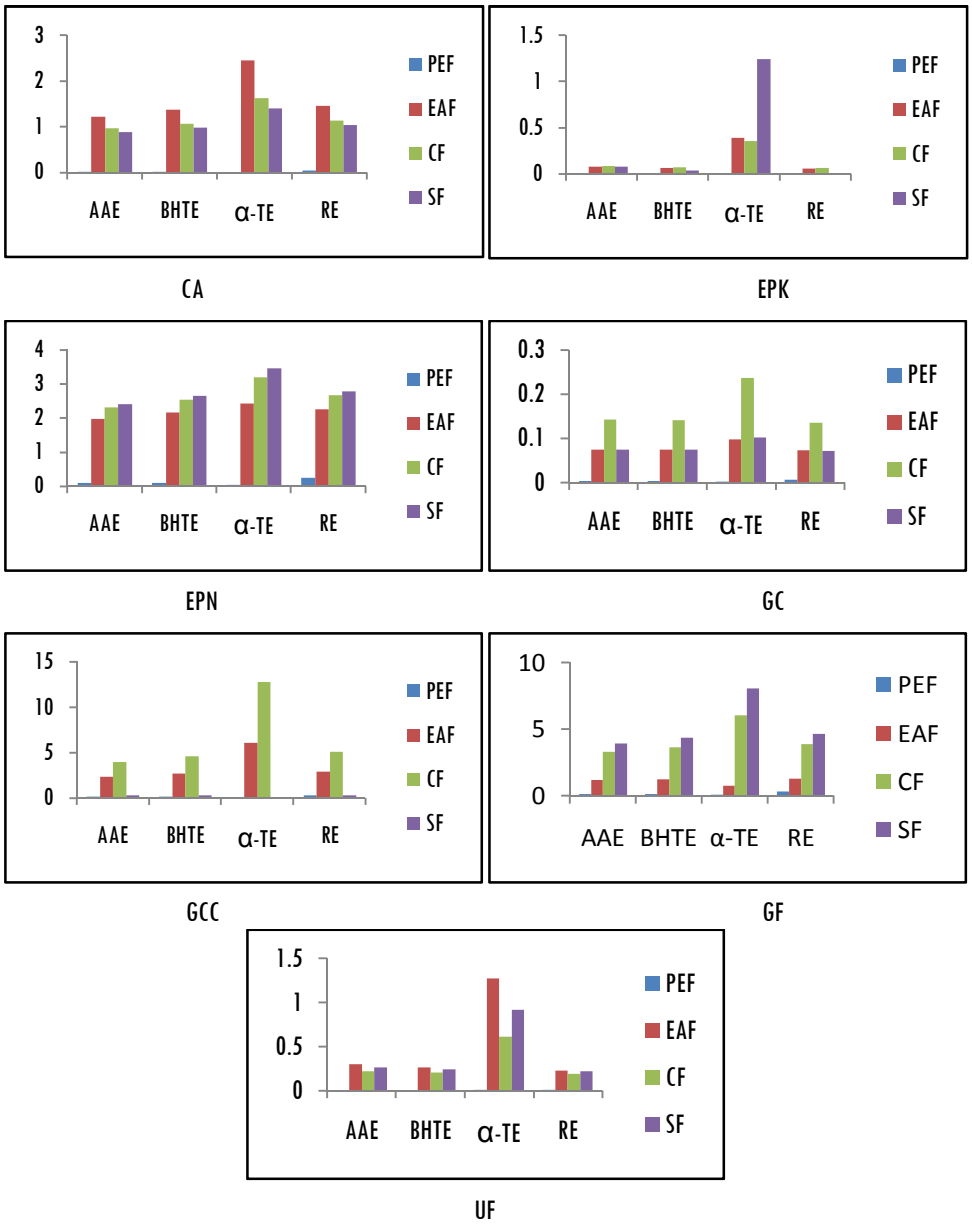


Fig. 5.3 Graphical representation of the DPPH free radical scavenging antioxidant activity of the total saponins fraction (SF), chloroform fraction (CF), ethyl acetate fraction (EAF), petroleum ether fraction (PEF), CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

Ferrous tartarate method indicates the potency of the extracts in acting as reducing agent. This activity was observed in general to the maximum in the SF

fractions (Table 5.5). *G. corticata* var. *cylindrica* and *C. antennina* exhibited the maximum activity with respect to all the four standards. Highest oxidising activity of the PEF and EAF was exhibited by the *C. antennina* (Fig. 5.4). Reducing activity of the CF was found highest in the *C. antennina*. PEF of *G. corticata* var. *cylindrica* exhibited highest ascorbic acid equivalence (0.05 %). PEF of *C. antennina* exhibited the highest BHT (0.06 %), α-tocopherol (0.07 %) and resorcinol (0.12 %) equivalences. EAF of *E. prolifera* of Njarakkal location exhibited highest ascorbic acid equivalence (0.48 %). EAF of *C. antennina* exhibited the highest BHT (0.76 %), α-tocopherol (0.75 %) and resorcinol (1.28 %) equivalences. CF of *G. corticata* var. *cylindrica* exhibited highest ascorbic acid equivalence (0.40 %). CF of *C. antennina* exhibited the highest BHT (0.64 %), α-tocopherol (0.63 %) and resorcinol (1.18 %) equivalences. SF of *G. corticata* var. *cylindrica* exhibited highest ascorbic acid (0.68 %), BHT (0.59 %), α-tocopherol (0.59 %) and resorcinol (0.88 %) equivalences. SF fraction of *C. antennina* also exhibited high α-tocopherol (0.59 %) equivalence. In general, the highest total reducing potential was observed for the extracts of *C. antennina*. Least potential was exhibited by the extracts of *G. foliifera*.

$KMnO_4$ titrimetric method indicates the total oxidation potential, which was observed to be in the maxima for the SF (Table 5.5). SF of *E. prolifera* from the Kayamkulam location and *G. corticata* showed highest oxidation potentials (Fig. 5.5). Ascorbic acid equivalence was seen highest in the SF of *E. prolifera* from the Kayamkulam location (0.37 %). The highest antioxidant potential with respect to the total oxidising capability in PEF was observed in *E. prolifera* from the Kayamkulam location, with 0.02 %, 0.28 %, 3.49 % and 0.33 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. The EAF of *E. prolifera* from the Kayamkulam location exhibited high total oxidation activities with 0.18 %, 1.79 %, 19.80 % and 2.59 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. Highest total oxidising activity for the CF was observed in the *G. corticata* var. *cylindrica* with 0.12 %, 1.23 %, 13.67 % and 1.79 % of ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. SF of *E. prolifera* from the Kayamkulam location possesed 0.37 %, 3.65 %, 40.43 % and 5.30 % of

ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. In general, *E. prolifera* from Kayamkulam location and *G. corticata* var. *cylindrica* exhibited the highest total oxidising capabilities.

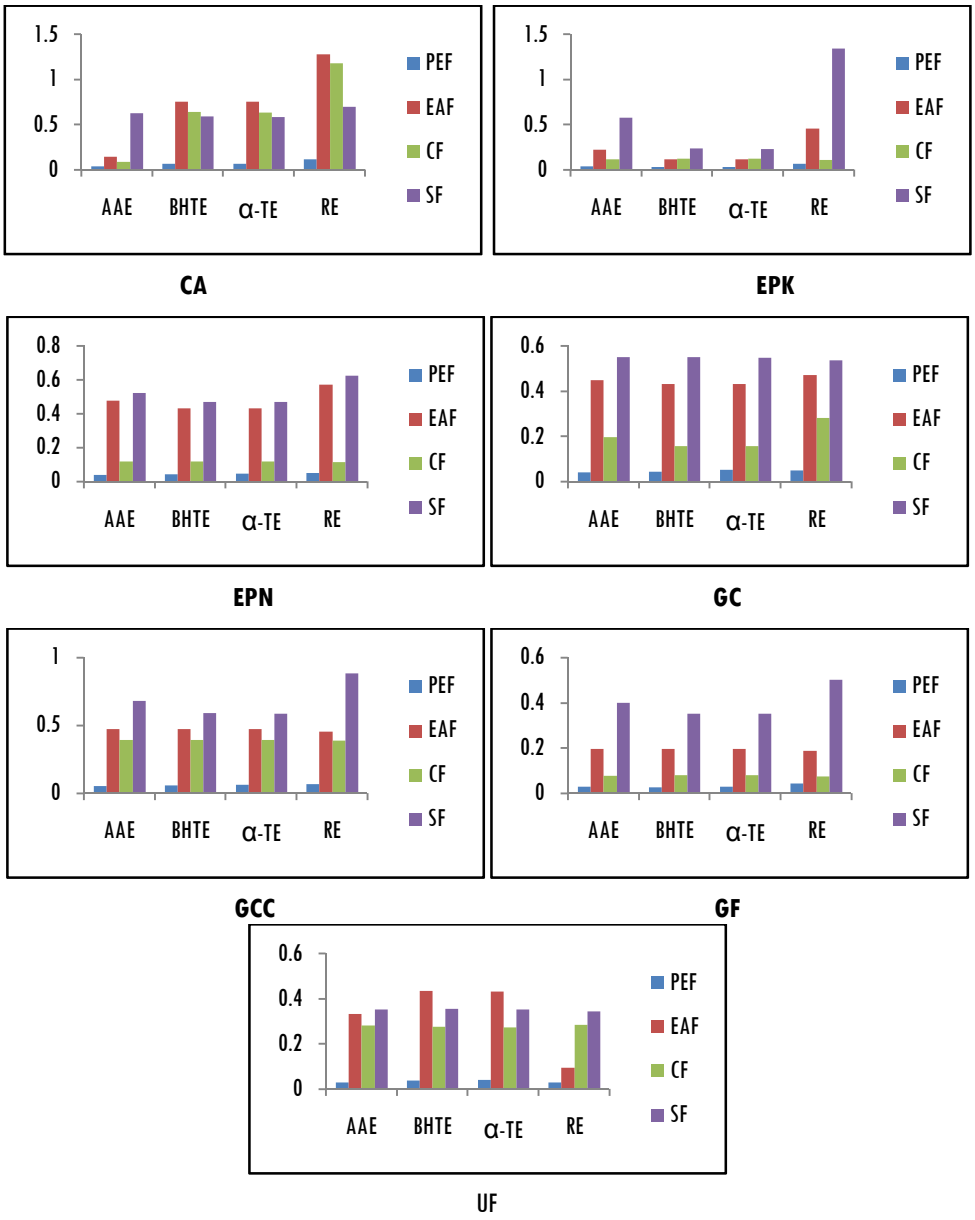


Fig. 5.4 Graphical representation of the ferrous tartarate antioxidant activity of the total saponins fraction (SF), chloroform fraction (CF), ethyl acetate fraction (EAF), petroleum ether fraction (PEF), CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

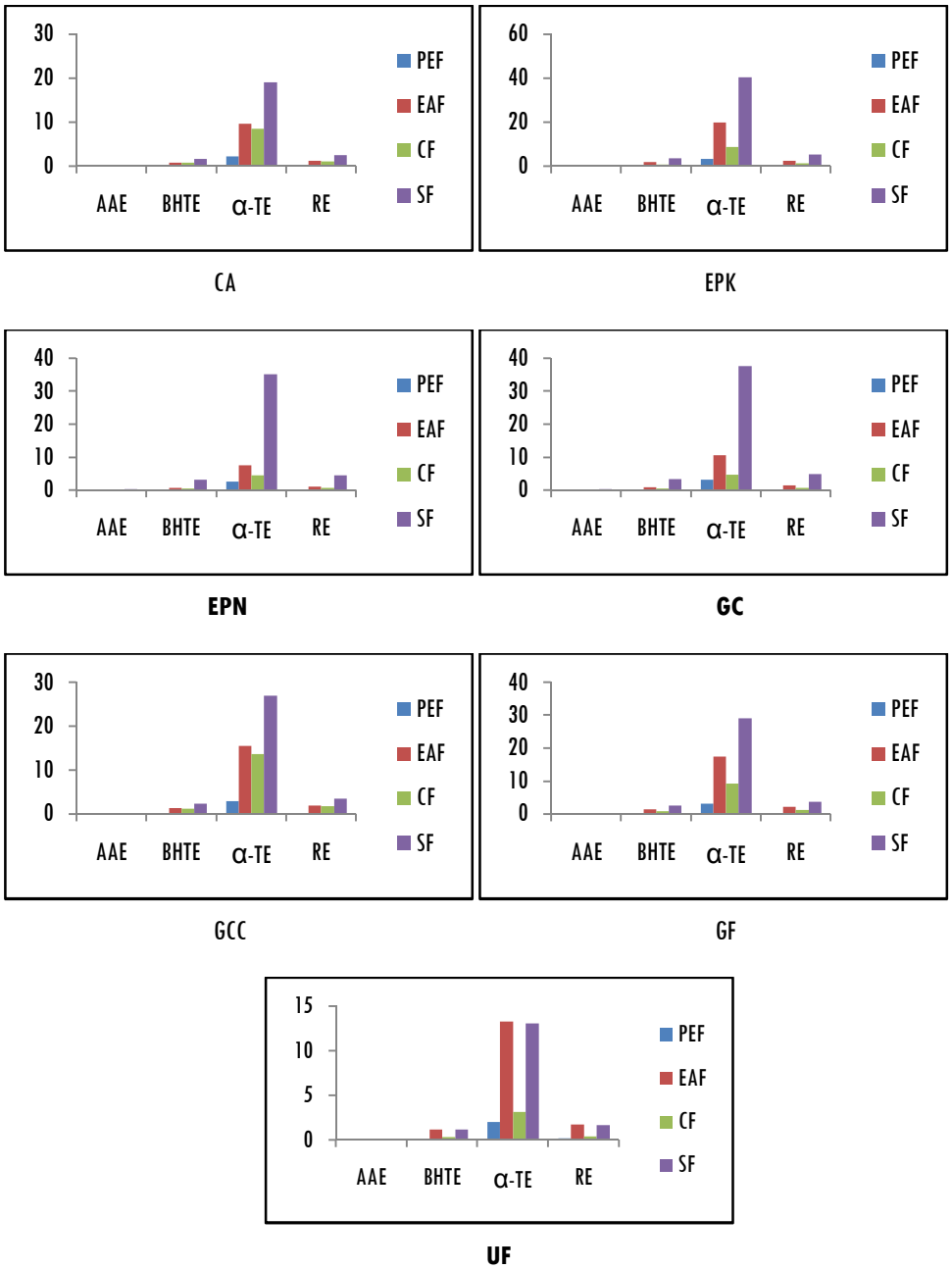


Fig. 5.5 Graphical representation of the $KMnO_4$ total antioxidant activity of the total saponins fraction (SF), chloroform fraction (CF), ethyl acetate fraction (EAF), petroleum ether fraction (PEF), CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

The observations of the three methods, as discussed above, showed remarkable antioxidant activities for all the fractions. The activities of free radical quenching were seen in the range of 0.01 to 0.31 % in PEF, 0.05 to 6.09 % in EAF, 0.06 to 12.77 % in CF and 0.01 to 8.03 % in SF. The reducing potential was seen in the range of 0.03 to 0.12 % in PEF, 0.10 to 1.28 % in EAF, 0.08 to 1.18 % in CF and 0.23 to 1.35 % in SF. The activities of PEF was in the range of 0.01 to 3.49 %, EAF was in the range of 0.07 to 19.80 %, CF was in the range of 0.03 to 13.67 % and SF was in the range of 0.12 to 40.43 % with respect to total oxidising potentials. The results therefore conclude that the SF and CF possess excellent anti oxidising capabilities which could be used up in the functional foods and nutraceuticals. The results of the current study were comparable to the previous reports on seaweeds. The methanolic extracts of seaweeds (Phaeophyta and Chlorophyta) collected from the Saurashtra coast, India showed appreciable radical scavenging activity (Kumar et al., 2011). Seaweeds such as *Eucheuma cottonii, Eucheuma spinosum, Halymenia durvillaea, Caulerpa lentillifera, Caulerpa racemosa, Dictyota dichotoma, Sargassum polycystum* and *Padina* sp., exhibited good free radical scavenging properties along with reducing powers (Matanjun et al., 2008). Antioxidant activities with respect to the free radical scavenging properties were observed in the extracts of *Gracilaria acerosa* (Devi et al., 2008).

5.3.5 Antibacterial Activity

Each fraction segregated by the solvent extractions, was observed to possess moderate antioxidant activities which could be incorporated into the anti microbial studies too. The cell walls of micro organisms are made of polysaccharides and lypophilic materials which could be easily oxidized upon with compounds having the antioxidant activities. Gram - negative bacteria are more resistant due to the additional presence of a lipid polysaccharide layer on the outer surface which protects it from easy attack of drugs and antibiotics. The four fractions, namely PEF, EAF, CF and SF were screened for the antimicrobial activity studies. The solvent impregnated disc (blank) indicated 2 mm diameter in

the streaked plates of *S. aureus* and *S. abony*, whereas 3 mm in *B. cereus* and *E.coli* (Table 5.3). Thus, 2 mm of inhibitory diameter was reduced as the blank correction upon the observations made on the activity against *S. aureus* and *S. abony* and 3 mm deduction against *B. cereus* and *E.coli*. Chloramphenicol exhibited an inhibitory diameter of 8 mm on *S. aureus*, 12 mm on *B. cereus*, 10 mm *on E.coli* and 12 mm on *S. abony*. Tetracycline exhibited the inhibitory diameter of 9 mm on *S. aureus* and *S. abony*, whereas 10 mm on *B. cereus* and 8 mm on *E.coli*. Both the inhibitory diameters of the positive controls were measured after deducting the solvent inhibition (Table 5.6).

With respect to chloramphenicol as the positive control (Table 5.7), the activity was observed almost in the similar pattern to that of tetracycline. The analytical conclusion thereby states the extracts of *U. fasciata* to be comprehensively active against a broad spectrum of pathogens. Selective extracts of seaweeds were seen to yield good and appreciable activities. Prominent bactericidal activity against the strains of *S. aureus* by the EAF of *U. fasciata, B. cereus* by the PEF of *G. corticata* var. *cylindrica, E. coli* by the SF of *G. corticata* var. *cylindrica* and *S.abony* by the EAF of *U. fasciata* were observed.

With respect to the tetracycline as the positive control (Table 5.8), highest antibacterial activity with broad spectrum activity on both Gram - positive and Gram - negative cells was exhibited by fractions of *U. fasciata* except PEF. *C. antennina* was observed to possess the detrimental effect on *E.coli* (Gram - negative) and *B. cereus* (Gram - positive). *G. corticata* var. *cylindrica* extracts had high inhibitory activity on *E. coli* whereas moderate and selective activity on the other strains. EAF showed high activity on *S. aureus* and Gram - negative bacteria. *E. prolifera* from the Kayamkulam location showed good inhibitory responses to *S. aureus* and *E. coli.* Similar activity was shown by the extracts of *G. corticata*, whereas the activity was low for the fractions of *E. prolifera* collected from Njarakkal location. Fractions of *G. foliifera*, except PEF exhibited moderate broad spectrum activity.

Table 5.6 Antimicrobial assays of the fractions of seaweed extracts and their activities against the positive controls, (mean ± SD), (n = 3).

Analyte	Fractions (100ppm)	Inhibition level (mm)			
		Gram +ve		Gram -ve	
		S.aureus	*B. cereus*	*E. Coli*	*S. abony*
CA	PEF	2.1 ± 0.1	3.0 ± 0.1	4.0 ± 0.2	2.0 ± 0.1
	EAF	1.5 ± 0.1	4.0 ± 0.2	4.0 ± 0.1	2.0 ± 0.1
	CF	2.0 ± 0.1	4.0 ± 0.2	4.0 ± 0.1	2.0 ± 0.1
	SF	2.1 ± 0.1	4.0 ± 0.1	4.0 ± 0.3	2.0 ± 0.2
EPK	PEF	4.0 ± 0.2	2.5 ± 0.1	3.5 ± 0.2	2.0 ± 0.1
	EAF	3.5 ± 0.2	3.0 ± 0.1	4.0 ± 0.2	1.5 ± 0.1
	CF	3.0 ± 0.2	3.0 ± 0.2	4.0 ± 0.1	1.5 ± 0.1
	SF	3.5 ± 0.1	3.0 ± 0.1	3.0 ± 0.2	2.0 ± 0.1
EPN	PEF	4.0 ± 0.1	3.0 ± 0.1	4.0 ± 0.2	1.5 ± 0.1
	EAF	3.0 ± 0.1	3.0 ± 0.1	4.0 ± 0.3	2.0 ± 0.1
	CF	2.0 ± 0.2	3.0 ± 0.1	3.0 ± 0.1	2.0 ± 0.1
	SF	2.0 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
GC	PEF	1.5 ± 0.1	3.0 ± 0.2	3.5 ± 0.2	2.0 ± 0.1
	EAF	2.5 ± 0.1	2.5 ± 0.1	4.0 ± 0.2	2.0 ± 0.1
	CF	3.0 ± 0.1	3.0 ± 0.2	4.0 ± 0.1	2.0 ± 0.1
	SF	3.0 ± 0.1	3.0 ± 0.2	4.0 ± 0.1	3.0 ± 0.1
GCC	PEF	2.0 ± 0.2	5.0 ± 0.2	4.0 ± 0.1	2.0 ± 0.2
	EAF	4.0 ± 0.3	3.0 ± 0.1	4.0 ± 0.1	3.5 ± 0.1
	CF	2.0 ± 0.2	4.0 ± 0.1	4.0 ± 0.1	2.0 ± 0.1
	SF	2.0 ± 0.1	4.5 ± 0.2	4.5 ± 0.1	2.0 ± 0.1
GF	PEF	2.0 ± 0.1	3.0 ± 0.1	4.0 ± 0.1	2.0 ± 0.1
	EAF	3.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1	3.5 ± 0.2
	CF	3.0 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.0 ± 0.1
	SF	3.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1	3.0 ± 0.1
UF	PEF	4.0 ± 0.2	4.0 ± 0.2	3.5 ± 0.1	1.5 ± 0.1
	EAF	5.0 ± 0.2	3.5 ± 0.1	3.5 ± 0.1	4.0 ± 0.2
	CF	4.0 ± 0.2	3.5 ± 0.2	4.0 ± 0.1	3.5 ± 0.3
	SF	3.5 ± 0.2	4.0 ± 0.2	4.0 ± 0.3	2.5 ± 0.1

PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction, SF - Saponins fraction, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

Table 5.7 Antimicrobial activity with respect to chloramphenicol as positive control, (mean ± SD), (n = 3).

Analyte	Fraction	% of Activity			
		Chloramphenicol equivalents			
		S. aureus	*B. cereus*	*E. coli*	*S. abony*
CA	PEF	0	0	10.1 ± 0.4	0
	EAF	0	8.0 ± 0.4	10.0 ± 0.5	0
	CF	0	8.1 ± 0.4	10.1 ± 0.5	0
	SF	0	8.2 ± 0.4	10.1 ± 0.4	0
EPK	PEF	25.3 ± 1.2	0	5.0 ± 0.2	0
	EAF	19.0 ± 0.9	0	10.0 ± 0.4	0
	CF	13.0 ± 0.6	0	10.1 ± 0.4	0
	SF	19.0 ± 0.9	0	0	0
EPN	PEF	25.2 ± 1.2	0	10.1 ± 0.2	0
	EAF	13.0 ± 0.9	0	10.2 ± 0.3	0
	CF	0	0	0	0
	SF	0	0	0	8.0 ± 0.5
GC	PEF	0	0	5.0 ± 0.2	0
	EAF	6.0 ± 0.3	0	10.1 ± 0.4	0
	CF	13.0 ± 0.6	0	10.0 ± 0.4	0
	SF	13.0 ± 0.6	0	10.0 ± 0.5	8.1 ± 0.3
GCC	PEF	0	17.0 ± 0.8	10.2 ± 0.5	0
	EAF	25.1 ± 1.2	0	10.1 ± 0.5	13.0 ± 0.6
	CF	0	8.1 ± 0.4	10.1 ± 0.5	0
	SF	0	13.0 ± 0.6	15.2 ± 0.7	0
GF	PEF	0	0	10.1 ± 0.2	0
	EAF	13.0 ± 0.4	8.0 ± 0.4	10.2 ± 0.1	13.0 ± 0.1
	CF	13.0 ± 0.4	4.0 ± 0.2	5.1 ± 0.2	8.0 ± 0.1
	SF	13.0 ± 0.5	8.0 ± 0.4	10.1 ± 0.1	8.2 ± 0.2
UF	PEF	25.1 ± 1.2	8.1 ± 0.4	5.0 ± 0.2	0
	EAF	38.0 ± 1.8	4.0 ± 0.2	5.3 ± 0.3	17.0 ± 0.8
	CF	25.1 ± 1.2	4.0 ± 0.2	10.1 ± 0.4	13.0 ± 0.6
	SF	19.0 ± 0.9	8.0 ± 0.4	10.2 ± 0.4	4.1 ± 0.2

PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction, SF - Saponins fraction, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

Table 5.8 Antimicrobial activity with respect to tetracycline as positive control, (mean ± SD), (n = 3).

Analyte	Fraction	% of Activity			
		Tetracycline equivalents			
		S. aureus	*B. cereus*	*E. coli*	*S. abony*
CA	PEF	0	0	13.0 ± 0.7	0
	EAF	0	10.0 ± 0.5	13.1 ± 0.5	0
	CF	0	10.0 ± 0.5	13.1 ± 0.6	0
	SF	0	10.0 ± 0.4	13.1 ± 0.6	0
EPK	PEF	22.0 ± 1.1	0	6.0 ± 0.3	0
	EAF	17.0 ± 0.8	0	13.0 ± 0.7	0
	CF	11.0 ± 0.5	0	13.1 ± 0.6	0
	SF	17.0 ± 0.6	0	0	0
EPN	PEF	22.0 ± 1.0	0	13.0 ± 0.5	0
	EAF	11.0 ± 0.4	0	13.0 ± 0.5	0
	CF	0	0	0	0
	SF	0	0	0	11.0 ± 0.5
GC	PEF	0	0	6.0 ± 0.3	0
	EAF	6.0 ± 0.3	0	13.0 ± 0.6	0
	CF	11.0 ± 0.5	0	13.0 ± 0.7	0
	SF	11.0 ± 0.5	0	13.0 ± 0.1	11.0 ± 0.5
GCC	PEF	0	20.0 ± 1.0	13.0 ± 0.6	0
	EAF	22.0 ± 1.0	0	13.0 ± 0.5	17.0 ± 0.8
	CF	0	10.0 ± 0.5	13.0 ± 0.6	0
	SF	0	15.0 ± 0.7	19.0 ± 0.9	0
GF	PEF	0	0	13.0 ± 0.3	0
	EAF	11.0 ± 0.5	10.0 ± 0.1	13.0 ± 0.6	17.0 ± 0.8
	CF	11.0 ± 0.5	5.0 ± 0.1	6.0 ± 0.6	11.0 ± 0.5
	SF	11.0 ± 0.4	10.0 ± 0.4	13.0 ± 0.8	11.0 ± 0.5
UF	PEF	22.0 ± 1.1	10.0 ± 0.5	6.0 ± 0.3	0
	EAF	33.0 ± 1.6	5.0 ± 0.2	6.0 ± 0.3	22.1 ± 1.0
	CF	22.0 ± 1.1	5.0 ± 0.3	13.0 ± 0.6	17.1 ± 0.8
	SF	17.0 ± 0.8	10.0 ± 0.5	13.0 ± 0.6	6.0 ± 0.3

PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction, SF - Saponins fraction, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

PEF of *C. antennina* exhibited > 10 % bactericidal activity against *E. coli* (Fig. 5.6). PEF of *E. prolifera* obtained from both the locations exhibited > 22 % bactericidal activity against *S. aureus*. PEF of *G. corticata* var. *cylindrica* exhibited bactericidal activity against *B. cereus* (> 17 %) and *E. coli* (> 10 %). PEF of *G. foliifera* was active against *E. coli* (> 10 %). PEF of *U. fasciata* exhibited bactericidal activity against *S. aureus* (> 22 %)

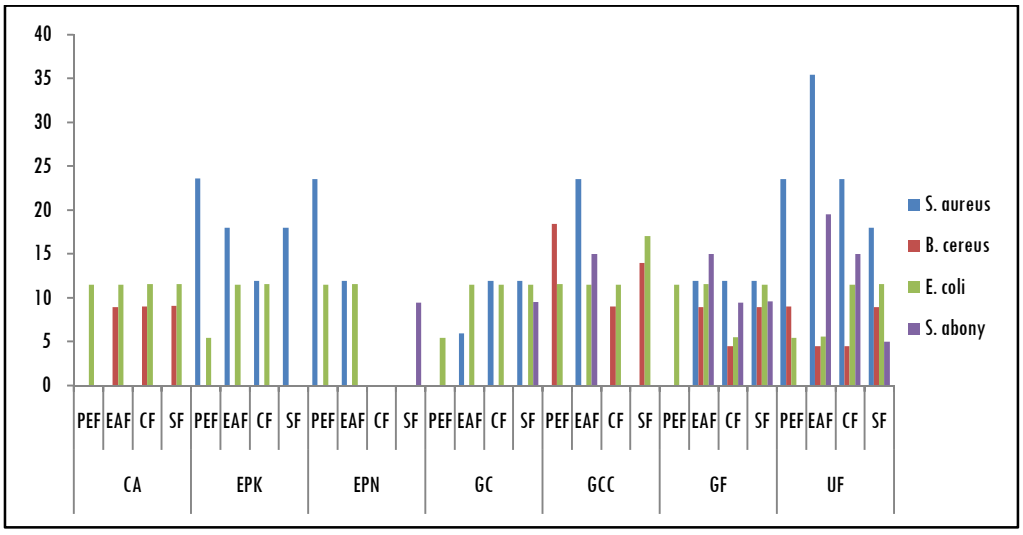


Fig. 5.6 Graphical representation of the antimicrobial activity of the total saponins fraction (SF), chloroform fraction (CF), ethyl acetate fraction (EAF), petroleum ether fraction (PEF), CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

EAF of *C. antennina* was active against *E. coli* (> 10 %). Greater than 10 % bactericidal activity against *S. aureus* and *E. coli* was exhibited by EAF of both *E. prolifera*. EAF of G. corticata exhibited > 10 % bactericidal activity against *E. coli*. EAF of *G. corticata* var. *cylindrica* exhibited > 22 % bactericidal activity against *S. aureus* and > 10 % activity against *E. coli* and *S. abony*. EAF of *G. foliifera* exhibited > 10 % bactericidal activity against *S. aureus*, *E. coli* and *S. abony*. EAF of *U. fasciata* exhibited > 33 % activity against *S. aureus*. Greater than 17 % activity was observed against *S. abony*.

CF of *C. antennina* exhibited > 10 % bactericidal activity against *E. coli*. CF of *E. prolifera* obtained from Kayamkulam location exhibited > 10 % activity against *S. aureus* and *E. coli*. Similar activity was exhibited by the CF of *G. corticata*. CF of *G. corticata* var. *cylindrica* exhibited > 10 % bactericidal activity against *E. coli*. CF of *G. foliifera* exhibited > 10 % activity against *S. aureus*. CF of *U. fasciata* exhibited > 22 % of bactericidal activity against *S. aureus* and > 10 % activity against *E. coli* and *S. abony*.

SF of *C. antennina* exhibited > 10 % bactericidal activity against *E. coli*. SF of *E. prolifera* obtained from Kayamkulam location exhibited > 17 % activity against *S. aureus*. SF of *G. corticata* exhibited > 10 % activity against *S. aureus* and *E. coli*. Greater than 13 % activity was observed in the SF of *G. corticata* var. *cylindrica* against *B. cereus* and *E. coli*. SF of *G. foliifera* exhibited > 10 % activity against *S. aureus* and *E. coli*. SF of *U. fasciata* exhibited > 17 % activity against *S. aureus* and > 10 % activity against *E. coli*.

The present result was an in-depth analysis on bactericidal activity after solvent speciation. The total antibacterial activity observed where similar to the previous reports. The ethyl acetate crude extract of *Caulerpa* sp. showed appreciable antibacterial inhibition activity on the strains of *E. coli, S. aureus, Streptococcus* sp. and *Salmonella* sp. (Nagappan & Vairappan, 2014). Methanolic extracts of the Irish seaweed *Himanthalia elongata* had high antioxidant properties (Gupta et al., 2012). The methanolic extract of *Sargassum marginatum* was found to have broad weedicidal and antialgal activity (Manilal et al., 2010a). 14 seaweeds collected from the southwest coast of India, which included *G. corticata, C. antennina, U. fasciata* etc., showed the extracts of toluene and methanol (1:3) with high antibacterial activity. The extract of *G. corticata* was

highly active against Gram - negative pathogen, *Proteus mirabilis*. The results showed better response to Gram - negative than Gram - positive due to the lipophylic nature of the active components in the crude extract (Shanmughapriya et al., 2008). Methanolic extracts of 15 red seaweed samples collected from the southwest coast of India exhibited *in vitro* antimicrobial activity against Gram - negative bacteria, like *E. coli*, and Gram - positive bacteria like *S. aureus*. Zone inhibition technique was used which was directly related to the concentration of extracts (Manilal et al., 2009). Potent antimicrobial activity was exhibited by the extracts of *Enteromorpha linza* on Gram - negative pathogens, *Prevotella intermedia* and *Porphyromonas gingivalis* which initiates periodontisis, a chronic inflammatory disease, without any side effects (Park et al., 2013).

Methanolic extract of *Laurencia brandenii,* collected from the south west coast of India exhibited antibacterial activity against *Colwellia* sp. and *Pseudoalteromonas* sp. (Manilal et al., 2010b). The petroleum ether fraction of *Laminaria japonica* was observed to be active against *Clavibacter michiganensis* (Cai et al., 2014). All the solvent extract fractions viz., benzene, acetic acid, hexane, dimethyl sulfoxide, diethyl ether and chloroform of *Sargassum longifolium* exhibited good antibacterial activities against *Streptococcus* sp., *Proteus* sp., *Bacillus subtilis*, *Klebsiella pneumonia* and *Enterococci* sp. (Kumar et al., 2014). Chlorophyta, Rhodophyta and Phaeophyta collected from the Kerala coast, were reported to have bactericidal activities against the Gram - negative bacteria (*E. coli, Proteus mirabilis* and *Pseudomonas aeruginosa*) and Gram - positive bacteria (*E. faecalis*, non-heamolytic *Streptococcus, M. luteus* and *S. epidermidis*) (Shanmughapriya et al., 2008). Non polar extracts of the red algae *G. corticata* was reported to inhibit the growth of *B. subtilis* (Lima-Filho et al.,

2002). Methanol extracts of Phaeophyta *Padina pavonica*, showed antibacterial activity against *Bacillus* (González del Val et al., 2001). Seaweeds were earlier reported to have antimicrobial activities against Gram - positive bacteria (Gerwick et al., 1985; Ballatine et al., 1987).

5.3.6 Chemical Composition

The four fractions (PEF, EAF, CF and SF) extracted were observed to have good antioxidant and antimicrobial activities. Hence, these bioactive fractions were subjected to GC-MS analysis, which revealed the major chemical compositions of these extracts (Table 5.9). Typical GC chromatogram is given in Fig. 5.7 and the MS pattern of some of the major compounds in comparison with the library data from NIST MS search 2.0, is given in Fig. 5.8. In general a wide range of alkanes viz., short chained (Fig. 5.9), medium chained (Fig. 5.10) and long chained (Fig. 5.11) alkanes, alkenes (Fig. 5.12), alkynes (Fig. 5.13), alcohols (Fig. 5.14), sterols (Fig. 5.15), SFAs (Fig. 5.16), MUFAs (Fig. 5.17) and PUFAs (Fig. 5.18). PEF of Chlorophyta exhibited alkanes, alkenes, alkynes and alcohols. High alkane content among the Chlorophyta was seen in PEF of *E. prolifera* collected from the Kayamkulam location (C-8 to C-43; 0.37 %) and *E. prolifera* collected from the Njarakkal location (C-8 to C-44; 0.45 %). In Rhodophyta, *G. foliifera* had 0.21 % of alkanes (C-11 to C-32). Alkenes were observed in the PEF of *E. prolifera* collected from the Njarakkal location (C-7 to C-17; 0.91 %). Alkynes were seen in PEF of *E. prolifera* collected from the Njarakkal location (C-18, C-19; 0.04 %) and *G. foliifera* (C-20; 0.02 %). Five alcohols were seen in the PEF of *E. prolifera* collected from the Njarakkal location (C-10 to C-20; 0.12 %). Phytol was the most common alcohol observed in all the analysed seaweeds.

Table 5.9 GC-MS profiling of the four extract fractions of nine seaweeds.

