Isolation and Characterization of Useful Secondary Metabolites with Antioxidant Activity from Seaweeds from Southeastern Coast of India

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DOCTOR OF PHILOSOPHY

in

CHEMISTRY

By

Mr. PRAVEEN N. K.
Central Marine Fisheries Research Institute (CMFRI)

Guided by : Dr. KAJAL CHAKRABORTY
SENIOR SCIENTIST (ORGANIC CHEMISTRY), MARINE BIOTECHNOLOGY DIVISION
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE (CMFRI)

Under the faculty of Science and Technology

MANGALORE UNIVERSITY
Mangalagangothri, Mangalore - 574 199

Central Marine Fisheries Research Institute
(Indian Council of Agricultural Research)
Post Box No. 1603, Cochin - 682 018

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DECLARATION

I hereby declare that the matter presented in this thesis is the result of investigations carried out by me in the Central Marine Fisheries Research Institute, Cochin-18, Kerala, India, under the guidance of Dr. Kajal Chakraborty, Senior Scientist (Organic Chemistry), Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin-18 and the same has not previously formed the basis for the award of any degree or diploma.

Whenever the work described is based on the findings of other researchers, due acknowledgement is made in keeping with the general practice of reporting scientific observations. However, errors and unintentional oversights, if any are regretted.

Place: Cochin **Praveen N. K.**

Date: 05.01.2015

केंद्रीय समुद्री मात्सिकी अनुसंधान संस्थान

कोच्ची - 682 018, केरल, भारत

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE

(Indian Council of Agricultural Research)

P.B No. 1603, EKM North PO,COCHIN 682018, Kerala, India

भाकृ अनुप
ICAR

केंस मा अनु सं
CMFRI

Telephone
Off: +91-484-2394867
Telegram: Cadalmin
Fax: +91-484-2394909
E-mail: chakrabortycmfri@gmail.com

Dr. Kajal Chakraborty
Senior Scientist (Organic Chemistry)

CERTIFICATE

This is to certify that this thesis entitled "ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES WITH ANTIOXIDANT ACTIVITY FROM SEAWEEDS FROM SOUTHEASTERN COASTS OF INDIA" submitted by Shri. Praveen N K, Junior Research Fellow of Marine Biotechnology Division of Central Marine Fisheries Research Institute, for the award of the degree of Doctor of Philosophy in Chemistry is the result of bonafide research work carried out by him in the Marine Biotechnology Division of Central Marine Fisheries Research Institute, Cochin-18, under my guidance and direct supervision. I further certify that this thesis or part thereof has not previously formed the basis for the award of any degree, diploma, associateship of any other University or Institution.

Place: Cochin
Date: 05.01.2015

Kajal Chakraborty

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ABBREVIATIONS

Abbreviations	-	**Expansions**
NMR	-	Nuclear Magnetic Resonance
2D-NMR	-	2-Dimensional-NMR
DEPT	-	Distortionless Enhancement By Polarization Transfer
COSY	-	Correlation Spectroscopy
HSQC	-	Heteronuclear Single Quantum Coherence
HMBC	-	Heteronuclear Multiple Bond Correlation
NOESY	-	Nuclear Overhauser Effect Spectroscopy
ppm	-	Parts Per Million
IR	-	Infra Red
γ_r	-	Rocking Vibration
ν	-	Stretching Vibration
δ	-	Bending Vibrations
ν_s	-	Symmetric Stretching Vibration
ν_a	-	Asymmetric Stretching Vibration
GC-MS	-	Gas Chromatography-Mass Spectrometry
m/e	-	Mass-to-Charge Ratio
HPLC	-	High-Performance Liquid Chromatography
DAD	-	Diode Array Detector
RP-HPLC	-	Reverse Phase High-Performance Liquid Chromatography
R_t	-	Retention Time
TLC	-	Thin Layer Chromatography
R_f	-	Retardation Factor
$OH^{\bullet}$	-	Hydroxyl Radical
H_2O_2	-	Hydrogen Peroxide
$NO_2^{\bullet}$	-	Nitric Dioxide
$^1O_2$	-	Singlet Oxygen
$O_2^{\bullet -}$	-	Superoxide Anion
OCl-	-	Hypochlorite
OONO -	-	Peroxynitrite
$R^{\bullet}$	-	Alkyl Radicals
$RO^{\bullet}$	-	Alkoxyl Radical
$ROO^{\bullet}$	-	Peroxyl Radical
ROS	-	Reactive Oxygen Species
COX	-	Cyclooxygenase

LOX	-	Lipoxygenases
PDA	-	Photodiode Array
SD	-	Standard Deviation
TFA	-	Trifluoroacetic Acid
NO	-	Nitric Oxide
DPPH	-	1,1-Diphenyl-2-Picrylhydrazyl
ABTS	-	2, 2-Azino-Bis-3ethylbenzothiozoline-6-Sulfonic Acid Diammonium Salt
TPC	-	Total Phenolic Content
TBARS	-	Thiobarbituric Acid-Reactive Substances Assay
EtOAc	-	Ethyl Acetate
MeOH	-	Methanol
MDC	-	Dichloromethane
IC_{50}	-	Inhibition Concentration At 50 %
EC_{50}	-	Effective Concentration At Which The Absorbance Was 0.5
GE	-	Gallic Acid Equivalence
BHA	-	Butylated Hydroxyanisole
BHT	-	Butylated Hydroxytoluene
EDTA	-	Ethylenediaminetetraacetic Acid
DMSO	-	Dimethyl Sulfoxide
TCA	-	Trichloroacetic Acid
TBA	-	Thiobarbituric Acid
MDA	-	Malonaldehyde
MDAEC	-	MDA Equivalent Compounds
EGCG	-	Epigalocatechin Gallate
EC	-	Epicatechin
ECG	-	Epicatechin Gallate
ANOVA	-	Analysis of Variance
SPSS	-	Statistical Program for Social Sciences
PCA	-	Principal Component Analysis

ABSTRACT

Seaweeds offer valuable bioactive molecules with antioxidative properties, and are abundantly available in the Gulf of Mannar region of the Southeastern coasts of India. This study aimed to evaluate the antioxidative properties of different seaweed species available in this region and isolating the compounds with potential radical scavenging activity. The methanol extract and solvent fractions (*n*-hexane, dichloromethane and ethyl acetate) of the brown seaweeds *Turbinaria conoides, Turbinaria ornata, Anthophycus longifolius, Sargassum plagiophyllum, Sargassum myriocystum, Padina tetrastomatica, Padina gymnospora* and *Stoechospermum marginatum* and the red seaweeds *Laurencia papillosa, Gelidiella acerosa* and *Acanthophora spicifera* collected from the Gulf of Mannar region of Mandapam were screened for potential antioxidant properties by different model systems. The antioxidant activities of these seaweeds have been evaluated using different *in vitro* assays, *viz* 1,1-dipheny1–2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (H_2O_2)/ hydroxyl radical (HO.) scavenging, ferrous ion (Fe^{2+}) chelating ability, thiobarbituric acid reactive species (TBARS) formation inhibition assay and reducing potential. A reversed-phase high-performance liquid chromatography method hyphenated to diode-array detection was also utilized to characterize the solvent extract fingerprints of phenolic acids in the seaweed species. These seaweed-derived crude extracts have been purified by a series of chromatographic purification steps based upon their antioxidant potential and further chromatographically purified to yield the antioxidant secondary metabolites.

It was observed that the ethyl acetate fractions of seaweeds exhibited higher radical scavenging potential and phenolic content as compared with the methanol extracts and other solvent fractions. A higher phenolic content (283 GE/g), DPPH (97%, 1 mg/mL) and ABTS (97%, 0.6 µg/mL) radical scavenging activities were recorded for the ethyl acetate fraction of *Laurencia papillosa*. The ethyl acetate fraction of *Padina tetrastomatica* registered higher hydroxyl radical potential (87%) followed by the dichloromethane fraction of *Padina gymnospora* (85%, 0.6 mg/mL). The dichloromethane fraction of *Padina gymnospora* and the ethyl acetate fractions of *Turbinaria conoides* and *Acanthophora spicifera* were proved to be equally effective towards scavenging hydrogen peroxide (18%, 1 mg/mL). Thiobarbituric acid reactive species formation was effectively hindered by the ethyl acetate fractions of *Gelidiella acerosa*, *Padina tetrastomatica* and *Sargassum myriocystum* (> 5 MDAEC/kg, 2 mg/mL). Higher reducing abilities were recorded with the ethyl acetate fractions of

Stoechospermum marginatum, *Acanthophora spicifera*, and *Anthophycus longifolius* (>1.48 A_{700nm}, 1 mg/mL). The ethyl acetate fraction of *Anthophycus longifolius* registered higher Fe^{2+} ion chelating ability (88%, 0.6 mg/mL) followed by that of *Stoechospermum marginatum* (82%, 0.6 mg/mL).

2, 5 dihydroxy benzoic acid (15 mg/g), epicatechin (36 mg/g) and epicatechin gallate (205 mg/g) were predominant in the methanol fraction of *Turbinaria conoides*, whilst chlorogenic acid (43 mg/g), salicylic acid (8 mg/g) and gallic acid (65 mg/g) were the main components in the ethyl acetate fraction of *Turbinaria ornata*. A higher amount of epigallocatechin gallate (84 mg/g) was identified in the ethyl acetate fraction of *Padina tetrastomatica*, whereas the methanol fraction of *Padina gymnospora* registered a higher amount of syringic acid (132 mg/g). Methanol fraction of *Gelidiella acerosa* exhibited a dominant amount of catechin (21 mg/g) than other seaweeds. Caffeic acid (12 mg/g), coumaric acid (21 mg/g), ferulic acid (27 mg/g) and quercetin (33 mg/g) were found to be predominant with the ethyl acetate fraction of *Stoechospermum marginatum*.