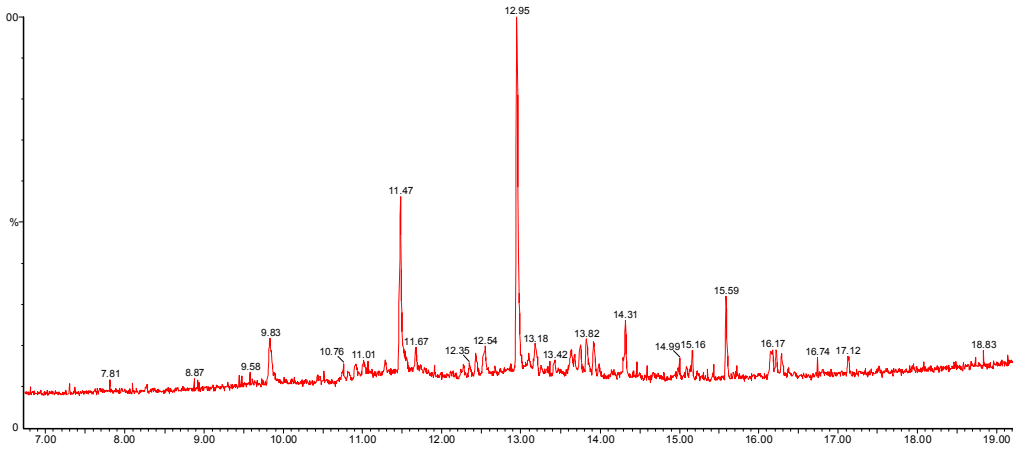
Seaweed	Fraction	Common name	Chemical group	Compounds	Conc. (%)
CA	PEF	Alkanes	C-13	2,6,8-trimethyl-decane	0.038
			C-13	6-ethyl-2-methyl-decane	0.024
			C-19	2,6-dimethyl-heptadecane	0.028
			C-19	Nonadecane	0.024
			C-21	2,6,10,14-tetramethyl-heptadecane	0.045
			C-21	2,6,10,15-tetramethyl-heptadecane	0.021
		Alcohols	C-15	(Z)6,(Z)9-pentadecadien-1-ol	0.025
			C-20	Phytol	0.023
	CF	SFAs	C-13	Tridecanoic acid	0.069
			C-15	Pentadecanoic acid	0.066
			C-16	4,8,12-trimethyl-tridecanoic acid	0.085
			C-16	Hexadecanoic acid	0.071
			C-16	14-methyl-pentadecanoic acid	0.060
			C-17	Heptadecanoic acid	0.065
			C-18	Octadecanoic acid	0.064
		MUFAs	C-16:1 ω-7	9-hexadecenoic acid	0.067
			C-18:1 ω-3	15-octadecenoic acid	0.069
		PUFAs	C-16:4 ω-3	4,7,10,13-hexadecatetraenoic acid	0.060
			C-18:2 ω-6	(Z,Z)-9,12-octadecadienoic acid	0.071
			C-18:2 ω-7	8,11-octadecadienoic acid	0.063
			C-18:2 ω-7	9,11-octadecadienoic acid	0.064
			C-20:4 ω-6	5,8,11,14-eicosatetraenoic acid	0.069
			C-20:5 ω-3	cis-5,8,11,14,17-eicosapentaenoic acid	0.088
EPK	PEF	Alkanes	C-08	Octane	0.023
			C-11	4,5-dimethyl-nonane	0.022
			C-13	2,6,7-trimethyl-decane	0.022
			C-13	2,5,6-trimethyl-decane	0.024
			C-13	2,4,6-trimethyl-decane	0.022
			C-18	7-methyl-hexadecane	0.022
			C-19	Nonadecane	0.029
			C-21	2,6,10,14-tetramethyl-heptadecane	0.020
			C-21	2,6,10,15-tetramethyl-heptadecane	0.028
			C-22	Docosane	0.022
			C-22	2,4-dimethyl-eicosane	0.021
			C-24	Cyclotetracosane	0.022
			C-26	3-ethyl-5-(2-ethylbutyl)-octadecane	0.019
			C-26	11-(1-ethylpropyl)-heneicosane	0.019
			C-28	Octacosane	0.023
			C-43	Tritetracontane	0.029
	EAF	Sterol	C-18	Estra-1,3,5(10)-tri-en-17-β-ol	0.020
			C-24	Scillarenin	0.029
			C-27	Cholesta-3,5-diene	0.030
	CF	SFAs	C-13	Tridecanoic acid	0.060
			C-14	Tetradecanoic acid	0.065
			C-16	Hexadecanoic acid	0.051
			C-17	Heptadecanoic acid	0.049
			C-17	15-methyl-hexadecanoic acid	0.046
			C-18	Octadecanoic acid	0.046
			C-27	Heptacosanoic acid	0.049
		MUFAs	C-16:1 ω-5	(Z)-11-hexadecenoic acid	0.049
			C-18:1 ω-9	(Z)-9-octadecenoic acid	0.043
		PUFAs	C-18:2 ω-6	9,12-octadecadienoic acid	0.052
			C-22:2 ω-5	6Z,8Z-dodecadienoic acid	0.051
EPN	PEF	Alkanes	C-08	3-methyl-heptane	0.027

			C-08	3,4-dimethyl-hexane	0.030
			C-09	Nonane	0.025
			C-10	2,6-dimethyl-octane	0.023
			C-12	5,6-dimethyl-decane	0.032
			C-13	4,7-dimethyl-undecane	0.041
			C-13	2,10-dimethyl-undecane	0.020
			C-13	2,6,6-trimethyl-decane	0.020
			C-15	2,6,11-trimethyl-dodecane	0.030
			C-18	2-methyl-heptadecane	0.022
			C-19	2,6-dimethyl-heptadecane	0.021
			C-21	3-methyl-eicosane	0.041
			C-21	2,6,10,14-tetramethyl-heptadecane	0.027
			C-24	2-methyl-tricosane	0.033
			C-24	2,21-dimethyl-docosane	0.020
			C-26	11-(1-ethylpropyl)-heneicosane	0.021
			C-44	Tetratetracontane	0.020
		Alkenes	C-07	2,4-dimethyl-pent-2-ene	0.021
			C-10	Z-(6)-tridecene	0.024
			C-14	2,6,10-trimethyl-undeca-1,3-diene	0.024
			C-17	6,9-heptadecadiene	0.022
		Alkynes	C-18	1-octadecyne	0.022
			C-19	2,4-nonadiyne	0.022
		Alcohols	C-10	3,7-dimethyl-2-octen-1-ol	0.022
			C-10	3,7-dimethyl-6-octen-1-ol	0.019
			C-14	E-11,13-tetradecadien-1-ol	0.021
			C-16	1-hexadecanol	0.027
			C-20	Phytol	0.026
	EAF	Sterols	C-30	26,26-dimethyl-5,23ergostadien-3β-ol	0.030
	CF	SFAs	C-13	Tridecanoic acid	0.022
			C-15	2-methyl-tetradecanoic acid	0.023
			C-16	Hexadecanoic acid	0.020
			C-17	15-methyl-hexadecanoic acid	0.021
		MUFAs	C-16:1 ω-7	9-hexadecenoic acid	0.066
			C-16:1 ω-9	7-hexadecenoic acid	0.074
			C-18:1 ω-7	11-octadecenoic acid	0.070
		PUFAs	C-16:4 ω-3	4,7,10,13-hexadecatetraenoic acid	0.050
			C-18:2 ω-6	10-trans-12-cis-octadecadienoic acid	0.044
			C-18:3 ω-3	(Z,Z,Z)-9,12,15-octadecatrienoic acid	0.044
			C-18:4 ω-3	Stearidoic acid	0.051
			C-20:3 ω-3	11,14,17-eicosatrienoic acid	0.060
			C-20:4 ω-6	5,8,11,14-eicosatetraenoic acid	0.041
GC	PEF	Alkanes	C-13	6-ethyl-2-methyl-decane	0.024
			C-21	2,6,10,14-tetramethyl-heptadecane	0.024
			C-26	11-(1-ethylpropyl)-heneicosane	0.032
		Alcohols	C-20	3,7,11,15-tetramethyl-2-hexadecen-1-ol	0.025
	EAF	Sterols	C-29	Cholest-5-en-3-ol-3β-acetate	0.054
	CF	SFAs	C-13	Tridecanoic acid	0.067
			C-16	Hexadecanoic acid	0.069
			C-16	14-methyl-pentadecanoic acid	0.085
		MUFAs	C-17:1 ω-9	7-hexadecenoic acid	0.066
			C-18:1 ω-8	10-octadecenoic acid	0.071
GCC	PEF	Alkanes	C-19	2-methyl-octadecane	0.018
			C-21	2,6,10,14-tetramethyl-heptadecane	0.024
		Alcohols	C-18	E,Z-2,13-octadecadien-1-ol	0.023
			C-20	Phytol	0.026
	CF	SFAs	C-13	Tridecanoic acid	0.030

			C-14	Tetradecanoic acid	0.027
			C-16	14-methyl-pentadecanoic acid	0.025
			C-16	Hexadecanoic acid	0.032
			C-27	Heptacosanoic acid	0.027
		MUFAs	C-18:1 ω-9	Z-9-octadecenoic acid	0.032
GF	PEF	Alkanes-10nos	C-11	Undecane	0.023
			C-13	6-ethyl-2-methyl-decane	0.021
			C-13	2,6,6 trimethyl-decane	0.021
			C-13	2,5,9 trimethyl-decane	0.020
			C-17	3-methyl-hexadecane	0.022
			C-18	2-methyl-heptadecane	0.021
			C-19	2,6 dimethyl-heptadecane	0.020
			C-21	2,6,10,14 tetra-methyl-heptadecane	0.022
			C-24	2-methyl-tricosane	0.020
			C-32	dotriacontane	0.021
		Alkynes	C-20	1-eicosyne	0.022
		Alcohols	C-14	1-tetradecanol	0.020
			C-20	Phytol	0.021
	EAF	Sterols	C-27	Cholesta-3,5-diene	0.065
			C-27	Cholesterol	0.067
			C-29	Cholest-5-en-3-ol-3β-acetate	0.064
	CF	SFAs	C-08	2-methyl heptanoic acid	0.088
			C-13	Tridecanoic acid	0.069
			C-16	14-methyl pentadecanoic acid	0.060
			C-17	15-methyl hexadecanoic acid	0.071
			C-27	Heptacosanoic acid	0.063
		MUFAs	C-14:1 ω-3	trans-11-tetradecenoic acid	0.074
			C-16:1 ω-7	9-hexadecenoic acid	0.064
			C-17:1 ω-9	7-hexadecenoic acid	0.066
			C-18:1 ω-9	9-octadecenoic acid	0.069
		PUFAs	C-25:2	10,12-pentacosadiynoic acid	0.070
UF	PEF	Alkanes	C-13	2,5,6-trimethyldecane	0.024
			C-13	2,6,7-trimethyl decane	0.021
			C-13	6-ethyl-2-methyl-decane	0.030
			C-16	2-methyl-pentadecane	0.023
			C-19	2,6-dimethyl-heptadecane	0.021
			C-19	Nonadecane	0.024
			C-20	Eicosane	0.023
			C-21	2,6,10,14-tetramethyl-heptadecane	0.022
			C-24	Tetracosane	0.020
			C-26	7-hexyl-eicosane	0.024
			C-30	Squalene	0.022
			C-34	11-decyl-tetracosane	0.020
			C-34	Tetratriacontane	0.022
			C-44	Tetratetracontane	0.020
		Alkene	C-24	2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene	0.024
			C-35	17-pentatriacontene	0.019
		Alcohol	C-14	E-11,13-tetradecadien-1-ol	0.032
			C-20	Phytol	0.022
	EAF	Sterols	C-27	Cholesterol	0.049
			C-29	Gamma-sitosterol	0.047
			C-29	Fucosterol	0.044
			C-30	Cholest-5-en-3-ol,24propylidene-3β	0.042
	CF	SFAs	C-13	Tridecanoic acid	0.042
			C-16	Hexadecanoic acid	0.061
			C-16	14-methyl-pentadecanoic acid	0.052
		MUFAs	C-12:1	11-dodecenoic acid	0.048

	C-16:1 ω-7	9-hexadecenoic acid	0.045
	C-18:1 ω-9	Z-9-octadecenoic acid	0.049
PUFAs	C-18:2 ω-3	Z,Z-9,15-octadecadienoic acid	0.045
	C-20:3 ω-3	11,14,17-eicosatrienoic acid	0.024

PEF - Petroleum ether fraction, EAF - Ethyl acetate fraction, CF - Chloroform fraction, SF - Saponins fraction, ω - omega fatty acid, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera*, UF - *U. fasciata*, SFAs - saturated fatty acids, MUFAs - monounsaturated fatty acids and PUFAs - polyunsaturated fatty acids.



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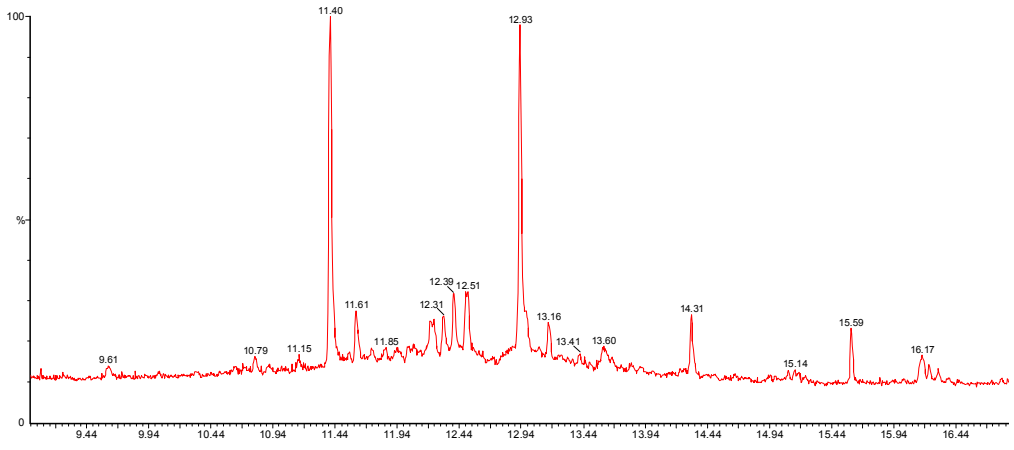

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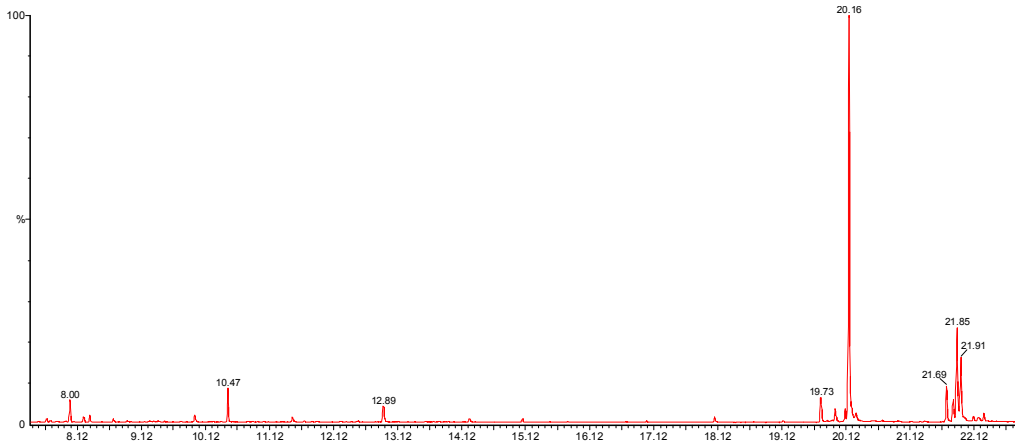
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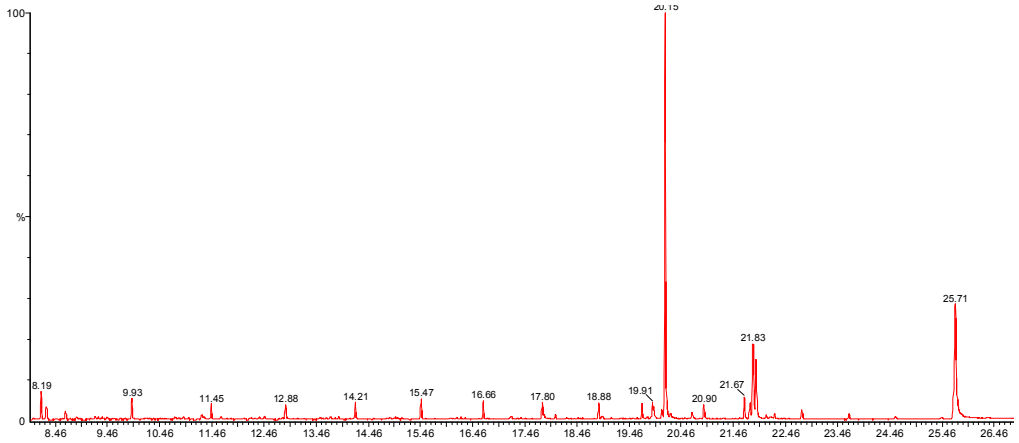
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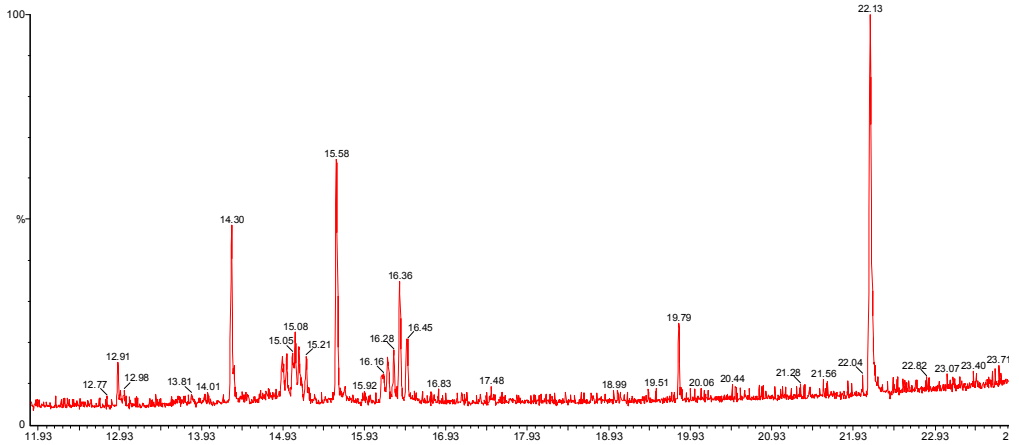
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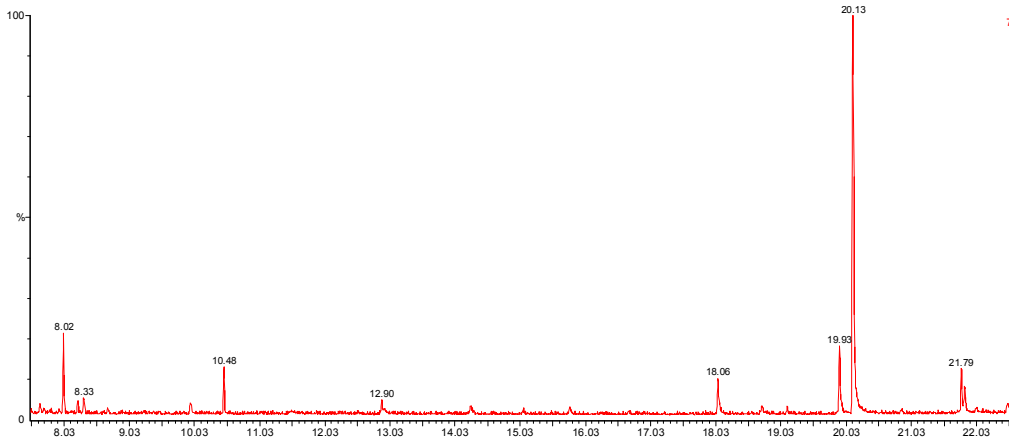

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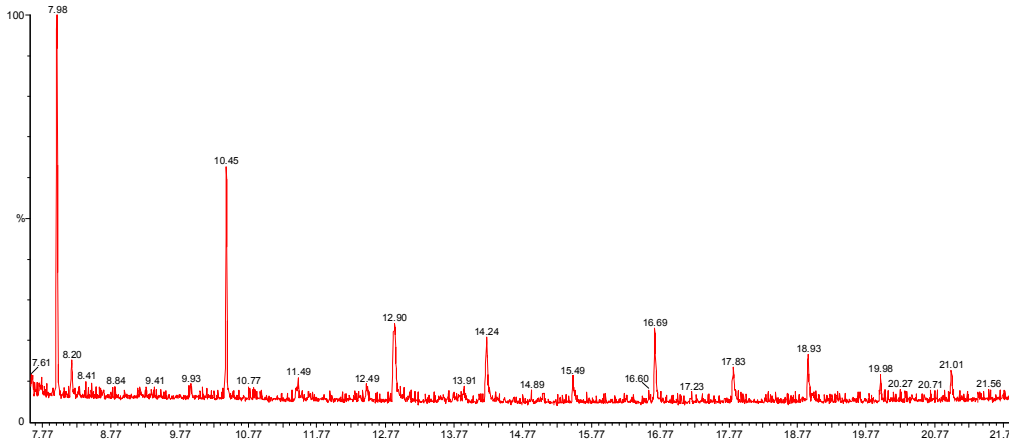

G

Fig. 5.7 Typical GC-MS Total ion chromatogram (TIC) of the nine seaweeds

A - TIC of petroleum ether fraction of *C. antennina*

B - TIC of petroleum ether fraction of *E. prolifera* obtained from Kayamkulam

C - TIC of chloroform fraction of *E. prolifera* obtained from Njarakkal

D - TIC of chloroform fraction of *U. fasciata*

E - TIC of ethyl acetate fraction of *G. corticata*

F - TIC of ethyl acetate fraction of *G. corticata* var. *cylindrica*

G - TIC of petroleum ether fraction of *G. foliifera*

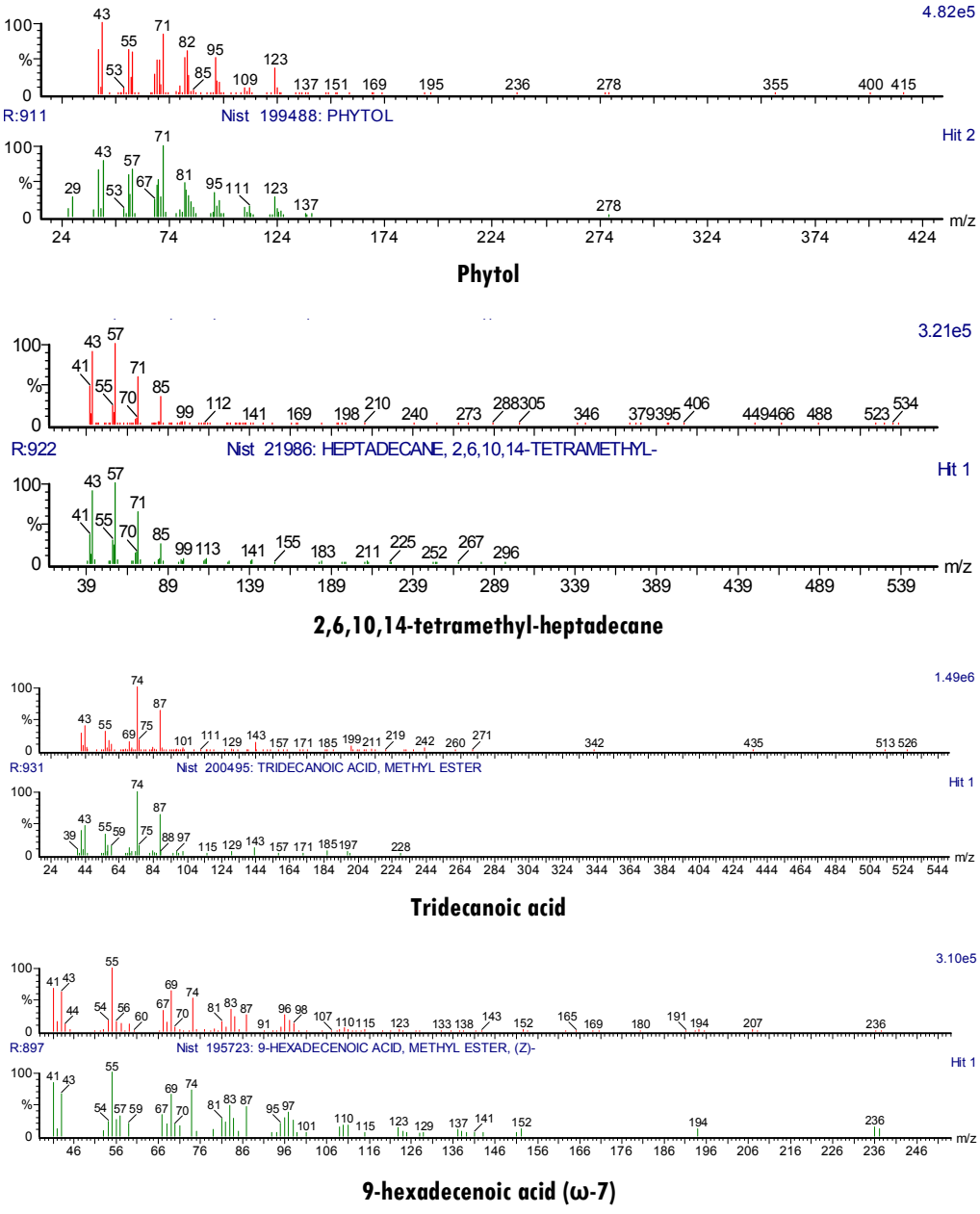

Fig. 5.8 Typical MS pattern of some of the major compounds detected in the nine seaweeds (top) in comparison with the standard MS data taken from NIST MS search 2.0 library (bottom).

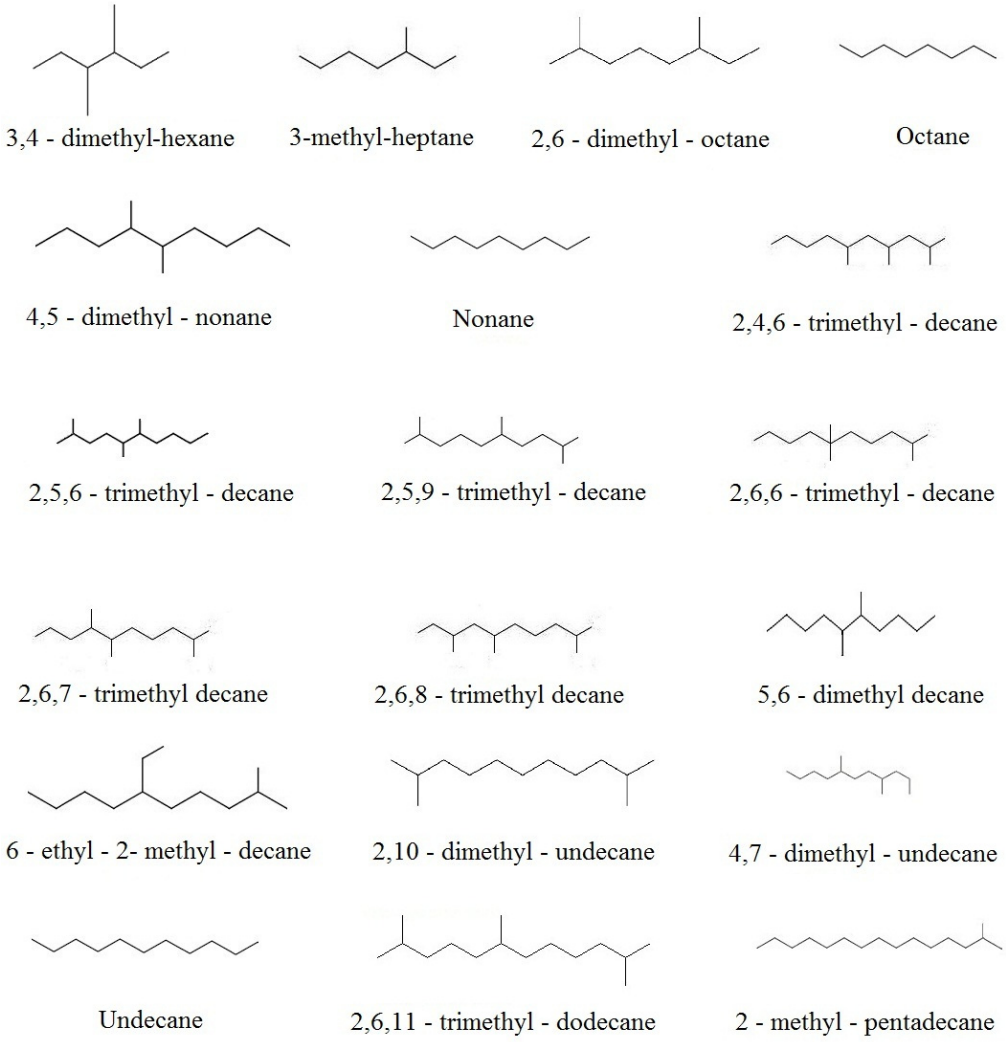

Fig. 5.9 Short chained alkanes observed in the current study by using GC-MS analyses.

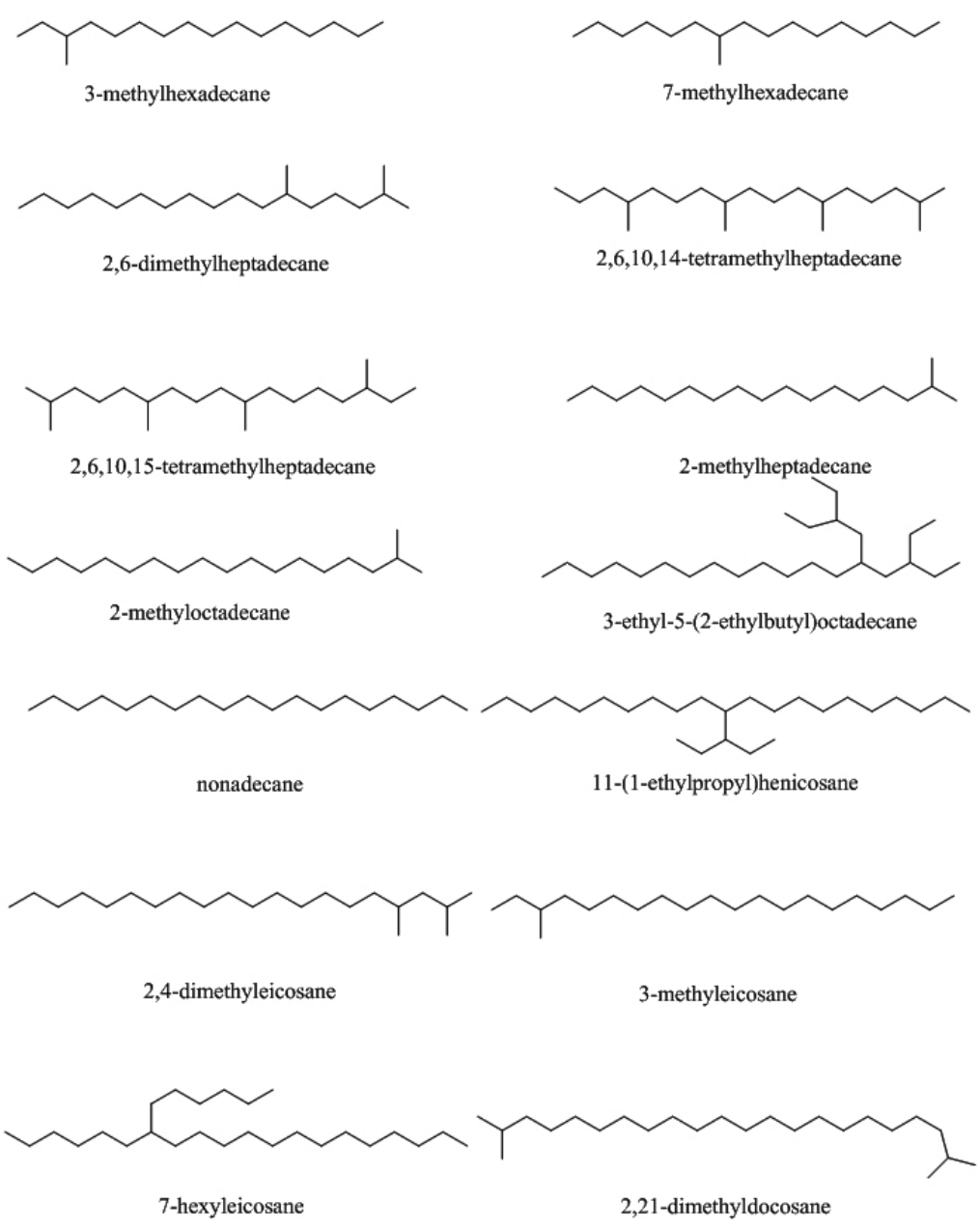

Fig. 5.10 Medium chained alkanes observed in the current study by using GC-MS analyses.

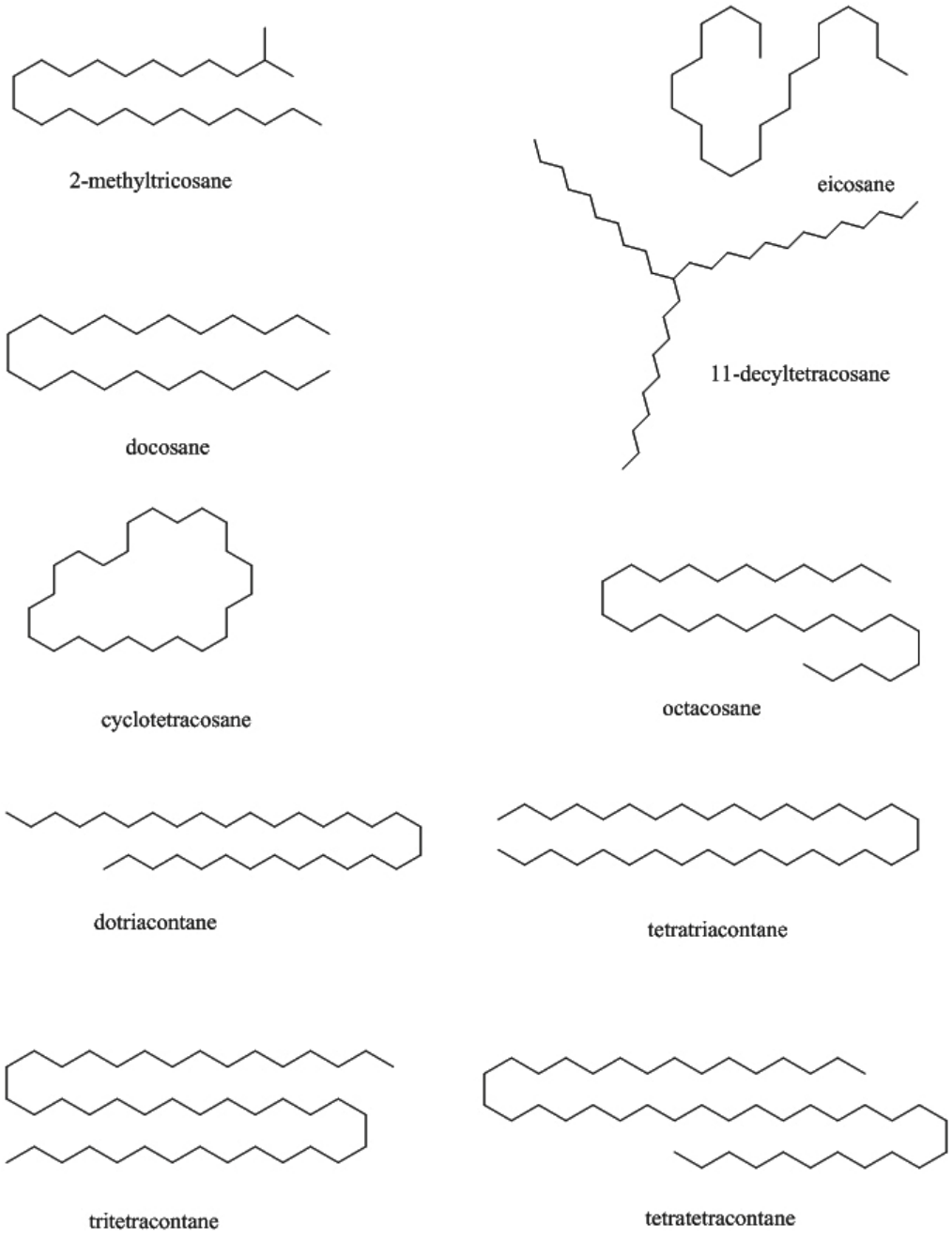

Fig. 5.11 Long chained alkanes observed in the current study by using GC-MS analyses

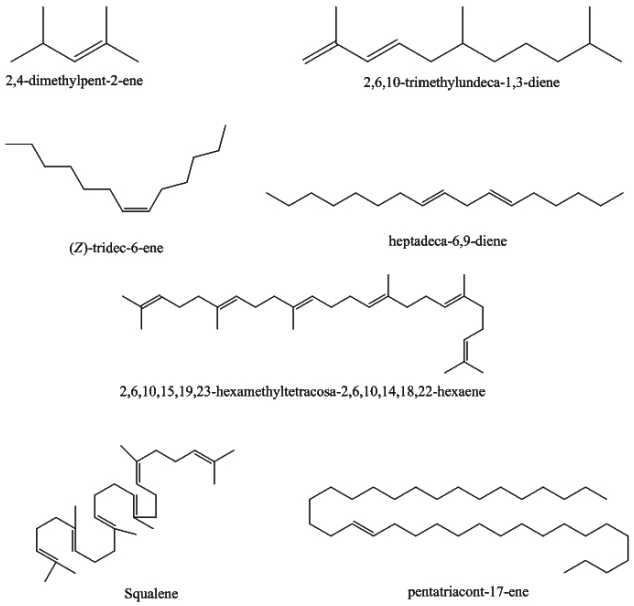

Fig. 5.12 Major alkenes observed in the current study by using GC-MS analyses

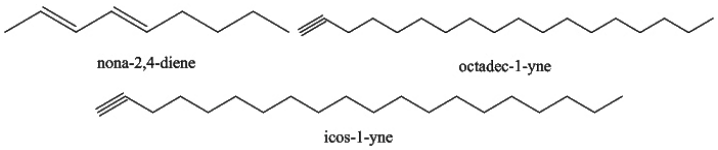

Fig. 5.13 Major alkynes observed in the current study by using GC-MS analyses

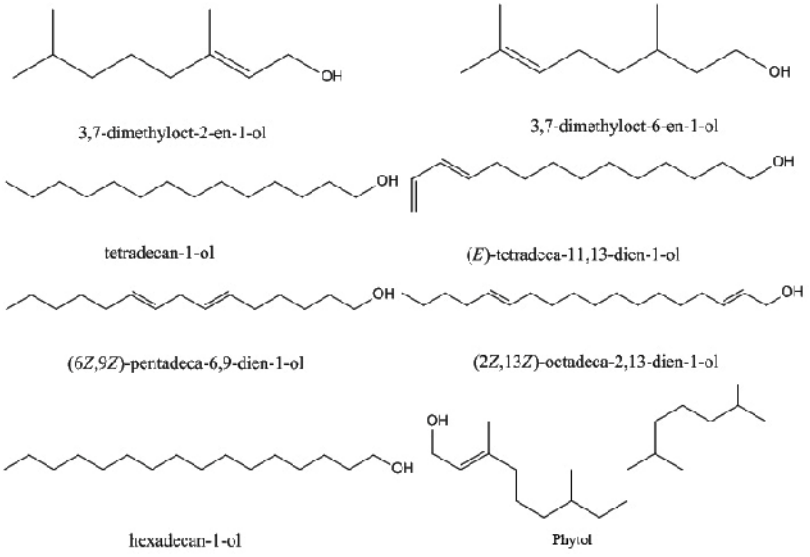

Fig. 5.14 Major alcohols observed in the current study by using GC-MS analyses.

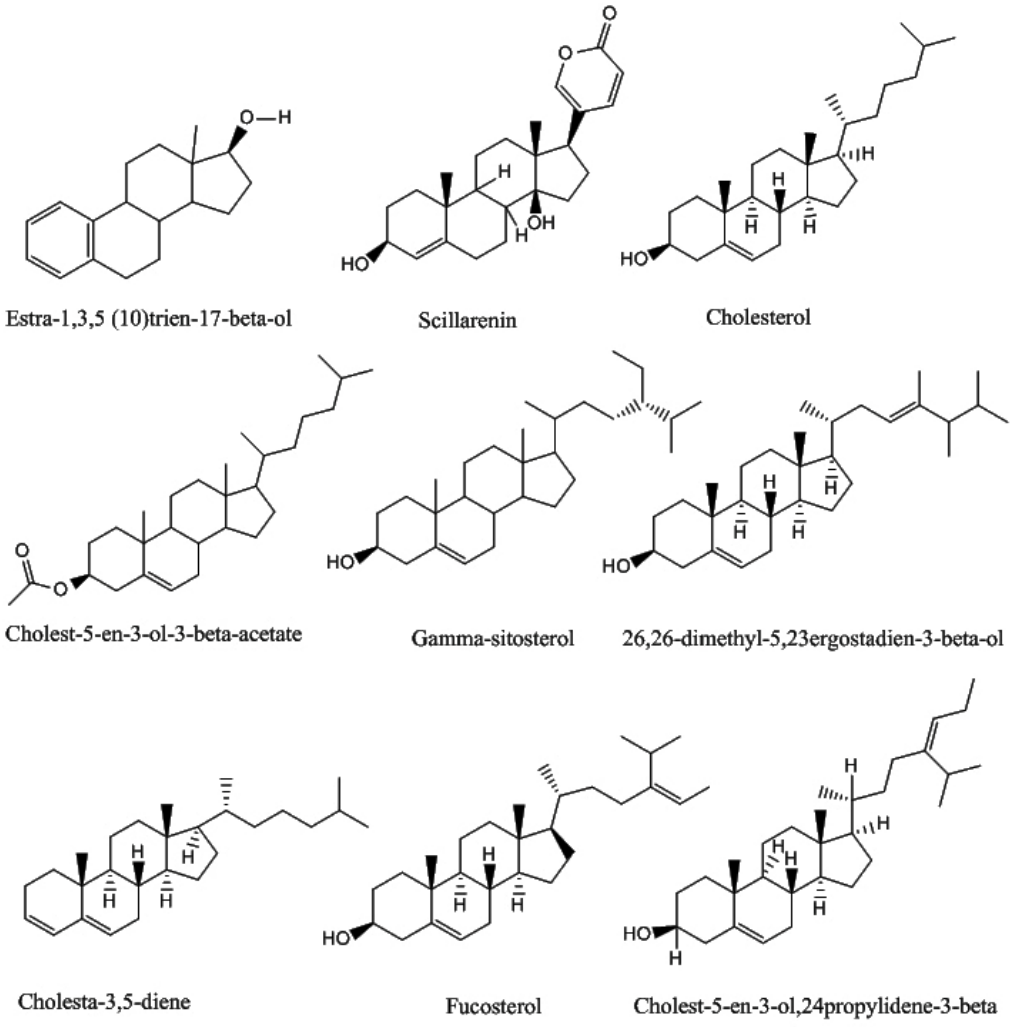

Fig. 5.15 Major sterols observed in the current study by using GC-MS analyses.

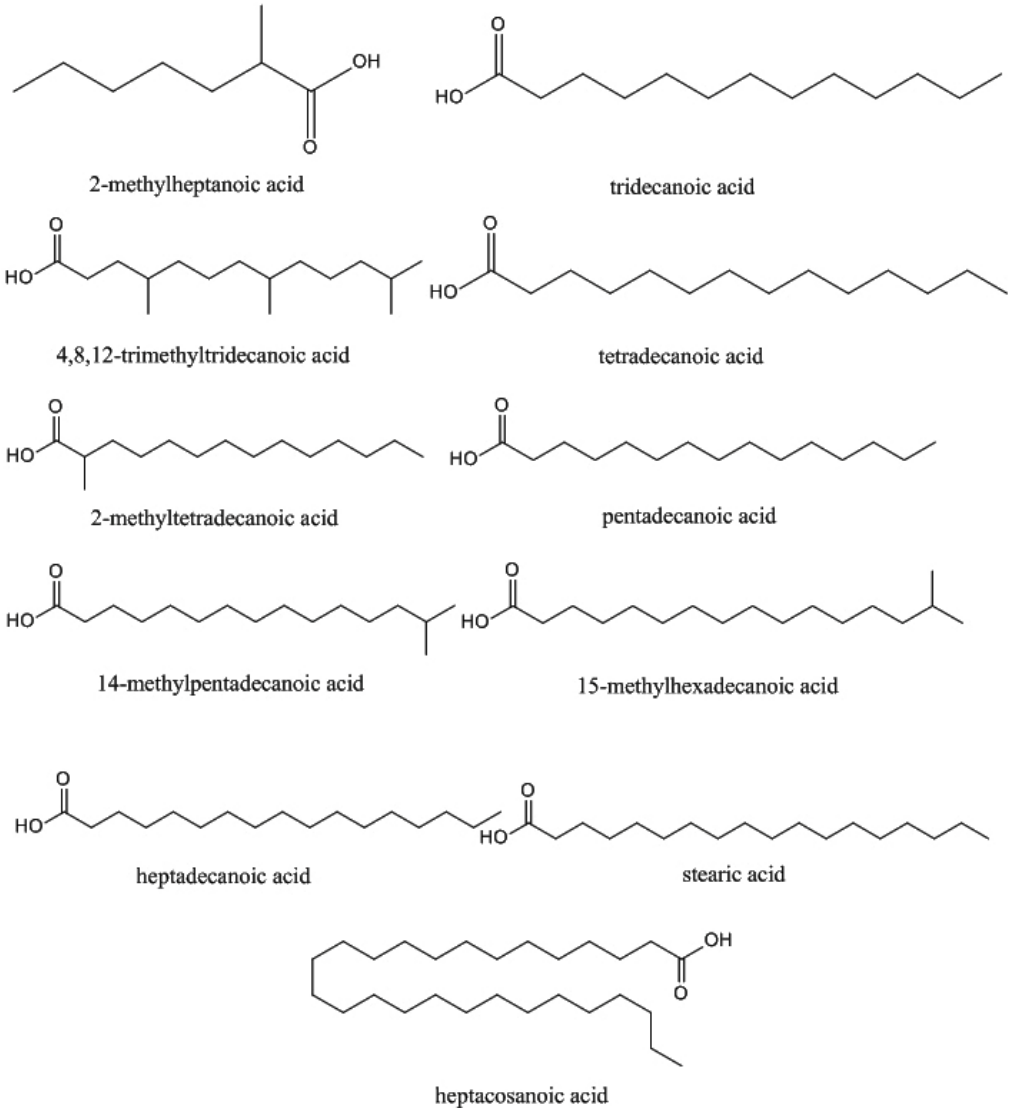

Fig. 5.16 Major saturated fatty acids observed in the current study by using GC-MS analyses.