Based upon the bioassay results the brown seaweeds *Anthophycus longifolius* and *Padina gymnospora* and the red seaweeds *Acanthophora spicifera* and *Laurencia papillosa* were selected for further isolation and chromatographic purification of antioxidant secondary metabolites. Bioassy guided chromatographic purification of the methanol extract of *Anthophycus longifolius* yielded (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate and 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid as major antioxidant secondary metabolites with higher DPPH (>76%, 0.1mg/mL) and ABTS (>30%, 0.1µg/mL) radical scavenging abilities, and ion chelating potential (>26%, 0.1mg/mL). The methanol extract of *Padina gymnospora* upon sequential bioassay guided chromatographic purification yielded two antioxidant secondary metabolites 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one and (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon -6-enoate (>70% DPPH and >23% ABTS radical scavenging activities, and >20% ion chelating potential). Two antioxidant secondary metabolites 3-hexyl-5,6-dihydro-6-undecylpyran-2-one and butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate were yielded by repeated bioassay guided chromatographic purification of the methanol extract of the red seaweed *Acanthophora spicifera.* The methanol extract of *Laurencia papillosa* upon continual bioassay guided chromatographic purification yielded two antioxidant secondary metabolites 12-tridecenyl 2-methylacrylate and 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-

oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal with >72% DPPH and >29% ABTS radical scavenging activities and >21% ion chelating potential. The natural antioxidative compounds identified from the seaweeds in the present study will serve as potential lead molecule for newer synthetic routes.

The natural antioxidative compounds identified from the seaweeds in the present study will serve as a potential substitute replacing the harmful synthetic alternatives and also open up new horizons for the development of safe synthetic antioxidants. The study stands as the first of its kind reported from the biodiversity rich habitat of Gulf of Mannar. We have explored the seaweeds which are abundantly available and their abundance is independent of seasonal variation. This Study will serve as the lead to understand the medicinal importance of the seaweeds, which, in turn, will be the natural template for synthesis of highly active molecules. The study also open up the importance of sea weed mariculture as the same will stand as raw material for the isolation of natural compounds with antioxidant activity.

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CHAPTER 1

Introduction

1.1. Oxidative Stress

Free radicals are generated in the living cells due to many biological processes that normally take place in our metabolic pool. These free radicals are highly reactive and can damage the living cells in many ways. But our body possesses suitable defense mechanisms to detoxify these deleterious radicals. When the production of these free radicals exceeds beyond a limit due to excessive oxidation, the natural defense system of body fails. This causes a state called oxidative stress which results in the denaturation of proteins, cellular membranes and genes. Oxidative stress is associated with a wide variety of diseases like atherosclerosis, diabetes mellitus, neurodegenerative disorders, cancers, rheumatic diseases, autoimmune disorders etc (Ilie & Marginã 2012), and also leads to a general feeling of illness, lethargy, lack of enthusiasm, depressed immune system leading to the loss in cell and organ functions (dedicated website http://www.neurogenol.co.uk/oxidativestress.html). The oxidative stress can also have harmful effects on foods as production of rancid flavours and odours, reducing the shelf-life, nutritional quality, and safety of food products (Zainol et al. 2003; Chanwitheesuk et al. 2005).

1.2. Reactive Oxygen Species (ROS) and Health Implications

1.2.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are chemically reactive molecules derived from the molecular oxygen. Superoxide anion ($O_2^{-\bullet}$) hydroxyl radical ($^{\bullet}OH$), lipid peroxyl radical ($LOO^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are some common examples of ROS. These reactive species originate from the environment, from other free radicals in chain reactions, and from many normal biological processes in *vivo*. The free radicals will enter into our cellular system through different endogenous and exogenous pathways. ROS are generated endogenously by different processes such as mitochondrial electron transport, endoplasmic reticulum oxidation, plurality of enzymatic activities, gluconolactone oxidase, prostaglandin synthesis, auto oxidation of biomolecules with unsaturated and electron rich centers, which are predominant in several biosynthetic path ways (Tandon et al. 2005). Exogenous sources such as drugs, halothene, paracetamol, bleomycine, doxorubicin, metrenidazole, ethanol, CCl_4, pesticides, transition metals, radiations, and high temperature (Tandon et al. 2005) also contribute significantly towards the generation of these deleterious free radicals.

The predominant types of ROS are distributed under the following heads

1.2.1.1. Superoxide Anion

The cellular processes like mitochondrial electron transport systems, microsomal electron transport systems, xanthine oxidase, xanthine dehydrogenase etc. are considered to be the major sources of superoxide anion molecule. They are also created by the cellular process where NADPH

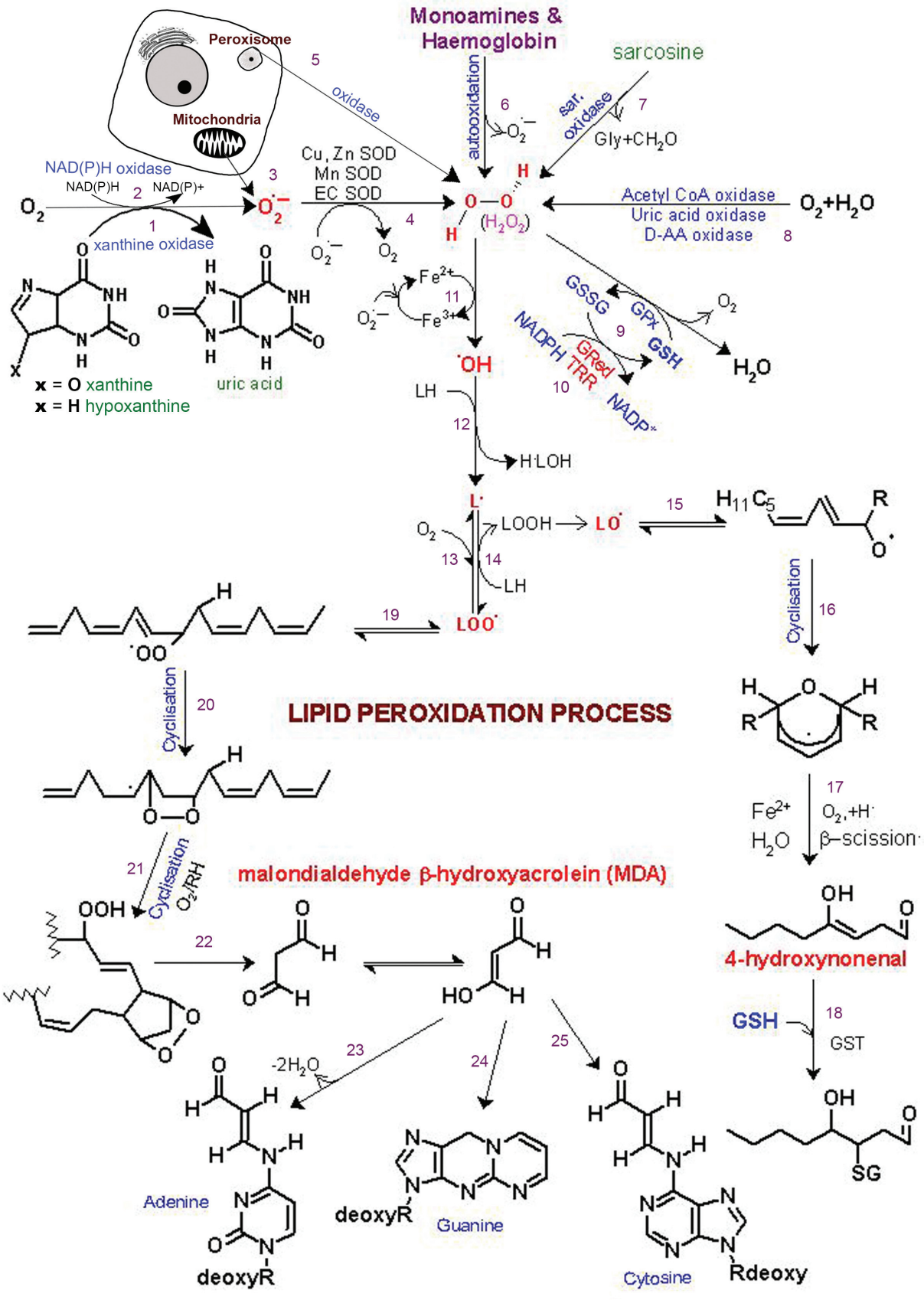

Figure 1.1. Schematic representation of the ROS formation and its mechanism of action

REACTIONS

1, 2 and 3	:	The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain.
4	:	Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide.
5, 6, 7 and 8	:	Generation of H_2O_2 from peroxisome, monoamines and haemoglobin, sarcosine and the reaction between water and oxygen
9	:	Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor.
10	:	The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase Gred) which uses NADPH as the electron donor.
11	:	Some transition metals (e.g. Fe^{2+}, Cu+ and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction).
12	:	The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•).
13	:	The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 19–25 and 15–18).
14 and 15	:	Formation of lipid alkoxyl radical (LO•) from lipid peroxyl radical (LOO•) and polyunsaturated fatty acid (LH) through lipid hydroperoxide (LOOH) path way
16	:	Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide.
17	:	Six-membered ring hydroperoxide udergoes further reactions (involving-scission) to from 4-hydroxy-nonenal.
18	:	4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione S-transferase).
19 and 20	:	A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical.
21	:	This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide.
22	:	Formed compound is an intermediate product for the production of malondialdehyde.
23, 24, 25	:	Malondialdehyde can react with DNA bases cytosine, adenine, and guanine to form adducts M1C, M1A and M1G, respectively.

oxidase reduces oxygen as the part of its defense mechanisms and the excess amount produced are converted to hydrogen peroxide by the enzyme superoxide dismutase. Even though superoxide anion is considered to be as a non-reactive species they are able to generate more reactive free radicals by the reaction with other molecules.

1.2.1.2. Hydrogen Peroxide (H_2O_2)

H_2O_2 is may not be termed as a true species of free radical because it bears no unpaired electrons. However being an oxidizing agent it can intensify and initiate the OH radical formation. They are produced by the enzyme superoxide dismutase during the cellular processes and can occupy both cytoplasmic and intracellular spaces in the body. They can also cross biological membranes by diffusion.

1.2.1.3. Hydroxyl Radical (OH)

Hydroxyl radicals are considered to be the most reactive free radical species formed in the cells by the transition ion catalyzed reaction (Haber-Weiss reaction) of H_2O_2. Lipid-peroxidation of microsomal, mitochondrial and cell membranes, modification of purines and pyrimidines (Fenton 1894; Haber 1934) or strand breakage (Oshima et al. 1996) are some of the major problems caused by the hydroxyl radicals. Hydroxyl radicals react with the biological molecules by number of ways viz, electron transfer, hydrogen transfer, addition to aromatic systems etc, to produce the secondary reactive species, which are even more deleterious than the parent $^{\bullet}OH$ radical.

1.2.1.4. Lipid Peroxide (LOOH)

Oxidation of fatty acids generated fatty acid free radicals, which can react with oxygen to from the peroxyl radicals that further react with other fatty acids to produce lipid hydroperoxides. The lipid hydroperoxides are considered to be more reactive as it can cause lipid peroxidation by the production of the more reactive species like lipid peroxyl, lipid alkoxyl and malondialdehyde (MDA) like compounds (Slater 1979).

1.2.1.5. Singlet Oxygen ($^1O_2$)

As H_2O_2, the singlet oxygen species is not a free radical in true sense, and is considered as oxidant species, which can cause the tissue damage in the biological system. The singlet oxygen species is formed by the spin reversal of electron present in the outer orbital of the O_2 molecule by enzymatic catalyzed reactions. Due to its electrophilic nature, it can readily react with the fatty acids and other molecules to produce peroxide radicals.

1.2.2. Effect of ROS on Body

The reactive oxygen species react with the protein molecules, unsaturated fatty acids, nucleic acids, carbohydrates etc to cause their oxidative damage, thereby leading to severe health problems. They are considered to be the major cause of inactivation of enzymes. The protein molecules are fragmented by the attack of free radicals or their metal binding sites may be affected, or can undergo many other modifications in their amino acid residues like proline, histidine, cysteine methionine, tryptophan, tyrosine, phenylalanine etc. Lipids especially polyunsaturated type readily undergoes peroxidation, leading to the formation of other free radicals, which act as chain propagators for further

lipid peroxidation process. This will also lead to the rancidity or off flavor and spoilage of the food products. Lipid peroxidation can also affect the cellular membrane functions. ROS are the primary cause for several genetic defects caused by the radical induced nitrogenous base modification of nucleic acids and mutations. Hyaluronic acid, a carbohydrate present in the synovial fluid can undergo free radical induced depolymerisation leading to the joint inflammation (Grootveld et al. 1991). Other macromolecules such as collagen, proteoglycans etc. are also fragmented by the action of free radicals.

1.3. Antioxidants

Our body possess innate defense mechanisms against these free radicals generated through a series of mechanisms involving different biomolecular reactions such as enzymes, amino acids, bioactive antioxidant molecules, selenium, vitamins etc to protect the cells from oxidative damage (Wojcik et al. 2010; Samaranayaka & Li-Chan 2011). Antioxidant enzyms like superoxide dismutases, catalase, glutathione peroxidase etc are the major common enzymes, which are involved in scavenging free radicals like superoxide anion, hydrogen peroxide, lipid hydroperoxides etc (Lobo et al. 2010). Ascorbic acid, glutathione, melatonin, tocotrienols, uric acid etc are the non enzymatic compounds used by our body to prevent from oxidative damage (Lobo et al. 2010). As these compounds are effectively used to scavenge free radicals they are called as antioxidants.

The antioxidants help to reduce the risk of free radical derived issues. Antioxidants generally inactivate free radicals (alkoxyl, peroxyl and alkyl) by donating its H atom to form stable compounds as illustrated below:-

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$

$$LOO^{\bullet} + A^{\bullet} \longrightarrow LOOA$$

Where $LOO^{\bullet}$ is a lipid peroxyl radical and AH is the representation of the antioxidant molecule able to donate its H atom. Transition metals like iron and copper are capable of producing very reactive hydroxyl radicals from peroxides, which can damage the living cells.

$$LOOH + Fe^{2+} \longrightarrow LO^{\bullet} + OH^{-} + Fe^{3+}$$

1.3.1. Synthetic Antioxidants

Butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG), teritiary butyl hydroquinone (TBHQ), octyl gallate (OG), 2,4,5-trihydroxy butapyranone, nordihydroguaiaretic acid and 4-hexyl resorcinol are some of the common examples of synthetic antioxidants (Carocho 2013, Aguillar et al. 2012, Gharavi & El-Kadi, 2005, Anton et al. 2004, Kubo et al. 2001, Astill et al. 1959, Evan & Gardner 1979, Chen et al. 2004) (Figure 1.2). They are extensively used to control rancidity in lipid-containing foods and formation of lipid oxidation or peroxidation products, cosmetic and pharma industries.

1.3.2. Drawbacks of Synthetic Antioxidants

BHT was reported to react with other ingested substances to cause the formation of carcinogens. BHT is banned in the UK (dedicated website: http://www.healthyeatingadvisor.com).

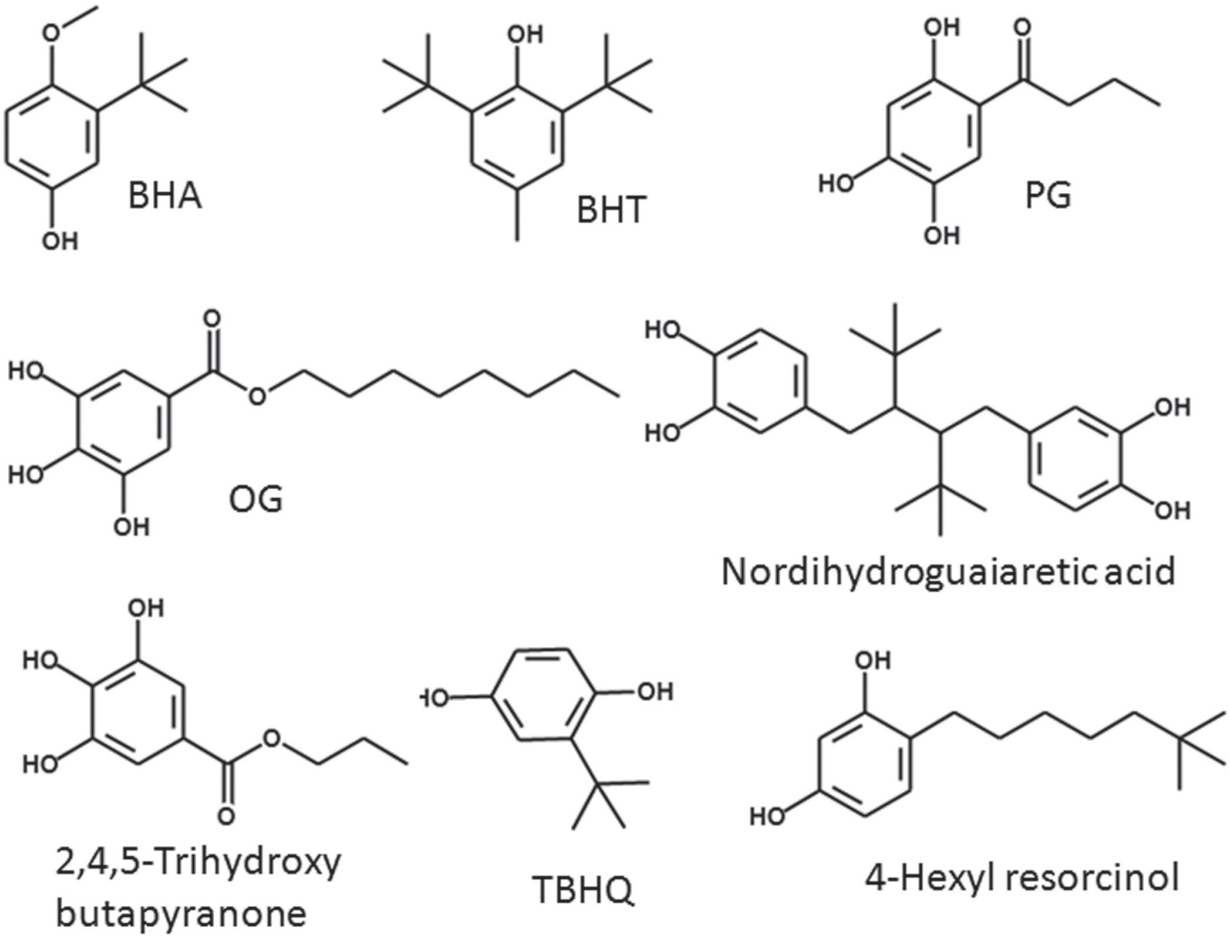

Figure 1.2. Structure of some common synthetic antioxidants

TBHQ is banned in Japan and certain European countries (Shahidi 1997). Many countries like Japan banned the production and use of BHA whereas some other countries like UK could not implement the recommendations of the officials to ban the same due to pressure from the industries. McDonald's eliminated BHT from their US products by 1986 (dedicated website: http://www.foodfigures.com/food_additive.htm). BHA and BHT are also known to cause cancer in humans. Therefore, in recent years, interest in finding naturally occurring antioxidant compounds in food or medicine to replace synthetic products has increased considerably, given that synthetic ones are being restricted due to consumer preference for natural products and concern about the potential toxic effects of synthetic medicines (Zheng et al. 2001).