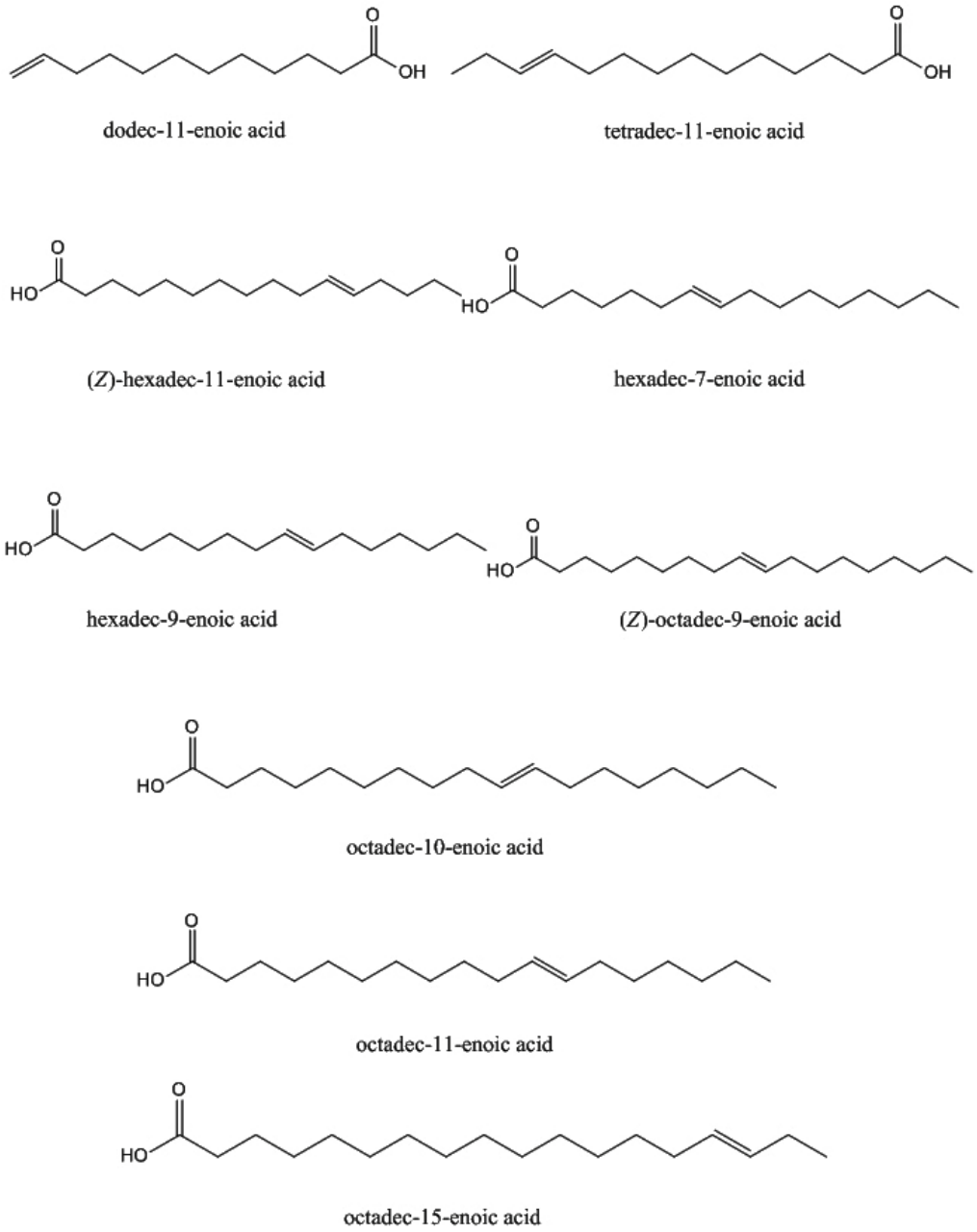

Fig. 5.17 Major mono unsaturated fatty acids observed in the current study by using GC-MS analyses.

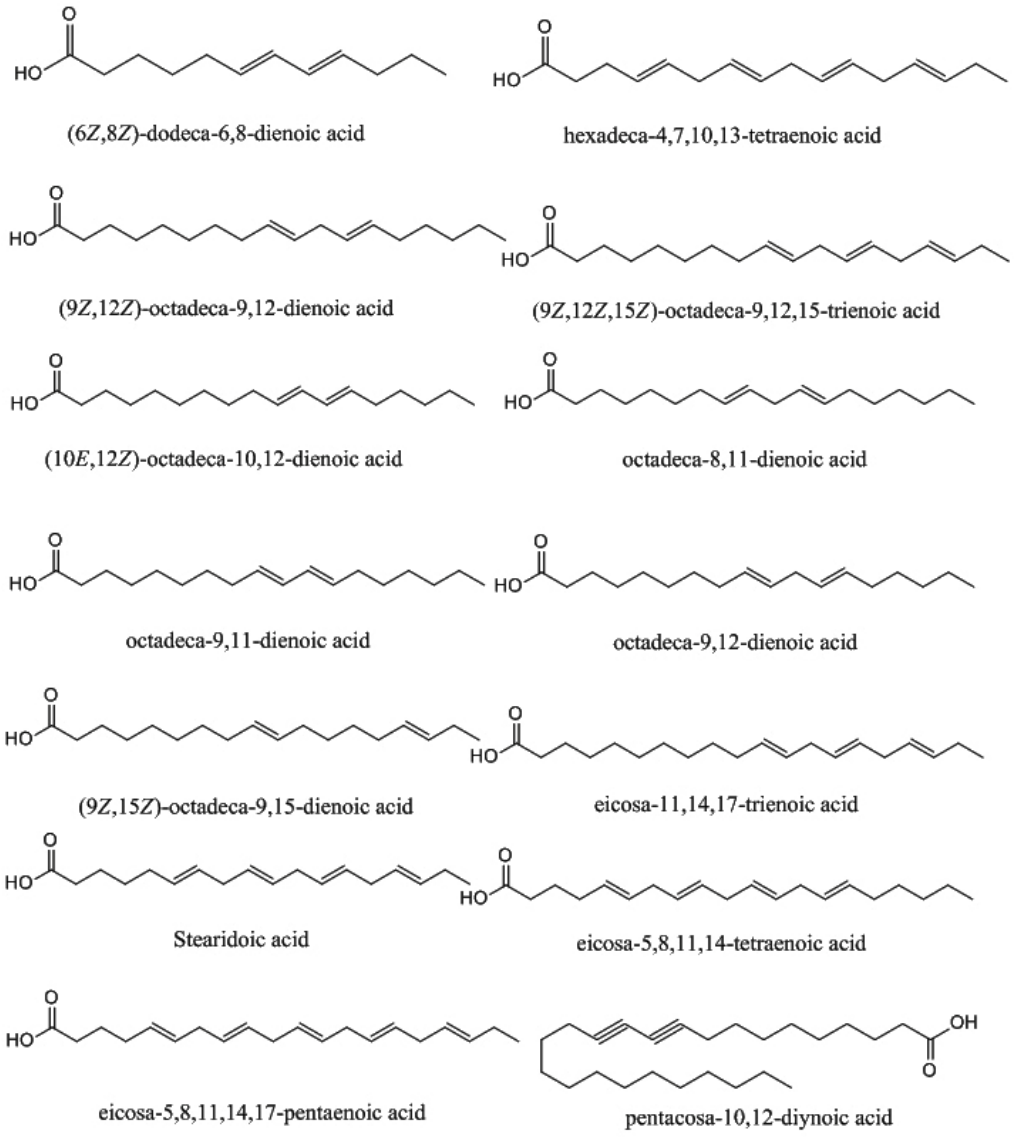

Fig. 5.18 Major poly unsaturated fatty acids observed in the current study by using GC-MS analyses.

EAF of the analysed seaweeds exhibited the presence of sterols with cholesterol and its analogues as the common occurrence. Sterols ranged from C-17 to C-30 in Chlorophyta. 0.18 % of sterols was seen in *U. fasciata*. 0.20 % of C-27 to C-29 sterols was seen in *G. foliifera* belonging to the Rhodophyta family. No specific peaks were observed upon the direct GC-MS injection of CF. Hence, it was subjected to esterification to yield the FAMEs. FAMEs were detected in the GC-MS and were found to possess with SFAs, MUFAs and PUFAs (Table 5.9). SFAs (C-13 to C-18) were seen highest in Chlorophyta with 0.48 % in *C. antennina*. *E. prolifera* collected from the Njarakkal location exhibited low SFAs (C-13 to C-16) with 0.09 %. Among the Rhodophyta, *G. foliifera* had the highest SFAs with 0.35 % (C-8 to C-27).

Relative concentrations of SFAs (Table 5.10) were observed to be high in *G. corticata* var. *cylindrica* (81.50 %) and *E. prolifera* obtained from Kayamkulam location (65.24 %). Comparable contents were seen in *G. corticata* (61.73 %). Low contents of SFAs were observed in *E. prolifera* obtained from Njarakkal location (14.68 %). Comparable contents were also observed in between *G. foliifera* (50.58 %), *C. antennina* (46.56 %) and *U. fasciata* (42.35 %). MUFAs were observed to be almost comparable in between *E. prolifera* obtained from Njarakkal location (35.84 %), *G. corticata* (38.27 %), *U. fasciata* (38.80 %) and *G. foliifera* (39.34 %). *G. corticata* var. *cylindrica* (18.50 %) and *E. prolifera* obtained from Kayamkulam location (16.40 %) also exhibited comparable MUFAs contents. Least was observed in *C. antennina* (13.19 %). PUFAs were observed only in the analysed five seaweeds (*C. antennina, E. prolifera* obtained from both of the locations, *G. foliifera* and *U. fasciata*). Among these, highest content was observed in *E. prolifera* obtained from Njarakkal location (49.48 %) and least in *G. foliifera* (10.08 %).

Comparable PUFAs contents were seen in between *E. prolifera* obtained from Kayamkulam location (18.36 %) and *U. fasciata* (18.85 %).

Pharmacologically important unsaturated fatty acids were seen highest in Chlorophyta. *E. prolifera* collected from the Njarakkal location had 0.21 % of MUFAs (C16:1 and C18:1) and 0.29 % of PUFAs (C16:4, C18:2, C18:3, C18:4, C20:3 and C20:4). Omega fatty acids were observed in majority with 41 % ω-3, 17 % ω-6, 27 % ω-7 and 15 % ω-9. Similarly, the *C. antennina* had 0.14 % MUFAs (C16:1 and C18:1) and 0.41 % PUFAs (C16:4, C18:2, C20:4 and C20:5). The fatty acids observed were also belonging to the omega fatty acids with 39 % ω-3, 26 % ω-6 and 35 % ω-7. 0.14 % of MUFAs (C12:1, C16:1 and C18:1) and 0.07 % of PUFAs (C18:2 and C20:3) were observed in *U. fasciata* with 23 % ω-3, 23 % ω-7, 21 % ω-9 and 23 % ω-11 fatty acids. *E. prolifera* collected from the Kayamkulam location was observed to have low contents of MUFAs and PUFAs. Rhodophyta had relatively low amounts of MUFAs and PUFAs. 0.27 % MUFAs (C14:1, C16:1, C17:1 and C18:1) (27 % ω-3, 23 % ω-7 and 50 % ω-9 fatty acids) and 0.07 % PUFAs (C25:2) were observed in *G. foliifera*. 0.03 % of MUFAs (C18:1, ω-9) was observed in *G. corticata* var. *cylindrica*. *G. corticata* possessed 0.14 % of MUFAs with 48 % C17:1 ω-9 and 52% C18:1 ω-8 fatty acids.

The CF of both *E. prolifera* collected from the Njarakkal location and *C. antennina* had the ω-6/ω-3 ratio (Table 5.10) within the WHO prescribed standards (<10) (Kumar et al., 2011). *C. antennina* (0.65) and *E. prolifera* obtained from Njarakkal location (0.41) had the omega fatty acids content as recommended by WHO. The ω-6/ω-3 ratio of the current study was comparable to the ratios established in previous studies in the same field. The ratio of ω-6 to ω-3 was also observed to be 0.71 in *Saccorhiza polyschides*, 0.81 to 1.32 in *Himanthalia elongata*, 0.83 in *Laminaria ochroleuca*, 0.49 in *Undaria*

pinnatifida, 0.13 in *Palmaria* sp., 1.21 in *Porphyra* sp. (Sánchez-Machado et al., 2004), 0.68 in *Nereocystis luetkeana*, 0.46 in *Porphyra perforata*, 1.38 in *Fucus distichus*, 1.49 in *Fucus* sp., 0.40 in *Pterigophora* sp., 0.11 in *Ulva fenestrata* (Colombo et al., 2006), 0.60 in *Porphyra* sp. from Japan and Korea, 1.8 in *Porphyra* sp. from China and 0.3 in *H. fusiforme* (Dawczynski et al., 2007). The ratio of ω-6 to ω-3 was 2.85, 3.25 and 2.90 in *Caulerpa lentillifera, Caulerpa racemosa* var. *clavifera* f. *microphysa* and *Caulerpa racemosa* var. *laetevirens* respectively (Nagappan & Vairappan, 2014).

Table 5.10 Fatty acid contents in the seven seaweeds. Concentrations is given as relative concentrations to total fatty acid contents.

Species	SFA	MUFA	PUFA	ω-3	ω-6	ω-6/ω-3
	Relative concentration (%)			Relative concentration (%)		
CA	46.56	13.19	40.25	21.05	13.58	0.65
EPK	65.24	16.40	18.36	0.00	9.27	-
EPN	14.68	35.84	49.48	34.98	14.50	0.41
GC	61.73	38.27	0.00	0.00	0.00	-
GCC	81.50	18.50	0.00	0.00	0.00	-
GF	50.58	39.34	10.08	10.66	0.00	0.00
UF	42.35	38.80	18.85	18.85	0.00	0.00

SFA - Saturated fatty acid, MUFA - Mono unsaturated fatty acid, PUFA - Poly unsaturated fatty acid, ω - omega fatty acid, CA - *C. antennina*, EPK - *E. prolifera* obtained from Kayamkulam, EPN - *E. prolifera* obtained from Njarakkal, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata*.

SF of all the analysed seaweeds couldn't be detected in GC-MS. This observation highlights the presence of non-volatile high molecular glycosidic linkages which requires advanced analytical procedures in future. The GC-MS profiling of the current study had similar patterns that were reported earlier on seaweeds. 22 tropical seaweeds collected from the Saurashtra coast, India, showed high level of ω-6 and ω-3 PUFAs with their ratios underlying with the WHO prescription limits (Kumar et al., 2011). The fatty acid composition was

with high palmitic acid (C16:0) content and traces of other SFAs. High contents of PUFAs with 11 to 65 % in Rhodophyta, 39 to 49% in Phaeophyta and 14 to 43 % in Chlorophyta were reported. ω-3 and ω-6 were seen in all analysed seaweeds. ω-6/ω-3 ratio was seen to be <10 except in *G. corticata, Gracilaria dura, Gracilaria debilis* and *Gracilaria fergusonii* where the values exceeded (Kumar et al., 2011). Methanolic extract of *Laurencia brandenii,* collected from the south west coast of India upon GC-MS analysis exhibited the presence of hexanols, dodecanol, hexadecanoic acid, 9-dodecanoic acid, PUFAs etc. (Manilal et al., 2009).

The total lipid contents varied from about 0.4 to 1.8% in *Palmaria palmata* (Mouritsen et al., 2013). Three edible species of *Caulerpa* genus exhibited high ω-3and ω-6 PUFAs (Nagappan & Vairappan, 2014). Two PUFAs that cannot be synthesized by verterbrates including humans are the linoleic (ω-6) and α-linoleic acids (ω-3). GC-MS analysis of the fractions of red and brown seaweeds evidenced 3-5 % of ω-9, 3-32 % of ω-6 and 8-63% of ω-3 PUFAs (van Ginneken et al., 2011). The GC-MS analysis of the methanolic extract of *Sargassum marginatum* showed the presence of tetradecanoic acid, n-hexadecanoic acid and phytols (Manilal et al., 2010a). The highly active methanolic extracts of the 15 red seaweed samples collected from the southwest coast of India, upon GC-MS analysis evidenced the presence of ω-9 PUFAs and some other fatty acids (Manilal et al., 2009). The ethanolic extract of the red seaweed *Hypnea musciformis* collected from the Pudumadam coast, Tamil Nadu, India exhibited richness in the bioactive compounds which upon GC-MS analysis revealed the presence of potentially bioactive major constituents like n-hexadecanoic acid, tetradecanoic acid, PUFAs such as the oleic acid, 9-octadecenoic acid and 6-octadecenoic acid (Balamurugan et al., 2013). SFAs content were observed in a range of 31 to 78

% in Rhodophyta, 36 to 46 % in phaeophyta and 48 to 63 % in Chlorophyta (Kumar et al., 2011). MUFAs were seen in the range of 3 to 25 % in Rhodophyta, 10 to 14 % in phaeophyta and 4 to 22 % in Chlorophyta. PUFAs were seen in the range of 11 to 65 % in Rhodophyta, 39 to 49 % in Phaeophyta and 14 to 42 % in Chlorophyta (Kumar et al., 2011). *Palmaria palmata* collected from the Atlantic region was observed to have 22 to 57 % saturated fatty acids, 6 to 16 % MUFAs and 3 to 61 % PUFAs. ω-6 was observed in the range of 0.65 to 4.22 % and ω-3 in the range of 2 to 60% (Mouritsen et al., 2013).

Caulerpa lentillifera collected from the Sabah coast had 42 % of saturated fatty acids, 18 % of MUFAs and 38 % PUFAs. *Caulerpa racemosa* var. *clavifera* f. *microphysa* had 66 % SFAs, 12 % MUFAs and 30 % PUFAs. *Caulerpa racemosa* var. *laetevirens* had 57 % SFAs, 12 % MUFAs and 29 % PUFAs (Nagappan & Vairappan, 2014). *Ulva rigida* collected from Chilka Lake had a relative content of 20.2 % PUFAs and 3.6 % MUFAs (Satpati & Pal, 2011). *Ulva lactuca* and *Durvillaea antarctica* collected from the coastal area of Chile, had high contents of PUFAs and MUFAs. The SFAs ranged from C-12 to C-24, MUFAs from C-14 to C-22 (ω-7 and 9) and PUFAs from C-16 to C-22 (ω-3,6 and 7). Total SFAs content was 33 % and 25 %, total MUFAs content was 36 % and 38 % and total PUFAs content was 18 % and 34 % respectively (Ortiz et al., 2006). *Porphyra* sp. collected from Japan, Korea and China, *U. pinnatifida, Laminaria* sp., and *Hizikia fusiforme* collected from China were reported to have the contents of SFAs in the range of C-12 to C-24, MUFAs in the range of C-14 to C-22 (ω-5,7,9,11 and 13) and PUFAs in the range of C-18 to C-22 (ω-3 and 6). The total SFA content was 35 %, 44 %, 17 %, 41 % and 28 % respectively. MUFAs content was 18 %, 20 %, 7 %, 17 % and 13 % respectively and PUFAs content was 44 %, 33 %, 73 %, 39 % and 57 % respectively (Dawczynski et al., 2007).

5.3.7 Statistical Correlation

The biochemical constituents upon correlation analysis with the bioactivities indicated appreciable positive correlations (Table 5.11). The amount of total extractives correlated with the antioxidant and antimicrobial activities. The alcohols, alkanes, alkenes and alkynes contents in the PEF correlated positively with the iodine value and microbiological activities. The presence of these compounds in PEF indicated a lethal effect on *E. coli, S. aureus* and *B. cereus*. The fatty acids such as the SFAs, MUFAs and PUFAs exhibited positive correlations with all the three antioxidant activities, iodine and saponification values. It was also active against *B. cereus* and *E. coli*. Sterol rich EAF correlated with the antimicrobial activities indicating its broad spectrum antimicrobial activity. Antioxidant activities were also seen to be correlated with the antimicrobial activities too. Both the DPPH and ferrous tartarate active fractions were seen to correlate with all the four microbes which indicate the broad spectral activities. The area of interest of the current study was upon the saponins content and it exhibited full segment antioxidant activity. It was positively correlated to the lethality of *B. cereus* and *S. abony*.

Table 5.11 Pearson's bivariate correlation analysis, (n=21), (Split into 10 sections for enhanced readability).

(a)

	YIELD	ALC	ALKA	ALKE	ALKY	MUFA	PUFA	SFA
YIELD	1							
ALC	-0.030	1						
ALKA	-0.050	0.780*	1					
ALKE	0.070	0.830*	0.740*	1				
ALKY	-0.030	0.790*	0.670*	0.800*	1			
MUFA	0.240	-0.230	-0.240	-0.130	-0.130	1		
PUFA	0.340	-0.160	-0.170	-0.090	-0.100	0.640*	1	
SFA	0.390	-0.220	-0.240	-0.130	-0.130	0.770*	0.700*	1

(b)

	YIELD	ALC	ALKA	ALKE	ALKY	MUFA	PUFA	SFA
STER	-0.140	-0.170	-0.180	-0.100	-0.100	-0.190	-0.140	-0.190
SC	0.220	-0.260	-0.280	-0.150	-0.150	-0.290	-0.210	-0.290
SV	0.510	-0.310	-0.310	-0.150	-0.190	0.850*	0.660*	0.820*
IV	0.020	0.150	0.360	0.220	0.390	0.390	0.470	0.320
DAAE	0.300	-0.330	-0.350	-0.200	-0.190	0.190	0.010	0.310
DBHTE	0.300	-0.330	-0.340	-0.190	-0.190	0.180	0.001	0.310
DTE	0.420	-0.290	-0.310	-0.170	-0.170	0.130	-0.030	0.240
DRE	0.300	-0.310	-0.320	-0.190	-0.180	0.170	0.001	0.300

(c)

	YIELD	ALC	ALKA	ALKE	ALKY	MUFA	PUFA	SFA
FTAAE	0.050	-0.510	-0.540	-0.290	-0.300	-0.300	-0.270	-0.290
FTBHTE	0.090	-0.520	-0.550	-0.310	-0.320	-0.170	0.110	0.001
FTTE	0.090	-0.510	-0.550	-0.310	-0.320	-0.180	0.110	0.001
FTRE	0.040	-0.410	-0.440	-0.240	-0.240	-0.140	0.180	0.060
KAAE	0.080	-0.430	-0.460	-0.250	-0.240	-0.250	-0.170	-0.250
KBHTE	0.080	-0.420	-0.450	-0.240	-0.230	-0.260	-0.170	-0.250
KTE	0.080	-0.410	-0.440	-0.240	-0.230	-0.260	-0.170	-0.260
KRE	0.080	-0.430	-0.460	-0.250	-0.240	-0.250	-0.170	-0.250

(d)

	YIELD	ALC	ALKA	ALKE	ALKY	MUFA	PUFA	SFA
TCSA	-0.050	0.060	0.260	0.330	0.130	-0.080	-0.240	-0.120
TCBC	0.210	-0.010	-0.190	-0.070	-0.220	-0.090	0.030	0.020
TCEC	-0.240	0.070	-0.080	0.010	0.140	-0.230	-0.160	0.050
TCSAB	-0.030	-0.290	-0.300	-0.170	-0.170	0.070	-0.120	-0.060
CPSA	-0.050	0.060	0.260	0.330	0.130	-0.070	-0.240	-0.110
CPBC	0.190	0.001	-0.190	-0.070	-0.210	-0.090	0.020	0.010
CPEC	-0.250	0.080	-0.070	0.010	0.130	-0.220	-0.170	0.050
CPSAB	-0.030	-0.280	-0.300	-0.160	-0.170	0.060	-0.120	-0.060

(e)

	STER	SC	SV	IV	DAAE	DBHTE	DTE	DRE
STER	1							
SC	-0.220	1						
SV	-0.240	-0.210	1					
IV	-0.150	-0.370	0.470	1				
DAAE	-0.040	0.090	0.330	-0.070	1			
DBHTE	-0.050	0.080	0.330	-0.070	0.999*	1		
DTE	-0.110	0.050	0.340	-0.130	0.930*	0.930*	1	
DRE	-0.060	0.070	0.320	-0.070	0.999*	0.999*	0.940*	1

(f)

	STER	SC	SV	IV	DAAE	DBHTE	DTE	DRE
FTAAE	0.080	0.720*	-0.150	-0.390	0.220	0.210	0.230	0.200
FTBHTE	0.080	0.430	-0.020	-0.340	0.250	0.250	0.240	0.240
FTTE	0.070	0.430	-0.030	-0.340	0.250	0.250	0.240	0.240
FTRE	-0.150	0.460	-0.040	-0.300	0.100	0.100	0.110	0.090
KAAE	0.040	0.810*	-0.210	-0.450	0.220	0.220	0.210	0.200
KBHTE	0.040	0.810*	-0.220	-0.440	0.220	0.210	0.210	0.200
KTE	0.030	0.810*	-0.220	-0.450	0.210	0.210	0.200	0.190
KRE	0.040	0.810*	-0.210	-0.440	0.220	0.220	0.210	0.200

(g)

	STER	SC	SV	IV	DAAE	DBHTE	DTE	DRE
TCSA	0.360	-0.120	-0.110	0.120	0.020	0.020	-0.020	0.001
TCBC	0.050	0.180	0.001	-0.380	0.130	0.140	0.150	0.140
TCEC	0.010	-0.050	-0.090	-0.130	0.040	0.050	0.030	0.050
TCSAB	0.510	0.100	-0.050	-0.270	0.150	0.140	0.160	0.130
CPSA	0.360	-0.120	-0.100	0.120	0.030	0.020	-0.020	0.010
CPBC	0.040	0.180	-0.010	-0.370	0.120	0.120	0.140	0.130
CPEC	0.010	-0.060	-0.100	-0.140	0.040	0.040	0.030	0.050
CPSAB	0.520	0.080	-0.050	-0.260	0.150	0.140	0.160	0.120

(h)

	FTAAE	FTBHTE	FTTE	FTRE	KAAE	KBHTE	KTE	KRE
FTAAE	1							
FTBHTE	0.680*	1						
FTTE	0.670*	0.999*	1					
FTRE	0.550	0.750*	0.750*	1				
KAAE	0.780*	0.490	0.490	0.580	1			
KBHTE	0.780*	0.480	0.480	0.580	0.999*	1		
KTE	0.770*	0.480	0.480	0.570	0.999*	0.999*	1	
KRE	0.780*	0.490	0.490	0.580	0.999*	0.999*	0.999*	1

(i)

	FTAAE	FTBHTE	FTTE	FTRE	KAAE	KBHTE	KTE	KRE
TCSA	0.010	-0.090	-0.090	-0.370	-0.080	-0.080	-0.080	-0.080
TCBC	0.060	0.280	0.280	0.180	0.010	0.010	0.010	0.010
TCEC	0.110	0.280	0.280	0.070	-0.170	-0.170	-0.170	-0.170
TCSAB	0.250	0.140	0.140	-0.040	0.310	0.300	0.300	0.310
CPSA	0.001	-0.100	-0.100	-0.370	-0.080	-0.080	-0.080	-0.080
CPBC	0.070	0.280	0.280	0.170	0.010	0.010	0.010	0.010
CPEC	0.100	0.270	0.270	0.060	-0.180	-0.180	-0.180	-0.180
CPSAB	0.250	0.140	0.140	-0.040	0.300	0.290	0.290	0.290

(j)

	TCSA	TCBC	TCEC	TCSAB	CPSA	CPBC	CPEC	CPSAB
TCSA	1							
TCBC	-0.230	1						
TCEC	-0.130	0.360	1					
TCSAB	0.500	0.020	-0.080	1				
CPSA	0.999*	-0.230	-0.130	0.510	1			
CPBC	-0.240	0.999*	0.360	0.010	-0.240	1		
CPEC	-0.120	0.360	0.999*	-0.080	-0.120	0.370	1	
CPSAB	0.500	0.020	-0.080	0.999*	0.510	0.010	-0.080	1

*. Correlation is significant at the 0.001 level (2-tailed).

YIELD - Yield of the fractions collected, ALC - Alcohols content, ALKA - Alkanes content, ALKE - Alkenes content, ALKY - Alkynes content, MUFA - Mono unsaturated fatty acid content, PUFA - Poly unsaturated Fatty acid content, SFA - Saturated fatty acid content, STER - Sterol content, SC - Saponins content, SV - Saponification value, IV - Iodine value, DAAE - DPPH Ascorbic acid equivalence, DBHTE - DPPH BHT equivalence, DTE - DPPH α-tocopherol equivalence, DRE - DPPH resorcinol equivalence, FTAAE - Ferrous tartarate ascorbic acid equivalence, FTBHTE - Ferrous tartarate BHT equivalence, FTTE - Ferrous tartarate tocopherol equivalence, FTRE - Ferrous tartarate resorcinol equivalence, KAAE - $KMnO_4$ ascorbic acid equivalence, KBHTE - $KMnO_4$ BHT equivalence, KTE - $KMnO_4$ tocopherol equivalence, KRE - $KMnO_4$ resorcinol equivalence, TCSA - Tetracycline - *S. aureus*, TCBC - Tetracycline - *B. cereus*, TCEC - Tetracycline - *E. coli*, TCSAB - Tetracycline - *S. abony*, CPSA - Chloramphenicol - S. *aureus*, CPBC - Chloramphenicol - *B. cereus*, CPEC - Chloramphenicol - *E.coli* and CPSAB - Chloramphenicol - *S. abony*.

5.4 Conclusion

This study showed that the seven seaweeds from the south west coast of India were a significant source of pharmacologically active saponins and fatty acids, which possessed high anti oxidation and antimicrobial activities. Additionally, all the seaweed extracts were potent bioactive resource by meeting the standard requirements. Further, it is indicated that the selective extraction of

bioactive compounds from seaweeds could be adopted in the emerging needs for natural bioactive compounds. In all the analysed seaweeds, the extracted fractions were observed to exhibit either antioxidant or antimicrobial activities or both. The saponins/cardiac glycosides which are considered as the pharmacological input of traditional medicines were also observed in the seaweeds. Broad spectrum antioxidant activity was seen in all the seaweeds. Broad spectrum antimicrobial activity was observed in *U. fasciata, G. corticata* and *G. foliifera.* Economically and pharmaceutically important omega fatty acids were seen in all seaweeds with *C. antennina* and *E. prolifera* having the ω-6/ω-3 ratio in line with the WHO prescribed standards. The biochemical constituents were correlated with bioactivities. In conclusion, the green seaweeds especially *U. fasciata, E. prolifera* and *C .antennina* representing important sources of bioactive constituents should be used up in cultivation and as an input source of functional food and nutraceuticals. The cultivation of the studied species like the *G. corticata* var. *cylindrica* and *C. antennina* as a resource for food may not be advisable as it may not necessarily be edible. But the cultivation would be viable where the species could be considered as a commercial raw material or as a nutraceutical extractive source.

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Chapter 6

POLYSACCHARIDES

Contents

6.1 Introduction

Seaweed biochemical composition studies have so far highlighted the presence of biologically important compounds viz. total protein, carbohydrate, lipid, iodine, ash and crude fibre contents, calorific value, vitamins, total phenolics, flavonoids, lutein, zeaxanthin, the antioxidant activity of the methanolic extracts, amino acids, saponins, volatile compounds, antioxidant and antimicrobial activity of the various solvent extracts etc. But still, some of the areas were left behind. Seaweed species vary mainly in its chemical and physical morphology. The physical morphological pattern is one among the unattended areas. It contributes to the shape and structure of the seaweeds. The individual algal cells which form the structure have its exoskeleton made of carbohydrate units and are the key area that determines the physical variations. These carbohydrate units are interconnected to form simple molecules which are termed as the disaccharide units and much more complex molecules which are stronger with respect to its tensile strength and termed as polysaccharides. Seaweed cell walls are made of interconnecting polysaccharides with higher amounts of active compound diversity. This extended diversity is difficult to degrade and are present as hydrogen bonded crystalline fibers with self

imposed sulfated and branched polysaccharides. They are attached to proteins and metals like the calcium and potassium (Shanmugham et al., 2013). In addition to this, the presence of sulfated polysaccharides have brought value additions in the food industry due to its gelling and thickening properties (Jiao et al., 2011b). Algal sulfated polysaccharides are renewable sources that are widespread in nature (Kaliaperumal et al., 1995; Hooper et al., 1996). They possess numerous biological activities with structural diversity and pharmacological features that finds therapeutic benefits. The isolated polysaccharides from seaweeds have antioxidant, anticoagulation (Athukorala et al., 2007; Li et al., 2012) and gelling abilities which in the recent years have been used up for economical and commercial growths (Souza et al., 2012).

The chemical characterization and biological activities of algal polysaccharides are seen to be dependent on the sampling time and site patterns (Marinho-Soriano & Bourret, 2003). The structure of the polysaccharides isolated from seaweeds had different type of substituted sulfate ester groups which vary with species (Chevolot et al., 1999). They comprises of a complex group of bioactive macromolecules whose chemical structure varies according to the algal species and diversity (Costa et al., 2010). This group possesses broad spectrum therapeutic properties (Patel, 2012) which exhibit strong antioxidant, antitumor, anti-inflammatory, antibacterial, immune stimulatory, antiviral and antiprotozoan applications (Jiao et al., 2011b). Recent researches focused on the natural polysaccharides isolated from the seaweeds with remarkable identification on several algal species belonging to Phaeophyta, Rhodophyta and Chlorophyta, as potential sources of sulfated polysaccharides (Patel, 2012). Sulfated fucans functions as an anticoagulant and antithrombotic active agent (Fonseca et al., 2008; Xie et

al., 2011; Qi et al., 2013). *E. linza* collected from China has been reported with sulfated polysaccharides possessing anticoagulant activities (Qi et al., 2013).

Sulfated polysaccharides present in the seaweed cell walls are extracted mainly by the precipitation method. Mainly three classifications are observed in phyco-polysaccharides namely, the fucans - extracted from Phaeophyta possessing a fucose back bone, ulvans - extracted from the Chlorophyta possessing a repeating disaccharide (sulfated rhamnose linked to either glucuronic acid, iduronic acid or xylose) backbone and carrageenans and agars extracted from Rhodophyta possessing a galactose backbone which exhibits a wide range of biological and physiological activities like the antiviral, anticoagulant, antitumor, etc. (Kalitnik et al., 2013). Among the three, red alga is a rich source of sulfated polysaccharides with sulfate contents varying among its species (Pomin & Mourão, 2008; Amorim et al., 2011). Thai seaweeds were reported to have appreciable polysaccharides yield which composed of sulfate, glucose, mannose, fucose and galactose units (Kantachumpoo & Chirapart, 2010). Japanese seaweeds were also reported with high sulfate containing polysaccharides which composed of glucose, fucose, galactose, mannose, xylose and ribose units (Men'shova et al., 2013).

Algal polysaccharides have shown 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and antioxidant activities. Recently sulfated polysaccharide isolated from *Gracilaria birdiae* was used as cheese coating to retard its spoilage (Cerqueira et al., 2009; Souza et al., 2012). Algal sulfated polysaccharides have proved to have prominent antioxidant activities (Rupérez et al., 2002; Qi et al., 2006; de Souza et al., 2007; Costa et al., 2010; Wang et al., 2010). Fucans from *Fucus vesiculosus* exhibited appreciable ferric reducing (Rupérez et al., 2002) and superoxide radical scavenging capabilities (de Souza et al., 2007). The antioxidant activities of sulfated

polysaccharides were observed to have correlations with the sulfate contents too (Jiao et al., 2011b). Sulfated polysaccharides with high sulfate content show high antibacterial activity (Chevolot et al., 1999). The sulfated polysaccharides isolated from brown seaweeds were reported with antimicrobial broad spectrum activity (Kantachumpoo & Chirapart, 2010).

Structural features of sulfated polysaccharides could not be determined up to perfection, which is due to their diverse, complex and heterogeneous structure (Mandal et al., 2007). The monosaccharide compositions of the polysaccharides collected from seaweeds depend on the method of isolation, purification, harvest season and species (Noseda et al., 1999; Rioux et al., 2010; Vishchuk et al., 2013). Infrared spectroscopy was the earliest method used to examine the seaweed polysaccharide structures (Matsuhiro & Rivas, 1993), which have now been replaced by HPLC, GC and NMR techniques. Mixed sulfated polysaccharides with mannose, glucose, galactose and xylose were observed in red seaweeds (Haines et al., 1990). *Sargassum fusiforme,* an edible algae whose polysaccharides have important biological activities and been used as food for thousands of years, showed the content of galactose, fucose, glucose, mannose and xylose as the major monosaccharide units (Wu et al., 2014). Since the polysaccharides are made up of sugar moieties that possess potential refractive index finger print, the study analysed the hydrolyzed polysaccharides for its monosaccharide compositions using the HPLC-RI techniques. Monosaccharides such as ribose, xylose, arabinose, fructose, mannose, glucose and galactose, and disaccharide sucrose were determined. Ribose, xylose, arabinose, mannose and galactose were reported as the monosaccharide present in sulfated polysaccharides of seaweeds (Rao & Ramana, 1991; Kolender & Matulewicz, 2002; Mandal et al., 2007; Yoshiie et al., 2012; Men'shova et al., 2013). Fructose, glucose and sucrose were

observed in seaweeds in their free forms (Bourne et al., 1972; Rupérez et al., 2002).

The heterogeneity, complexity and diversity observed has paved to conduct a bioactivity potential study on the isolatable polysaccharides from the seven seaweeds collected from the south west coast of India (Kerala coast). The total polysaccharide contents, iodine and saponification value, antioxidant assay with respect to three methods, antimicrobial assay against two Gram - positive and two Gram - negative bacteria and the HPLC - RI monosaccharide (ribose, xylose, arabinose, fructose, mannose, glucose and galactose) and disaccharide (sucrose) profiling were done to substantiate the purpose of this study.

6.2 Materials and Methods

6.2.1 Chemicals Used

All the reference standards used were brought from Sigma (USA) and positive controls from Chromadex (USA). Chemicals were of analytical grade and the solvents were purchased from Merck (Germany) in HPLC grade. Water used was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Chloramphenicol and tetracycline were purchased from Sigma (USA). Mannitol salt agar (MSA), nutrient agar, Levin eosin methylene blue agar (L - EMBA), xylose lysine deoxycholate agar (XLDA) and soya bean casein digest agar were purchased from Difco, USA.

6.2.2 Pathogen Culture Used

Lyophilised cultures (live strains) of *Escherichia coli* ATCC 25922 - Gram-negative rod, *Salmonella abony* NCTC 6017 - Gram-negative rod, *Bacillus cereus* ATCC 10876 - Gram-positive rod and *Staphylococcus aureus* ATCC 6538 - Gram-positive cocci were purchased and used in this study.

6.2.3 Sampling

Seven seaweeds, comprising of four Chlorophyta and three Rhodophyta were collected from the Kerala coast, South India (Table 5.1). The samples were cleaned in salt water thrice followed up with fresh water and then dried under shade, ground and sifted through 300 microns nylon sieves. The fine powder was stored at room temperature maintaining the moisture level < 2 %. The ground samples were used up in further analysis.

6.2.4 Extraction of Isolatable Polysaccharides

Polysaccharides were extracted in water at 100 ^{o}C following the methods of Asker et al. (2007). 10 g of the dried alga powder was repeatedly refluxed at 65 ^{o}C with 100 mL pure methanol until the extract was colourless. The extract was discarded and the residue was dried in a hot air oven at 100 ^{o}C for 3 h, and mixed with 40 mL of distilled water and transferred to a water bath at 100 ^{o}C in a 250 mL beaker. The mixture was stirred and allowed to boil for 2 h with intermittent water addition to compensate the water evaporation lose. The liquid was filtered out using Whatman filter paper No. 40. The extraction was repeated thrice and the liquid collected after filtration was pooled, and concentrated to 10 mL. The liquid concentrate was then added to 100 mL of absolute ethanol, where the total isolatable polysaccharides were precipitated. The precipitate was collected by centrifugation at 10,000 rpm, washed thrice with 25 mL of absolute ethanol, centrifuged and dried at 60 ^{o}C under vacuum. The yield of polysaccharides was reported as the % to dry weight of the seaweed.

6.2.5 Determination of Iodine and Saponification Values

The iodine and saponification values provide information regarding the extent of acidic matter and unsaturation. Estimations were done based on the

analytical procedures described in the Indian Pharmacopoeia (Indian Pharmacopoeia, 1996a; Indian Pharmacopoeia, 1996b). Iodine value was reported as the weight of iodine absorbed per gram of the sample and saponification value was reported as the mg of KOH required per gram of the sample.

6.2.6 Specific Rotation

Specific rotation, an intensive property, is the change in orientation of monochromatic plane polarized light (sodium D line - 589 nm) as it passes through a compound in solution. Rotation towards right is defined as dextro rotatory with + ve magnitude and towards left is defined as levo rotatory with - ve magnitude. Specific rotation gives an idea on the enantiomeric excess in the analyte. Specific rotation of the aqueous solutions of 1 % polysaccharide samples were measured at 20 ^{o}C, using a 10 cm cylindrical cell and the sodium D line with a optics manual polarimeter. Rotation of the compound as a solution in water is measured at an angle of 0 ^{o} to 360 ^{o} where the instrument is preset to 0 ^{o} with HPLC grade water.

6.2.7 Determinations of Sulfate Content in Algal Polysaccharides

Phyco-polysaccharides are observed invariably in its sulfated forms to various extends. The sulfate content of the polysaccharide was estimated according to the modified $BaCl_2$ turbidimetric method (Craigie et al., 1984; Wang & Luo, 2007). 20 mg of the isolated polysaccharide was mixed with 0.5 mL of 2N HCl, flushed with nitrogen and hydrolysed for 2 h in a sealed tube at 100 ^{o}C. The cooled hydrolysate was transferred to a 10 mL standard flask and made up to volume with distilled water. The solution was treated with activated charcoal to remove the colour. 1 mL of the decolorized solution was mixed with 0.5 mL of 6M HCl solution, 2.5 mL of 70 % sorbitol solution and

0.5 g $BaCl_2.H_2O$ in a test tube. The solution was ultrasonicated for 1 min and absorbance of the suspension was measured immediately at 470 nm. The standard curve was plotted with series of concentration (50 to 500 μg g^{-1}) of potassium sulfate (Table 6.1). The results were reported as % of sulfate content in the sample.

Table 6.1 Sulfate content and antioxidant activity multipoint calibration data.

Standard	Calibration equations	r^2	Concentration (ppm)				
			1	2	3	4	5
TOTAL SULFATE CONTENT-UV/VIS SPECTROPHOTOMETER							
K_2SO_4	y = 0.0031x -0.0036	0.981	163	326	489	652	815
DPPH RADICAL SCAVENGING ACTIVITY-UV/VIS SPECTROPHOTOMETER							
ASE	y = -3.2176e-05x -0.081	0.995	403.36	806.72	1210.08	1613.44	-
RES	y = -3.1709e-05x - 0.088	0.999	406.56	813.12	1219.68	1626.24	-
TOC	y = -4.9594e-05x + 0.008	0.999	409.36	818.72	1228.08	1637.44	-
BHT	y = -2.9294e-05x - 0.064	0.996	405.32	810.64	1215.96	1621.28	-
FERROUS TARTARATE OXIDISING ACTIVITY-UV/VIS SPECTROPHOTOMETER							
ASE	y = 5.6376e-04x -0.013	0.978	403.36	806.72	1210.08	1613.44	-
RES	y = 7.2978e-06x - 0.012	0.992	405.60	811.20	1216.80	1622.40	-
TOC	y = 1.808e-03x + 0.556	0.995	409.36	818.72	1228.08	1637.44	-
BHT	y = 2.0351e-03x + 0.421	0.994	406.56	813.12	1219.68	1626.24	-
TOTAL ANTIOXIDANT POTENTIAL-TITRIMETRY							
Standard	**Weight of standards (g/50mL)**	**Blank readings**	**Titre Readings**			**Equivalence of 0.01N $KMnO_4$(g)**	
			1mL	**2mL**	**3mL**		
ASE	0.504	0.9	23.0	41.0	61.3	0.00046	
RES	0.507	0.9	2.4	4.0	6.0	0.05069	
TOC	0.507	0.9	1.1	1.3	1.4	0.00664	
BHT	0.503	0.9	3.1	5.9	6.8	0.00457	

ASC- Ascorbic acid, RES- Resorcinol, TOC- Tocopherol and BHT- Butylatedhydroxytoluene.

6.2.8 Antioxidant Activity

In vitro antioxidant activities of the isolated polysaccharides from the seaweeds using DPPH radical scavenging method (establishes the potency of

the extracts in free radical quenching), UV-Vis ferrous tartarate method (evaluates the reduction potential) and $KMnO_4$ titration method (estimates the efficiency of the extracts in undergoing complete oxidation, which in return exhibits the extend of its antioxidant potential) were carried out. The activity study was reported as the percentage of equivalence of four standards - ascorbic acid, α-tocopherol, butylatedhydroxytoluene (BHT) and resorcinol.

6.2.8.1 Antioxidant Assay - DPPH Method

The assay was done as per the method illustrated by Brand-Williams et al. (1995). DPPH is a stable free radical with a purple colour. In the presence of a hydrogen donor, the DPPH radical is reduced which leads to a yellow color formation with reduction in absorbance at 517 nm (Chen et al., 2008; Wang et al., 2010). This reduction in absorbance is estimated as the corresponding antioxidant activity. The standard curve was observed to be linear between 403 to 1613 μg g^{-1} for ascorbic acid, 409 to 1637 μg g^{-1} for α-tocopherol, 405 to 1621 μg g^{-1} of BHT and 406 to 1626 μg g^{-1} of resorcinol. The r^2 values were > 0.995 (Table 6.1). Results were expressed as the percentage of equivalence to the corresponding standard. Additional dilutions were done upon the requirement, when observed to be deviating from linear range.

6.2.8.2 Antioxidant Assay - UV-Vis Ferrous Tartarate Method

Antioxidant activity was determined by the modified UV-Visible spectro-photometric methods (Li et al., 2005). The extent of formation of the purple colour complex is measured. The standard curve was observed to be linear between 403 to 1613 μg g^{-1} for ascorbic acid, 406 to 1626 μg g^{-1} for BHT, 409 to 1637 μg g^{-1} for α-tocopherol and 405 to 1622 μg g^{-1} for resorcinol. The r^2 values were > 0.950 (Table 6.1). Results were expressed as

the percentage of equivalence to the corresponding standard. Additional dilutions were done upon the requirement, when observed to be deviating from linear range.

6.2.8.3 Antioxidant Assay - $KMnO_4$ Method

The antioxidant assay was determined by the Ribereau-Gayon-Maurié titrimetric method with $KMnO_4$ and indigo carmine dye as an indicator (Daničić, 1973; Radovanović, 1986). The change from blue to golden yellow colour is the extent of antioxidant activity. The results were calculated with the estimated observations against the standards (ascorbic acid, α-tocopherol, BHT and resorcinol) and were expressed in percentage of equivalence to the corresponding standard (Table 6.1).

6.2.9 Antibacterial Study

The antibacterial activity study was done based on the Kirby-Bauer disc diffusion method (Bauer et al., 1966). Positive cultures purchased, were re-cultured in the respective selective culture medium and the log phase of the culture was subsequently swabbed into soya bean casein digest agar plates. The polysaccharide was dissolved in sterilised water at a concentration of 100 µg mL^{-1} and kept in an autoclave at 121 ^oC for 10 min. The sample solution impregnated discs were then screened against pathogenic bacteria (two Gram positive - *Staphylococcus aureus* and *Bascillus cereus* and two Gram negative - *Escherichia coli* and *Salmonella abony*) with 100 µg mL^{-1} of chloramphenicol and tetracycline in methanol as the positive controls. Methanol and water was used as the analytical blank/negative control. The activity was measured in mm of the clearance area around the disc, discarding the diameter of the disc and deducting the inhibition zones of the negative

control. The activity is reported as % of inhibition against the positive controls.

6.2.10 Hydrolysis of the Polysaccharides

Polysaccharides are complex organo molecules whose composition cannot be determined as such. Hence, hydrolysis is done where H_2SO_4 is the main hydrolysis agent used. The drawbacks such as contamination of chromatographic columns and presence of residues have made the search for an alternative agent. Triflouroacetic acid (TFA) was observed to cater the function (Aman et al., 1981; Wu et al., 2014). 20 mg of the isolated polysaccharide was dissolved in 2 ml of 2M TFA in a 5 mL ampoule (Fengel & Wegener, 1979). The ampoule was sealed under nitrogen atmosphere and kept in boiling water bath for 10 h inorder to hydrolyze the polysaccharide into component monosaccharide. After being cooled to room temperature, the reaction mixture was centrifuged at 10000 rpm for 10 min. The supernatant was collected and dried under vacuum and then dissolved into 1 mL distilled water to make the sample solution for HPLC analysis.

6.2.11 HPLC Analysis of the Hydrolysed Polysaccharides

The monosaccharide composition of the hydrolysed polysaccharides was estimated using the HPLC-RI techniques. Shimadzu UFLC-20AD equipped with RID-20A at 22 ^{o}C and Shodex SP-0810, 300 mm x 8 mm x 7 µ column was used. Monosaccharides such as the ribose, xylose, arabinose, fructose, mannose, glucose and galactose, and disaccharide sucrose were used as the standards (Fig. 6.1). The mobile phase was HPLC grade water at a flow rate of 0.6 mL/min. 20 min was the run time. All the standard solutions were prepared in series concentration (10, 25, 50, 75 and 100 µg g^{-1}) and injected. The spike and recovery was > 99 % and calibration coefficient of $r^2 > 0.999$

(Table 6.2). The results were reported as the relative % of sugars in the polysaccharides.

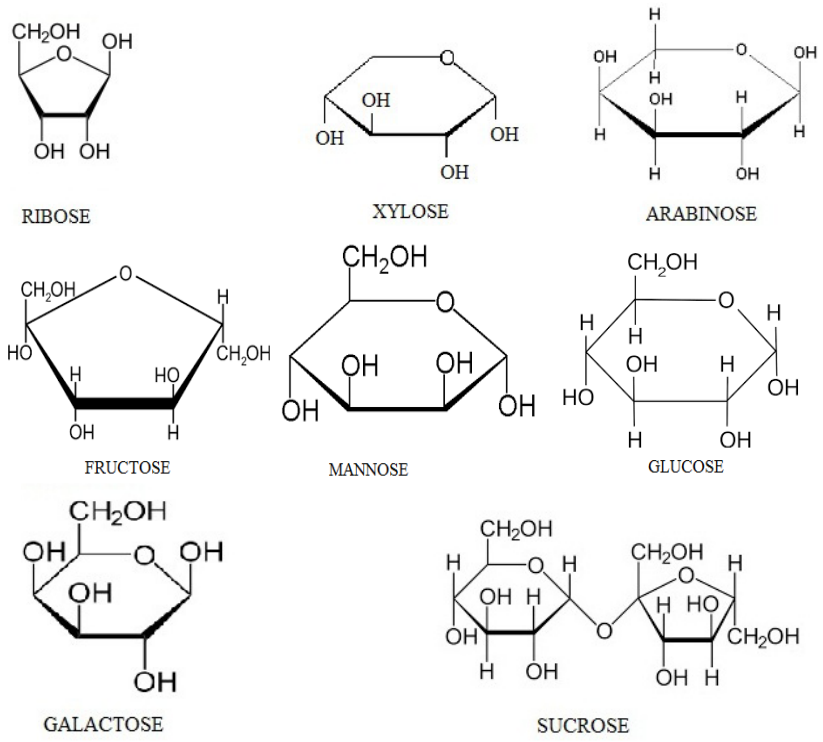


Fig. 6.1 Monosaccharides - ribose, xylose, arabinose, fructose, mannose, glucose and galactose and disaccharide - sucrose analysed in the study.

Table 6.2 HPLC-RI monosaccharide and disaccharide multipoint calibration data.

Standard	Calibration equations	r^2	Concentration (ppm)					Retention time
			1	2	3	4	5	
SUGARS-HPLC								
Ribose	y = 0.09571x + 0.64	0.999	10	25	50	75	100	6.777
Xylose	y = 0.09341x + 0.21	0.999	10	25	50	75	100	7.235
Arabinose	y = 0.02423 + 0.05	0.999	10	25	50	75	100	8.268
Fructose	y = 0.19280x - 0.41	0.999	10	25	50	75	100	8.533
Maltose	y = 0.07056x - 0.98	0.999	10	25	50	75	100	9.134
Glucose	y = 0.19304x + 0.05	0.999	10	25	50	75	100	9.834
Galactose	y = 0.16073x + 0.03	0.999	10	25	50	75	100	10.274
Sucrose	y = 0.29498 + 0.09	0.999	10	25	50	75	100	12.909

6.2.12 Statistical Correlation

The current study was an interdisciplinary approach. Both the chemical activities and biological activities were studied on the isolated polysaccharides. The chemical parameters such as the, iodine value, saponification value, sulfate content, *in vitro* antioxidant activities and HPLC profiling of the monosaccharides that composited the isolated polysaccharides were estimated. The *in vitro* antimicrobial activities were also estimated. Thus, the activity to compositional correlation was observed to be inevitable. The bioactivity and bioactive constituents quantified are means of three replicate determinations (± standard deviations). The mean values were taken for the discussion of this chapter. The data of this chapter was subjected to pearson bivariate correlation analysis in the SPSS 16.0 for Windows. The positive r^2 values explained the relation between the chemical constituents and activities.

6.3 Results and Discussions

6.3.1 Sampling

Four Chlorophyta and three Rhodophyta were collected from the south west coast of India (Kerala coast). The samples were identified as in Table 5.1.

6.3.2 Polysaccharides from Seaweeds

Rhodophyta exhibited the maximum polysaccharide contents (Table 6.3). 31.66 % of polysaccharides were obtained from *G. corticata* var. *cylindrica*. Comparable polysaccharide content was observed in between *G. corticata* (13.83 %) and *G. foliifera* (13.53 %) (Fig. 6.2). Chlorophyta had comparatively low polysaccharide contents with *E. prolifera* obtained from the two locations possessing higher concentrations [*E. prolifera* from Kayamkulam location (5.96 %) and *E. prolifera* from Njarakkal location (6.73 %)] of polysaccharides.

Least polysaccharide contents were seen in *U. fasciata* (0.07 %). Earlier observations stating the heterogeneity, diversity and complexity of polysaccharides underline the current observation.

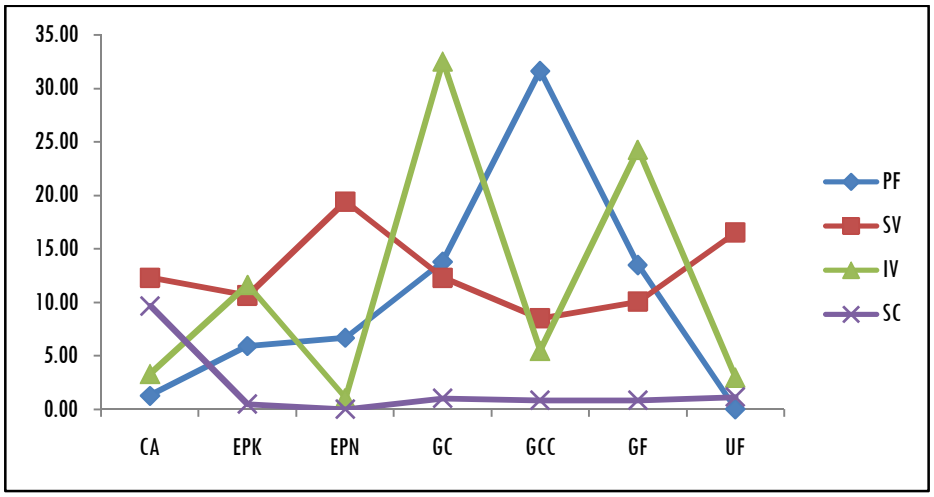


Fig. 6.2 Graphical representation of the polysaccharide yield (PF (%)), saponification value (SV), iodine value (IV) and sulfate content (SC (%)) of the seven seaweeds.

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

Table 6.3 Total polysaccharide content, saponification value, iodine value, specific rotation and sulfate content of the seven seaweeds, (mean ± SD), (n=3).

Species	Polysaccharide yield (%)	Saponification value	Iodine value	Specific rotation $[\alpha]_D^{20}$	SO_4^{2-} content (%)
CA	1.30 ± 0.05	12.34 ± 1.13	3.34 ± 0.31	-94°	9.69 ± 0.88
EPK	5.96 ± 0.11	10.67 ± 0.97	11.67 ± 1.07	-90°	0.55 ± 0.05
EPN	6.73 ± 0.08	19.44 ± 1.77	1.09 ± 0.10	-55°	0.05 ± 0.05
GC	13.83 ± 0.96	12.34 ± 1.13	32.55 ± 2.97	-65°	1.07 ± 0.10
GCC	31.66 ± 0.85	8.55 ± 0.78	5.49 ± 0.50	-85°	0.90 ± 0.08
GF	13.53 ± 0.76	10.12 ± 0.92	24.31 ± 2.22	-95°	0.86 ± 0.08
UF	0.07 ± 0.01	16.55 ± 1.51	3.02 ± 0.28	-76°	1.16 ± 0.11

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

Comparable yields were reported in various studies done earlier in this field. 24.2% of sulfated polysaccharide was isolated from *Codium tomentosum* collected from the south east coast of India (Shanmugham et al., 2013). *G. corticata* collected from Madagascan south island reported to have comparable yield to current study (Andriamanantoanina et al., 2007). The cell wall polymer (polysaccharides) extracted from red alga *Grateloupia filicina* collected from south-east coast of India, was observed to have a yield of 35.12% (Sahu & Kumar, 2014). Japanese seaweed *Eisenia bicyclis* were reported to have a polysaccharide yield of 0.93 to 2.09 % (Men'shova et al., 2013). Thai brown seaweeds, *Colpomenia sinuosa, Hydroclathrus clathratus, Dictyota dichotoma, Padina australis, Padina minor, Sargassum polycystum* and *Turbinaria conoides* were reported earlier with a polysaccharide yield of 1.97 to 19.69 % (Kantachumpoo & Chirapart, 2010).

6.3.3 Iodine and Saponification Values

Iodine value exhibits the extent of unsaturation in the isolated polysaccharides and was observed highest in *G. corticata* (32.55) (Table 6.3). *G. foliifera* (24.31) and *E. prolifera* collected from Kayamkulam location (11.67) also had higher unsaturation extends (Fig. 6.2). Comparable iodine value was observed in between *C. antennina* (3.34) and *U. fasciata* (3.02). Least was observed in *E. prolifera* collected from Njarakkal location (1.09). The iodine value indicates the presence of sugar esters/glycolipids, which require advanced studies in future.

Seaweeds possess high molecular weight sulfated polysaccharides that have a complex and heterogeneous structure and high electro negativity (Alves et al., 2012). Polysaccharides generally exhibit acidic nature which is due to the presence of the carboxylic acid or ionisable sulfate end groups. The extent

of this acidic nature was determined by the saponification value which elucidated an appreciable extent of acid nature. *E. prolifera* (19.44) collected from the Njarakkal location was observed to have more acidic nature polysaccharides (Table 6.3). The least acidic nature was observed in *G. corticata* var. *cylindrica* (8.55). Polysaccharides isolated from all the seaweeds exhibited comparable acidic nature (8.55 to 19.44) (Fig. 6.2). Comparable saponification value was exhibited by *E. prolifera* (10.67) obtained from Kayamkulam location and *G. foliifera* (10.12), and *C. antennina* (12.34) and *G. corticata* (12.34). Studies on polysaccharides collected from seaweeds especially *Gracilaria* sp. have shown the higher complexity with a range of neutral to highly charged galactans (Andriamanantoanina et al., 2007). This property is observed due to the presence of sulfated esters, which increases their applicability as food ingredients that are capable of gel formation.

6.3.4 Specific Rotation

All the isolated polysaccharides analysed were observed to have - ve specific rotation (Table 6.3). Higher levo rotations were seen in the polysaccharides isolated from *G. foliifera* ($[\alpha]_D^{20}$ -95^o), *C. antennina* ($[\alpha]_D^{20}$ -94^o) and *E. prolifera* obtained from Kayamkulam location ($[\alpha]_D^{20}$ -90^o). The least negative specific rotation was seen in *E. prolifera* collected from Njarakkal location ($[\alpha]_D^{20}$ -55^o). Levo rotation of the analyte indicates the presence of excess levo-rotatory units in the polysaccharides which could be the presence of ribose or fructose or any other sugars in the levo rotatory forms. The observations were seen to be in line with the studies done on specific rotations on phyco-polysaccharides. Specific rotations of the sulfated polysaccharides obtained from *Ulva pertusa* and *Ulva conglobata* were observed to be $[\alpha]_D^{20}$ -77.8^o and -26.3^o respectively (Yamamoto et al., 1980). The sulfated polysaccharide isolated from *Gracilaria ornata* collected from Brazil showed

negative specific rotation in the range of $[\alpha]_D^{20}$ -56.5° to -19.0° (Amorim et al., 2012). The specific rotation was seen in the range of $[\alpha]_D^{20}$ -35.0° to 49.7° in *Bostrychia montagnei* collected from Brazil (Noseda et al., 1999).

6.3.5 Sulfate Content

The current study on the isolated polysaccharides for the extend of sulfate content established the presence of sulfated groups in all the seaweeds (Table 6.3), with 9.69 % of sulfate in *C. antennina*, 1.07 % in *G. corticata* and 1.16 % in *U. fasciata*. All the other seaweeds exhibited moderate amounts of sulfate contents. Low level of sulfation was observed in polysaccharides isolated from *E. prolifera* collected from the Njarakkal location (0.05 %) (Fig. 6.2). *Gracilaria birdiae* collected from Brazil was observed to have 8.4 % of sulfate content (Souza et al., 2012). The sulfur content of *Spirulina platensis* was found to be 2.13 to 5.40 % (Abd El Baky et al., 2013). Sulfate contents of 13.3 % in *Gelidium* sp., 7.84 % in *Ulva sp*. and 11.13 % in *Sargassum* sp. were observed in the samples collected from Tamil Nadu (Padmanaban et al., 2013). Polysaccharides of Thai brown seaweeds, *C. sinuosa, H. clathratus, D. dichotoma, P. australis, P. minor, S. polycystum* and *T. conoides* were reported earlier with sulfate content in the range of 5.83 to 11.09 % (Kantachumpoo & Chirapart, 2010). Japanese seaweed *E. bicyclis* was reported to contain 13.5 % of sulfate (Men'shova et al., 2013).

6.3.6 Antioxidant Activity

DPPH free radical scavenging activity (Table 6.4) was observed highest in the polysaccharides isolated from *G. foliifera* possessing high ascorbic acid, BHT, α-tocopherol and resorcinol equivalences. The activity of *G. foliifera* was

observed to be comparable with *G. corticata* var. *cylindrica*. Least activity was observed in *C. antennina* and *U. fasciata*. Activity was moderately acceptable for the isolated polysaccharide of *E. prolifera* collected from Njarakkal location. DPPH ascorbic acid equivalence was seen high in *G. foliifera* (13.39 %) (Fig. 6.3). DPPH ascorbic acid equivalence was observed to be comparable in between the polysaccharides of *C. antennina* (0.23 %), *U. fasciata* (0.26 %), *G. corticata* (0.26 %) and *E. prolifera* obtained from Kayamkulam location (0.31 %). *E. prolifera* (10.83 %) obtained from Njarakkal location and *G. corticata* var. *cylindrica* (10.78 %) also exhibited comparable DPPH ascorbic acid equivalence. DPPH BHT equivalence was observed high in the polysaccharides isolated from *G. foliifera* (15.78 %). DPPH BHT equivalence was observed to be comparable in between *C. antennina* (0.24 %), *G. corticata* (0.25 %) and *E. prolifera* (0.27 %) obtained from Kayamkulam location. *G. corticata* (12.65 %) and *G. corticata* var. *cylindrica* (12.97 %) also exhibited comparable DPPH BHT equivalence. Least DPPH BHT equivalence was exhibited by *U. fasciata* (0.04 %). DPPH α-tocopherol equivalence was observed high in the polysaccharides obtained from *G. foliifera* (48.18 %). The activity was observed to be comparable with *G. corticata* var. *cylindrica* (46.04 %). Least DPPH α-tocopherol equivalence was observed in *C. antennina* (0.03 %). DPPH resorcinol equivalence was observed to be high in *G. foliifera* (17.41 %). *E. prolifera* (13.88 %) obtained from Njarakkal location and *G. corticata* var. *cylindrica* (14.51 %) had comparable activity. Comparable activity was also exhibited by *C. antennina* (0.24 %), *E. prolifera* obtained from Kayamkulam location (0.23 %) and *G. corticata* (0.23 %). Least resorcinol equivalence was exhibited by *U. fasciata* (0.15 %).

Table 6.4 Antioxidant activity study of the isolated polysaccharides from seaweeds, (mean ± SD), (n=3).

Species	AAE	BHTE	TE	RE
DPPH Method - % of Inhibition				
CA	0.23 ± 0.01	0.24 ± 0.01	0.03 ± 0.01	0.24 ± 0.01
EPK	0.31 ± 0.01	0.27 ± 0.02	1.46 ± 0.07	0.23 ± 0.01
EPN	10.83 ± 0.52	12.65 ± 0.60	35.89 ± 1.71	13.88 ± 0.66
GC	0.26 ± 0.01	0.25 ± 0.01	0.65 ± 0.03	0.23 ± 0.01
GCC	10.78 ± 0.51	12.97 ± 0.62	46.04 ± 2.19	14.51 ± 0.69
GF	13.39 ± 0.64	15.78 ± 0.75	48.18 ± 2.29	17.41 ± 0.83
UF	0.26 ± 0.01	0.04 ± 0.01	6.20 ± 0.29	0.15 ± 0.01
Ferrous Tartarate Method - % of Activity				
CA	0.76 ± 0.04	0.19 ± 0.01	0.19 ± 0.01	2.03 ± 0.10
EPK	0.59 ± 0.03	0.47 ± 0.02	0.47 ± 0.02	0.84 ± 0.04
EPN	0.62 ± 0.03	0.51 ± 0.02	0.51 ± 0.02	0.85 ± 0.04
GC	0.60 ± 0.03	0.47 ± 0.02	0.47 ± 0.02	0.87 ± 0.04
GCC	0.78 ± 0.04	0.59 ± 0.03	0.58 ± 0.03	1.18 ± 0.06
GF	0.56 ± 0.03	0.27 ± 0.01	0.27 ± 0.01	1.21 ± 0.06
UF	0.35 ± 0.02	0.28 ± 0.01	0.27 ± 0.01	0.51 ± 0.02
$KMnO_4$ method - % of Activity				
CA	0.32 ± 0.02	3.19 ± 0.15	35.34 ± 1.68	4.63 ± 0.22
EPK	0.39 ± 0.02	3.91 ± 0.19	43.40 ± 2.07	5.68 ± 0.27
EPN	0.36 ± 0.02	3.59 ± 0.17	39.78 ± 1.89	5.21 ± 0.25
GC	0.46 ± 0.02	4.58 ± 0.22	50.79 ± 2.42	6.65 ± 0.32
GCC	0.37 ± 0.02	3.66 ± 0.17	40.59 ± 1.93	5.32 ± 0.25
GF	0.44 ± 0.02	4.36 ± 0.21	48.32 ± 2.30	6.33 ± 0.30
UF	0.32 ± 0.02	3.20 ± 0.15	35.50 ± 1.69	4.65 ± 0.22

AAE - Ascorbic acid equivalence, BHTE - BHT equivalence, TE - α-tocopherol equivalence and RE - Resorcinol equivalence.

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

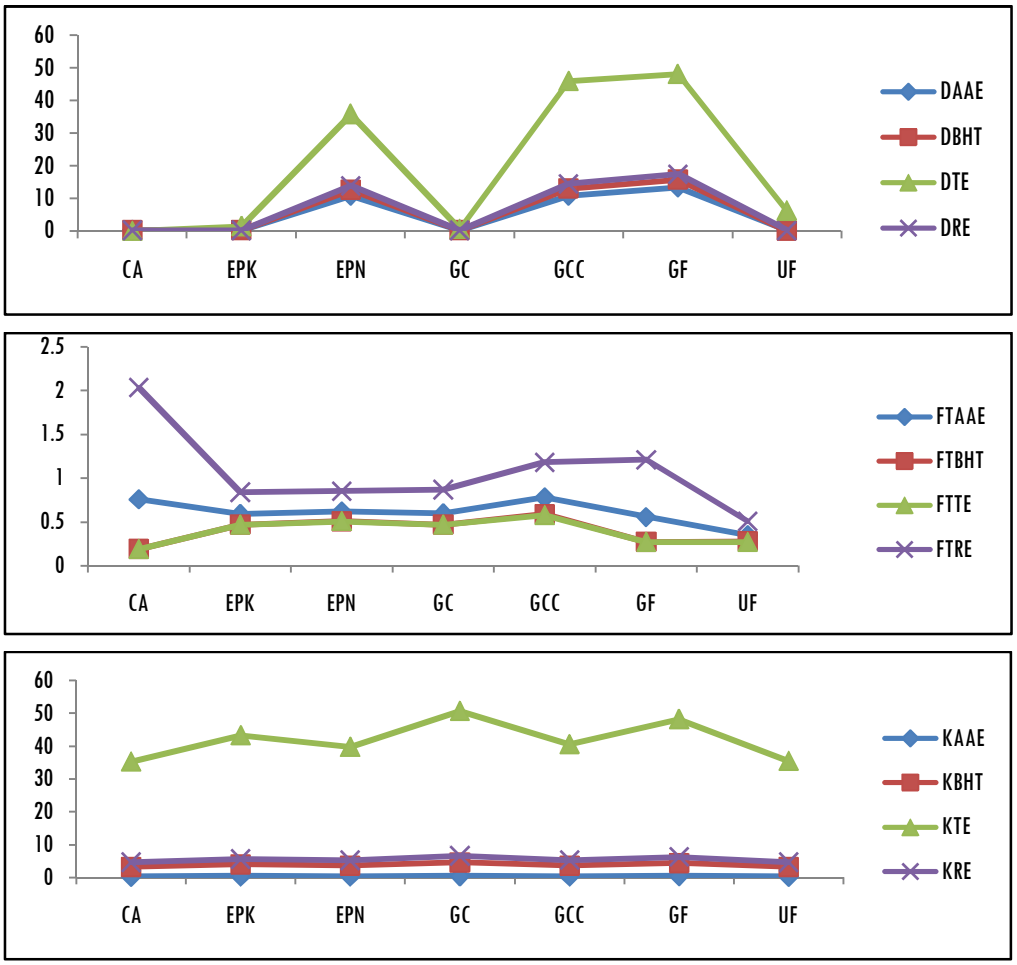


Fig. 6.3 Graphical representation of the antioxidant activities (%) of the polysaccharide fractions of the seven seaweeds.

DAAE - DPPH ascorbic acid equivalence activity, DBHT - DPPH BHT equivalence activity, DTE - DPPH α-tocopherol equivalence activity, DRE - DPPH resorcinol equivalence activity, FTAAE - Ferrous-tartrate ascorbic acid equivalence activity, FTBHT - Ferrous-tartrate BHT equivalence activity, FTTE - Ferrous-tartrate α-tocopherol equivalence activity, FTRE - Ferrous-tartrate resorcinol equivalence activity, KAAE - $KMnO_4$ ascorbic acid equivalence activity, KBHT - $KMnO_4$ BHT equivalence activity, KTE - $KMnO_4$ α-tocopherol equivalence activity and KRE - $KMnO_4$ resorcinol equivalence activity.

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

The metal reducing capability (Table 6.4) was observed highest in the polysaccharides of *G. corticata* var. *cylindrica* with appreciable ascorbic acid, BHT and α-tocopherol equivalence. *C. antennina* exhibited high resorcinol equivalence activities. The antioxidant activities of *G. corticata* var. *cylindrica* were almost comparable with *E. prolifera* collected from the Njarakkal location. Least ascorbic acid equivalence was shown by the polysaccharides of *U. fasciata*. BHT and α-tocopherol equivalence activities were seen to the minimum in the polysaccharides of *C. antennina* whereas resorcinol activity was seen lowest in *U. fasciata* (Fig. 6.3).

High ferrous tartarate ascorbic acid equivalence antioxidant activity was exhibited by the polysaccharides obtained from *G. corticata* var. *cylindrica* (0.78 %) and *C. antennina* (0.76 %). Comparable activity was exhibited by *E. prolifera* obtained from Kayamkulam location (0.59 %), *E. prolifera* obtained from Njarakkal location (0.62 %), *G. corticata* (0.60 %) and *G. foliifera* (0.56 %). Least Ferrous tartarate ascorbic acid equivalence antioxidant activity was exhibited by *U. fasciata* (0.35 %). High ferrous tartarate BHT equivalence antioxidant activity was exhibited by the polysaccharides obtained from *G. corticata* var. *cylindrica* (0.59 %) and was comparable with *E. prolifera* obtained from Njarakkal location (0.51 %). Comparable activity was observed in between *G. foliifera* (0.27 %) and *U. fasciata* (0.28 %) and in between *E. prolifera* obtained from Kayamkulam location (0.47 %) and *G. corticata* (0.47 %). Least activity was observed in *C. antennina* (0.19 %). High ferrous tartarate α-tocopherol equivalence antioxidant activity was observed in the polysaccharides obtained from *G. corticata* var. *cylindrica* (0.58 %). It was observed to be comparable with the activity of *E. prolifera* obtained from Njarakkal location (0.51 %). Comparable activity was also observed in between *G. foliifera* (0.27 %) and *U.*

fasciata (0.27 %) and in between *E. prolifera* (0.47 %) obtained from Kayamkulam location and *G. corticata* (0.47 %). Least activity was observed in the polysaccharides obtained from *C. antennina* (0.19 %). Ferrous tartarate resorcinol equivalence activity was observed to be high in *C. antennina* (2.03 %). Comparable activities were observed in between *G. corticata* var. *cylindrica* (1.18 %) and *G. foliifera* (1.21 %) and in between *E. prolifera* obtained from Kayamkulam location (0.84 %), *E. prolifera* obtained from Njarakkal location (0.85 %) and *G. corticata* (0.87 %). Least activity was seen in *U. fasciata* (0.51 %).

The total oxidising capability of the polysaccharides with respect to all the four standards, which was estimated using the $KMnO_4$ method (Table 6.4), was observed in the maxima in *G. corticata*. Comparable activity was also observed in *G. foliifera*. Least total oxidising capability was seen in *C. antennina* (Fig. 6.3). $KMnO_4$ ascorbic acid equivalence antioxidant activity was seen in higher levels in polysaccharides obtained from *G. corticata* (0.46 %). It was almost comparable with the activity of *G. foliifera* (0.44 %). Comparable activities were also exhibited by the polysaccharides of *E. prolifera* obtained from Njarakkal location (0.36 %), Kayamkulam location (0.39 %) and *G. corticata* var. *cylindrica* (0.37 %) and in between *C. antennina* (0.32 %) and *U. fasciata* (0.32 %). $KMnO_4$ BHT equivalence antioxidant activity was observed to be high in the polysaccharides obtained from *G. corticata* (4.58 %). It was almost comparable with the activity exhibited by *G. foliifera* (4.36 %). Comparable activities were also exhibited by *E. prolifera* obtained from Njarakkal location (3.59 %) and *G. corticata* var. *cylindrica* (3.66 %) and in between *C. antennina* (3.19 %) and *U. fasciata* (3.20 %). $KMnO_4$ α-tocopherol equivalence antioxidant activity was observed to be high in *G. corticata* (50.79 %). This was seen to be comparable with the activity exhibited by *G. foliifera* (48.32 %). Comparable activities were also exhibited by

E. prolifera obtained from Njarakkal location (39.78 %) and *G. corticata* var. *cylindrica* (40.59 %) and in between *C. antennina* (35.34 %) and *U. fasciata* (35.50 %). $KMnO_4$ resorcinol equivalence antioxidant activity was seen to be high in the polysaccharides obtained from *G. corticata* (6.65 %). It was almost comparable with *G. foliifera* (6.33 %). Comparable activity was also observed in between *E. prolifera* obtained from Njarakkal location (5.21 %) and *G. corticata* var. *cylindrica* (5.32 %) and in between *C. antennina* (4.63 %) and U. fasciata (4.65 %).

Even though the antioxidant activities exhibited large variations among the seaweeds analysed in comparison with the standards, all the sulfated polysaccharides isolated were possessing good antioxidant capabilities. Polysaccharides of Chlorophyta, *C. antennina* and *U. fasciata* exhibited low activities with exception in *E. prolifera* collected from Njarakkal location. In Rhodophyta, all the *Gracilaria* sp. exhibited high antioxidant activity. Sulfated polysaccharides were reported to be *in vitro* antioxidative in various studies. Moderate DPPH free radical scavenging effect was observed in the sulfated polysaccharides obtained from the Rhodophyta *Gracilaria birdiae* (Souza et al., 2012). Antioxidant activity rich polysaccharides composing of galactose and xylose were observed in *Corallina officinalis* (Yang et al., 2011). Rhamnose rich polysaccharides were isolated from *Undaria pinnatifida* which possessed *in vitro* antioxidant activities (Hu et al., 2010). *In vitro* antioxidant studies done on the isolated sulfated polysaccharides from *G. birdiae* showed appreciable activity as ascorbic acid equivalence (Fidelis et al., 2014). Sulfated polysaccharides extracted from *Hypnea musciformis* is a commercially important galactan and the samples collected from Brazil exhibited a concentration depended hydroxyl radical scavenging, DPPH scavenging, superoxide scavenging and metal chelating activity (Alves et al., 2012).

Polysaccharides obtained from the green alga *Ulva rigida* have been reported to have antioxidant activities (Godard et al., 2009). In addition, the polysaccharides obtained from mushrooms and macro algae have free radical scavenging abilities (Duan et al., 2006; Fan, et al., 2007). The sulfated polysaccharides extracted from *Spirulina platensis* showed appreciable DPPH radical scavenging activity against butylatedhydroxyanisole (BHA) and BHT as standards (Abd El Baky et al., 2013).

6.3.7 Antimicrobial Activity

Water and methanol were used as the negative controls. Water responded 0 mm inhibition zone against all the four analysed pathogens (Table 6.5, Fig. 6.4). Methanol exhibited 2 mm inhibitory zone against *S. aureus* and *S. abony* and 3 mm against *B. cereus and E. coli*. The methanol inhibition zone was deducted from the positive control zones in order to derive the activity.

Table 6.5 Antibacterial activity of the isolated polysaccharides. Zone inhibition data of the seven seaweed polysaccharides against the four pathogen colonies, (mean ± SD), (n=5).

Concentration of solutions - 100 μg mL^{-1}	Gram positive		Gram negative	
Species analysed	*S. aureus*	*B. cereus*	*E. coli*	*S. abony*
	in mm			
Water	0	0	0	0
Methanol	2.0 ± 0.09	3.0 ± 0.14	3.0 ± 0.15	2.0 ± 0.10
Tetracycline	9.0 ± 0.43	10.0 ± 0.48	8.0 ± 0.38	9.0 ± 0.43
Chloramphenicol	8.0 ± 0.38	12.0 ± 0.57	10.0 ± 0.43	12.0 ± 0.37
CA	2.0 ± 0.09	3.5 ± 0.17	3.0 ± 0.11	2.0 ± 0.03
EPK	4.0 ± 0.20	3.0 ± 0.06	3.5 ± 0.11	1.5 ± 0.01
EPN	1.5 ± 0.06	2.5 ± 0.22	2.5 ± 0.12	2.0 ± 0.13
GC	3.0 ± 0.07	2.5 ± 0.12	3.5 ± 0.19	1.5 ± 0.08
GCC	3.5 ± 0.30	3.5 ± 0.10	4.0 ± 0.11	3.5 ± 0.13
GF	2.5 ± 0.12	3.5 ± 0.09	3.5 ± 0.07	2.5 ± 0.12
UF	4.0 ± 0.24	3.5 ± 0.11	4.0 ± 0.27	3.0 ± 0.11

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

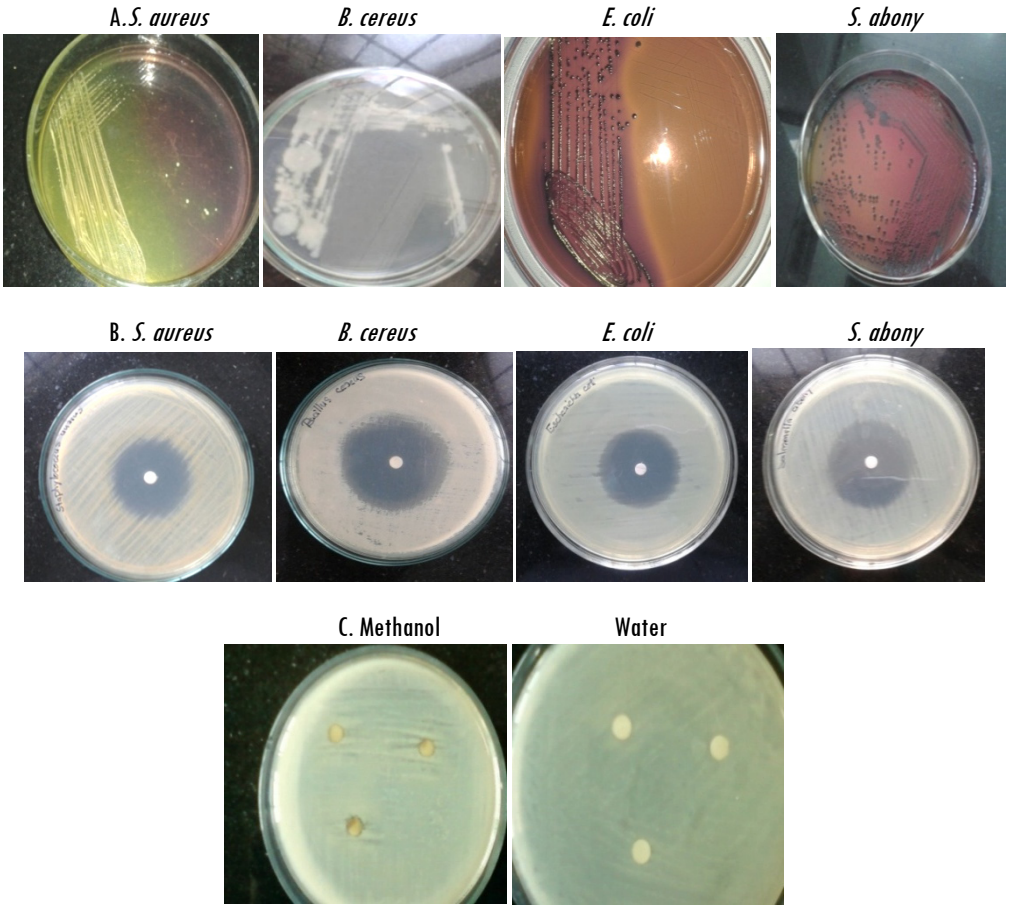


Fig. 6.4. Photographs of the antimicrobial activity studies (A. Selective culture of *S. aureus, B. cereus, E. coli* and *S. abony*. B. Positive control disc placed on the culture plates and C. Negative control disc placed on the culture plates).

The antimicrobial activities of the sulfated polysaccharides at a concentration level of 100 µg mL^{-1} in water were observed to be appreciable in comparison (> 50%) with the positive controls (concentration of 100 µg mL^{-1} in methanol) used against the *E. coli* strains. Higher resemblance was shown towards the tetracycline activity. Chloramphenicol equivalence was seen to be > 35 %. The sulfated polysaccharides of *U. fasciata* exhibited higher extend of broad spectrum antimicrobial activity with respect to both the standards. Similarity of activity was seen in *G. corticata* var. *cylindrica* and *E. prolifera* collected from Kayamkulam location. In general, the sulfated polysaccharides

exhibited moderate activities against *S. aureus* and *B. cereus* and low activity against *S. abony* with respect to both the standards.