1.3.3. Green Alternatives

Because of the possible harmful effects of synthetic antioxidants, the demand for a natural alternatives are increasing. Hence the pharmaceutical and agri-food industries have been concentrating in developing and marketing functional foods with green and natural antioxidant alternatives as ingredients. Marine flora constitutes the potential natural sources with pluralities of bioactive compounds having antioxidant properties. Seaweeds constitute a major share of marine flora, and they were reported to possess structurally diverse compounds of various bioactivities endowed with antioxidant, antibacterial, anti-inflammatory, and anticarcinogenic activities (Kornprobst, 2005). Since there is an increased interest in the antioxidants of natural origin in recent times in place of synthetic derivatives, it is rational to explore the seaweeds as natural sources to isolate antioxidant principles for use as nutraceutical supplements. The potential applications offered by these valuable resources as ingredients in functional foods are significant because of their richness in bioactive principles, particularly antioxidants.

In high light environments like the sea, energy absorbs faster than it can be dissipated, producing the free radicals and promoting lipid oxidation. Surprisingly the lack of structural damage in seaweed cells even after the regular exposure to light and high oxygen, attribute to the role of natural antioxidant compounds found in them in protecting the cell content (Swanson & Druehl, 2002; Burritt et al. 2002). Therefore, these marine floras may be considered as a potential resource of unexplored natural antioxidant molecules, which need to be studied further. It was reported that seaweeds are rich source of bioactive compounds, such as terpenoids, phloroglucinol phenolics, fucoidans, sterols and glycolipids, and the extracts or isolated components from seaweeds posses a wide range of pharmacological properties such as anticancer, antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, hypoglycaemic, hypolipidemic, hepatoprotective and neuroprotective activities (Liu et al. 2012; Chakraborty et al. 2013). Extracts from several brown and red seaweeds harvested in France (Le Tutour, 1990), Spain (Jiménez-Escrig et al. 2001), Indonesia (Anggadiredja et al.1997), Korea (Han et al. 1999), China (Yan et al. 1998) and Japan (Yan et al. 1999 and Sekikawa et al. 1986) have demonstrated antioxidant activity *in vitro*. The solvent extracts of brown seaweeds from Indian waters were reported to contain high levels of hydrophilic components, such as polyphenols and soluble polysaccharides with strong antimicrobial and antioxidant activities

(Chandini et al. 2008; Ananthi et al. 2010; Rajauria et al. 2012; Chakraborty et al. 2013). Although antioxidant properties of seaweeds have been demonstrated by numerous studies during the past few decades, only scarce reports are available from the Gulf of Mannar region, rich in biodiversity leading to the isolation and characterization of antioxidant secondary metabolites. The study was planned based on the hypothesis that the seaweed species distributed in the Gulf of Mannar region may have potential bioactive compounds with pharmaceutical and neutraceutical properties. The study also anticipate to shortlist potential seaweed species with natural bioactive compounds.

Standard methodologies were followed to collect and screen seaweeds for natural bioactive compounds. Antioxidant assays were used to find out the sea weeds species containing potential natural products. The promising fractions were further purified by different chromatographic procedures and the compounds purified to homogeneity were used to elucidate the structural characteristics. Further, different spectroscopic methods like NMR, IR, GC-MS etc were employed to decipher the structural confirmation.

Based on the previous studies demonstrating that the seaweeds are potential source of antioxidative compounds, the present work has been focused based on the following objectives,

1. To evaluate the antioxidant potential of the crude extracts and solvent fractions of seaweeds by a battery of *in vitro* reactive oxygen species scavenging assay.
2. To purify the molecules having potential antioxidant activity from the crude extracts by using various chromatographic techniques based on their bioassay results.
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Chapter 5 - Summary: This chapter is dealt with the sailent results of the study and their practical application in the functional food industry.

CHAPTER 1

Introduction

1.1. Oxidative Stress

Free radicals are generated in the living cells due to many biological processes that normally take place in our metabolic pool. These free radicals are highly reactive and can damage the living cells in many ways. But our body possesses suitable defense mechanisms to detoxify these deleterious radicals. When the production of these free radicals exceeds beyond a limit due to excessive oxidation, the natural defense system of body fails. This causes a state called oxidative stress which results in the denaturation of proteins, cellular membranes and genes. Oxidative stress is associated with a wide variety of diseases like atherosclerosis, diabetes mellitus, neurodegenerative disorders, cancers, rheumatic diseases, autoimmune disorders etc (Ilie & Marginã 2012), and also leads to a general feeling of illness, lethargy, lack of enthusiasm, depressed immune system leading to the loss in cell and organ functions (dedicated website http://www.neurogenol.co.uk/oxidativestress.html). The oxidative stress can also have harmful effects on foods as production of rancid flavours and odours, reducing the shelf-life, nutritional quality, and safety of food products (Zainol et al. 2003; Chanwitheesuk et al. 2005).

1.2. Reactive Oxygen Species (ROS) and Health Implications

1.2.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are chemically reactive molecules derived from the molecular oxygen. Superoxide anion ($O_2^{-\bullet}$) hydroxyl radical ($^{\bullet}OH$), lipid peroxyl radical ($LOO^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are some common examples of ROS. These reactive species originate from the environment, from other free radicals in chain reactions, and from many normal biological processes in *vivo*. The free radicals will enter into our cellular system through different endogenous and exogenous pathways. ROS are generated endogenously by different processes such as mitochondrial electron transport, endoplasmic reticulum oxidation, plurality of enzymatic activities, gluconolactone oxidase, prostaglandin synthesis, auto oxidation of biomolecules with unsaturated and electron rich centers, which are predominant in several biosynthetic path ways (Tandon et al. 2005). Exogenous sources such as drugs, halothene, paracetamol, bleomycine, doxorubicin, metrenidazole, ethanol, CCl_4, pesticides, transition metals, radiations, and high temperature (Tandon et al. 2005) also contribute significantly towards the generation of these deleterious free radicals.

The predominant types of ROS are distributed under the following heads

1.2.1.1. Superoxide Anion

The cellular processes like mitochondrial electron transport systems, microsomal electron transport systems, xanthine oxidase, xanthine dehydrogenase etc. are considered to be the major sources of superoxide anion molecule. They are also created by the cellular process where NADPH

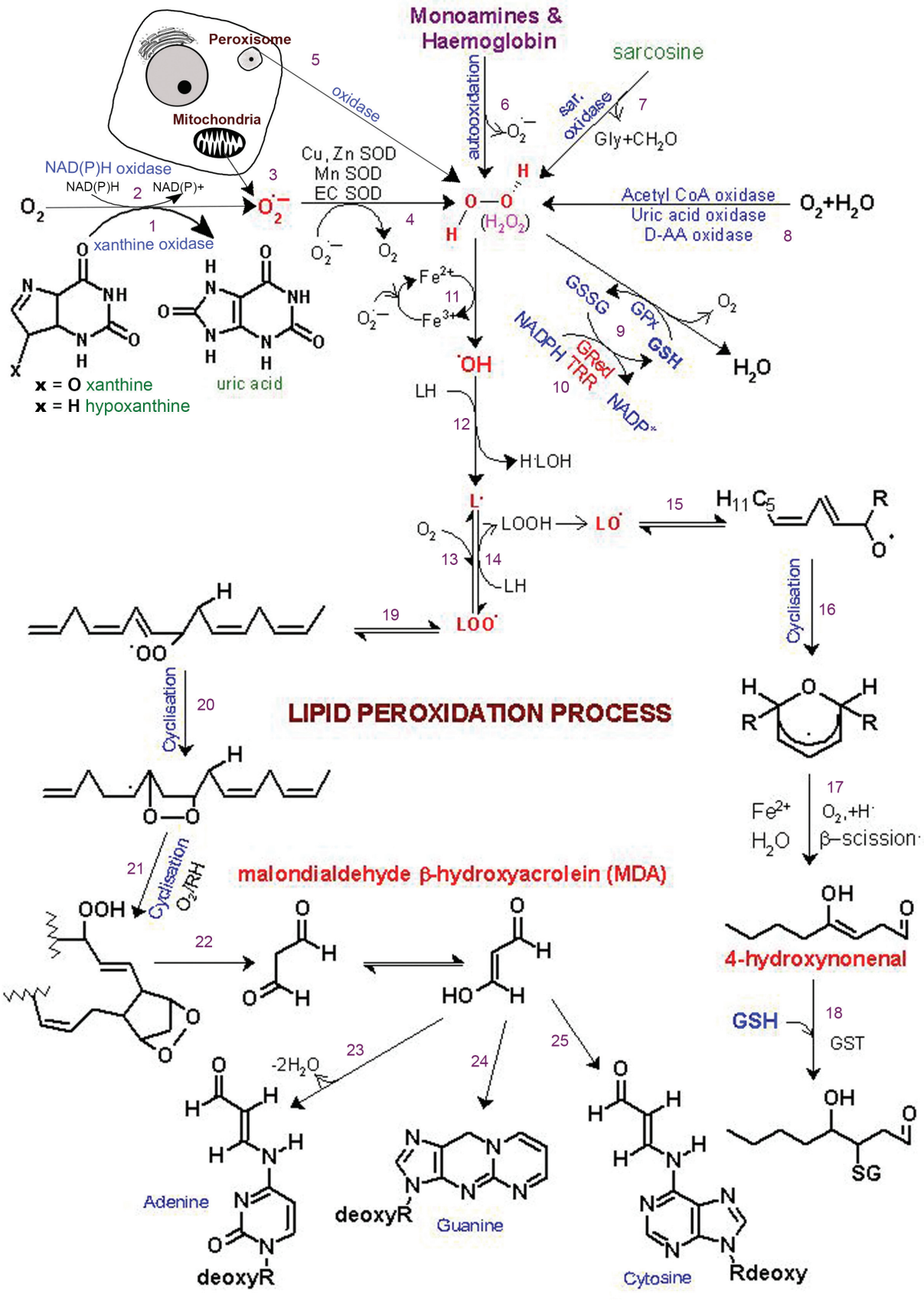

Figure 1.1. Schematic representation of the ROS formation and its mechanism of action