Sulfated polysaccharides of *U. fasciata* and *E. prolifera* collected from the Kayamkulam location were observed to be lethal to *S. aureus*. *B. cereus* growth was highly inhibited by the sulfated polysaccharides of *C. antennina, U. fasciata* and *G. corticata* var. *cylindrica.* Sulfated polysaccharides of *G. corticata* var *cylindrica* were observed to be lethal to *S. abony* and *E. coli. E. coli* inhibition was seen highest in the *U. fasciata* sulfated polysaccharides.

With tetracycline as the positive control (Table 6.6), the polysaccharide of *E. prolifera* obtained from Kayamkulam location (57 %) and *U. fasciata* (57 %) exhibited highest *S. aureus* lethal activity (Fig. 6.5). Least *S. aureus* lethal activity was observed in *E. prolifera* obtained from Njarakkal location (21 %). *B. cereus* lethal activity was observed high in the polysaccharides of *C. antennina* (50 %), *G. corticata* var. *cylindrica* (50 %), *G. foliifera* (50 %) and *U. fasciata* (50 %). Least activity was observed in the polysaccharides of *E. prolifera* obtained from Njarakkal location (36 %) and *G. corticata* (36 %). *E. coli* bactericidal activity was observed high in the polysaccharides obtained from *G. corticata* var. *cylindrica* (80 %) and *U. fasciata* (80 %). Comparable activity was exhibited by *E. prolifera* obtained from Kayamkulam location (70 %), *G. corticata* (70 %) and *G. foliifera* (70 %). Least activity was shown by *E. prolifera* obtained from Njarakkal location (50 %). *S. abony* bactericidal activity was observed high in the polysaccharides extracted from *G. corticata* var. *cylindrica* (50 %). Comparable activities were exhibited by *C. antennina* (29 %) and *E. prolifera* obtained from Njarakkal location (29 %). *E. prolifera* obtained from Kayamkulam location (21 %) and *G. corticata* (22 %) also exhibited comparable activities.

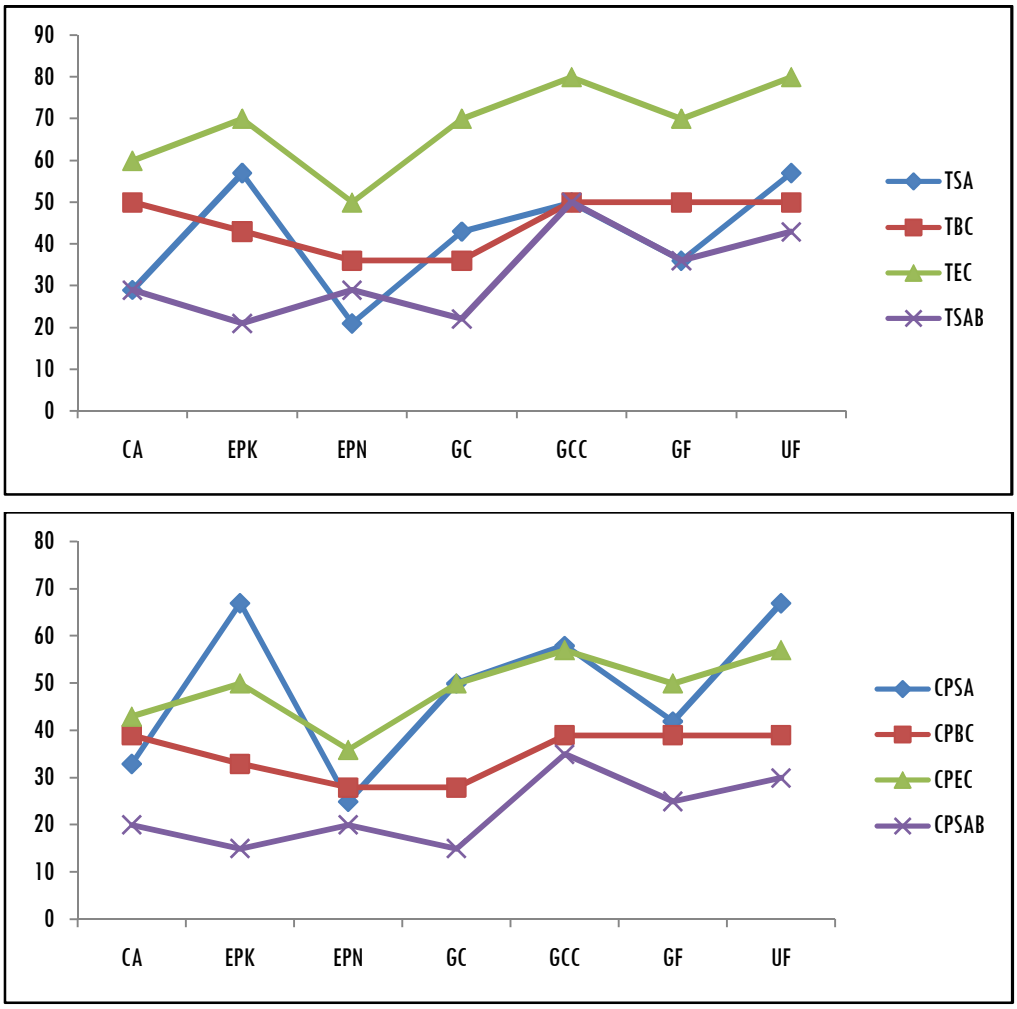


Fig. 6.5 Graphical representation of the antimicrobial activities (%) of the polysaccharide fractions of the seven seaweeds.

TSA - Tetracycline compared *S. aureus* activity, TBC - Tetracycline compared *B. cereus* activity, TEC - Tetracycline compared *E. coli* activity, TSAB - Tetracycline compared *S. abony* activity, CPSA - Chloramphenicol compared *S. aureus* activity, CPBC - Chloramphenicol compared *B. cereus* activity, CPEC - Chloramphenicol compared *E. coli* activity, CPSAB - Chloramphenicol compared *S. abony* activity.

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

Table 6.6 Antibacterial activity of the isolated polysaccharides. Activity based on tetracycline as the positive control (%), (mean ± SD), (n=5).

Species	Gram +ve		Gram -ve	
	S. aureus	*B. cereus*	*E. coli*	*S. abony*
CA	29 ± 1.26	50 ± 0.24	60 ± 0.75	29 ± 1.05
EPK	57 ± 1.06	43 ± 0.63	70 ± 0.23	21 ± 0.85
EPN	21 ± 0.93	36 ± 0.37	50 ± 0.39	29 ± 1.35
GC	43 ± 0.56	36 ± 0.78	70 ± 0.65	22 ± 0.61
GCC	50 ± 0.81	50 ± 0.26	80 ± 0.63	50 ± 0.79
GF	36 ± 0.70	50 ± 0.54	70 ± 0.84	36 ± 0.96
UF	57 ± 1.11	50 ± 0.23	80 ± 0.60	43 ± 0.53

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

With chloramphenicol as the positive control (Table 6.7), high *S. aureus* bactericidal activity was exhibited by *E. prolifera* obtained from Kayamkulam location (67 %) and *U. fasciata* (67 %) (Fig. 6.5). *B. cereus* bactericidal activity was high in *C. antennina* (39 %), *G. corticata* var. *cylindrica* (39 %), *G. foliifera* (39 %) and *U. fasciata* (39 %). Least activity was observed in *E. prolifera* obtained from Njarakkal location (28 %) and *G. corticata* (28 %). High *E. coli* lethal activity was observed in *G. corticata* var. *cylindrica* (57 %) and *U. fasciata* (57 %). Comparable activities were observed in *E. prolifera* obtained from Kayamkulam location (50 %), *G. corticata* (50 %) and *G. foliifera* (50 %). Least activity was seen in *E. prolifera* obtained from Njarakkal location (36 %). High *S. abony* lethal activity was seen in *G. corticata* var. *cylindrica* (35 %). Comparable activities were seen in *C. antennina* (20 %) and *E. prolifera* obtained from Njarakkal location (20 %). Least activity was seen in *E. prolifera* obtained from Kayamkulam location (15 %) and *G. corticata* (15 %).

Table 6.7 Antibacterial activity of the isolated polysaccharides. Activity based on chloramphenicol as the positive control (%), (mean ± SD), (n=5).

Species	Gram +ve		Gram -ve	
	S. aureus	*B. cereus*	*E. coli*	*S. abony*
CA	33 ± 0.64	39 ± 0.20	43 ± 0.86	20 ± 0.15
EPK	67 ± 1.21	33 ± 0.51	50 ± 0.26	15 ± 0.28
EPN	25 ± 0.73	28 ± 0.43	36 ± 0.71	20 ± 0.59
GC	50 ± 0.59	28 ± 0.78	50 ± 0.57	15 ± 0.32
GCC	58 ± 0.91	39 ± 0.21	57 ± 0.44	35 ± 0.63
GF	42 ± 0.47	39 ± 0.11	50 ± 0.08	25 ± 0.17
UF	67 ± 1.22	39 ± 0.21	57 ± 0.42	30 ± 0.38

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

Sulfated polysaccharide obtained from the red alga *Kappaphycus alvarezii* and brown alga *Padina boergessenii* were reported to exert antimicrobial activity (Kumaran et al., 2010). Thai brown seaweeds were reported to have polysaccharides possessing antimicrobial activities (Kantachumpoo & Chirapart, 2010). Seaweed polysaccharides were reported to act as protective agents against plant pathogens (Vera et al., 2011). The crude sulfated polysaccharides extracted from the Brazilian seaweed *G. ornata* were observed to have antimicrobial effects on *Escherichia coli, Bacillus subtilis, Enterobacter aerogens, Salmonella typhi, Salmonella choleraesuis, Staphylococcus aureus* and *Pseudomonas aeruginosa* (Amorim et al., 2012). Antibacterial activities against the strains of *Staphylococcus epidermidis, S. aureus, Enterococcus feacalis, E. coli and P. aeruginosa* were exhibited by the polysaccharides obtained from the red algae *Corallina* sp. collected from Lebanese coast (Sebaaly et al., 2014). Green seaweed *Caulerpa sertulariodes* was reported to have antibacterial activities (Pushparaj et al., 2014). Polysaccharides extracted from *G. corticata* were reported to have antimicrobial activity (Govindasamy et al., 2012). Fucoidan extracted from *Sargassum wightii* were reported to have *E. coli, Klebsiella*

pneumoniae, Vibrio cholerae, Proteus sp., *Shigella sonnie, Proteus aeruginosa, Salmonella typhi* and *Klebsiella* sp. bactericidal activity (Marudhupandi & Kumar, 2013).

6.3.8 HPLC Profiling of the Polysaccharides

Polysaccharides are composed of large amounts of interconnected monosaccharide units. In seaweeds, polysaccharides are classified based on the source of extractions and chemical compositions. Generally, phyco polysaccharides are classified as carrageenans and agars [isolated from red seaweeds (Rhodophyta)], fucans [isolated from brown seaweeds (Phaeophyta)], and ulvans [from green seaweeds (Chlorophyta)]. In this study, discussions on the monosaccharide compositions of the polysaccharides isolated from Chlorophyta and Rhodophyta were observed to be relevant.

Chlorophyta has starch as the major storage product. The similarity in Chlorophyta and other green seaweeds is greater, with both linear chains of α-1,4 linked units, as in amylase, and branched chains which have α-1,6 glycosydic bonds, as in amylopectin. Polysaccharides of Chlorophyta constitute of sulfated rhamnose linked to either glucuronic acid or iduronic acid and have major monosaccharides constituting of glucose and xylose.

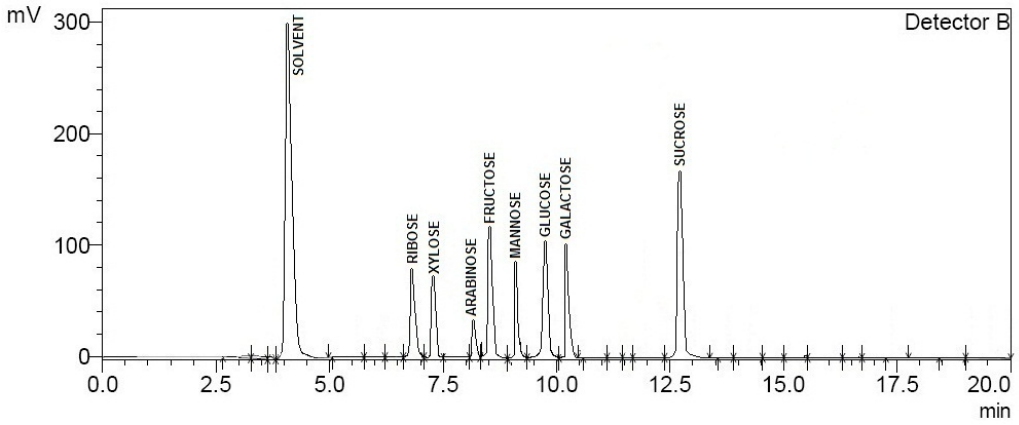
Rhodophyta has floridean starch as the major storage product. Carrageenans have long been known to be the highly sulfated galactans. The common structural feature in all carrageenans was verified to be a linear polymer alternating with the repeating disaccharide units consisting of 1, 3-linked-β-D-galactopyranose and 1,4-linked-α-D-galactopyranose or 1,4-linked 3,6-anhydrogalactopyranose. Carrageenans are distinguished from agars based on the optical rotations of the second units. Carrageenan are in the D form whereas agars are in the L form. Carrageenans are composed of galactose, ester sulfate, xylose, glucose, and uronic acids.

The polysaccharides isolated in this study constituted of both the storage and cell wall sugar forms. The isolated polysaccharides upon hydrolysis were subjected to HPLC analysis (Fig. 6.6) with refractive index detection against multipoint calibrated analysis. The study aimed in determining the chemical properties of the complex crude polysaccharides isolated from seaweeds. The results obtained were interesting and not following the general trends reported earlier (Table 6.8). Ribose and arabinose was observed as the major monosaccharide units (Fig. 6.7). Xylose and galactose were observed in successive concentrations. The presence of fructose and glucose in seaweeds indicated the availability of those as free form sugars or as easily hydrolysable forms or as disaccharide units of sucrose.

Table 6.8 Monosaccharide and disaccharide compositions of the polysaccharides isolated from the seven seaweeds, (relative %), (mean ± SD), (n=3).

Species	Ribose	Xylose	Arabinose	Fructose
CA	11.20 ± 0.53	5.37 ± 0.26	36.45 ± 1.74	-
EPK	20.26 ± 0.96	10.33 ± 0.49	27.10 ± 1.29	2.48 ± 0.12
EPN	19.29 ± 0.92	4.52 ± 0.22	19.60 ± 0.93	12.43 ± 0.59
GC	16.99 ± 0.81	9.41 ± 0.45	28.13 ± 1.34	2.10 ± 0.10
GCC	10.09 ± 0.48	19.97 ± 0.95	33.27 ± 1.58	3.90 ± 0.19
GF	22.97 ± 1.09	13.17 ± 0.63	23.90 ± 1.14	2.38 ± 0.11
UF	18.23 ± 0.87	11.73 ± 0.56	25.85 ± 1.23	2.68 ± 0.13
Species	**Mannose**	**Glucose**	**Galactose**	**Sucrose**
CA	-	6.41 ± 0.31	26.31 ± 1.25	4.19 ± 0.20
EPK	0.68 ± 0.03	2.75 ± 0.13	10.71 ± 0.51	7.67 ± 0.37
EPN	37.61 ± 1.79	0.11 ± 0.01	2.31 ± 0.11	1.08 ± 0.05
GC	2.28 ± 0.11	2.97 ± 0.14	9.24 ± 0.44	7.60 ± 0.36
GCC	7.88 ± 0.38	4.97 ± 0.24	12.46 ± 0.59	3.15 ± 0.15
GF	1.08 ± 0.05	3.30 ± 0.16	9.08 ± 0.43	6.41 ± 0.31
UF	2.41 ± 0.11	4.09 ± 0.19	10.31 ± 0.49	8.12 ± 0.39

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*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200
100
0
Detector B
SOLVENT
RIBOSE
XYLOSE
ARABINOSE
FRUCTOSE
MANNOSE
GLUCOSE
GALACTOSE
SUCROSE
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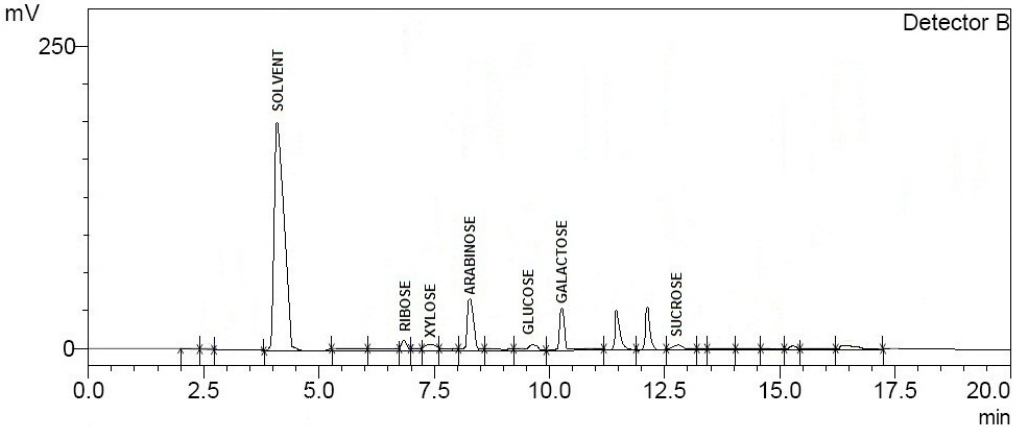
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
min

A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10.0
12.5
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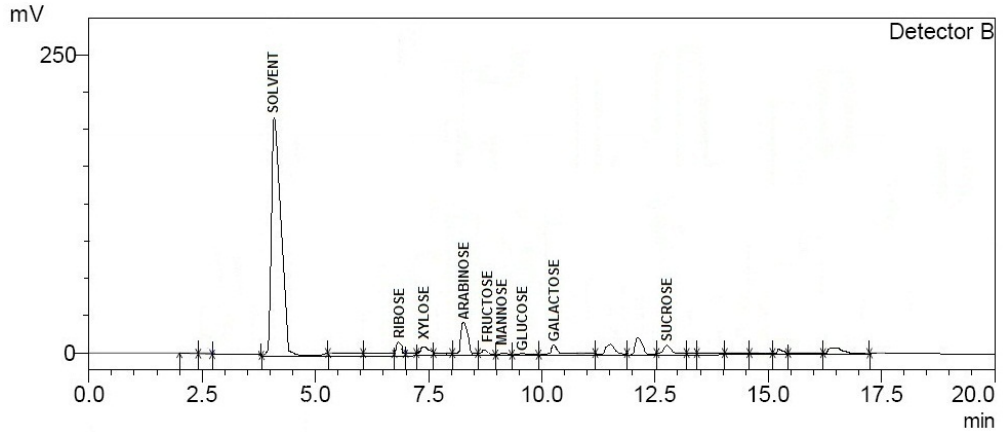
17.5
20.0
min

B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ARABINOSE
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5.0
7.5
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12.5
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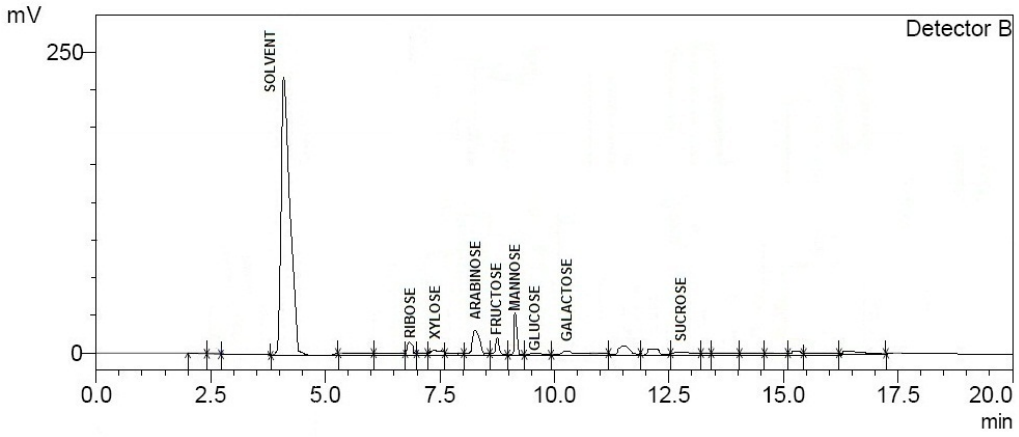
20.0
min

C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ARABINOSE
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MANNOSE
GLUCOSE
GALACTOSE
SUCROSE
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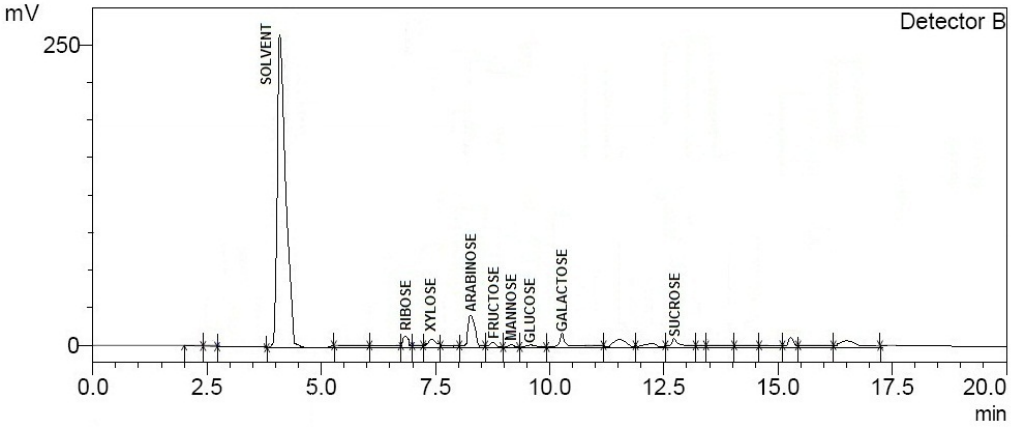
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
min

D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RIBOSE
XYLOSE
ARABINOSE
FRUCTOSE
MANNOSE
GLUCOSE
GALACTOSE
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5.0
7.5
10.0
12.5
15.0
17.5
20.0
min

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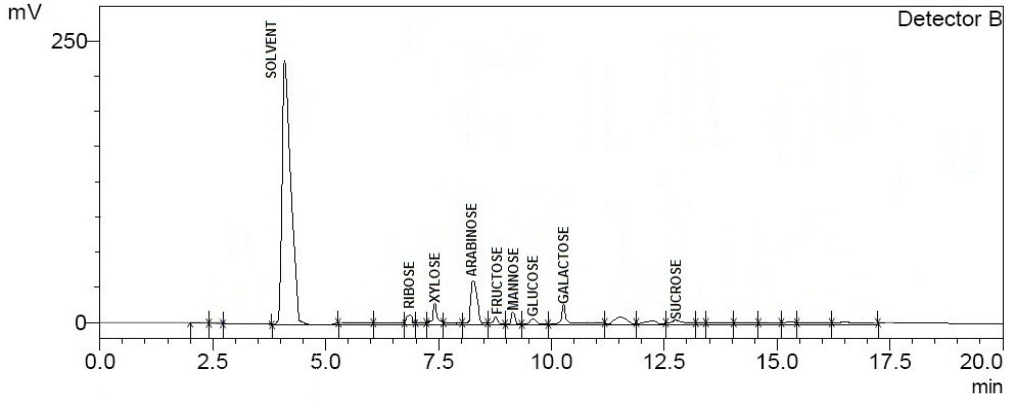
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RIBOSE
XYLOSE
ARABINOSE
FRUCTOSE
MANNOSE
GLUCOSE
GALACTOSE
SUCROSE
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2.5
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7.5
10.0
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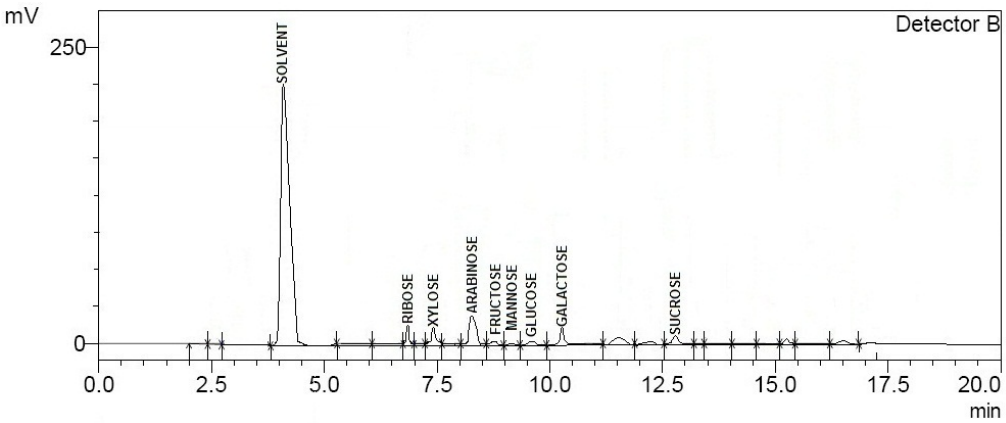
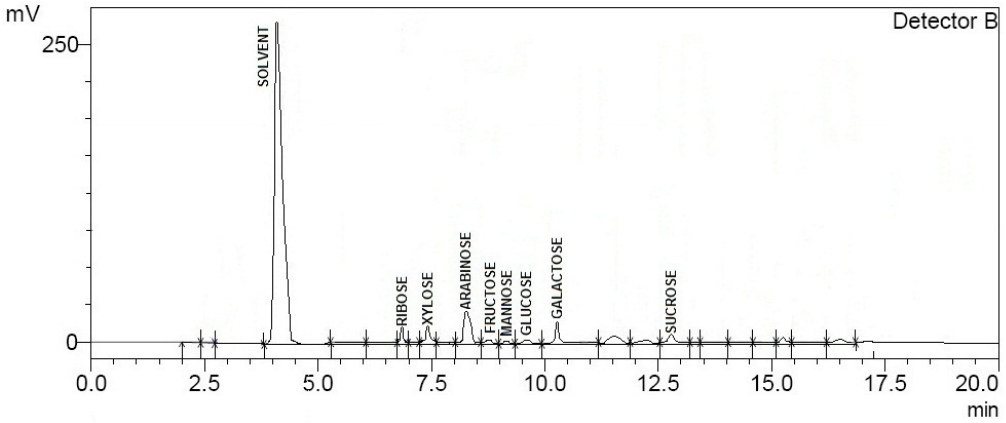
15.0
17.5
20.0
min

F

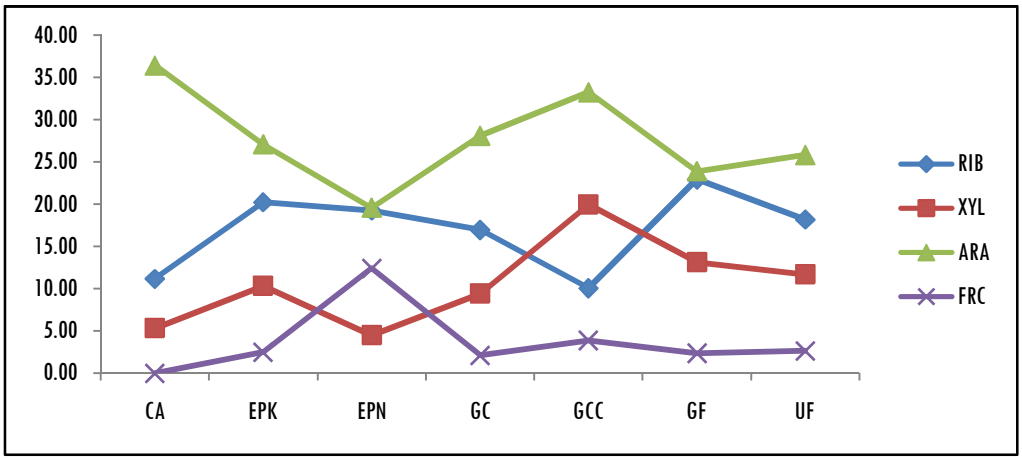


G

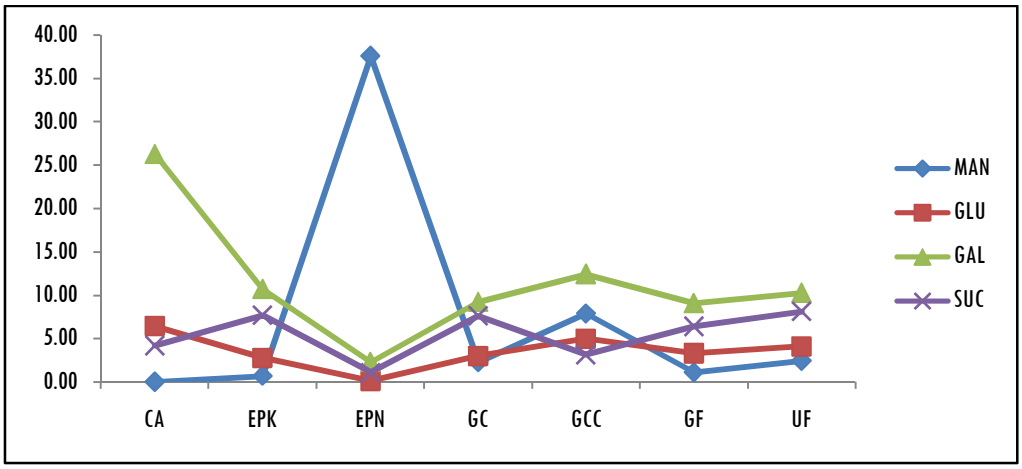


H

Fig. 6.6 HPLC chromatogram of the monosaccharides and disaccharide (A - Standards, B - *C. antennina*, C - *E. prolifera* obtained from Kayamkulam location, D - *E. prolifera* obtained from Njarakkal location, E - *G. corticata*, F - *G. corticata* var. *cylindrica*, G - *G. foliifera*, H - *U. fasciata*).



A



B

Fig. 6.7 Graphical representation of the monosaccharide and disaccharide contents in the polysaccharide fractions of the seven seaweeds estimated by HPLC-RI.

RIB-Ribose content, XYL-Xylose content, ARA-Arabinose content, FRC- Fructose content, MAN-Mannose content, GLU-Glucose content, GAL-Galactose content and SUC-Sucrose content (%).

CA - *C. antennina*, EPK - *E. prolifera* collected from Kayamkulam location, EPN - *E. prolifera* collected from Njarakkal location, GC - *G. corticata*, GCC - *G. corticata* var. *cylindrica*, GF - *G. foliifera* and UF - *U. fasciata.*

Gracilaria sp. had high arabinose content succeeded by ribose and xylose content. Chlorophyta had arabinose as major sugar succeeded with galactose, mannose and sucrose units. Ribose was seen highest in *G. foliifera*, xylose in *G. corticata* var. *cylindrica*, arabinose, glucose and galactose in *C. antennina*, fructose and mannose in *E. prolifera* collected from Njarakkal location and sucrose in *U. fasciata*.

With respect to the ribose content, no particular distinguishing pattern was observed in between the Chlorophyta and Rhodophyta. In Rhodophyta, the high ribose containing polysaccharide was obtained from *G. foliifera* (22 %) whereas in Chlorophyta, it was the *E. prolifera* obtained from Kayamkulam location (20 %). Least ribose containing Rhodophyta was the *G. corticata* var. *cylindrica* (10 %), whereas in Chlorophyta it was the *C. antennina* (11 %). Irrespectively, comparable ribose content was observed in between *E. prolifera* obtained from Kayamkulam location (20 %) and *E. prolifera* obtained from Njarakkal location (19 %). With respect to the xylose content, remarkable variation was observed in between the Rhodophyta and Chlorophyta. Rhodophyta had high levels of xylose contents. *G. corticata* var. *cylindrica* (19 %) had the high xylose content among the analysed seaweeds. Among the Chlorophyta, *U. fasciata* had high xylose contents (11 %). Comparable contents were observed in between *C. antennina* (5.37 %) and *E. prolifera* obtained from Njarakkal location (4.52 %), and in between *E. prolifera* obtained from Kayamkulam location (10 %) and *G. corticata* (9.41 %). Arabinose contents had no specific distinguishing patterns between the Rhodophyta and Chlorophyta. Among the Rhodophyta, *G. corticata* var. *cylindrica* (33 %) and among the Chlorophyta, *C. antennina* (36 %) exhibited high contents of arabinose. Least arabinose content among the analysed seaweeds was observed in the Chlorophyta, *E. prolifera* obtained from

Njarakkal location (19 %). Among the Rhodophyta, the least arabinose containing polysaccharide was obtained from *G. foliifera* (23 %).

High contents of fructose were observed in *E. prolifera* obtained from Njarakkal location (12 %). *C. antennina* didn't exhibit the presence of fructose. Comparable fructose content was seen in between *E. prolifera* obtained from Kayamkulam location (2.48 %) and *G. foliifera* (2.38 %). Among the detected seaweeds, *G. corticata* (2.10 %) had the least fructose contents. The pattern of mannose content in the isolated polysaccharides exhibited similarity with the fructose contents. Irrespective of the divisions, *E. prolifera* obtained from the Njarakkal location (37 %) had the high mannose contents. *C. antennina* didn't exhibit mannose content in its polysaccharide. Comparable mannose contents were observed in between *G. corticata* (2.28 %) and *U. fasciata* (2.41 %). Least mannose content among the Chlorophyta was observed in *E. prolifera* obtained from Kayamkulam location (0.68 %). Among the Rhodophyta, the least mannose content was observed in *G. foliifera* (1.08 %). Glucose content was seen high in Chlorophyta with *C. antennina* exhibiting 6.41 %. Among the Rhodophyta, it was exhibited by *G. corticata* var. *cylindrica* (4.97 %). *G. corticata* (2.97 %) and *G. foliifera* (3.30 %) exhibited comparable glucose content. Least glucose content was exhibited by *E. prolifera* obtained from Njarakkal location (0.11 %). High galactose content was seen in the Chlorophyta *C. antennina* (26 %). Among the Rhodophyta, it was observed in *G. corticata* var. *cylindrica* (12 %). Comparable galactose content in the Chlorophyta was observed in between *E. prolifera* obtained from Kayamkulam location (10 %) and *U. fasciata* (10 %). In Rhodophyta, it was observed in between the *G. corticata* (9.24 %) and *G. foliifera* (9.08 %). Least galactose content was observed in *E. prolifera* obtained from Njarakkal location (2.31 %).

With respect to sucrose content, no specific patterns that could distinguish between the Chlorophyta and Rhodophyta were observed. The sucrose content was seen high in *U. fasciata* (8.12 %). Comparable sucrose content was seen in between *E. prolifera* obtained from Kayamkulam location (7.67 %) and *G. corticata* (7.60 %). Least sucrose content was seen in *E. prolifera* obtained from Njarakkal location (1.08 %).

Monosaccharide speciation in previous studies had similar constituents. Xylose was observed in the sulfated polysaccharides of the red seaweed *Georgiella confluens* (Kolender & Matulewicz, 2002) and *Nemalion helminthoides* (Pérez Recalde et al., 2009). Xylose and galactose were reported in the sulfated galactans obtained from the brown seaweed *Chaetomorpha indica* (Mandal et al., 2007) and *Padina gymnospora* (Silva et al., 2005). Glucose, galactose and xylose were reported in the sulfated polysaccharides obtained from the edible marine brown seaweed *Fucus vesiculosus* (Rupérez et al., 2002) and in the polysaccharides extracted from the red seaweed *Bostrychia montagnei* (Noseda et al., 1999). Arabinose (57 %) and galactose (38 %) was observed in the polysaccharides isolated from *C. antennina* (Rao & Ramana, 1991). Arabinose (8.9 %), xylose (1.0 %), galactose (12 %) and glucose (1.0 %) were reported in the sulfated polysaccharides extracted from the green seaweed *Spongomorpha indica* (Rao et al., 1991). Arabinose (31 %), galactose (58 %) and xylose (10 %) were observed in the green seaweed *Cladophora socialis* (Ramana & Rao, 1991).

Glucose, fructose and sucrose were reported in the green seaweed *Acetabularia crenulata* (Bourne et al., 1972). Mannose was reported in *Sargassum fulvellum* and *Zostera marina* (Yoshiie et al., 2012). It was also reported in various other studies done on seaweeds (Abdel-Fattah et al., 1974; Maruyama & Yamamoto, 1984; Chizhov et al., 1999; Duarte et al., 2001; Ponce et al., 2003; Hemmingson et al., 2006; Li et al., 2006; Cumashi et al.,

2007; Ale et al., 2012). Glucose was also reported in earlier studies conducted on seaweeds (Maruyama & Yamamoto, 1984; Chizhov et al., 1999; Nagaoka et al., 1999; Duarte et al., 2001; Marais & Joseleau, 2001; Ponce et al., 2003; Hemmingson et al., 2006; Li et al., 2006; Cumashi et al., 2007; Ale et al., 2012). Galactose as the major sugar, along with mannose and glucose containing fucoidan was reported from *Turbinaria decurrens* collected from the Gulf of Mannar, India (Shanthi et al., 2014). The red seaweed, *Porphyra umbilicalis* was reported to have the corresponding sulfated polysaccharides with galactose as the major sugar moiety (Turvey & Rees, 1961). Fructose, glucose and sucrose were observed in the fermented beverage made from the red seaweed *Gracilaria fisheri* (Hayisama-ae et al., 2014). Galactose, glucose, xylose and ribose were observed in the galactans isolated from the red seaweed *Laurencia papillosa* (Abdel-Fattah & Edrees, 1973).

Cultivated *Ulva clathrata* was reported to have xylose (10 - 13 %) and glucose (10 - 16%) (Peña-Rodríguez et al., 2011). Thai brown seaweeds, *C. sinuosa, H. clathratus, D. dichotoma, P. australis, P. minor, S. polycystum* and *T. conoides* were reported with its sulfated polysaccharides compositing of monosaccharide sugars such as glucose, mannose, fucose and galactose (Kantachumpoo & Chirapart, 2010). Japanese seaweed *E. bicyclis* was reported to have a polysaccharide which consisted of glucose, fucose, galactose, mannose, xylose and ribose as their monosaccharide units (Men'shova et al., 2013).

Fucoidans isolated from *F. vesiculosus,* showed the dominance of fucose and galactose as the main components (Rodriguez-Jasso et al., 2013). Five different polysaccharide fractions isolated from *G. birdiae* showed the presence of galactose, glucose, arabinose and xylose as the major monosaccharide composition (Fidelis et al., 2014). Sulfated polysaccharide isolated from *Padina pavonia*, had mannose and fucose as the main constituents. Xylose, galactose and glucose were also observed in low concentrations (Mohamed & Agili, 2013).

Sulfated polysaccharides isolated from *S. platensis* collected from USA, showed the dominance of glucouronic acid and galactose, followed by rhamnose, arabinose, glucose and ribose (Abd El Baky et al., 2013). Monosaccharide speciation of the polysaccharides isolated from the Atlantic Canadian seaweeds exhibited glucose, galactose and xylose in the three red algae *Polysiphonia lanosa, Furcellaria lumbricalis* and *Palmaria palmata* (Jiao et al., 2011a). Mannose, glucose, galactose, xylose and fucose in the two brown algae *Ascophyllum nodosum* and *F. vesiculosus*, and rhamnose, glucose, galactose, xylose and arabinose in the green alga *Ulva lactuca* was reported (Jiao et al., 2011a). Japanese brown seaweeds *Saccharina cichorioides, Fucus evanescens* and *U. pinnatifida* were observed to have the fucose as major monosaccharide. Presence of galactose, mannose, xylose, rhamnose and glucose was also observed (Vishchuk et al., 2013).

Even though the presence of all the monosaccharide units and sucrose analysed in this study were previously reported in seaweeds, the overall sugar compositions of seaweeds are reported for the first time. Uncertainty remains on the source of these sugars. In consideration of this aspect, the correlations studies were relied upon. The observations of the correlation studies would aid in forming a final conclusion upon the controversy that whether they are present in the polysaccharide backbone or were isolated from the seaweed as a free form.

6.3.9 Correlation Studies

The correlation studies in consideration with the biochemical constituents and *in vitro* bioactivity studies were carried out (Table 6.9). The isolated polysaccharide fraction had positive correlations with iodine value. It exhibits the tendency of absorbance of iodine by the polysaccharide units which could be due to the presence of glycolipids or glycoesters. It also has positive correlations with xylose, arabinose, fructose, mannose and glucose.

Ribose, galactose and sucrose had negative correlations with the polysaccharide fractions. The positive values indicate their undoubted presence in the polysaccharide units. Overall, seaweeds have the presence of all the seven monosaccharides and one disaccharide. The negative values thereby indicate that majority of the estimated sugar moieties are present in free form in seaweeds, which may or may not be attached to the complex polysaccharide units. The polysaccharide units also had positive correlations with the DPPH, ferrous tartarate and $KMnO_4$ antioxidant activity. It also exhibited positive correlations with the antimicrobial activities too.

Table 6.9 Pearson's bivariate correlation analysis, (n=21), (Split into 10 sections for enhanced readability).

(a)

	PF	SV	IV	SC	RIB	XYL	ARA	FRC
PF	1							
SV	-0.604	1						
IV	0.233	-0.414	1					
SC	-0.355	-0.108	-0.255	1				
RIB	-0.363	0.265	0.380	-0.561	1			
XYL	0.783*	-0.664*	0.126	-0.389	-0.220	1		
ARA	0.219	-0.593	-0.108	0.724*	-0.839*	0.247	1	
FRC	0.047	0.669*	-0.355	-0.490	0.237	-0.273	-0.698*	1

(b)

	PF	SV	IV	SC	RIB	XYL	ARA	FRC
MAN	0.012	0.694*	-0.408	-0.327	0.112	-0.366	-0.591	0.982*
GLU	0.094	-0.562	-0.112	0.714*	-0.673*	0.328	0.912*	-0.813*
GAL	-0.170	-0.401	-0.187	0.930*	-0.639*	-0.076	0.893*	-0.718*
SUC	-0.278	-0.273	0.533	-0.131	0.384	0.166	0.028	-0.679*
DAAE	0.565	-0.083	-0.021	-0.390	0.172	0.348	-0.379	0.518
DBHT	0.578	-0.098	-0.019	-0.384	0.158	0.358	-0.364	0.510
DTE	0.636	-0.144	-0.056	-0.412	0.099	0.481	-0.311	0.458
DRE	0.583	-0.102	-0.023	-0.384	0.151	0.368	-0.358	0.506

(c)

	PF	SV	IV	SC	RIB	XYL	ARA	FRC
FTAAE	0.520	-0.462	-0.096	0.434	-0.668*	0.077	0.591	-0.018
FTBHT	0.655*	-0.076	0.025	-0.644*	-0.127	0.353	-0.191	0.521
FTTE	0.647*	-0.075	0.038	-0.641*	-0.114	0.332	-0.199	0.526
FTRE	0.031	-0.382	-0.110	0.864*	-0.540	-0.174	0.691*	-0.382
KAAE	0.410	-0.404	0.935*	-0.467	0.444	0.195	-0.275	-0.086
KBHT	0.389	-0.393	0.936*	-0.471	0.458	0.177	-0.284	-0.086
KTE	0.389	-0.394	0.936*	-0.472	0.458	0.179	-0.283	-0.087
KRE	0.391	-0.395	0.936*	-0.472	0.457	0.181	-0.282	-0.087

(d)

	PF	SV	IV	SC	RIB	XYL	ARA	FRC
TSA	0.143	-0.398	0.095	-0.340	0.009	0.617	0.185	-0.480
TBC	0.083	-0.454	-0.299	0.383	-0.289	0.507	0.503	-0.567
TEC	0.397	-0.539	0.187	-0.261	-0.147	0.839*	0.310	-0.566
TSAB	0.480	-0.149	-0.391	-0.118	-0.367	0.716*	0.177	0.005
CPSA	0.133	-0.378	0.090	-0.360	0.032	0.608	0.158	-0.459
CPBC	0.089	-0.442	-0.297	0.387	-0.298	0.506	0.502	-0.559
CPEC	0.397	-0.539	0.187	-0.261	-0.147	0.839*	0.310	-0.566
CPSAB	0.482	-0.160	-0.398	-0.127	-0.363	0.727*	0.179	0.001

(e)

	MAN	GLU	GAL	SUC	DAAE	DBHT	DTE	DRE
MAN	1.000							
GLU	-0.730	1.000						
GAL	-0.589	0.897	1.000					
SUC	-0.771	0.199	0.061	1.000				
DAAE	0.480	-0.324	-0.442	-0.583	1.000			
DBHT	0.473	-0.313	-0.433	-0.585	1.000	1.000		
DTE	0.413	-0.238	-0.418	-0.541	0.987	0.988	1.000	
DRE	0.469	-0.306	-0.430	-0.584	1.000	1.000	0.990	1.000

(f)

	MAN	GLU	GAL	SUC	DAAE	DBHT	DTE	DRE
FTAAE	0.092	0.314	0.443	-0.621	0.243	0.258	0.225	0.258
FTBHT	0.458	-0.475	-0.568	-0.329	0.284	0.290	0.310	0.292
FTTE	0.464	-0.489	-0.570	-0.332	0.284	0.290	0.305	0.291
FTRE	-0.234	0.631	0.818	-0.358	0.035	0.044	0.000	0.044
KAAE	-0.163	-0.339	-0.411	0.315	0.235	0.238	0.191	0.233
KBHT	-0.164	-0.351	-0.415	0.330	0.213	0.215	0.167	0.211
KTE	-0.166	-0.351	-0.416	0.333	0.211	0.213	0.165	0.208
KRE	-0.166	-0.350	-0.416	0.332	0.213	0.215	0.168	0.210

(g)

	MAN	GLU	GAL	SUC	DAAE	DBHT	DTE	DRE
TSA	-0.598	0.264	-0.015	0.709	-0.410	-0.406	-0.288	-0.399
TBC	-0.560	0.769	0.569	0.148	0.093	0.097	0.190	0.105
TEC	-0.661	0.470	0.071	0.600	-0.144	-0.138	0.001	-0.128
TSAB	-0.017	0.369	0.019	-0.214	0.454	0.457	0.579	0.468
CPSA	-0.580	0.237	-0.040	0.709	-0.404	-0.401	-0.283	-0.394
CPBC	-0.549	0.771	0.568	0.134	0.105	0.109	0.202	0.117
CPEC	-0.661	0.470	0.071	0.600	-0.144	-0.138	0.001	-0.128
CPSAB	-0.025	0.370	0.018	-0.205	0.449	0.453	0.576	0.463

(h)

	FTAAE	FTBHT	FTTE	FTRE	KAAE	KBHT	KTE	KRE
FTAAE	1							
FTBHT	0.294	1						
FTTE	0.302	0.999*	1					
FTRE	0.737*	-0.429	-0.421	1				
KAAE	0.015	0.291	0.307	-0.193	1			
KBHT	0.001	0.291	0.307	-0.206	0.999*	1		
KTE	-0.001	0.292	0.309	-0.209	0.999*	0.999*	1	
KRE	0.001	0.292	0.309	-0.208	0.999*	0.999*	0.999*	1

(i)

	FTAAE	FTBHT	FTTE	FTRE	KAAE	KBHT	KTE	KRE
TSA	-0.396	0.164	0.146	-0.491	0.020	0.028	0.033	0.032
TBC	0.016	-0.522	-0.542	0.387	-0.418	-0.434	-0.434	-0.432
TEC	-0.286	0.060	0.036	-0.312	0.086	0.080	0.082	0.083
TSAB	-0.016	0.044	0.016	-0.042	-0.361	-0.385	-0.385	-0.383
CPSA	-0.414	0.171	0.153	-0.514	0.021	0.030	0.034	0.033
CPBC	0.018	-0.527	-0.547	0.392	-0.419	-0.435	-0.435	-0.434
CPEC	-0.286	0.060	0.036	-0.312	0.086	0.080	0.082	0.083
CPSAB	-0.019	0.053	0.024	-0.050	-0.364	-0.388	-0.388	-0.385

(j)

	TSA	TBC	TEC	TSAB	CPSA	CPBC	CPEC	CPSAB
TSA	1							
TBC	0.306	1						
TEC	0.873*	0.539	1					
TSAB	0.237	0.672*	0.563	1				
CPSA	0.999*	0.292	0.864*	0.231	1			
CPBC	0.287	0.999*	0.534	0.686*	0.273	1		
CPEC	0.873*	0.539	0.999*	0.563	0.864*	0.534	1	
CPSAB	0.258	0.679*	0.575	0.999*	0.253	0.692*	0.575	1

*. Correlation is significant at the 0.001 level (2-tailed).

PF - Polysaccharide content, SV - Saponification value, IV - Iodine value, SC - Sulfate content, RIB - Ribose content, XYL - Xylose content, ARA - Arabinose content, FRC - Fructose content, MAN - Mannose content, GLU - Glucose content, GAL - Galactose content, SUC - Sucrose content, DAAE - DPPH Ascorbic acid equivalence, DBHTE - DPPH BHT equivalence, DTE - DPPH α-tocopherol equivalence, DRE - DPPH resorcinol equivalence, FTAAE - Ferrous tartarate ascorbic acid equivalence, FTBHTE - Ferrous tartarate BHT equivalence, FTTE - Ferrous tartarate tocopherol equivalence, FTRE - Ferrous tartarate resorcinol equivalence, KAAE - $KMnO_4$ ascorbic acid equivalence, KBHTE - $KMnO_4$ BHT equivalence, KTE - $KMnO_4$ tocopherol equivalence, KRE - $KMnO_4$ resorcinol equivalence, TCSA - Tetracycline - *S. aureus*, TCBC - Tetracycline - *B. cereus*, TCEC - Tetracycline - *E. coli*, TCSAB - Tetracycline - *S. abony*, CPSA - Chloramphenicol - *S. aureus*, CPBC - Chloramphenicol - *B. cereus*, CPEC - Chloramphenicol - *E.coli* and CPSAB - Chloramphenicol - *S. abony*.

Saponification value exhibited positive correlation with ribose, fructose and mannose contents. Iodine value had positive correlations with ribose, xylose and sucrose contents. It also exhibited positive correlations with ferrous tartarate BHT equivalence, ferrous tartarate α-tocopherol equivalence, $KMnO_4$ antioxidant activities and the antimicrobial activity studies against the *S. aureus* and *E. coli* strains. The sulfate content had positive correlations with arabinose, glucose and galactose contents. It also exhibited positive relations with the ferrous tartarate ascorbic acid equivalence and ferrous tartarate resorcinol equivalence antioxidant activities and *B. cereus* bactericidal activities.

Ribose had positive correlations with the fructose, mannose and sucrose contents. It also exhibited positive correlations with both the DPPH and ferrous tartarate antioxidant activities and the *S. aureus* bactericidal activities. Xylose had positive correlations with the arabinose, glucose and sucrose contents. Xylose also had positive correlations with the three antioxidant activities except ferrous tartarate resorcinol equivalence. Xylose exhibited high positive correlations against all the bactericidal activity of the four strains of pathogens. Arabinose had positive correlations with glucose, galactose and sucrose. Among the antioxidant activity studies, only the ferrous tartarate ascorbic acid equivalence and ferrous tartarate resorcinol equivalence exhibited positive correlation with arabinose. Irrespective of the low correlation with antioxidant activities, arabinose was observed to have positive correlations with all the four strains bactericidal activities. Fructose had positive correlations with mannose content, DPPH and ferrous tartarate BHT equivalences and ferrous tartarate α-tocopherol equivalence antioxidant activities, and the *S. abony* bactericidal activities. Mannose had positive correlations with DPPH and ferrous tartarate antioxidant activities with exception in the ferrous tartarate resorcinol equivalence. Glucose exhibited

positive correlations with galactose and sucrose contents, ferrous tartarate ascorbic acid equivalence and ferrous tartarate resorcinol equivalence antioxidant activities and all the four strain bactericidal activities. Galactose had positive correlations with sucrose content. It also had high positive correlations with ferrous tartarate resorcinol equivalence activity and *B. cereus, E. coli* and *S. abony* bactericidal activities. Sucrose had high positive correlations with $KMnO_4$ antioxidant activities. It also had *S. aureus*, *B. cereus* and *E. coli* bactericidal activities.

DPPH antioxidant activities were observed to have positive correlations with ferrous tartarate and $KMnO_4$ antioxidant activities. But the antioxidant activities exhibited by the DPPH method were found to be positively correlated with the antimicrobial activity against the *B. cereus* and *S. abony*. This indicates the presence of the bactericidal antioxidant component in the polysaccharides. Ferrous tartarate ascorbic acid equivalence antioxidant activities had positive correlations with BHT, α-tocopherol and resorcinol equivalences, $KMnO_4$ activities except α-tocopherol equivalence and *B. cereus* bactericidal activities. Ferrous tartarate BHT equivalence antioxidant activities exhibited positive correlations with α-tocopherol equivalence, $KMnO_4$ activities and *S. aureus*, *E. coli* and *S. abony* activities. Ferrous tartarate α-tocopherol equivalence antioxidant activities had positive correlations with $KMnO_4$ activities and *S. aureus*, *E. coli* and *S. abony* activities. Ferrous tartarate resorcinol equivalence antioxidant activities had positive correlations with B. cereus bactericidal activities. $KMnO_4$ antioxidant activities had positive correlations with *S. aureus* and *E. coli* bactericidal activities. The bactericidal activities had specific inter-bacterial positive correlations with specific correlations between each of the organisms.

6.4 Conclusion

This study evaluated the overall sugars isolated from Chlorophyta and Rhodophyta. High contents of isolatable polysaccharides were isolated from Rhodophyta (*Gracilaria* sp.). The saponification and iodine values indicated the probability for glycolipids / glycoesters seaweeds. Sulfate contents indicated the presence of sulfate esters. Negative optical rotation indicated the probability of negative optical isomers, which was evidenced by the monosaccharide composition profiling. Arabinose, ribose, galactose and xylose were the major monosaccharides. The isolated sugars had appreciable antioxidant and antimicrobial activities too.

The previous studies on algal polysaccharides have substantiated these observations. The polysaccharides yield, its saponification and iodine value and sulfate content of the current study highlights its importance. It plays a vital role in human health and nutrition which also acts as a natural chemo preventive agent. The antioxidant and antimicrobial activities exhibited by the polysaccharides in the current study play an important role as an effective bio medicinal tool to cure degenerative diseases in the upcoming future. In addition, they are considered to be low toxic and less irritating which with continued efforts in future could be utilized to develop novel types of polymers that could be useful as a drug or for human health care systems.

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Chapter 7

ISOLATION AND CHARACTERIZATION OF A KETOSTEROID –*E*-GUGGULSTERONE

Contents

7.1 Introduction

Previous chapters had a detailed discussion on the biochemical constitutions of the selected seaweeds from the Kerala coast. In addition to those, seaweeds are renowned for their structurally diverse bioactive compounds that possess antioxidant and antimicrobial activities too (Thirunavukkarasu et al., 2013). This diversity is observed as a response of seaweeds towards oxidation and extreme environmental conditions in which they live. The revolutionized therapy of degenerative and infectious diseases on humans by the use of chemical antioxidants and antimicrobial drugs has certain limitations. The limitations include the adverse effects of chemicals and mutation of microorganisms. This effect resulted in the demand for improved pharmacokinetic compounds from renewable vegetative sources (Al-Haj et al., 2009; Jeeva et al., 2012). Seaweeds which are one of the important marine food resources are remarked as the most accessible marine resource of the coastal zone which has pharmaceutical importance.

Seaweeds possess an extensive profile of secondary metabolites like the terpenes, fatty acids, sterols etc. (Rajasulochana et al., 2012; El-Shoubaky &

Salem, 2014). Sterols such as cholesterol in Caribbean red seaweeds (Beastall et al., 1971; Govindan et al., 1993), stigmasterol in *Kappaphycus* sp. (Rao et al., 1991), cholesterol and desmosterol in brown and red seaweeds (Goad & Goodwin, 1972; Sánchez-Machado, 2004), fucosterol in brown seaweeds, desmosterol in red seaweeds (Dhamotharan, 2002; Simonsen et al., 2009), cholesterol in red algae and fucosterol in brown algae (Combaut et al., 1985), camposterol, cholesterol, stigmasterol, *E*-sistosterol and methylenechole sterol in *Kappaphycus* sp. (Rajasulochana et al., 2009), 24-propylidenecholesta-5,25-dien-3β-ol, 24-propylidenecholesterol and 26,26-dimethyl-24-methylenecholesterol in *Pelagophyceae* (Giner et al., 2009), poriferast-5-en-3β-ol (clionasterol), 3β-hydroxyporiferast-5-en-7-one, poriferast-5-en-3β,7α-diol and poriferasta-3,5-diene-7-one from *Gracilaria edulis* were reported earlier (Das & Srinivas, 1992b).

Sterol compositions of seaweeds were observed to vary with algal classes. Red algae had cholesterol and desmosterol as the dominated sterols (Kamenarska et al., 2003). It also had 24-methyl-cholesta-5, 24(28)-dien-3β-ol (24-methylenecholesterol). 5α-chlolestanol was found in low concentrations (Kamenarska et al., 2003). Cholesterol and clinoasterol were observed in *Gracilaria crassa* and *Gracilaria coronopifolia* (Das & Srinivas, 1992a; Das & Srinivas, 1992b; Combres et al., 1986), and in *Gracilaria longa* (Henriquez et al., 1972; Fusetani & Hashimoto, 1984; Vilalta et al., 1984; Castedo et al., 1985; Toffanin et al., 1992) and *Gracilaria dura* (de Almeida et al., 2011). The methanol extracts of *Laurencia papillosa* had been reported with antimicrobial effects against the Gram negative *Escherichia coli*, *Pseudomonas aerugenosa*, *Klebsiella pneumoniae* and *Shigella flexineri* strains (Kavita et al., 2014). A cholesterol derivative, 24-propylidenecholest-5-en-3β-ol was identified as its active compound (Kavita et al., 2014). Other steroids such as 3-β-hydroxy-

poriferast-5-en-7-one, 3-β-7-α-diol-poriferast-5-ene and 5-α-poriferast-9(11)-en-3-β-ol are isolated from *Gracilaria dura* (Das & Srinivas, 1992a). Cholestane-3-β-5-diol,5-α:24(S)-ethyl (Combres et al., 1986), poriferastene 8 (Das & Srinivas, 1992a), poriferast-5-ene-3-β-7-β-diol (Das & Srinivas, 1992b) and poriferast-5-ene-3-β-7-α-diol (Das & Srinivas, 1992b) were identified in *Gracilaria coronopifolia. Gracilaria longa* had 5-dehydroavenasterol, fucosterol, desmosterol and 5-α-24(*S*)-ethyl-cholestane-3-β-6-β-diol (Das & Srinivas, 1993). *Gracilaria domigensis* had steroids such as stigmasterol, sitosterol, campesterol, cholest-7-en-3-β-ol and brassicasterol (Combres et al., 1986).

Brown algae had fucosterol as the major sterol (Kamenarska et al., 2003). Brown algae collected from the Black sea were reported to possess sterols such as 24-nor-chol-5-en-3β-ol, 24-nor-cholesta-5,22-dien-3β-ol, 24-nor-cholest-22-en-3β-ol, 27-nor-24-methyl-cholesta-5,22-dien-3β-ol, (22-*E*)-cholesta-5,22-dien-3β-ol, (22-*E*)-cholest-22-en-3β-ol, cholest-5,24(25)-dien-3β-ol, cholesterol, 5α-cholestan-3β-ol, 24-methyl-cholesta-5,22-dien-3β-ol, 5α-cholest-7-en-3β-ol, 24-methyl-cholest-5-en-3β-ol, 24-methylcholest-22-en-3-one, 24-methyl-cholesta-5,24(28)-dien-3β-ol, 24-methyl-cholest-24(28)-en-3β-ol, cholest-4-en-3-one, 24-ethyl-cholesta-5,24(28)*E*-dien-3β-ol (fucosterol), 24-ethyl-cholesta-5,24(28)-*Z*-dien-3β-ol (isofucosterol), 24-ethyl-cholesta-5,22-dien-3β-ol, 24-ethyl-cholest-5-en-3β-ol and 24-methyl-cholesta-4,24(28)-dien-3-one sterols (Kamenarska et al., 2003). Brown seaweed *Cystoseria* sp. reported to contain fucosterol, 22-*E*-dehydrocholesterol, brassicasterol, 24-methylenecholesterol and cystosterol (Francisco et al., 1977). Brown alga *Sargassum* sp. was observed to have antioxidant, cytotoxic and antitumor active steroids such as the fucosterol ((24-*E*)-stigmasta-5, 24(28)-diene-3β-ol), saringosterone (24-vinylcholest-4-ene-3-one) and saringosterol (24-vinylcholest-5-ene-3β, 24-diol) (Ayyad et al., 2011). Marine benthic brown alga *Iyengaria stellata* was reported to have sterols such as

cholesterol, 24-methylcholesterol and ergosterol (Usmanghani et al., 1987). Cytotoxic steroidal ketones such as the ergosta-4,24(28)-diene-3-one, ergosta-4,24(28)-diene-3,6-dione, stigmasta-4,24(28)-diene-3-one and stigmasta-4,24(28)-diene-3,6-dione were isolated from the holdfast of cultivated *Laminaria japonica* (Nishizawa et al., 2003).

Green algae had isofucosterol and sitosterol as the major sterols (Kamenarska et al., 2003). Green alga *Chaetomorpha basiretorsa* had been reported with the sterols such as β-lawsaritol, saringosterol, 24-hydroperoxy-24-vinyl-cholesterol, β-stigmasterol, stigmast-4-en-3α,6β-diol and 29-hydroxystigmasta-5,24(28)-dien-3β-ol (Shi Dayong et al., 2008). *Caulerpa lentillifera* was observed to have sterols such as cholesta-5,22-*E*-dien-3β-ol, cholest-5-en-3β-ol, 24-methylcholesta-5,22-*E*-dien-3β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol, 24-methylcholest-5-en-3β-ol, 24-ethylcholesta-5,22-*E*-dien-3β-ol and 24-*S*-ethylcholest-5-en-3β-ol (clionasterol) (Shevchenko et al., 2009). Green seaweeds such as the *Ulva lactuca* and *Cystoseira adriatica* were reported to possess sterols such as cholesterol, (*Z*)-stigmasta-5,24(28)-dien-3β-ol, (24R)-stigmast-5-en-3β-ol, stigmasta-5,22-dien-3β-ol, 22-dehydrocholesterol, ergosta-5(22)-dien-3β-ol, ergost-5-en-3β-ol and (*E*)-stigmasta-5,24(28)-dien-3β-ol (Kapetanović et al., 2005).

The previous studies on seaweeds as discussed above, highlight the presence of many ketosteroids such as 3β-hydroxyporiferast-5-en-7-one, poriferasta-3,5-diene-7-one, 24-methylcholest-22-en-3-one, cholest-4-en-3-one, 24-methyl-cholesta-4,24(28)-dien-3-one, saringosterone (24-vinyl cholest-4-ene-3-one), ergosta-4,24(28)-diene-3-one, ergosta-4,24(28)-diene-3,6-dione, stigmasta-4,24(28)-diene-3-one and stigmasta-4,24(28)-diene-3,6-dione. Most of these ketosteroids were observed to have antioxidant and antimicrobial properties.

This chapter deals with the isolation, characterization and activity studies of steroids from the seaweed *Gracilaria foliifera* which was abundantly available in the Kerala coast. *G. foliifera* sp. is observed to be confined to Red, Arabian and Indian seas (Guiry & Freamhainn, 2009). It has been already reported to possess sterols (Govindan *et al.,* 1993; Das & Srinivas, 1992a; Das & Srinivas, 1992b) such as fucosterol, (24*E*)-stigmasta-5,24(28)-diene-3β-ol and isofucosterol, (24*Z*)-stigmasta-5,24(28)-diene-3β-ol (Alarif et al., 2010). This is the first study on the isolation, characterization and bioactivity of a ketosteroid, *E*-guggulsterone obtained from *G. foliifera*, collected from the Kerala coast.

E-guggulsterone [Pregna-4,17-diene-3,16-dione ((8R,9S,10R,13S,14S)-17-ethylidene-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15-decahydrocyclopenta [a]phenanthrene-3,16-dione)] is a prominent plant steroid that has been reported to be present in the resin of the tree *Commiphora wightii* and has not been reported in any of the seaweed sources. Guggulsterol III has been reported earlier from a marine cnidarian, *Leptogorgia sarmentosa* (Benvegnu et al., 1982). Guggulsterone possess many therapeutic benefits (Dave & Chopda, 2013). Guggulsterone has anti-inflammatory (Shishodia et al., 2008; Deng, 2007), antioxidant (Singh et al., 1994; Chander et al., 2003; Shah et al., 2012), anti-arthritic (Sharma, 1977; Lee et al., 2008; Shah et al., 2012), antiosteoarthritic (Shah et al., 2012), cardioprotective (Chander et al., 2003; Wang et al., 2004; Ojha et al., 2008), anticholesterolic (Chander et al., 1996; Cui et al., 2003; Urizar & Moore, 2003; Deng et al., 2007), anticarcinogenic (Shah et al., 2012), antiobesic (Yang et al., 2008), antidiabetic (Cornick et al., 2009), neuroprotective (Saxena et al., 2007) and antimicrobial (Ishnava et al., 2010) effects.

E-guggulsterone was isolated from the saponins fraction of *G. foliifera*. UV scans, HPLC, specific rotation, melting point, FTIR, GC-MS, LC-MS/MS, ^{13}C and ^{1}H NMR analyses were carried out. NMR studies were further processed using the 2 dimensional COSY, HMBC and HSQC analytical techniques, to conclude the proposed structure. The bioactivity was studied, with the antioxidant activity using the DPPH free radical scavenging, ferrous tartarate and $KMnO_4$ methods and antimicrobial activities on Gram positive and Gram negative strains.

7.2 Materials and Methods

7.2.1 Apparatus and Chemicals

Chemicals used were of analytical grade and the solvents were purchased in HPLC grade (Merck (Germany)). Water used was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Chloramphenicol and tetracycline were purchased from sigma (USA). Mannitol salt agar (MSA), nutrient agar, Levin eosin methylene blue agar (L-EMBA) and soyabean casein digest agar were purchased from Difco, USA.

7.2.2 Pathogen Culture used

Lyophilised cultures (live strains) of *Escherichia coli* ATCC 25922 - Gram - negative rod, and *Staphylococcus aureus* ATCC 6538 - Gram - positive cocci were used.

7.2.3 Samples

Among the collected nine seaweeds, *G. foliifera* was observed to be the most abundant species. Initially 1,750 g of dry, coarse ground *G. foliifera* was

used in this study. The saponins fraction of *G. foliifera* was taken for the study as all other solvent fractions had previously reported compounds (Chapter 5). The study was counter checked with freshly collected 1,500 g of *G. foliifera* collected from a different location.

7.2.4 Extraction of Steroid

7.2.4.1 Extraction and Purification of Saponins

Saponins content of the seaweed was extracted by the double solvent gravimetric method (Harbone, 1973). 1,750 g of the dry, coarse ground *G. foliifera* was mixed with 5 L of 20 % ethanol solution and heated at 50 - 60 ^{o}C in a water bath for 90 min (Fig. 7.1). The supernatant solution was concentrated and filtered off through a Whatman no. 40 filter paper. The residue was re-extracted twice and the supernatants were combined and filtered. The supernatant was reduced to 200 mL at 90 ^{o}C and transferred to a 1 L separating funnel. 200 mL of diethyl ether was added and vigorously shaken. The ether layer was separated off. The aqueous layer was re-extracted twice with 200 mL of diethyl ether. The diethyl ether layer was discarded. The aqueous layer was extracted further with 600 mL of n-butanol thrice. The combined n-butanol layer was subjected to distillation to remove the solvent layer and dried in a pre weighed 500 mL glass beaker at 60 ^{o}C. The weight of residue was estimated and reported as the weight of crude saponins.

The above residue was repeatedly extracted using 500 mL each of 90 % methanol-water until the supernatant was clear. The aqueous methanolic solution was then reduced to 100 mL. 100 mL 6N methanolic KOH was added and refluxed for 3 h. The extract volume was again reduced to one fourth

under vacuum at 50 ^{o}C and transferred to a separating funnel. 50 mL of petroleum ether was added and shaken vigorously. The petroleum ether layer was separated. The aqueous layer was re-extracted with 50 mL of petroleum ether until the petroleum layer was colourless. The petroleum ether layer was discarded. The aqueous layer was then kept in a water bath to remove the traces of petroleum ether. It was then transferred into a separating funnel and extracted with 50 mL ethyl acetate. The process was repeated until the ethyl acetate layer was colourless. The ethyl acetate layer was discarded. The aqueous layer was then kept in boiling water bath for 5 min, cooled and the pH of the solution was adjusted to 2 - 2.5 using drops of concentrated hydrochloric acid. The aqueous layer was again transferred to a separating funnel and extracted with 50 mL chloroform. The process was repeated until the chloroform layer was colourless. The chloroform layer was discarded. The pH of the aqueous layer was raised to 7.0 with 10 % NaOH solution. This layer was transferred to a 250 mL round bottom flask and refluxed for 3h with 1 % acetic acid solution. The aqueous layer was allowed to cool to room temperature (30 ^{o}C) and transferred to 500 mL separating funnel. It was extracted thrice with both ethyl acetate and dichloromethane. The aqueous layer was discarded. The ethyl acetate and dichloromethane layers were desolventized, dried and weighed. The combined weight was reported as the weight of purified saponins.

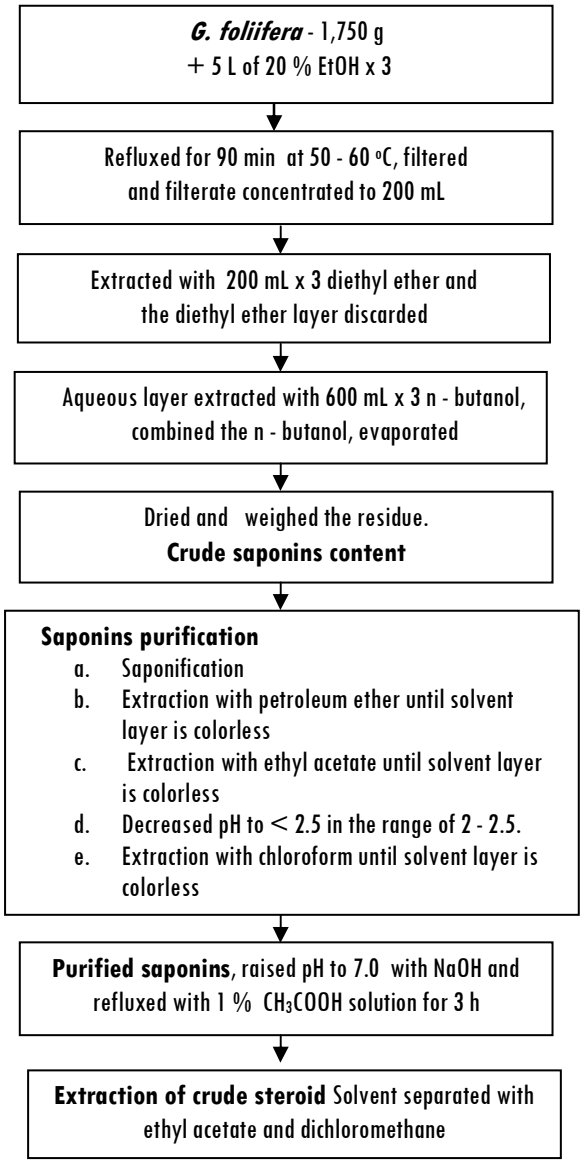


Fig. 7.1 Flow diagram of the sequential extraction and purification processes involved in the extraction of crude steroids from *G. foliifera*.

Ethanolic extract of the fresh 1,500 g *G. foliifera* was collected following the above methods using proportionate amount of solvents. The extract was dried and kept for counter checking of the final results.

7.2.4.2 Extraction of Steroids

7.2.4.2.1 Characterization using thin Layer Chromatography (TLC)

Thin layer chromatography screening was done with both the solvent extracts on the 10 cm x 5 cm, chromatographic plates coated with silica gel H (10 - 40 µm). Both the extracts were also screened with the counterpart solvents too (Fig. 7.2).

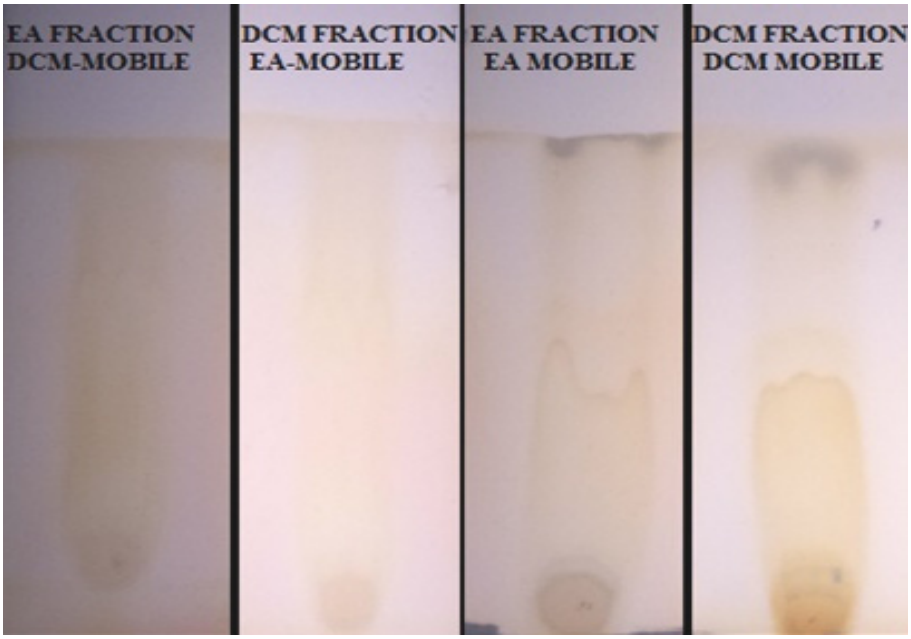


Fig. 7.2 Thin layer chromatogram of the crude steroid fraction collected from *G. foliifera*.

7.2.4.2.2 Characterization using UV-Vis Scanning

UV-Vis spectrophotometric scanning from 1100-190 nm was carried out on Carry-60 UV-Vis spectrophotometer (Fig. 7.3). The above extract exhibited an absorption maximum at 269 nm. The maximum absorption ranged from 270.5 to 230.0 nm. The TLC and UV pattern was similar and thus was pooled together and desolventized. The volume of extract was made to 10 mL.

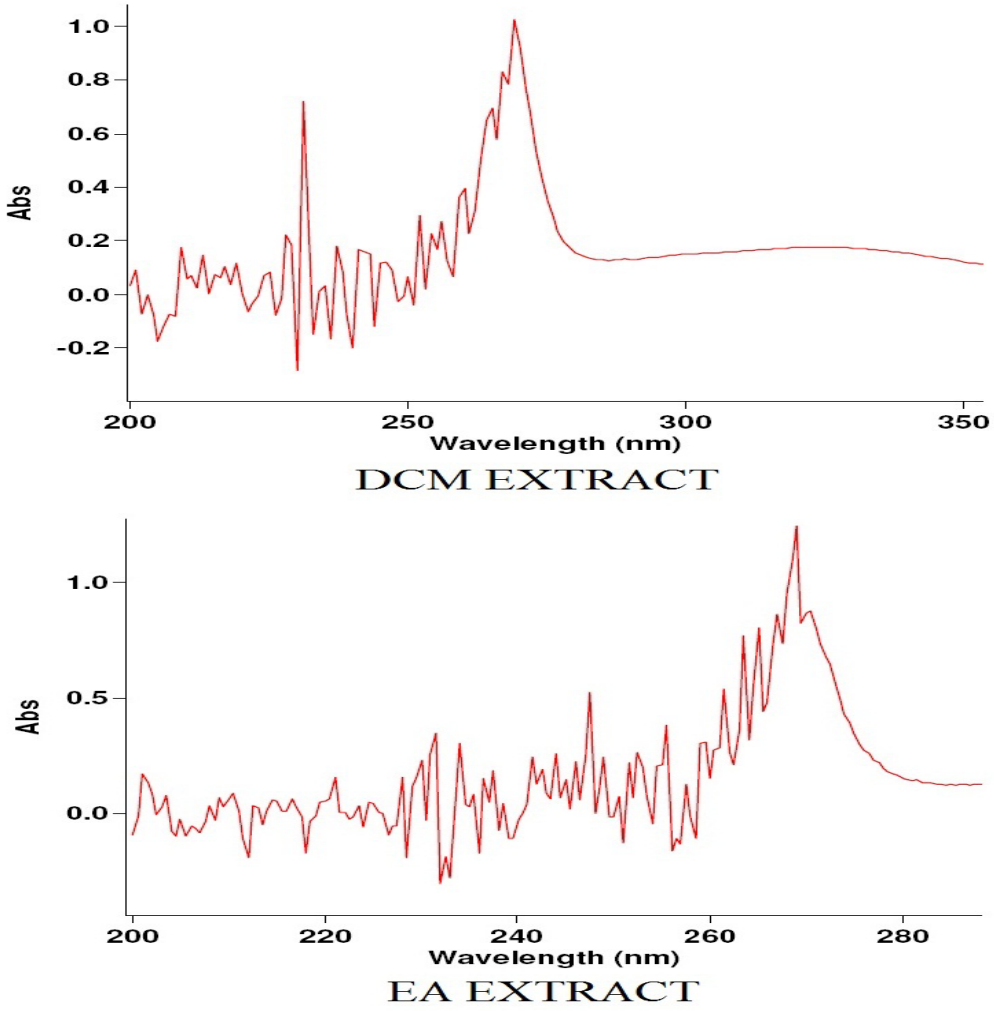


Fig. 7.3 UV-Vis spectrum scan of the crude steroid fraction (Ethyl acetate and DCM fractions) collected from *G. foliifera*.

7.2.4.2.3 Characterization using HPLC

The extract was injected to Shimadzu LC-20AD equipped with SPD-20A UV-Vis detector. The column used was phenomenex C-18, 250 mm x 4.6 mm x 5 µ column. Peak separation and integration was observed when the solvent system was 70:30 acetonitrile-water and detection at 254 nm (Fig.

7.4). The run time was 30 min, at a flow rate of 1.2 mL min^{-1}. The numerous numbers of peaks indicated the presence of many compounds.

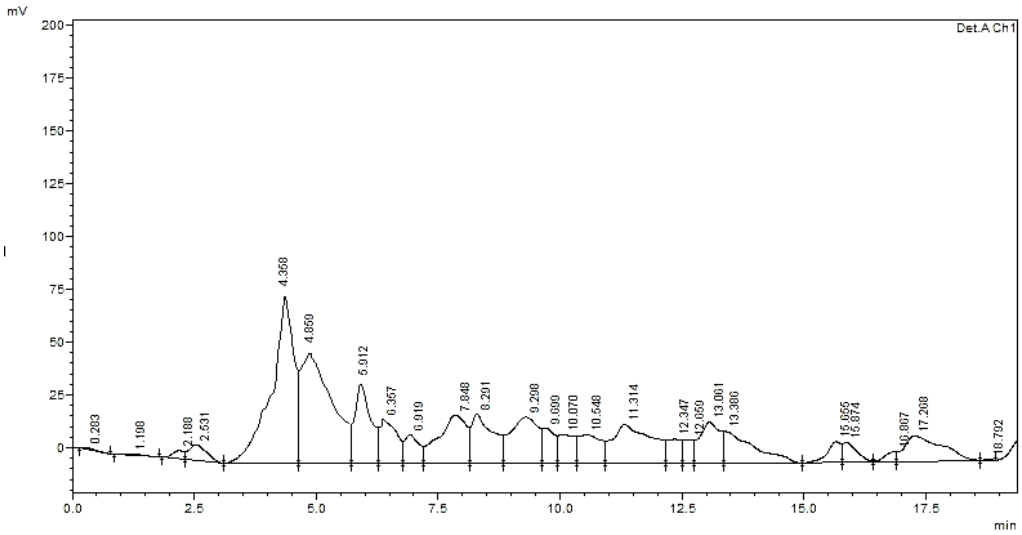


Fig. 7.4 HPLC chromatogram of the crude steroid fraction (Ethyl acetate and DCM fractions mixed) collected from *G. foliifera*. (detected at 254 nm).

7.2.4.2.4 Resolution using Column Chromatography and TLC

The extract was concentrated and mixed with 1 g silica gel and 5 mL 100 % hexane for column chromatography (70 - 230 µm). Silica gel column (125 g in 50 cm x 1 cm column) packed in 100 % hexane was used for resolution. The extract mixture was poured on the top of the column and eluted successively with hexane, hexane-ethyl acetate, ethyl acetate and dichloromethane. All the fractions were subjectively screened with TLC. The fractions obtained during 40:60, 50:50 and 60:40 hexane-ethyl acetate elution had maximum separation and distinctive spots. All the three fractions had similar TLC patterns (Fig. 7.5) and so were pooled together.

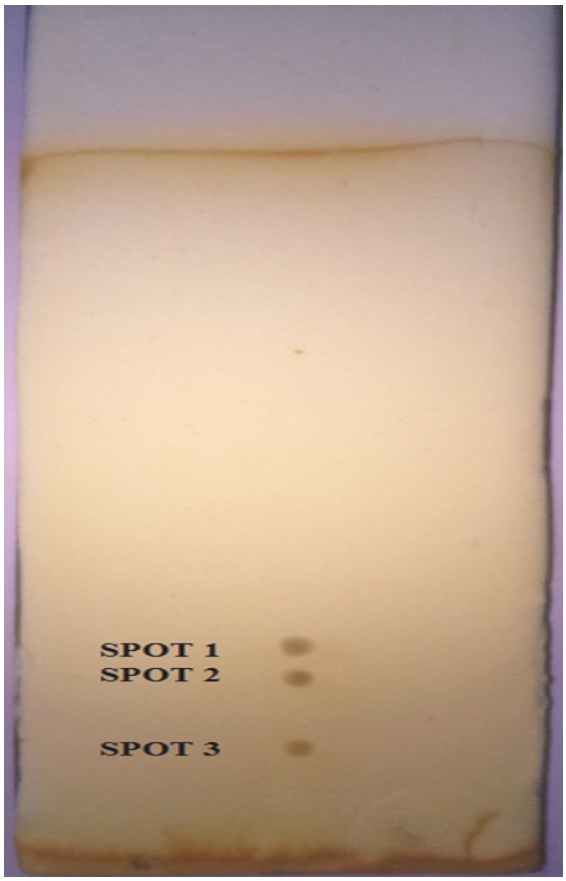
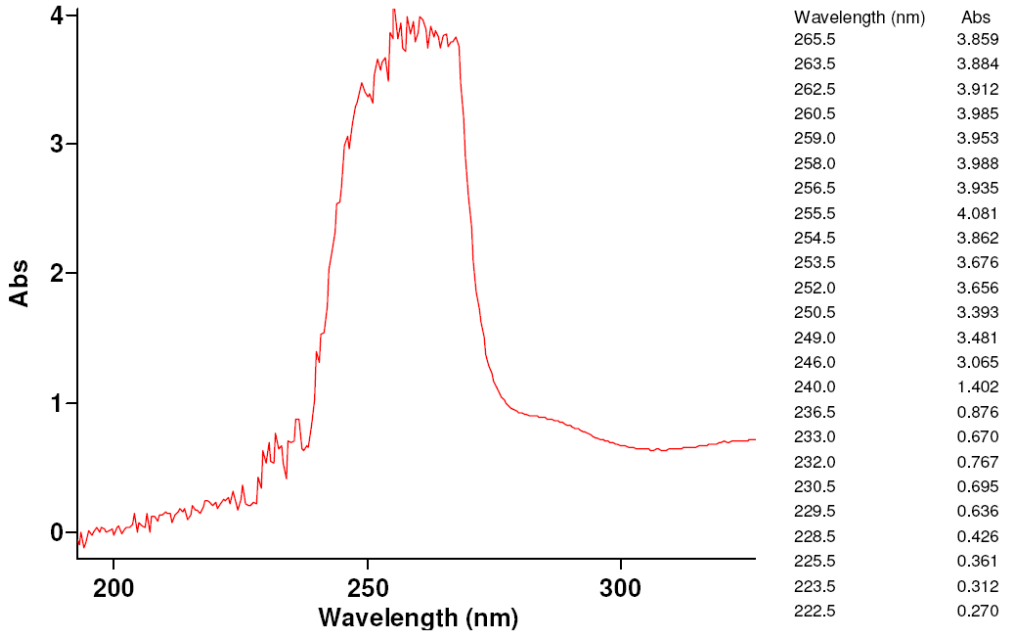


Fig.7.5 Thin layer chromatogram of the combined resolution fraction obtained after column chromatography, isolated from *G. foliifera*.