REACTIONS

1, 2 and 3	:	The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain.
4	:	Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide.
5, 6, 7 and 8	:	Generation of H_2O_2 from peroxisome, monoamines and haemoglobin, sarcosine and the reaction between water and oxygen
9	:	Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor.
10	:	The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase Gred) which uses NADPH as the electron donor.
11	:	Some transition metals (e.g. Fe^{2+}, Cu+ and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction).
12	:	The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•).
13	:	The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 19–25 and 15–18).
14 and 15	:	Formation of lipid alkoxyl radical (LO•) from lipid peroxyl radical (LOO•) and polyunsaturated fatty acid (LH) through lipid hydroperoxide (LOOH) path way
16	:	Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide.
17	:	Six-membered ring hydroperoxide udergoes further reactions (involving-scission) to from 4-hydroxy-nonenal.
18	:	4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione S-transferase).
19 and 20	:	A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical.
21	:	This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide.
22	:	Formed compound is an intermediate product for the production of malondialdehyde.
23, 24, 25	:	Malondialdehyde can react with DNA bases cytosine, adenine, and guanine to form adducts M1C, M1A and M1G, respectively.

oxidase reduces oxygen as the part of its defense mechanisms and the excess amount produced are converted to hydrogen peroxide by the enzyme superoxide dismutase. Even though superoxide anion is considered to be as a non-reactive species they are able to generate more reactive free radicals by the reaction with other molecules.

1.2.1.2. Hydrogen Peroxide (H_2O_2)

H_2O_2 is may not be termed as a true species of free radical because it bears no unpaired electrons. However being an oxidizing agent it can intensify and initiate the OH radical formation. They are produced by the enzyme superoxide dismutase during the cellular processes and can occupy both cytoplasmic and intracellular spaces in the body. They can also cross biological membranes by diffusion.

1.2.1.3. Hydroxyl Radical (OH)

Hydroxyl radicals are considered to be the most reactive free radical species formed in the cells by the transition ion catalyzed reaction (Haber-Weiss reaction) of H_2O_2. Lipid-peroxidation of microsomal, mitochondrial and cell membranes, modification of purines and pyrimidines (Fenton 1894; Haber 1934) or strand breakage (Oshima et al. 1996) are some of the major problems caused by the hydroxyl radicals. Hydroxyl radicals react with the biological molecules by number of ways viz, electron transfer, hydrogen transfer, addition to aromatic systems etc, to produce the secondary reactive species, which are even more deleterious than the parent $^{\bullet}OH$ radical.

1.2.1.4. Lipid Peroxide (LOOH)

Oxidation of fatty acids generated fatty acid free radicals, which can react with oxygen to from the peroxyl radicals that further react with other fatty acids to produce lipid hydroperoxides. The lipid hydroperoxides are considered to be more reactive as it can cause lipid peroxidation by the production of the more reactive species like lipid peroxyl, lipid alkoxyl and malondialdehyde (MDA) like compounds (Slater 1979).

1.2.1.5. Singlet Oxygen ($^1O_2$)

As H_2O_2, the singlet oxygen species is not a free radical in true sense, and is considered as oxidant species, which can cause the tissue damage in the biological system. The singlet oxygen species is formed by the spin reversal of electron present in the outer orbital of the O_2 molecule by enzymatic catalyzed reactions. Due to its electrophilic nature, it can readily react with the fatty acids and other molecules to produce peroxide radicals.

1.2.2. Effect of ROS on Body

The reactive oxygen species react with the protein molecules, unsaturated fatty acids, nucleic acids, carbohydrates etc to cause their oxidative damage, thereby leading to severe health problems. They are considered to be the major cause of inactivation of enzymes. The protein molecules are fragmented by the attack of free radicals or their metal binding sites may be affected, or can undergo many other modifications in their amino acid residues like proline, histidine, cysteine methionine, tryptophan, tyrosine, phenylalanine etc. Lipids especially polyunsaturated type readily undergoes peroxidation, leading to the formation of other free radicals, which act as chain propagators for further

lipid peroxidation process. This will also lead to the rancidity or off flavor and spoilage of the food products. Lipid peroxidation can also affect the cellular membrane functions. ROS are the primary cause for several genetic defects caused by the radical induced nitrogenous base modification of nucleic acids and mutations. Hyaluronic acid, a carbohydrate present in the synovial fluid can undergo free radical induced depolymerisation leading to the joint inflammation (Grootveld et al. 1991). Other macromolecules such as collagen, proteoglycans etc. are also fragmented by the action of free radicals.

1.3. Antioxidants

Our body possess innate defense mechanisms against these free radicals generated through a series of mechanisms involving different biomolecular reactions such as enzymes, amino acids, bioactive antioxidant molecules, selenium, vitamins etc to protect the cells from oxidative damage (Wojcik et al. 2010; Samaranayaka & Li-Chan 2011). Antioxidant enzyms like superoxide dismutases, catalase, glutathione peroxidase etc are the major common enzymes, which are involved in scavenging free radicals like superoxide anion, hydrogen peroxide, lipid hydroperoxides etc (Lobo et al. 2010). Ascorbic acid, glutathione, melatonin, tocotrienols, uric acid etc are the non enzymatic compounds used by our body to prevent from oxidative damage (Lobo et al. 2010). As these compounds are effectively used to scavenge free radicals they are called as antioxidants.

The antioxidants help to reduce the risk of free radical derived issues. Antioxidants generally inactivate free radicals (alkoxyl, peroxyl and alkyl) by donating its H atom to form stable compounds as illustrated below:-

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$

$$LOO^{\bullet} + A^{\bullet} \longrightarrow LOOA$$

Where $LOO^{\bullet}$ is a lipid peroxyl radical and AH is the representation of the antioxidant molecule able to donate its H atom. Transition metals like iron and copper are capable of producing very reactive hydroxyl radicals from peroxides, which can damage the living cells.

$$LOOH + Fe^{2+} \longrightarrow LO^{\bullet} + OH^{-} + Fe^{3+}$$

1.3.1. Synthetic Antioxidants

Butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG), teritiary butyl hydroquinone (TBHQ), octyl gallate (OG), 2,4,5-trihydroxy butapyranone, nordihydroguaiaretic acid and 4-hexyl resorcinol are some of the common examples of synthetic antioxidants (Carocho 2013, Aguillar et al. 2012, Gharavi & El-Kadi, 2005, Anton et al. 2004, Kubo et al. 2001, Astill et al. 1959, Evan & Gardner 1979, Chen et al. 2004) (Figure 1.2). They are extensively used to control rancidity in lipid-containing foods and formation of lipid oxidation or peroxidation products, cosmetic and pharma industries.

1.3.2. Drawbacks of Synthetic Antioxidants

BHT was reported to react with other ingested substances to cause the formation of carcinogens. BHT is banned in the UK (dedicated website: http://www.healthyeatingadvisor.com).

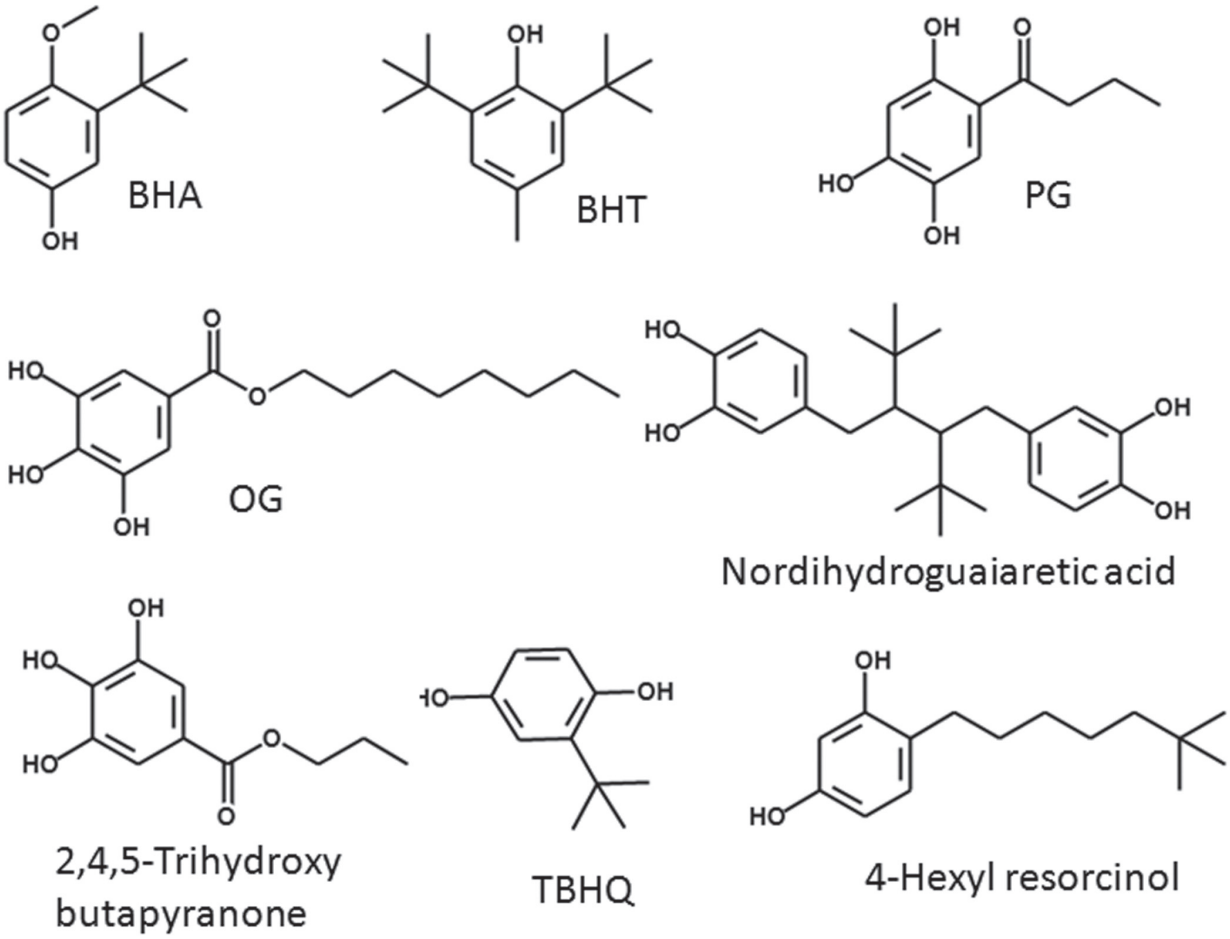

Figure 1.2. Structure of some common synthetic antioxidants

TBHQ is banned in Japan and certain European countries (Shahidi 1997). Many countries like Japan banned the production and use of BHA whereas some other countries like UK could not implement the recommendations of the officials to ban the same due to pressure from the industries. McDonald's eliminated BHT from their US products by 1986 (dedicated website: http://www.foodfigures.com/food_additive.htm). BHA and BHT are also known to cause cancer in humans. Therefore, in recent years, interest in finding naturally occurring antioxidant compounds in food or medicine to replace synthetic products has increased considerably, given that synthetic ones are being restricted due to consumer preference for natural products and concern about the potential toxic effects of synthetic medicines (Zheng et al. 2001).

1.3.3. Green Alternatives

Because of the possible harmful effects of synthetic antioxidants, the demand for a natural alternatives are increasing. Hence the pharmaceutical and agri-food industries have been concentrating in developing and marketing functional foods with green and natural antioxidant alternatives as ingredients. Marine flora constitutes the potential natural sources with pluralities of bioactive compounds having antioxidant properties. Seaweeds constitute a major share of marine flora, and they were reported to possess structurally diverse compounds of various bioactivities endowed with antioxidant, antibacterial, anti-inflammatory, and anticarcinogenic activities (Kornprobst, 2005). Since there is an increased interest in the antioxidants of natural origin in recent times in place of synthetic derivatives, it is rational to explore the seaweeds as natural sources to isolate antioxidant principles for use as nutraceutical supplements. The potential applications offered by these valuable resources as ingredients in functional foods are significant because of their richness in bioactive principles, particularly antioxidants.

In high light environments like the sea, energy absorbs faster than it can be dissipated, producing the free radicals and promoting lipid oxidation. Surprisingly the lack of structural damage in seaweed cells even after the regular exposure to light and high oxygen, attribute to the role of natural antioxidant compounds found in them in protecting the cell content (Swanson & Druehl, 2002; Burritt et al. 2002). Therefore, these marine floras may be considered as a potential resource of unexplored natural antioxidant molecules, which need to be studied further. It was reported that seaweeds are rich source of bioactive compounds, such as terpenoids, phloroglucinol phenolics, fucoidans, sterols and glycolipids, and the extracts or isolated components from seaweeds posses a wide range of pharmacological properties such as anticancer, antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, hypoglycaemic, hypolipidemic, hepatoprotective and neuroprotective activities (Liu et al. 2012; Chakraborty et al. 2013). Extracts from several brown and red seaweeds harvested in France (Le Tutour, 1990), Spain (Jiménez-Escrig et al. 2001), Indonesia (Anggadiredja et al.1997), Korea (Han et al. 1999), China (Yan et al. 1998) and Japan (Yan et al. 1999 and Sekikawa et al. 1986) have demonstrated antioxidant activity *in vitro*. The solvent extracts of brown seaweeds from Indian waters were reported to contain high levels of hydrophilic components, such as polyphenols and soluble polysaccharides with strong antimicrobial and antioxidant activities

(Chandini et al. 2008; Ananthi et al. 2010; Rajauria et al. 2012; Chakraborty et al. 2013). Although antioxidant properties of seaweeds have been demonstrated by numerous studies during the past few decades, only scarce reports are available from the Gulf of Mannar region, rich in biodiversity leading to the isolation and characterization of antioxidant secondary metabolites. The study was planned based on the hypothesis that the seaweed species distributed in the Gulf of Mannar region may have potential bioactive compounds with pharmaceutical and neutraceutical properties. The study also anticipate to shortlist potential seaweed species with natural bioactive compounds.

Standard methodologies were followed to collect and screen seaweeds for natural bioactive compounds. Antioxidant assays were used to find out the sea weeds species containing potential natural products. The promising fractions were further purified by different chromatographic procedures and the compounds purified to homogeneity were used to elucidate the structural characteristics. Further, different spectroscopic methods like NMR, IR, GC-MS etc were employed to decipher the structural confirmation.

Based on the previous studies demonstrating that the seaweeds are potential source of antioxidative compounds, the present work has been focused based on the following objectives,

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CHAPTER 2

Review of Literature

2.1. Background

Antioxidants play an important role in controlling many of the free radical induced diseases such as cancer, stroke, myocardial infarction, diabetes, septic and hemorrhagic shock, Alzhemer's and Parkinsons diseases (Chew et al. 2008). Currently, the reactive oxygen species and lipid oxidation in the food industry are being controlled or minimized by the addition of synthetic antioxidants (Gray et al. 1996). However, with the safety concerns about synthetic antioxidants (Wichi et al. 1998), considerable interest has arisen in finding alternative sources of natural antioxidants for use in food systems.

2.2. Why Seaweeds

Since there is an increased interest in the antioxidants of natural origin in recent times in place of the synthetic derivatives, it is rational to explore seaweeds as natural sources to isolate antioxidant principles for use as nutraceutical supplements. During the last few decades, agri-food and nutraceutical industries have been at the origin of a great expansion in the demand of seaweeds. Seaweeds constitute a major share of marine flora, and they were reported to possess structurally diverse compounds of various bioactivities endowed with anticancer, antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, hypoglycaemic, hypolipidemic, hepatoprotective and neuroprotective activities (Liu et al. 2012; Chakraborty et al. 2013). The potential applications offered by these valuable resources as ingredients in functional food are significant because of their richness in bioactive principles, particularly antioxidants. In the high light environments like the sea, energy absorbs faster than it can be dissipated, producing free radicals and promoting lipid oxidation. Seaweeds as photosynthetic organisms are exposed to a combination of light and high O_2 concentration at the origin of the formation of free radicals and other oxidative reagents. Surprisingly the lack of structural damage in seaweed cells even after the regular exposure to light and high oxygen, attribute to the role of natural antioxidant compounds found in them in protecting the cell content (Swanson & Druehl, 2002; Burritt et al. 2002). These soft-bodied sessile organisms typically lack the protection and provide a rich environment to house defensive mechanisms as a means to shield themselves from the oxidative stress conditions in oceanic ecosphere, and therefore, natural product chemists are looking at alternative natural compounds to be used in health supplements against stress induced disorders pharmaceuticals and health food against oxidative stress-induced disorders. It is interesting to note that over the time of evolution and adaptation,

these sessile organisms developed strategies to decompose the potentially harmful oxidants through a cascade of biochemical reactions thereby preventing oxidative damage in their structural components. The antioxidant properties of seaweeds could be effectively exploited to prevent free radical accumulation and to promote the immune system in eliminating the proliferation of radicals (Skibola 2004). Therefore, much research attention has been focused on the free-radical scavenging activity of metabolites from seaweeds. Essentially, the antioxidative defenses of seaweeds include water-soluble reductants (antioxidants) and fat-soluble reductants. The biofunction of small-molecule antioxidants in the photophysiology of marine organisms is yet poorly understood. So, it is imperative to explore the small biomolecules from seaweeds as potent antioxidants. Red and brown seaweeds have been identified in both inter-tidal and deep water regions, which have proven to be rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potential (Tutour et al. 1998; Satoru et al. 2003). There is scanty information regarding structural information of antioxidant molecules in seaweeds from Indian waters. The knowledge on the structural features responsible for antioxidant activity will guide us to synthesize the molecules in commercial scale, and enable to describe their mode of action. A systematic search for the development of new sources of chemical compounds from seaweeds will be helpful for the design and development of antioxidant molecules to be used for increasing the shelf life of food in the functional food industry.

2.3. Type of Seaweeds

Seaweeds belong to a very diverse group of marine plants with no roots, leaves, and stems, but a hold fast, blade and stipe instead and the complete body is called as a thallus. The hold fast makes them to hold on the rocks or thick surfaces, the stipe provide support to seaweeds. Even though all the cells are capable of photosynthesis, blades are the mainly designed region for this. Most of the seaweeds also contain an air bladder called float which will enable them to float and stay upright in the wavy waters to get exposed to sunlight.