7.2.4.2.5 Characterization using UV-Vis Scanning and HPLC

The pooled fractions were concentrated by vacuum distillation to a volume of 1 mL, and subjected to UV-Vis spectrophotometric scanning. Absorption maxima were observed at 255.5 nm (Fig. 7.6). The samples were injected to the Shimadzu LC-20AD equipped with SPD-20A UV-Vis detector. One major peak along with many minor peaks was observed at 254 nm (Fig. 7.7). The major peak was carefully eluted using the preparative HPLC techniques and later identified as a steroid compound.



Wavelength (nm)	Abs
265.5	3.859
263.5	3.884
262.5	3.912
260.5	3.985
259.0	3.953
258.0	3.988
256.5	3.935
255.5	4.081
254.5	3.862
253.5	3.676
252.0	3.656
250.5	3.393
249.0	3.481
246.0	3.065
240.0	1.402
236.5	0.876
233.0	0.670
232.0	0.767
230.5	0.695
229.5	0.636
228.5	0.426
225.5	0.361
223.5	0.312
222.5	0.270

Fig. 7.6 UV-Vis spectrum scan of the combined resolution fraction obtained after column chromatography, isolated from *G. foliifera*.

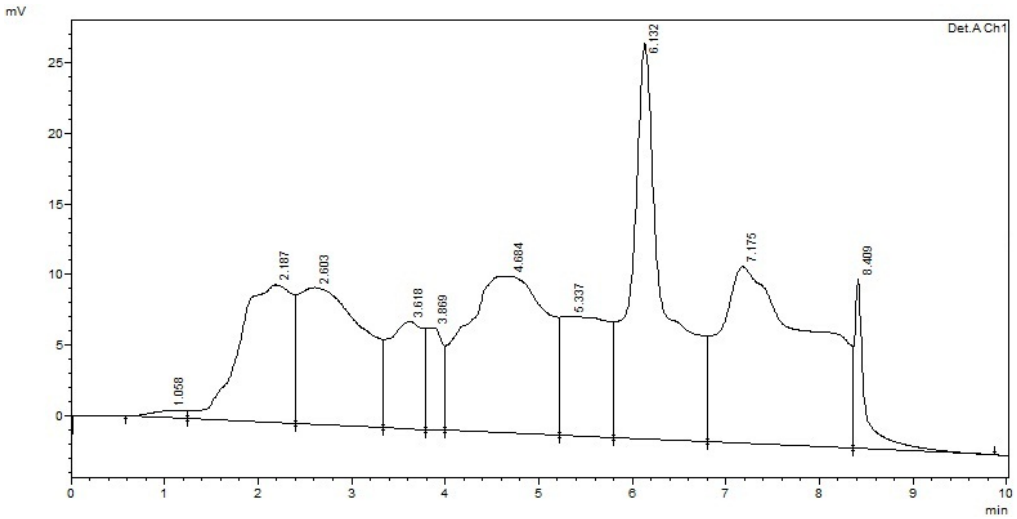


Fig. 7.7 HPLC chromatogram of the combined resolution fraction obtained after column chromatography, isolated from *G. foliifera* (detected at 254 nm).

7.2.4.2.6 Further Characterization and Resolution

50 % of steroid containing fraction was dried. The melting point and specific rotation was estimated. Melting point was estimated in the conventional melting point analysis instrument. Specific rotation was determined using the 0.1 % sample solution in HPLC methanol at 25 ^{o}C (using a 10 cm cylindrical cell and the sodium D line with an optics manual polarimeter). Again, the methanol solution of the steroid compound was injected to the Shimadzu LC-20AD equipped with SPD-20A UV-Vis detector (254nm) to confirm its purity (Fig. 7.8).

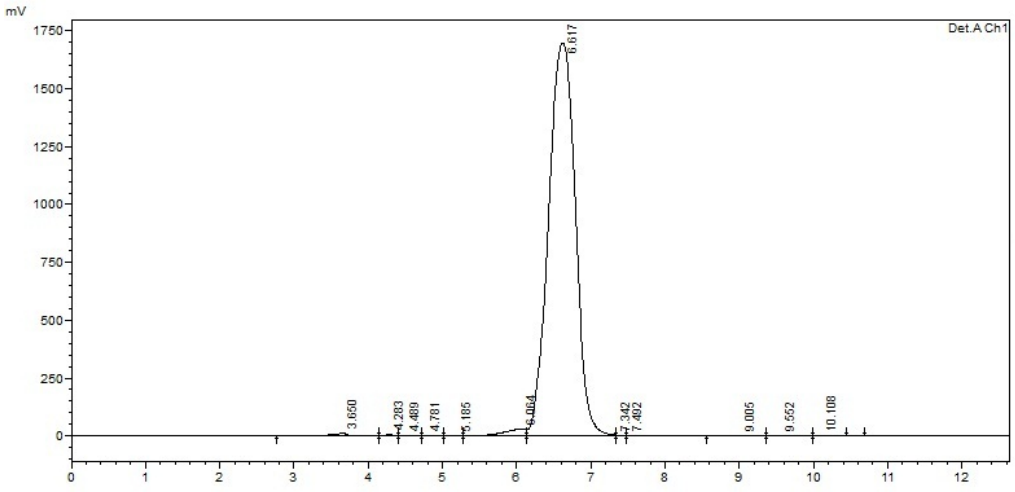


Fig. 7.8 HPLC chromatogram of the resolution fraction collected through preparative HPLC technique (detected at 254 nm).

7.2.5 FTIR, GC and GC-MS Characterization Studies

The steroid compound was dried and subjected to FTIR analysis. Analysis was done on Perkin Elmer Spectrum 100 FTIR spectrometer. The compound was dissolved in HPLC grade methanol and subjected to GC analysis to confirm its purity. The purity was checked on Agilent GC-7890A equipped with HP-5 30m x 0.32 mm, 0.25 µm column and FID 300 ^{o}C. High

pure nitrogen was used as the carrier gas. Splitless injection was used to inject the sample. The run time was 20 min ramping from 100 ^{o}C to 280 ^{o}C.

The GC-MS analysis was done to determine the mass (m/z) and subsequent fragmentation patterns. GC-MS analysis was done on Perkin Elmer Clarus 680 gas chromatograph equipped with mass spectrometer with electron ionization as mass identification source. Dimethyl polysiloxane with 5 % diphenyl (Elite 5MS) column was used for the separation having 30 m length and 250 μm internal diameters. High pure helium was used as the carrier gas. Pneumatic split/splitless injection was used to inject the sample having a split ratio 50:1. The oven temperature was initially kept at 35 ^{o}C and increased up to 280 ^{o}C at the rate of 10 ^{o}C per min keeping hold 10 min in the maximum temperature. The injector temperature was kept at 280 ^{o}C. Inlet and source temperature of the mass spectrometer was kept at 200 ^{o}C, scan range 4-500 a.m.u. and solvent delay was 4 min.

7.2.6 LC-MS/MS Characterization Studies

LC-MS/MS analysis was done to confirm the mass. The isolated compound was subjected to LC-MS/MS analysis. The ethanolic extract of fresh G. foliifera was also injected to confirm the presence of the isolated steroid compound in the extract. The analysis was done on AB SCIEX 4000 QTRAP LC-MS/MS connected to WATERS ACQUITY UPLC equipped with WATERS ACQUITY UPLC BEH C-18 2.8 cm x column.

7.2.7 ^{1}H and ^{13}C NMR studies

The proton (^{1}H) and ^{13}C NMR data were studied in order to derive the structural pattern of the compound isolated. Bruker 400 MHz NMR spectrometer was used for the analysis. $CDCl_3$ was used as the solvent. DEPT (Distortionless Enhancement by Polarization Transfer) analysis was done to

determine the presence of primary (positive in DEPT-135 other than the peaks in DEPT-90), secondary (negative in DEPT-135), tertiary (positive in DEPT-90) and quaternary carbon atoms (left over peaks from DEPT-90 and DEPT-135). The 2-Dimensional data such as COSY (Correlation Spectroscopy - defines which all H atoms are coupling with each other), HSQC (Heteronuclear Single Quantum Correlation - defines the correlation between the aliphatic carbon and its attached protons) and HMBC (*Heteronuclear Multiple Bond Correlation* - defines correlation between carbons and protons that are separated by two, three, and sometimes in conjugated systems, four bonds. Direct one-bond correlations are suppressed) were also interpreted.

7.2.8 Bioactivity Studies

7.2.8.1 Antioxidant Activity

Antioxidant activity studies were done based on three prominent methods such as the DPPH, UV-Vis ferrous tartarate and $KMnO_4$ methods. The results were reported as the percentage of equivalence to the corresponding four standard (ascorbic acid, butylatedhydroxytoluene (BHT), α-tocopherol and resorcinol) concentrations. The sample solution of the compound isolated was prepared by dissolving 0.001 g of the sample in 1mL methanol.

7.2.8.1.1 Antioxidant Assay - DPPH Method

The assay was done as per the method given by Brand-Williams et al. (1995) with some modifications. DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 mL HPLC grade methanol and stored at -20 ^{o}C until needed. The working solution was obtained by mixing a 10 mL stock solution with 45 mL HPLC grade methanol to obtain an absorbance of 1.170 ± 0.02 units at 515 nm using Cary-60 UV-Vis Spectrophotometer. 0.15 mL of

the sample solution was allowed to react upon mixing with 2.85 mL of DPPH solution for 24 h in dark. The absorbance was measured at 515 nm against the reagent blank. The standard curve was observed to be linear between 403 to 1613 µg $g^{-1}$for ascorbic acid, 409 to 1637 µg g^{-1} for α-tocopherol, 405 to 1621 µg g^{-1} of BHT and 406 to 1626 µg $g^{-1}$of resorcinol. Results were expressed as the percentage of equivalence to the corresponding standard concentrations. The r^2 values were > 0.995 (Table 7.1).

Table 7.1 Multipoint calibration data of the antioxidant assays.

Standard	Calibration equations	r^2	Concentration (ppm)			
			1	2	3	4
DPPH radical scavenging activity						
ASC	y = -3.2176e-05x -0.081	0.995	403.36	806.72	1210.08	1613.44
RES	y = -3.1709e-05x - 0.088	0.999	406.56	813.12	1219.68	1626.24
TOC	y = -4.9594e-05x + 0.008	0.999	409.36	818.72	1228.08	1637.44
BHT	y = -2.9294e-05x - 0.064	0.996	405.32	810.64	1215.96	1621.28
Ferrous tartarate oxidising activity						
ASC	y = 5.6376e-04x -0.013	0.978	403.36	806.72	1210.08	1613.44
RES	y = 7.2978e-06x - 0.012	0.992	405.60	811.20	1216.80	1622.40
TOC	y = 1.808e-03x + 0.556	0.995	409.36	818.72	1228.08	1637.44
BHT	y = 2.0351e-03x + 0.421	0.994	406.56	813.12	1219.68	1626.24

Total antioxidant potential						
Standard	Weight of standards (g/50mL)	Blank readings	Titre Readings			Equivalence of 0.01N $KMnO_4$(g)
			1mL	2mL	3mL	
ASC	0.504	0.9	23.0	41.0	61.3	0.00046
RES	0.507	0.9	1.1	1.3	1.4	0.00664
TOC	0.503	0.9	3.1	5.9	6.8	0.00457
BHT	0.507	0.9	2.4	4.0	6.0	0.05069

ASC- Ascorbic acid, RES- Resorcinol, TOC- Tocopherol and BHT- Butylatedhydroxytoluene.

7.2.8.1.2 Antioxidant assay - UV-VIS Ferrous Tartarate Method

Antioxidant activity was determined by using the modified UV-visible spectrophotometric methods (Liang et al., 2003; Li et al., 2005). Analysis was

carried out by taking 0.4 mL of the sample solution into a 10 mL standard flask, to which 1.6 mL of HPLC grade methanol was added followed with 2 mL of tartaric acid ferrous sulfate solution and made up to the volume using phosphate buffer solution. The absorbance was measured at 540 nm nullifying the reagent blank. The standard curve was observed to be linear between 403 to 1613 μg g^{-1} for ascorbic acid, 406 to 1626 μg g^{-1} for BHT, 409 to 1637 μg g^{-1} for α-tocopherol and 405 to 1622 μg g^{-1} for resorcinol. Results were expressed as the percentage of equivalence to the corresponding standard concentration. The r^2 values were > 0.950 (Table 7.1).

7.2.8.1.3 Antioxidant assay - $KMnO_4$ Method

The antioxidant assay was estimated adopting the Ribereau-Gayon-Maurié titrimetric method with $KMnO_4$ and indigo carmine dye as an indicator (Daničić, 1973; Radovanović, 1986). 0.3 mL of the sample solution was taken into a conical flask, 1.5 mL methanol and 0.1 mL indigo carmine dye, were added and titrated against 0.1N standard $KMnO_4$ solution taken in a micro burette, against reagent blanks. The results were calculated with the estimated observations against the standards such as ascorbic acid, α-tocopherol, BHT and resorcinol and were expressed in percentage of equivalence to the corresponding standard concentration to the dry weight of the sample (Table 7.1).

7.2.8.2 Antimicrobial Activity

Kirby-Bauer disc diffusion method (Bauer et al., 1966) with some modifications was adopted for the antimicrobial activity determination. Instead of disc diffusion, the well method was used. Small wells of 1 mm depth and 5 mm width were made on the streaked plates. Sample for analysis was poured carefully into the well and allowed to dry. After drying the test organism was swabbed into the plates and the inhibition area was studied for determining the

bioactivity. Pathogenic gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*) forms of bacteria were used for the study. Chloramphenicol and tetracycline were used as the positive controls, dissolved in methanol at a level of 100 mg L^{-1}. Methanol was used as the analytical blank whose activity observations (Table 7.2) were deducted as blank correction in results. The activity was measured as in mm of the clearance area around the disc, discarding the diameter of the disc and reported as % of inhibition against the positive controls.

Table 7.2 Antimicrobial assays - inhibition area of positive controls.

Positive control (100 mg L^{-1})	Inhibition level (mm)	
	Gram +ve	Gram -ve
	S. aureus	*E. Coli*
Solvent	2.0 ± 0.1	3.0 ± 0.2
Tetracycline	9.0 ± 0.4	8.0 ± 0.4
Chloramphenicol	8.0 ± 0.4	10.0 ± 0.4

7.3 Results and Discussions

7.3.1 Extraction of Steroids

21.054 g of crude saponins were obtained from 1750 g of the dry seaweed sample. It corresponded to 1.203 % of the dry weight of the seaweed. The crude saponins upon purification yielded 19.159 g which constituted of 1.095 % to the dry weight of the seaweed. The purified saponins on acid hydrolysis and subsequent column chromatography and HPLC resolution yielded 12.462 mg of steroid compound (7.121×10^{-4} % to dwt of seaweed).

7.3.2 Melting point and Specific Rotation

The compound had a melting point of 171.8 to 172.2 ^{o}C. The specific rotation ($[\alpha]_D^{25}$) was observed to be -29.5^{o}.

7.3.3 UV-Vis Characterization

The maximum absorption was observed at 255.5 nm (Fig. 7.6). Absorption pattern was observed in between 267.5 to 240 nm. The absorption maxima at 255.5 nm indicated the absence of aromatic compounds.

7.3.4 FTIR Characterization

The compound had absorption (Fig. 7.9) at the wavelength 2935.5 cm^{-1} which indicated the presence of methylene CH asymmetrical/symmetrical stretching, 1715.14 cm^{-1} which indicates the presence of ketones and 1673.04 cm^{-1} which indicated the presence of alkenyl C=C stretching. The absorptions at 2058 cm^{-1}, 2026.97 cm^{-1} and 1976.33 cm^{-1} indicated that the compound is constituted of allenes or ketenes or having conjugation properties that are similar to aromatic compounds.

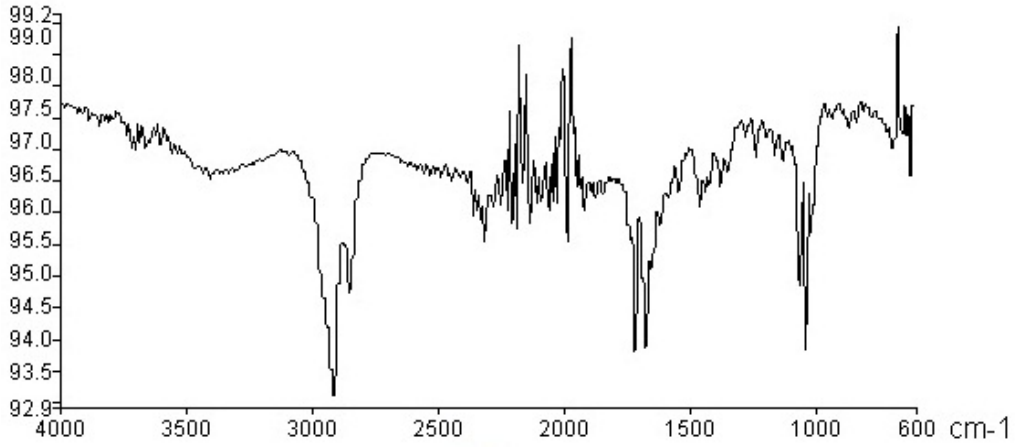


Fig. 7.9 FTIR spectrum of the isolated steroid.

7.3.5 GC and GC-MS Analysis

The compound upon GC analysis (Fig. 7.10) indicated the presence of pure compound. GCMS analysis exhibited (Fig. 7.11) the major base peak m/z value as 297. Second highest peak was with m/z value as 312. GCMS

fractionation pattern was (m/z) 312, 297, 279, 255, 241, 227, 213, 201, 189, 173, 161, 147, 135, 121, 105, 91, 79, 67, 55, 41, 28 and 18. The difference of m/z values in between the fractions indicated the presence of three -CH_3, two -OH, seven -CH_2 and multiple possibilities of both quaternary and tertiary carbon groups.

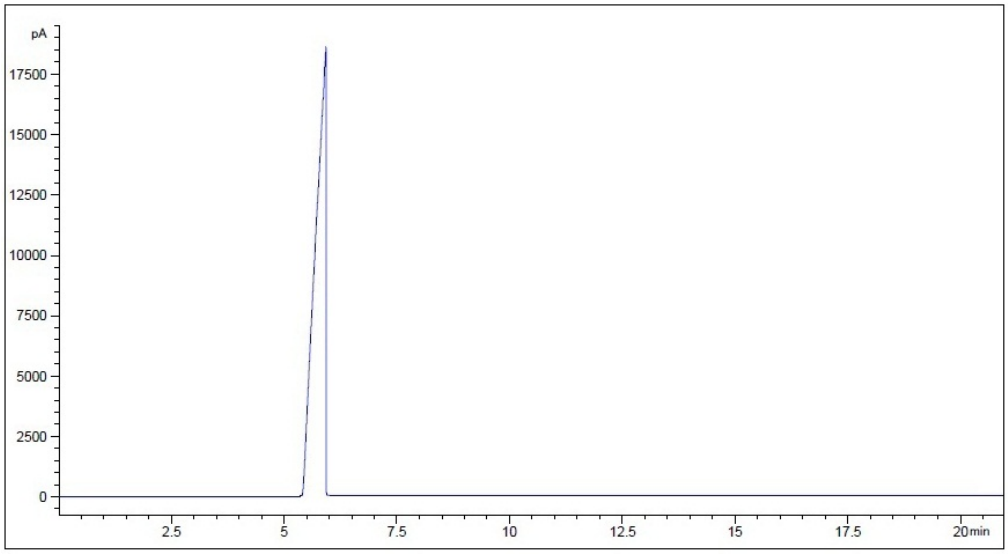
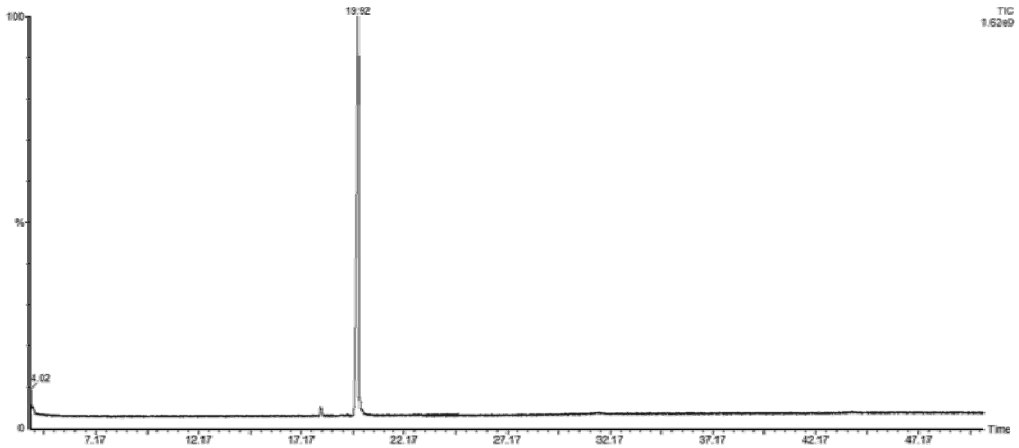


Fig. 7.10 Gas chromatogram of the isolated steroid compound.



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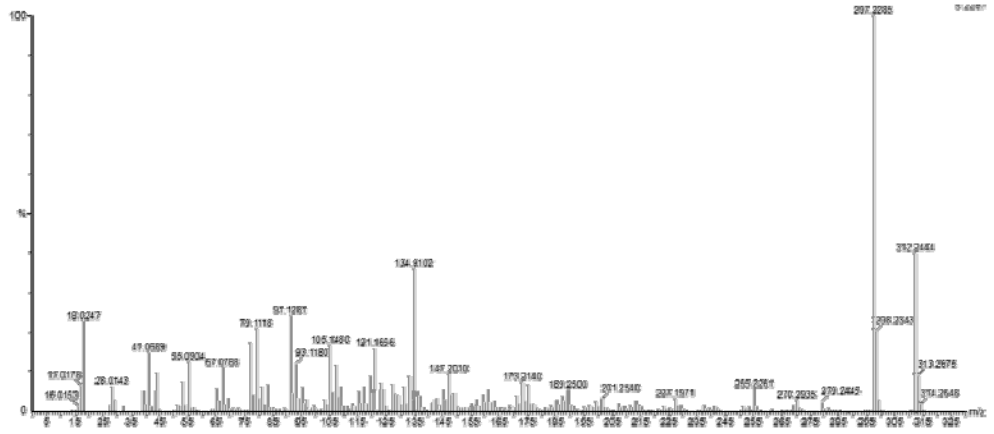


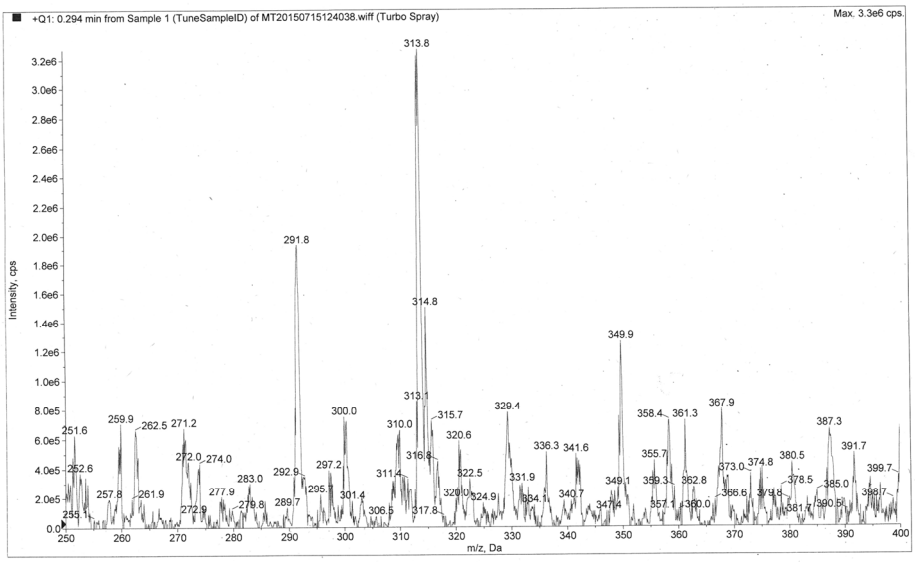
B

Fig. 7.11 GC - MS chromatogram of the isolated steroid compound.

A. Total ion chromatogram of isolated steroid compound.
B. Mass spectra indicating the fragmentation of isolated steroid compound.

7.3.6 LC-MS/MS Characterization

LC-MS/MS characterization (Fig. 7.12) confirmed the molecular mass m/z as $(M+H)^+$ 313.8. The ethanolic extract of *G. foliifera* collected from a different location also exhibited the presence of peaks corresponding to the m/z value $(M+H)^+$ 313.7.



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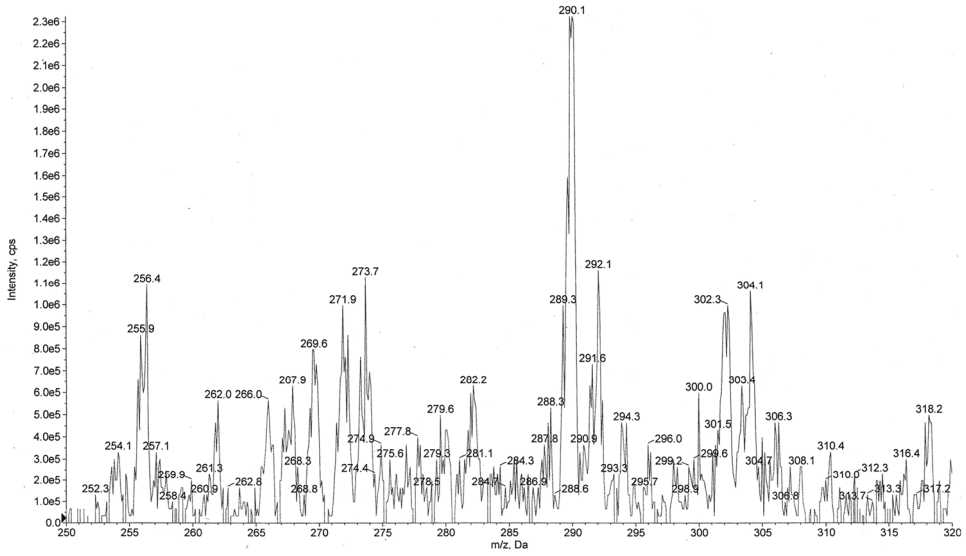


Fig. 7.12 LC-MS/MS chromatogram.

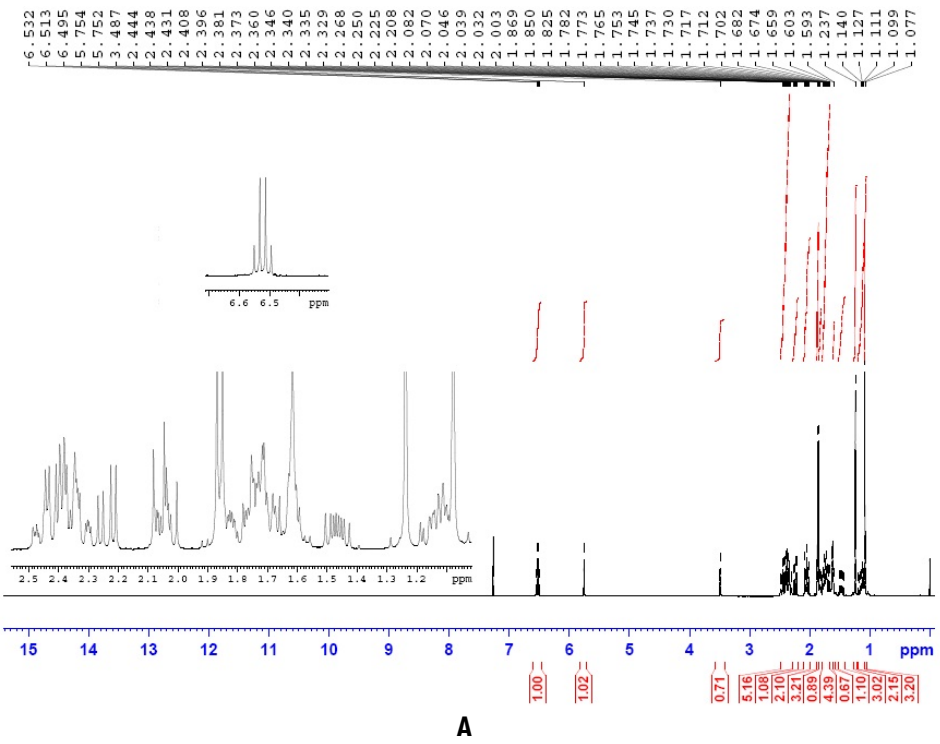
A. LC-MS/MS data of the isolated steroid compound.

B. LC-MS/MS data confirming the presence of the steroid compound in crude ethanolic extract of *G. foliifera* obtained from another location.

7.3.7 NMR Characterization

7.3.7.1 Proton (^{1}H) NMR

The compound upon proton NMR analysis (Fig. 7.13) indicated the presence of 29 hydrogen atoms. The chemical shift pattern and J value was as follows, δ 6.52 (q, J = 7.5 Hz, 1H), 5.75 (d, J = 1.8 Hz, 1H), 3.49 (s, 1H), 2.51 - 2.27 (m, 5H), 2.24 (dd, J = 17.1, 6.9 Hz, 1H), 2.12 - 1.98 (m, 2H), 1.89 - 1.53 (m, 9H), 1.`47 (ddd, J = 14.3, 10.8, 6.9 Hz, 1H), 1.24 (s, 3H), 1.20 - 1.07 (m, 2H), 1.08 (s, 3H).



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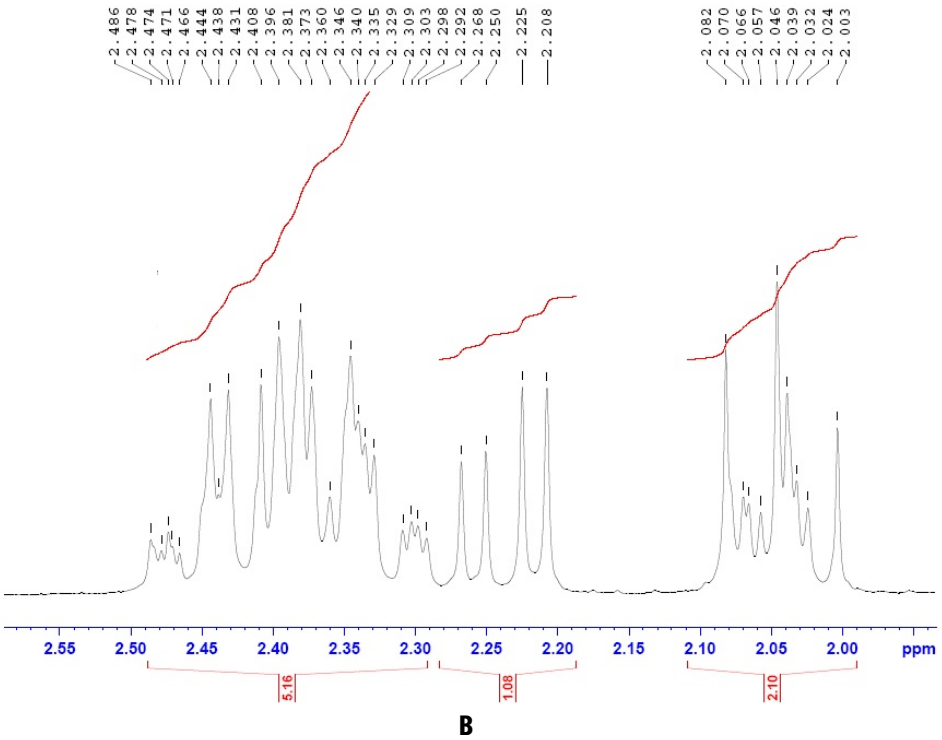

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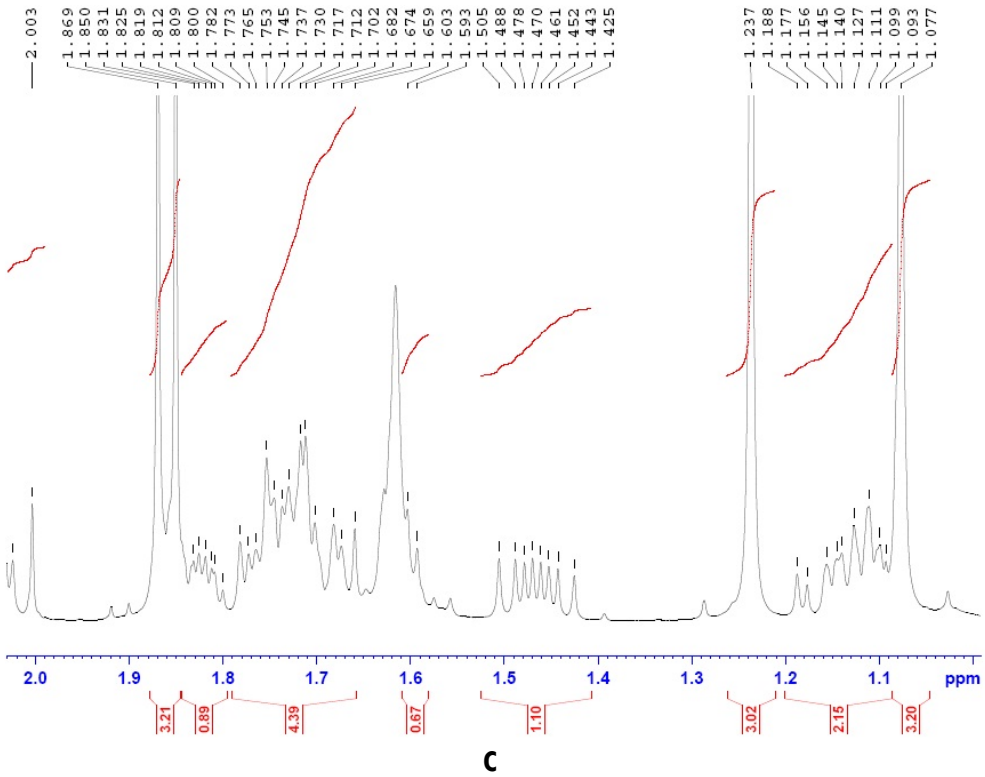


Fig. 7.13 ^{1}H proton NMR of isolated steroid compound (Split into A, B & C for enhanced readability).

7.3.7.2 ^{13}C NMR

^{13}C NMR analysis (Fig. 7.14) indicated the presence of 21 carbon atoms. The chemical shift patterns were as follows, δ 205.54, 199.18, 170.12, 147.43, 129.46, 124.15, 53.38, 49.55, 43.08, 38.63, 37.78, 35.99, 35.47, 34.32, 33.89, 32.55, 31.87, 20.71, 17.54, 17.31 and 13.16.

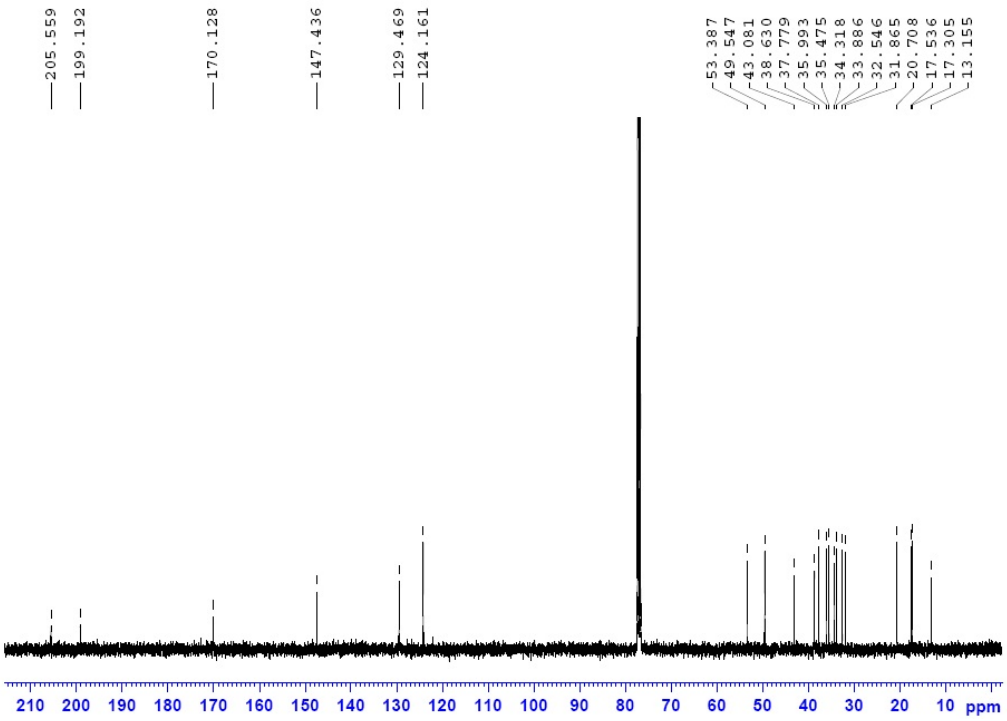


Fig. 7.14 ^{13}C NMR of isolated steroid compound.

7.3.7.3 DEPT NMR Analysis

The DEPT analysis was carried out (Fig. 7.15). The DEPT-90 indicated the presence of five -CH groups. DEPT-135 indicated the presence of three $-CH_3$ groups and seven $-CH_2$ groups. The above observations were deducted from the ^{13}C data which derived the presence of quaternary carbon atoms which commuted to six numbers.

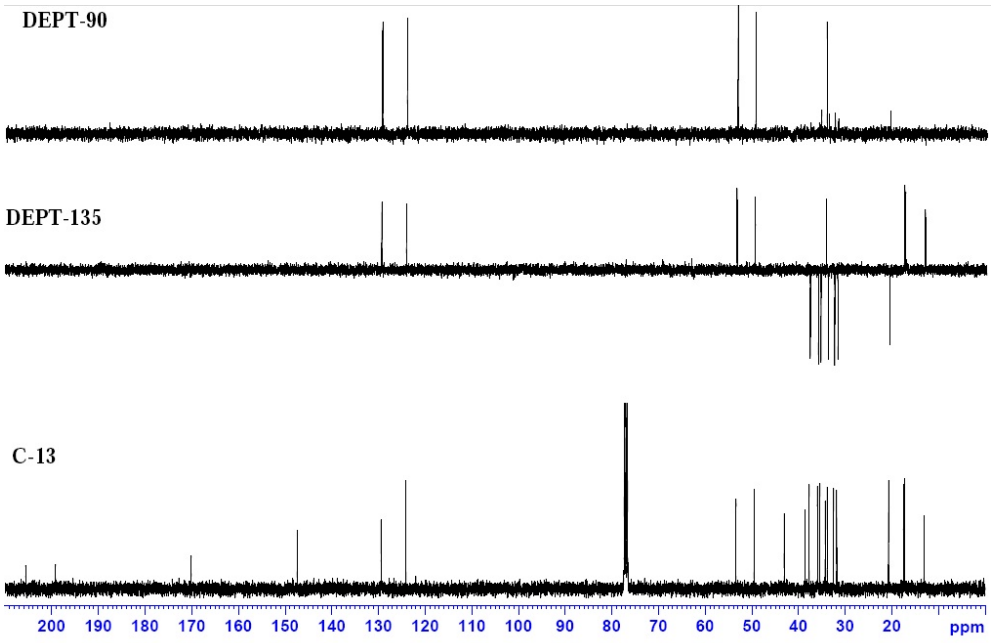


Fig. 7.15 DEPT analysis of isolated steroid compound.

7.3.7.4 2-dimensional correlation analysis (COSY, HSQC and HMBC)

COSY analysis (Fig. 7.16) indicated the ^{1}H-^{1}H correlations of δ 6.52 with δ 1.89-1.53, δ 2.51-2.27 with δ 2.12-1.98 and δ 1.89-1.53, δ 2.24 with δ 2.12-1.98, δ 2.12-1.98 with δ 1.47, δ 1.89-1.53 with δ 1.47, δ 1.20-1.09 and δ 1.07, and δ 1.20-1.09 with δ 1.07. Significant COSY data is given in Table 7.3.

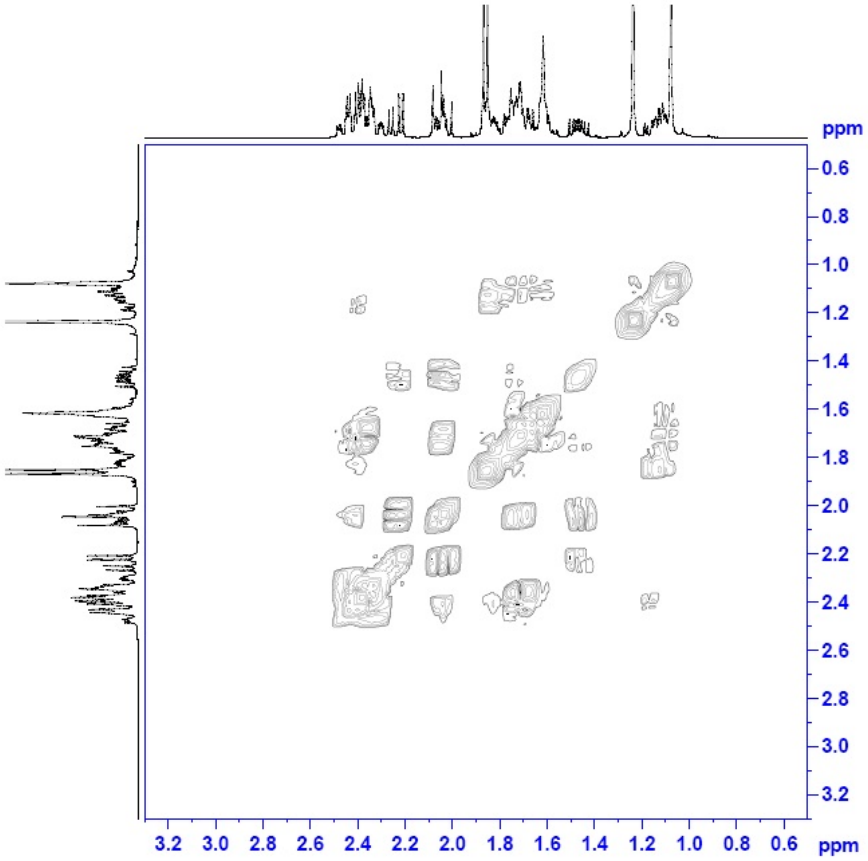


Fig. 7.16 COSY analysis of isolated steroid compound.

Table 7.3 Significant COSY (^{1}H - ^{1}H) correlation data of the isolated steroid compound.

^{1}H δ	^{1}H δ
1.08	1.23, 1.70
1.11	1.70, 1.86
1.47	1.75, 2.03, 2.04, 2.22,2.30
1.72	2.37, 2.39
1.75	2.37, 2.39
1.70	2.37, 2.39
1.86	6.52
2.03	2.22, 2.30
2.04	2.22, 2.30, 2.44

HSQC analysis (Fig. 7.17) exhibited short range inter correlations of ^{13}C δ 129.46 with ^{1}H δ 6.52, ^{13}C δ 124.15 with ^{1}H δ 5.75, ^{13}C δ 53.38 with ^{1}H δ 1.10, ^{13}C δ 49.55 with ^{1}H δ 1.47, ^{13}C δ 37.78 with ^{1}H δ 2.22 and ^{1}H δ 2.04, ^{13}C δ 35.99 with ^{1}H δ 2.37and ^{1}H δ 1.70, ^{13}C δ 35.47 with ^{1}H δ 1.75 and ^{1}H δ 2.03, ^{13}C δ 34.32 with ^{1}H δ 1.75, ^{13}C δ 33.89 with ^{1}H δ 2.39 and ^{1}H δ 2.44, ^{13}C δ 32.55 with ^{1}H δ 2.30 and ^{1}H δ 2.47, ^{13}C δ 31.87 with ^{1}H δ 1.15 and ^{1}H δ 1.83, ^{13}C δ 20.71 with ^{1}H δ 1.62 and ^{1}H δ 1.72, ^{13}C δ 17.54 with ^{1}H δ 1.08, ^{13}C δ 17.31 with ^{1}H δ 1.23 and ^{13}C δ 13.16 with ^{1}H δ 1.86. Significant HSQC data is given in Table 7.4.

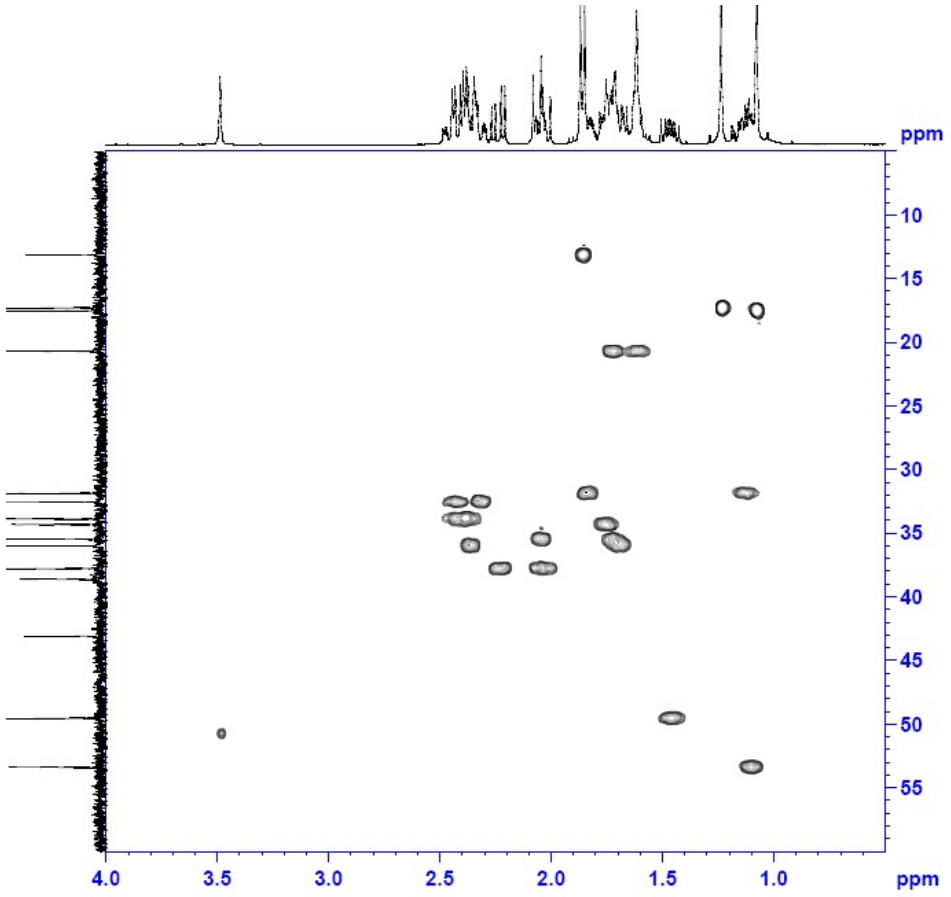
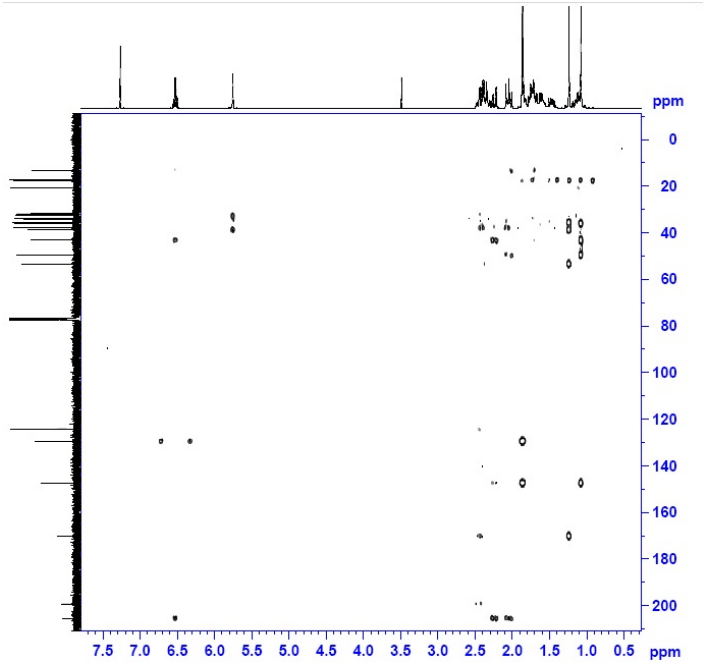


Fig. 7.17 HSQC analysis of isolated steroid compound.

Table 7.4 Significant HSQC (^{13}C - ^{1}H) correlation data of the isolated steroid compound.

^{13}C δ	^{1}H δ
129.46	6.52
124.15	5.75
53.38	1.10
49.55	1.47
37.78	2.04, 2.22
35.99	2.37, 1.70
35.47	1.75, 2.03
34.32	1.75
33.89	2.39, 2.44
32.55	2.30, 2.47
31.87	1.15, 1.83
20.71	1.62, 1.72
17.54	1.08
17.31	1.23
13.16	1.86

HMBC analysis (Fig. 7.18) exhibited long range inter correlations. Prominent correlations were observed in between ^{13}C δ 170.12 and ^{1}H δ 1.23, ^{13}C δ 147.43 and ^{1}H δ 1.08, 1.86, ^{13}C δ 129.46 and ^{1}H δ 1.86, ^{13}C δ 53.38 and ^{1}H δ 1.23, ^{13}C δ 49.55 and ^{1}H δ 1.15, ^{13}C δ 43.08 and ^{1}H δ 1.08, ^{13}C δ 38.63 and ^{1}H δ 1.10, ^{13}C δ 35.99 and ^{1}H δ 1.08, and ^{13}C δ 35.47 and ^{1}H δ 1.23. Significant HMBC data is given in Table 7.5.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100
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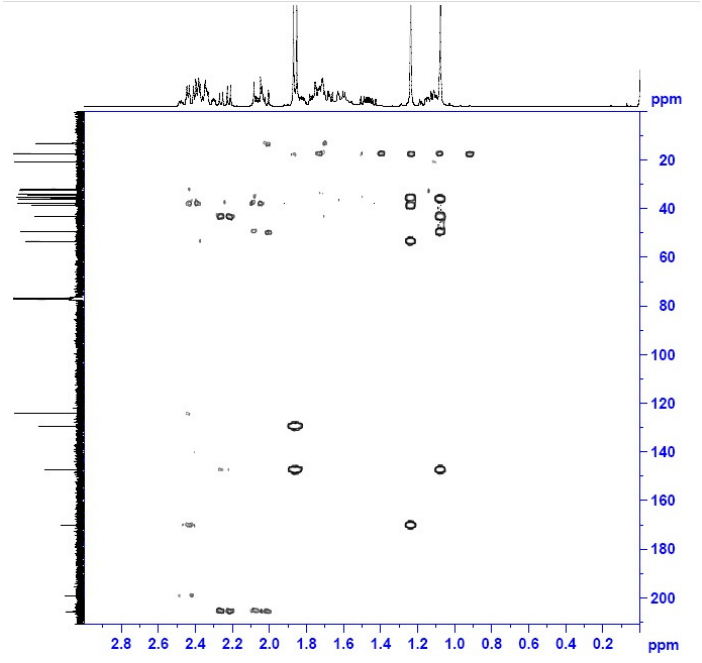
ppm

A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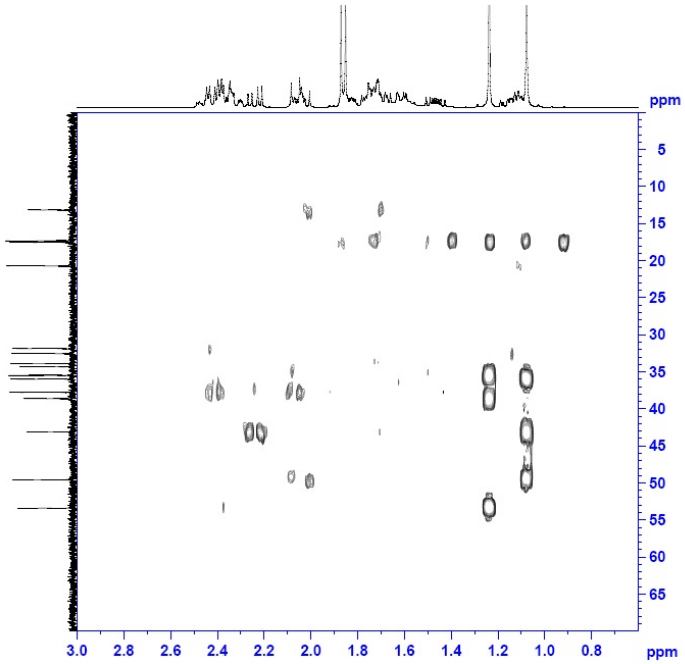
ppm

B



C

Fig. 7.18 HMBC analysis of isolated steroid compound (Split into A, B & C for enhanced readability).

Table 7.5 Significant HMBC (^{13}**C** - ^{1}**H**) correlation data of the isolated steroid compound.

^{13}C δ	^{1}H δ
205.54	2.22, 2.04, 6.52
199.18	2.39, 2.44
170.12	1.23, 2.47
147.43	1.08, 1.86, 2.22
129.46	1.86
124.15	2.47
53.38	1.23, 2.37
49.55	1.15, 2.04,
43.08	1.08, 2.04, 6.52
38.63	1.1, 2.3, 5.75
37.78	1.15
35.99	1.08, 1.10, 1.23
35.47	1.23, 5.75
34.32	1.10, 1.15, 1.23
33.89	5.75
32.55	5.75
31.87	5.75
20.71	1.10
17.54	1.47
17.31	1.10
13.16	1.70

7.3.8 Structure Elucidation

Based on the ^{13}C NMR and its DEPT analysis data, ^{1}H NMR and the mass obtained from the GC-MS and LC-MS/MS analysis, the compound was observed to have the molecular formula $C_{21}H_{28}O_2$. ^{1}H NMR data indicated the data for M+H. The fragmentation pattern of GC-MS analysis and the DEPT substantiated the molecular formula derivation. Both derived the presence of three -CH_3 groups, seven -CH_2 groups, five -CH groups, six quaternary carbon atoms and presence of two oxygen atoms. The calculated mass of $C_{21}H_{28}O_2$ was 312.4458 g mol^{-1}. The observed mass in GC-MS was 312.2443 g mol^{-1} and LC-MS/MS was $[M+H]^+$ mass 313.8 g mol^{-1}.

COSY, HSQC and HMBC data along with the above derivations where correlated and the structure was derived. The compound had a phenanthrene basic structure and structure correlates to that of a steroid. The steroid compound composited of C-1 CH_2 (^{13}C δ 35.47, ^{1}H δ 1.75, 2.03), C-2 CH_2 (^{13}C δ 33.89, ^{1}H δ 2.39, 2.44), C-3 C=O (^{13}C δ 199.18), C-4 CH (^{13}C δ 124.15, ^{1}H δ 5.75), C-5 C (^{13}C δ 170.12), C-6 CH_2 (^{13}C δ 32.55, ^{1}H δ 2.30, 2.47), C-7 CH_2 (^{13}C δ 31.87, ^{1}H δ 1.15, 1.83), C-8 CH (^{13}C δ 34.32, ^{1}H δ 1.75), C-9 CH (^{13}C δ 53.38, ^{1}H δ 1.10), C-10 C (^{13}C δ 38.63), C-11 CH_2 (^{13}C δ 20.71, ^{1}H δ 1.62, 1.72), C-12 CH_2 (^{13}C δ 35.99, ^{1}H δ 1.70, 2.37), C-13 C (^{13}C δ 43.08), C-14 CH (^{13}C δ 49.55, ^{1}H δ 1.47), C-15 CH_2 (^{13}C δ 37.78, ^{1}H δ 2.04, 2.22), C-16 C=O (^{13}C δ 205.54), C-17 C (^{13}C δ 147.43), C-18 CH_3 (^{13}C δ 17.54, ^{1}H δ 1.08), C-19 CH_3 (^{13}C δ 17.31, ^{1}H δ 1.23), C-20 CH (^{13}C δ 129.46, ^{1}H δ 6.52) and C-21 CH_3 (^{13}C δ 13.16, ^{1}H δ 1.86) (Table 7.6; Fig. 7.19; Fig. 7.20). Significant correlation observed in HMBC are given in Fig. 7.21.

Table 7.6 Carbon and hydrogen tagging of the isolated steroid compound.

Carbon No.	Carbon type (from DEPT)	^{13}C δ	^{1}H δ
1	CH_2	35.47	1.75, 2.03
2	CH_2	33.89	2.39, 2.44
3	C	199.18	
4	CH	124.15	5.75
5	C	170.12	
6	CH_2	32.55	2.30, 2.47
7	CH_2	31.87	1.15, 1.83
8	CH	34.32	1.75
9	CH	53.38	1.10
10	C	38.63	
11	CH_2	20.71	1.62, 1.72
12	CH_2	35.99	1.70, 2.37
13	C	43.08	
14	CH	49.55	1.47
15	CH_2	37.78	2.04, 2.22
16	C	205.54	
17	C	147.43	
18	CH_3	17.54	1.08
19	CH_3	17.31	1.23
20	CH	129.46	6.52
21	CH_3	13.16	1.86

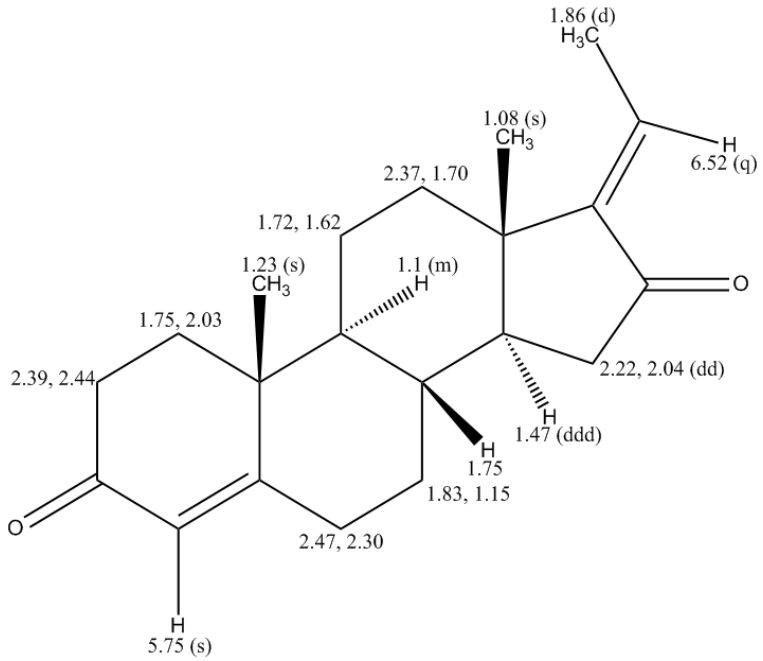


Fig. 7.19 ^{1}H NMR data, denoted on the probable structure of the isolated steroid compound.

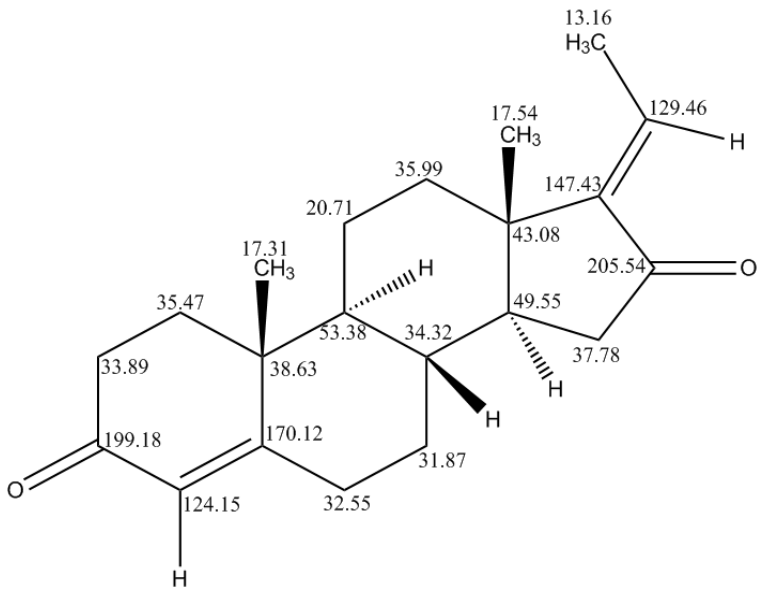


Fig.7.20 ^{13}C NMR data, denoted on the probable structure of the isolated steroid compound.

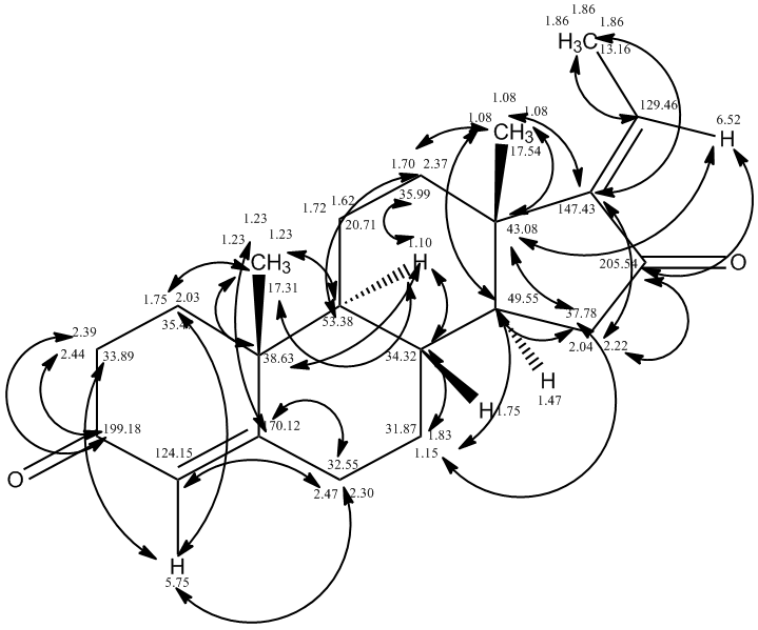


Fig. 7.21 Significant correlations observed in HMBC data of *E*-guggulsterone.

The elucidated structure could explain the observed FTIR data (probability of ketones, alkenyl groups and ketenes). The UV-Vis spectra of the compound followed the Woodwards rule. The expected absorbance was λmax 245 to 275 nm and the observed was in the range of 240-267.5 nm.

The compound was named as ((8R,9S,10R,13S,14S)-17-Ethylidene (*E*) -10,13-dimethyl-1,2,6,7,8,9,11,12,14,15-decahydrocyclopenta[a] phenanthrene-3, 16-dione or trans-Pregna-4,17-diene-3,16-dione or *E*-Guggulsterone (Fig. 7.22). The structure was confirmed as *E*-guggulsterone based on the ^{13}C δ value (205.54) of C-16 and ^{1}H δ of C-20 (6.52). The geometrical isomer *Z*-guggulsterone has ^{13}C δ value (207.8 to 208.2) for C-16 and has no ^{1}H δ value around 6.52 (Ham et al., 2011; Gioiello et al., 2012). In addition to the above, *E*-guggulsterone has been reported to have specific rotation around - 34.5 ^{o} (Ham et al., 2011) and - 27.4 ^{o} (Gioiello et al., 2012) and melting point in the range of 168 - 171 ^{o}C (Ham et al., 2011; Gioiello et al., 2012). Present study on E-

guggulsterone isolated from *G. foliifera* exhibited specific rotation of $[\alpha]_D^{25}$ - 29.5 ^{o} and melting point of 171.8 to 172.2 ^{o}C. *Z*-guggulsterone had been reported to exhibit specific rotation around - 50.8 ^{o} and - 54.8 ^{o} and melting point of 191 - 193 ^{o}C (Ham et al., 2011; Gioiello et al., 2012). The structure elucidation of *E*-guggulsterone was validated with the studies done by Ham et al. (2011). Similar NMR data were observed in earlier studies too (Hung et al., 1995; Ham et al., 2011; Gioiello et al., 2012).

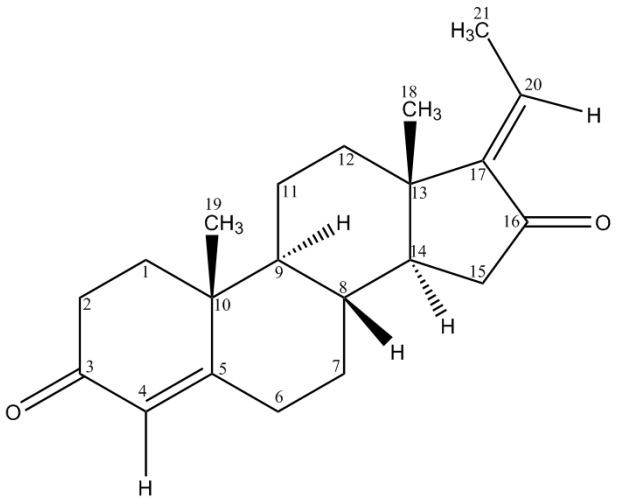


Fig. 7.22 *E*-Guggulsterone - structure derived upon analysing the NMR, GC - MS and LC-MS/MS data.

7.3.9 Antioxidant Activities

Guggulsterone have been reported as a major bioactive component due to its pharmaceutical applications (Dev, 1997). Guggul lipids were reported to have hypolipidemic and antiobesitic activities (Jain & Gupta, 2006). Guggul lipid has been reported for its astringent, antiseptic, antiarthritic, antimicrobial, antiinflammatory, anticancerous and antithrombotic effects (Singh et al., 1997; Sultana et al., 2005; Musharraf et al., 2011; Dave & Chopda, 2013; Jain & Nadgauda, 2013; Soni et al., 2013).

Isolated *E*-Guggulsterone exhibited good antioxidant activities in comparison with the four standards and three methods (Table 7.7). DPPH free radical scavenging activity and $KMnO_4$ activity was observed high in comparison with α-tocopherol equivalence. Ferrous tartrate was high in comparison with resorcinol activity.

Table 7.7 Antioxidant activity of *E*-Guggulsterone, (mean ± SD), (n=3).

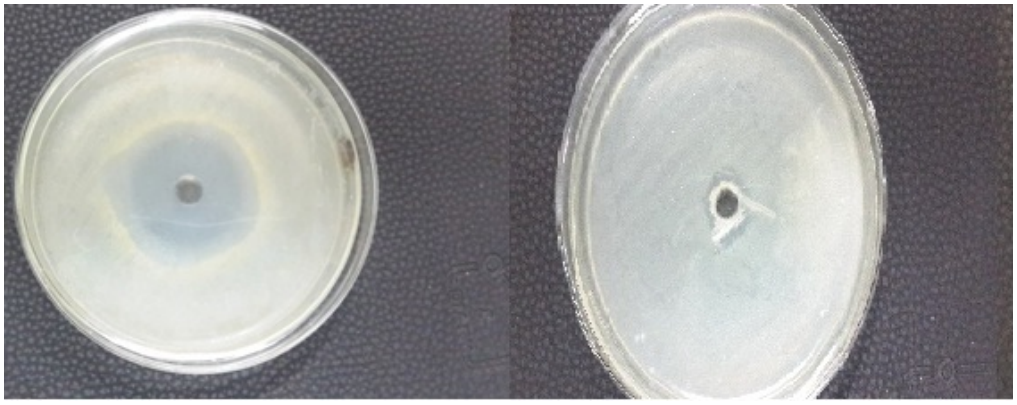
AAE	BHTE	α-TE	RE
DPPH method-% of inhibition			
90.31 ± 0.66	91.57 ± 0.84	93.18 ± 0.76	91.82 ± 0.42
Ferrous tartarate method-% of activity			
95.19 ± 0.69	93.87 ± 0.84	94.64 ± 0.72	97.22 ± 0.97
$KMnO_4$ method-% of activity			
72.25 ± 0.58	80.98 ± 0.35	83.29 ± 0.16	81.68 ± 0.94

AAE - Ascorbic acid equivalence, BHTE - Butylatedhydroxytoluene equivalence, α - TE - α - tocopherol equivalence and RE - Resorcinol equivalence.

7.3.10 Antimicrobial Activity

Guggulsterone have been reported to be used in various topical applications (Jain & Gupta, 2006). It has been reported for its antiseptic and antimicrobial effects too (Kasera et al., 2002; Ishnava et al., 2010; Jain & Nadgauda, 2013).

Isolated *E*-Guggulsterone had bactericidal activity against both Gram positive and negative bacteria (Fig. 7.23). But high extend of activity was seen towards Gram positive with about > 250 % of activity in comparison with tetracycline and chloramphenicol (Table 7.8). Gram negative bactericidal activity was relatively low (> 10 % against the standards). Similar activities were reported by Ishnava et al. (2010) against both *S. aureus* and *E. coli*.



A B

Fig. 7.23 Photographs of the antimicrobial activity studies.

A. *S. aureus* inhibitory antimicrobial activity of *E*-Guggulsterone.

B. *E.coli* inhibitory antimicrobial activity of *E*-Guggulsterone.

Table 7.8 Antimicrobial activity of *E*-Guggulsterone, (mean ± SD), (n=3).

Inhibition level (mm)		
Gram +ve	Gram -ve	
S. aureus	*E. Coli*	
23.0 ± 0.3	1.0 ± 0.1	
Inhibition level (%)		
Comparison positive control	Gram +ve	Gram -ve
	S. aureus	*E. Coli*
Tetracycline	255.6 ± 1.3	12.5 ± 0.1
Chloramphenicol	287.5 ± 3.0	10.0 ± 0.1

7.4 Conclusion

This is the first report on the presence of guggulsterones from seaweeds. The isolated compound was named as *E*-guggulsterone and is the natural form of guggulsterone that exists in the seaweed. The study reported to have low yield of isolatable *E*-guggulsterone (12.462 mg; 7.121 ppm), but the bioactivity and its commercial importance is a remarkable milestone. *E*-Guggulsterone was 2.5 times effective as that of tetracycline and chloramphenicol against *S. aureus*. The study revealed that *G. foliifera* had

potential antioxidant and antimicrobial properties which is due to the presence of novel bioactive components like *E*-guggulsterone.

7.5 References

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SUMMARY

Seaweeds or marine macroalgae are the most diverse, biochemically rich and biologically active marine living resources inhabiting the rocky and sandy shores. They are being used as food, live stock feed, in agriculture and as fertilizer for the past decades or centuries. The biochemical constituents (proteins, carbohydrates, lipids, crude fibres, iodine, calorific value, vitamins, carotenoids, phenolics, flavonoids, amino acids, fatty acids, sterols, polysaccharides, antioxidant and antimicrobial activities) in seaweeds, mark them as commercially, pharmaceutically and nutraceutically important resources. The present study investigates the biochemical composition and biological activities of seaweeds collected from two locations (Njarakkal and Kayamkulam) of the Kerala coast. The study quantifies and highlights the concentration and bioactivities of the primary and secondary metabolites in seaweeds. The information supports the potentiality of the seaweeds in their utilization as sources of food for human and animal consumption, and as a commercial raw material. The study also evaluates the structure and bioactivities of a steroid (ketosteroid) compound isolated from *G. foliifera*. The highlights of this study are summarized in the following paragraphs.

Nine seaweed species were collected from Kerala coast. Njarakkal location had a large algal diversity. Seven species of seaweeds (five Rhodophyta and three Chlorophyta) were collected from Njarakkal. Collection volume, in terms of weight, had the following pattern. *G. foliifera* > *C. antennina* > *G. corticata* var. *cylindrica* > *G. corticata* > *E. prolifera* > *G. pusillum* > *A. spicifera*. Relatively, lower algal diversity was observed at Kayamkulam location. Two Chlorophyta were collected from Kayamkulam and their collection pattern, in terms of weight, was *E. prolifera* > *U. fasciata*.

The algal species investigated in this study were found to contain appreciable amounts of proteins and carbohydrates. Chlorophyta exhibited 8.99 to 25.12 % and Rhodophyta exhibited 5.98 to 9.37 % of protein contents. With respect to carbohydrates content, Chlorophyta exhibited 33.17 to 50.83 % and Rhodophyta exhibited 31.02 to 75.95 %. Lipids content were relatively low. 2.09 to 10.76 % in Chlorophyta and 1.49 to 2.65 % in Rhodophyta. Species wise variations were observed in iodine concentrations. Rhodophyta had 137.54 to 185.38 µg g^{-1} of iodine content, whereas, Chlorophyta had 41.46 to 62.49 µg g^{-1}. Ash content was almost uniform throughout. Chlorophyta had 20.43 to 27.71 % and Rhodophyta had 13.06 to 23.12 % of ash content. Crude fibre content had no specific patterns. Chlorophyta had 37.57 to 61.59 % and Rhodophyta had 40.07 to 63.39 % of crude fibre content. The average energy contents varied from 1.39 to 3.49 kcal g^{-1} for Chlorophyta and 1.67 to 3.52 kcal g^{-1} for Rhodophyta.

Proximate composition analysis of the investigated algal species, in comparison with recommended daily intake levels suggested the utilization of *U. fasciata* and *E. prolifera* as protein supplements, *G. pusillum* and *G. corticata* var. *cylindrica* was carbohydrate source and, *E. prolifera* and *U. fasciata* as lipids supplement. *Gracilaria* sp. was suggested as an iodine supplement. All the nine investigated algal species were observed to be a rich source of crude fibre. *U. fasciata*, *A. spicifera*, *G. pusillum*, *E. prolifera* and *G. foliifera* had remarkable proximate compositions.

Study on 1:1 aqueous methanolic extractives of the algal species exhibited the presence of 13 vitamins, two carotenoids, phenolics and five flavonoids contents. Chlorophyta exhibited 12.04 to 13.14 % and Rhodophyta exhibited 11.16 to 17.21 % of 1:1 aqueous methanolic yields. Among the 13 vitamins quantified, high amount of ascorbic acid and α – tocopherol were

observed throughout the nine seaweeds. Commendable concentrations of pantothenic acid, niacin, phylloquinone, riboflavin and thiamine were observed. Biotin, ergocalciferol and cyanocobalamin contents were observed to be low. *G. pusillum* had high contents of vitamins (9.15 %). Chlorophyta had 2.78 to 4.31 % and Rhodophyta had 0.51 to 9.15 % of total vitamin contents. Two carotenoids, viz. lutein and zeaxanthin were observed to be high in *G. pusillum* (4.91 μg g^{-1}) and *G. corticata* var. *cylindrica* (1.06 μg g^{-1}) respectively. Overall, 0.3 to 1.66 μg g^{-1} of lutein and 0.09 to 0.2 μg g^{-1} of zeaxanthin were observed in Chlorophyta. 0.43 to 4.91 μg g^{-1} of lutein and 0.14 to 1.06 μg g^{-1} of zeaxanthin were observed in Rhodophyta.

Comparatively low total phenolics content were observed. Chlorophyta had 1.97 to 81.10 μg g^{-1} and Rhodophyta had 2.34 to 91.18 μg g^{-1} of total phenolic contents. Five flavonoids viz. myricetin, quercetin, luteolin, kaempferol and apigenin were observed in all the analysed algal samples except in *G. pusillum*. Chlorophyta exhibited 0.02 to 57.88 μg g^{-1} and Rhodophyta exhibited 0.09 to 42.41 μg g^{-1} of myricetin content. Quercetin content was in the range of 0.02 to 5.69 μg g^{-1} in Chlorophyta and 0.14 to 8.57 μg g^{-1} in Rhodophyta. Luteolin content was in the range of 0.05 to 7.53 μg g^{-1} in Chlorophyta and 0.07 to 6.65 μg g^{-1} in Rhodophyta. Kaempferol content was in the range of 0.27 to 1.80 μg g^{-1} in Chlorophyta and 0.09 to 11.64 μg g^{-1} in Rhodophyta. 0.02 to 11.46 μg g^{-1} and 0.09 to 13.38 μg g^{-1} of apigenin were observed in Chlorophyta and Rhodophyta respectively. Flavonoid contents were almost proportional to the total phenolics content. With respect to the vitamins, carotenoids and phenolic contents, the 1:1 aqueous methanolic extracts exhibited potential antioxidant activities too. All the algal species extracts exhibited commendable antioxidant activities with respect to the three

methods against four standards. Extracts of *G. foliifera*, *C. antennina* and *E. prolifera* exhibited greater extend of antioxidant activities.

The appreciable levels of major secondary metabolites in the analysed algal species, with special emphasis on vitamins, in comparison with the recommended daily intake levels evidenced the recommendation of *G. pusillum*, *G. foliifera* and *E. prolifera* as vitamin supplements. *G. foliifera*, *G. pusillum* and *A. spicifera* could also be used to cater the vitamin deficiencies.

Study on the amino acid compositions of algal species showed the presence of both essential and non essential amino acids. In addition to these, the presence of free amino acids and L-dopa were the highlights of this study. Both PITC and OPA pre column derivatization HPLC methods exhibited comparable amino acid contents. Among the 16 amino acids, threonine, histidine and alanine were the major EAA forms, and aspartic acid and proline were the major NEAA forms. Amino acid contents were high in *E. prolifera* and *U. fasciata*. L-dopa was high in *G. pusillum*. Comparatively, low amounts of amino acids were observed in Rhodophyta. EAA/NEAA and EAA/P were observed to be high in *U. fasciata*, *G. corticata* and *G. corticata* var. *cylindrica*. Free amino acid contents ranged from 0.96 to 2.66 % in Chlorophyta and 0.58 to 1.39 % in Rhodophyta. Comparison to the recommended daily intake levels of essential amino acids suggests the utilization of *U. fasciata*, *E. prolifera* and *A. spicifera* as an amino acid rich food source.

Study on the biologically important metabolites evidenced the presence of commercially important saponins, fatty acids viz. saturated fatty acids, mono unsaturated fatty acids (MUFAs) and poly unsaturated fatty acids (PUFAs), alkanes, alkenes, alkynes, alcohols and sterols. Saponin contents

varied from 1.19 to 1.72 % in Chlorophyta and 0.27 to 1.21 % in Rhodophyta. All the extracted fractions exhibited good antioxidant and antimicrobial activities. Chemical speciation of the extracted fractions showed the presence of phytol as a common alcohol. Alkanes ranging from 8 to 44 carbon atoms, alkenes ranging from 7 to 17 carbon atoms and alkynes ranging from 18 to 20 carbon atoms were observed. Alcohols ranged from 10 to 20 carbon atoms. Among sterols, cholesterol and its analogues were a common occurrence.

SFAs ranged from 13 to 18 carbon atoms in Chlorophyta and 8 to 27 carbon atoms in Rhodophyta. SFAs were high in *G. corticata* var. *cylindrica*. MUFAs ranged from 12 to 20 carbon atoms and were relatively high in in *G. foliifera*. PUFAs ranged from 16 to 25 carbon atoms with relatively high contents in *E. prolifera*. Omega fatty acids were seen high in Chlorophyta.

Studies on total polysaccharides isolated from the algal species evidenced the presence of sulfated polysaccharides. Saponification and iodine value indicated the possibilities of glycolipids or glycoesters. Negative optical rotation evidenced the presence of negative optically active monosaccharide compositions which was quantified by HPLC-RI techniques. Polysaccharides isolated from *Gracilaria* sp. exhibited high antioxidant activities. Rhodophyta exhibited high antioxidant active polysaccharides. Antimicrobial activity was relatively high for Chlorophyta. The monosaccharide composition analysis of the total isolatable polysaccharides exhibited high contents of ribose and arabinose. Disaccharide sucrose was also quantified which is available in its free form.

Algal species were observed to be rich in various primary and secondary metabolites. Irrespective of the quantified biochemical constituents, the study investigated for the presence of novel secondary metabolites too. Abundantly available seaweed species such as *G. foliifera* was selected for this study. The unattended saponin fraction was chemically investigated with

analytical techniques such as sequential extractions, TLC, UV-Vis scanning, HPLC, preparative HPLC, GC, GC-MS, optical rotation, melting point, LC-MS/MS, ^{1}H NMR, ^{13}C NMR and 2-D NMR. The structure of isolated steroid compound was derived and confirmed to be *E*-guggulsterone. No previous studies were observed for the isolation and quantification of *E*-guggulsterone from seaweeds. Hence, this study is the first report with respect to isolation of *E*-guggulsterone from seaweeds. Isolated *E*-guggulsterone exhibited potent antioxidant and antimicrobial activities.

In short, the thesis highlights the scope of Chlorophyta and Rhodophyta (especially *E. prolifera* and *U. fasciata* from Chlorophyta and *Gracilaria* sp. and *G. pusillum* from Rhodophyta) as potential sources of proteins, carbohydrates, lipids, crude fibres, iodine, calorific value, vitamins, carotenoids, phenolics, flavonoids, amino acids, fatty acids, sterols, polysaccharides, antioxidant and antimicrobial activities, which are needed for human and animal nutrition and well being. Data obtained in this investigation also supports the utilization of these seaweeds as bio important renewable raw material sources that are suitable for nutraceutical extraction industries.

In future, with respect to the scenario of declining land resources, the study strongly supports the possibilities of utilization of seaweeds in day to day diets. Known edible seaweeds such as *G. pusillum*, *G. corticata*, *U. fasciata*, *E. prolifera* and *A. spicifera* could be used as an alternative and nutritive food resource or as food supplements. They can also serve as a source of important secondary metabolites that would cater the agricultural sectors. In addition to this, *G. foliifera* occurring at Njarakkal can be developed as a commercial crop for the extraction of many more bioactive compounds in future.

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LIST OF RESEARCH ARTICLES – PUBLISHED/COMMUNICATED

Kailas, A. P., & Nair, S. M. (2016). HPLC profiling of antimicrobial and antioxidant phyco-sugars isolated from the South West coast of India. *Carbohydrate Polymers, DOI 10.1016/J.CARBPOL.2016.05.111.* Impact factor 4.568.

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Kailas, A. P., & Nair, S. M. (2016). Amino acid composition of some tropical seaweeds - Comparison of o-phthalaldehyde (OPA) and phenylisothiocyanate HPLC pre-derivatization methods and amino acids contribution to recommended daily index (RDI). *Algal Research, Under review after 1st revision*. Impact factor 5.027.

Kailas, A. P., & Nair, S. M. (2016). Nutraceutical potentiality of some seaweeds - quantification of carotenoids, vitamins, flavonoids and total phenolic contents, and a comparative evaluation of the antioxidant activities of the extracts. *Food Chemistry*, *Communicated*.