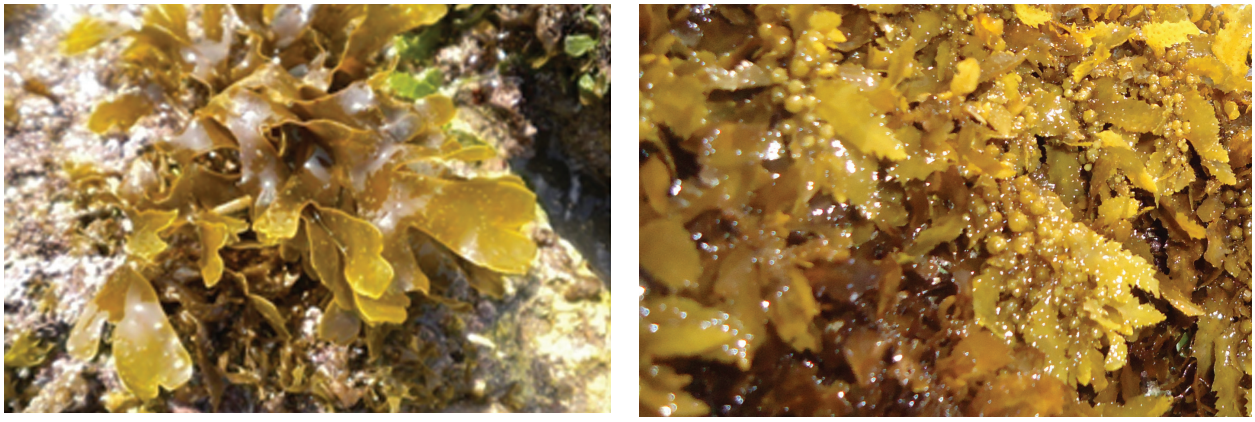
Seaweeds belong to the three classes such as green (about 1500 species), brown (about 1800 species) and red (about 6500 species) (dedicated website: http://www.seaweed.ie/). The classification is based on pigmentation of the seaweeds.

2.3.1. Green Seaweed (Phylum Chlorophyta)

They contain photosynthetic pigments such as chlorophyll a, and b and carotenoids. They also contain starch as storage product and cellulose as cell wall component. *Ulva*, *Bryopsis* and *Caulerpa* are some common examples (Figure 2.1.).

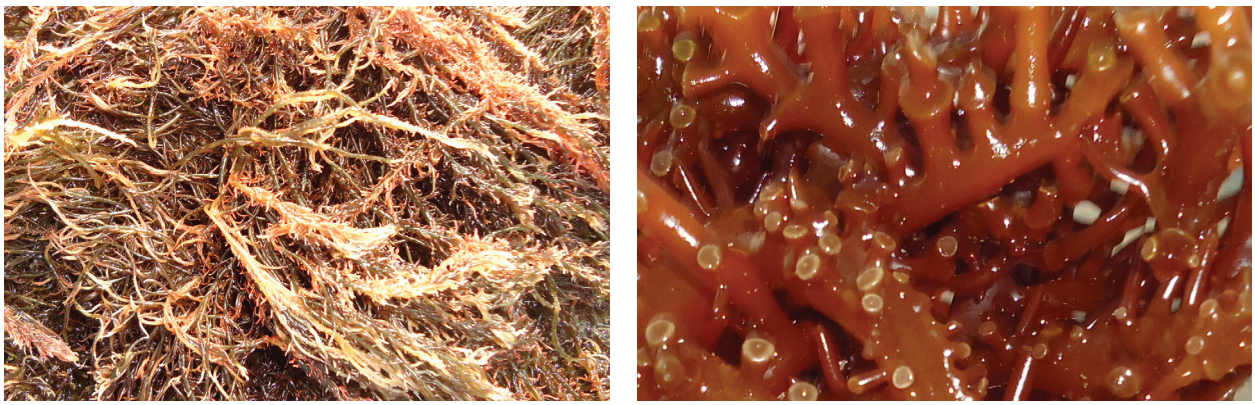
Bryopsis plumosa *Caulerapa cetruloidis*

Figure 2.1. Examples of green seaweeds



Stoechospermum marginatum *Sargassum wightii*

Figure 2.2. Examples of brown seaweeds



Hypnia musciformis *Kappaphycus alverazil*

Figure 2.3. Examples of red seaweeds

2.3.2. Brown Seaweed (Phylum Heterokontophyta: Class Phaeophyta)

They are brown because of the presence of carotenoid fucoxanthin. They contain photosynthetic pigments chlorophyll a, and c. Laminarin is their major storage product and cellulose is the major cell wall component. *Sargassum, Padina* and *Stoechospermum* are some common examples (Figure 2.2).

2.3.3. Red Seaweed (Phylum Rhodophta)

The pigment phycoerythrin is the reason for its red colour. Its main photosynthetic pigment is chlorophyll a, and major storage product is starch. Cellulose, agar and carrageenan are its major cell wall product. *Gelidiella, Hypnia* and *Kappaphycus* are some common examples (Figure 2.3).

2.4. Antioxidant Potential and Phenolic Contents of Seaweeds

Among the seaweed natural antioxidants, phenolic antioxidants (phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids) are in the forefront (Duan et al. 2006). The polyphenols in marine brown seaweed termed as phlorotannins are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and known to act as potent antioxidants (Ragan & Glombitza, 1986). Jiménez-Escrig et al. (2001), reported antioxidant activity of fresh and processed edible seaweeds and indicated strong antioxidative activity of brown seaweed *Fucus vesiculosus* using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay. The antioxidant activity of the crude extract and sub fractions derived from a red seaweed, *Polysiphonia urceolata*, was established using DPPH radical scavenging assay and the β-carotene–linoleate assay systems (Duan et al. 2006). Antioxidant effect was observed with a sulfoglycolipid fraction isolated from the red seaweed, *Porphyridum creuntum*, which was found to inhibit the production of superoxide anion radicals (Bergé et al. 2002). There are some reports about fucoxanthin-related compounds in *Petalonia binghamiae* having inhibitory activities against free radicals (Murakami et al. 2002). Sargaquinoic acid isolated from the brown seaweed *Sargassum macrocarpum* has been found to possess antioxidant activity (Tsang & Kamei 2004).

Seaweeds have protective enzymes and antioxidative molecules (phlorotannins, ascorbic acid, tocopherols, carotenoids, phospholipids, chlorophyll related compounds, bromophenols, catechins, mycosporine-like amino acids, polysaccharides, etc.), which are similar to those of vascular plants (Fujimoto 1990; Le Tutour et al. 1998; Rupérez et al. 2002; Yuan et al. 2005; Pavia & Aberg 1996), and were reported to possess antioxidant activities (Ragan & Glombitza 1986). A water extract of a brown seaweed *Scytosiphon lomentaria* was reported to have antioxidant activity (Kuda et al. 2005).

Compounds of extracts from brown seaweed *Petalonia binghamiae* was reported to have antioxidant activities due to phenolic compounds (Murakami et al. 2002). Brown seaweed *Fucus vesiculosus* was known to have antioxidant importance and normalized lipid peroxidation status, thereby preventing membrane injury and free radical formation (Veena et al. 2007). The extracts of the brown seaweed *Taonia atomaria* were reported to exhibit high radical scavenging activity (Mayer & Lehmann 2000). Hydrogen peroxide scavenging activity of many seaweed extracts was predominant and those samples showed significant inhibitory effects against DNA damage (Heo et al. 2005).

Among the crude solvent extracts prepared from the ten *Phaeophyta* species collected from Brittany coasts, the crude extracts from *Bifurcaria bifurcata*, *Cystoseira tamariscifolia*, *Fucus ceranoides* and *Halidrys siliquosa*, displayed higher antioxidant activity (Zubia et al. 2009). Extracts of Japanese edible brown seaweeds, *Eisenia bicyclis*, *Kjellmaniella crassifolia*, *Alaria crassifolia*, *Sargassum horneri*, and *Cystoseira hakodatensis* were reported to posses potential radical scavenging activities (DPPH, peroxyl radical, and ABTS), and antioxidant activity in a liposome system (Airanthi et al. 2011). Fabian et al. (2013) assayed the extracts of 16 species of seaweeds collected along the Danish coasts for antioxidant activities, and found that *Polysiphonia fucoides* and all the *Fucus* species showed higher radical scavenging activity, reducing power, and were high in phenolic content.

Indu & Seenivasan (2013) assayed the antioxidant potential of *Chaetomorpha linum*, *Grateloupia lithophila* and *Sargassum wightii* collected from the Mandapam coast in Rameswaram, and identified that the ethanol extract of *S. wightii* as the best with highest antioxidant potential. Vijayabaskar & Shiyamala (2013) identified that the methanol extract of the brown seaweed *Turbinaria ornata* collected from the Mandapam coast exhibited good DPPH and hydroxyl radical scavenging potentials.

Wang et al. (2009) studied the potential antioxidant activities of ten species of Icelandic seaweeds and found that the extracts from three *Fucoid* species had the highest phenolic content, radical scavenging activities and ferrous ion-chelating activity. Audibert et al. (2010) reported that in *Ascophylllum nodosum* the fraction containing phenolic compounds appeared to be with high antioxidant potential.

The antioxidant potential of methanol extracts of brown seaweeds collected from Galway (Ireland), were assessed and found that *Ascophyllum nodosum, Pelvetia canaliculata*, and *Fucus serratus* contained the higher phenol concentrations (O'Sullivan et al. 2011). The authors indentified that the methanol extracts of *Fucus vesiculosus* and *F. serratus* exhibited the highest ferric reducing

power, whlist *F. vesiculosus* and *A. nodosum* were highly effective towards scavenging DPPH radicals and preventing β-carotene bleaching (O'Sullivan et al. 2011). The study made by Le Lann et al. (2012) investigated 18 brown seaweed samples belonging to *Turbinaria* and *Sargassum* from three archi-pelagos of the South West Pacific Ocean, and found that the phenolic content of *Turbinaria* sp were higher than those of *Sargassum* sp tested.

The brown seaweed *Sargassum* sp collected from the coastlines at Java Island Indonesia, Gunung Kidul (Yogyakarta) and Jepara (Central Java) and found higher phenolic content and DPPH radical scavenging potential (Budhiyanti et al. 2012). The antioxidant activity of *Eucheuma cottonii*, *E. spinosum* and *Halymenia durvillaei, Caulerpa lentillifera*, *C. racemosa, Dictyota dichotoma, Sargassum polycystum* and *Padina* sp obtained from Sabah waters were determined by Matanjun et al. (2008), and found that the methanol extracts of *C. lentillifera*, *C. racemosa* and *S. polycystum* showed better radical-scavenging and reducing power ability, and higher phenolic content.

The antioxidant activity of organic extracts of 37 seaweed samples, comprising of 30 species of Hawaiian seaweed from 27 different genera was determined and the extract of *T. ornata* was found to be the most active Kelman et al. (2012). The bioassay-guided fractionation of this extract led to the isolation of a variety of different carotenoid fucoxanthin as the active principles.

Mhadhebi et al. (2011) reported that the various organic (chloroform, ethyl acetate and methanol) extracts of the brown seaweed *Cystoseira crinite* exhibited significant radical scavenging activity and reducing power. Nahas et al. (2007) studied the radical scavenging potential of thirteen seaweed species collected from the Aegean Sea and identified that the brown seaweed *Taonia atomaria* exhibited higher potential. Nahas et al. (2007) further isolated taondiol, isoepitaondiol, stypodiol, stypoldione, sargaquinone and sargaol, which possess the radical scavenging ability.

Aoun et al. (2010) reported that the organic extracts from *Dictyopteris membranacea* collected from the Tunisian Mediterranean coast exhibited high radical scavenging potential and reducing potential. It was found that the crude polyphenolic fractions of *Ecklonia cava* exhibited high reducing power and the capacity to scavenge superoxide anion, hydrogen peroxide and hydroxyl radical (Athukorala et al. 2006). Senevirathne et al. (2006) reported that the methanol extract of *Ecklonia cava* showed significant antioxidant activities (DPPH, superoxide anion, hydrogen peroxide, hydroxyl radical, ferrous ion chelating, reducing power and lipid peroxidation inhibition and contained high phenolic content. There are other reports that showed that the phlorotannin-rich extracts of *Ecklonia cava* showed significant antioxidant activities such as DPPH radical scavenging, ferric ion reduction and inhibition of LDL oxidation (Shin et al. 2006).

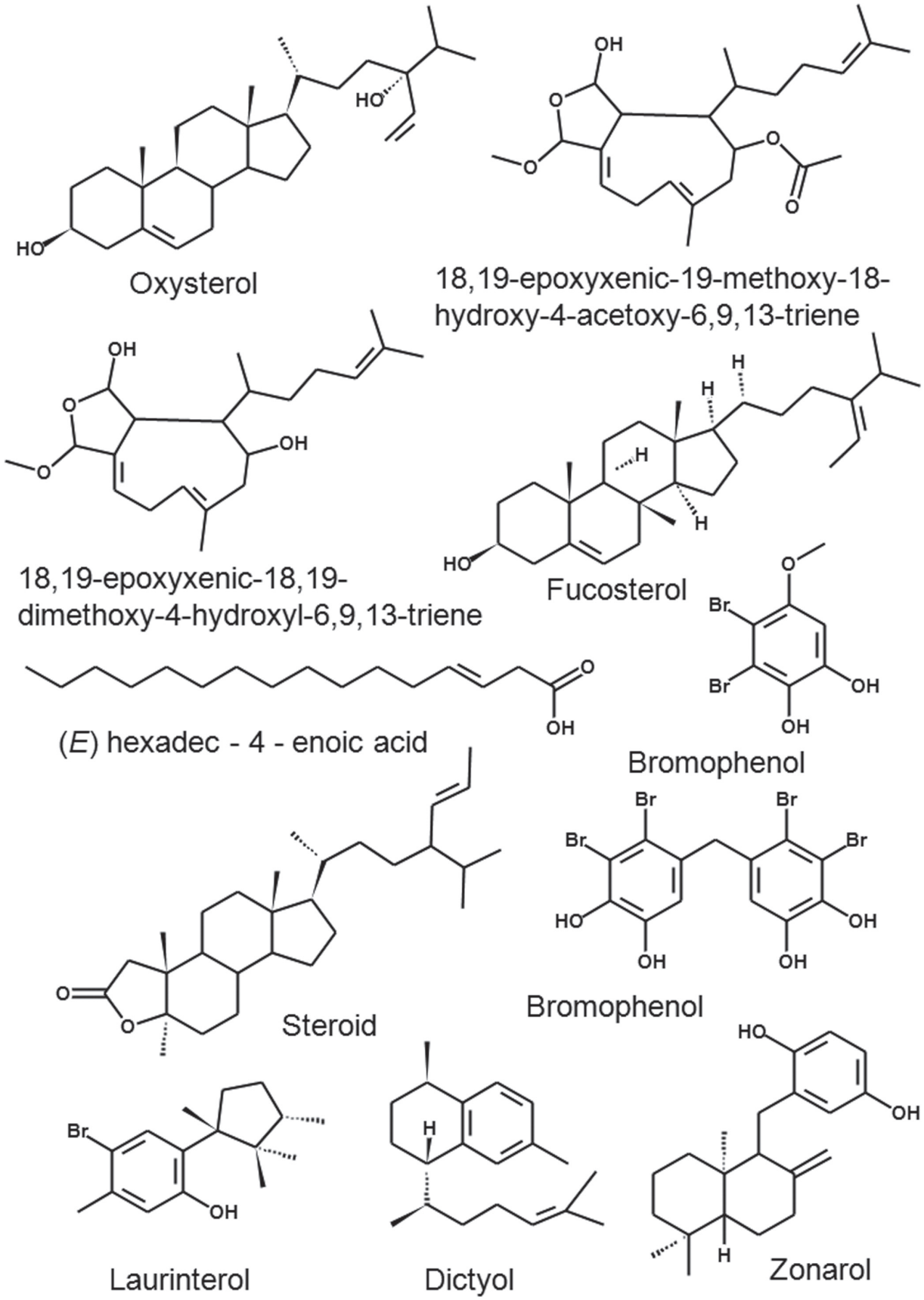


Figure 2.4. Structure of some bioactive compounds isolated from seaweeds

Kuda et al. (2006) reported that the extract of brown seaweed *Petalonia binghamiae* showed high phenolic content and antioxidant activities as established by the reducing power, DPPH radical and superoxide anion radical scavenging assays. Kuda et al. (2007) reported that the total phenolic content and the antioxidant activities of *E. stolorifera* and *E. kurome* products were higher than other seaweeds. Heo et al. (2005) reported that from the assayed enzymatic extracts from seven species of brown seaweeds exhibited prominent effects in hydrogen peroxide scavenging activity. Kindleysides et al. (2012) studied the effect of the addition of the extracts from two brown seaweeds (*Ecklonia radiata*, *Macrocystis pyrifera*) and two red species (*Champia* sp. and *Porphyra* sp.), to hoki (*Macruronus novaezelandiae*) oil, and assessed by the production of oxidation products in an elevated temperature (60 °C) storage trial. They found that the extracts from *E. radiata* performed best with significantly lower primary, secondary and total oxidation products, and higher DPPH radical scavenging ability than the commercial antioxidant, BHT. The extracts of *Padina antillarum, Caulerpa racemosa* and *Kappaphycus alvarezzi* were assayed for its phenolic contents and antioxidant activity using various assays and found that *P. antillarum* was found to have the higher phenolics, reducing power, and ferrous ion chelating ability (Chew et al. 2008). Among the extract of four species of seaweed, *Sargassum binderi*, *Amphiroa* sp., *Turbinaria conoides* and *Halimeda macroloba*, collected from the Gulf of Thailand, *T. conoides* extract showed the higher phenolic content, ABTS and DPPH radicals antioxidation activity (Boonchum et al. 2011).

Zahra et al. (2007) reported that the extract of *Sargassum boveanum* exhibited high phenolic content, inhibition of peroxidation of linoleic acid and high DPPH radical scavenging potential. It was reported that the crude solvent extracts of *Sargassum hystrix* exhibited higher total phenolic compound, radical scavenging activity, ferrous ion-chelating ability and singlet oxygen quenching activity (Budhiyanti et al. 2011). Luo et al. (2010) evaluated the antioxidant activities of methanol/chloroform extracts and fractions of five brown seaweeds (*Sargassum fusiforme, S. kjellmanianum, S. pallidum, S. thunbergii and S. horneri*) collected from China, and found that the methanol/chloroform extract of *S. kjellmanianum* showed higher DPPH/hydroxyl radical-scavenging activity and reducing power. In an *in vitro* study conducted by Mori et al. (2003), it was observed that the methanol extract, chloroform/methanol (3:1) extract and ethyl acetate fraction of brown seaweed *Sargassum micracanthum* inhibited lipid peroxidation and DPPH radical scavenging potential. Seo et al. (2004) isolated sargahydroquinoic acid, sargaquinoic acid and sargachromenol from *Sargassum thunbergii* which exhibited peroxynitrite-scavenging activities. Among the lipophilic extracts from 16 species of seaweeds collected along the Qingdao coastline, it was found higher for *Rhodomela confervoides* and *Symphyocladia latiuscula* and were comparable with that of the well-known antioxidant butylated hydroxytoluene and greater than that of propyl gallate (Huang & Wang 2004). Some of the common bioactive compounds isolated from seaweeds are given by Figure 2.4.

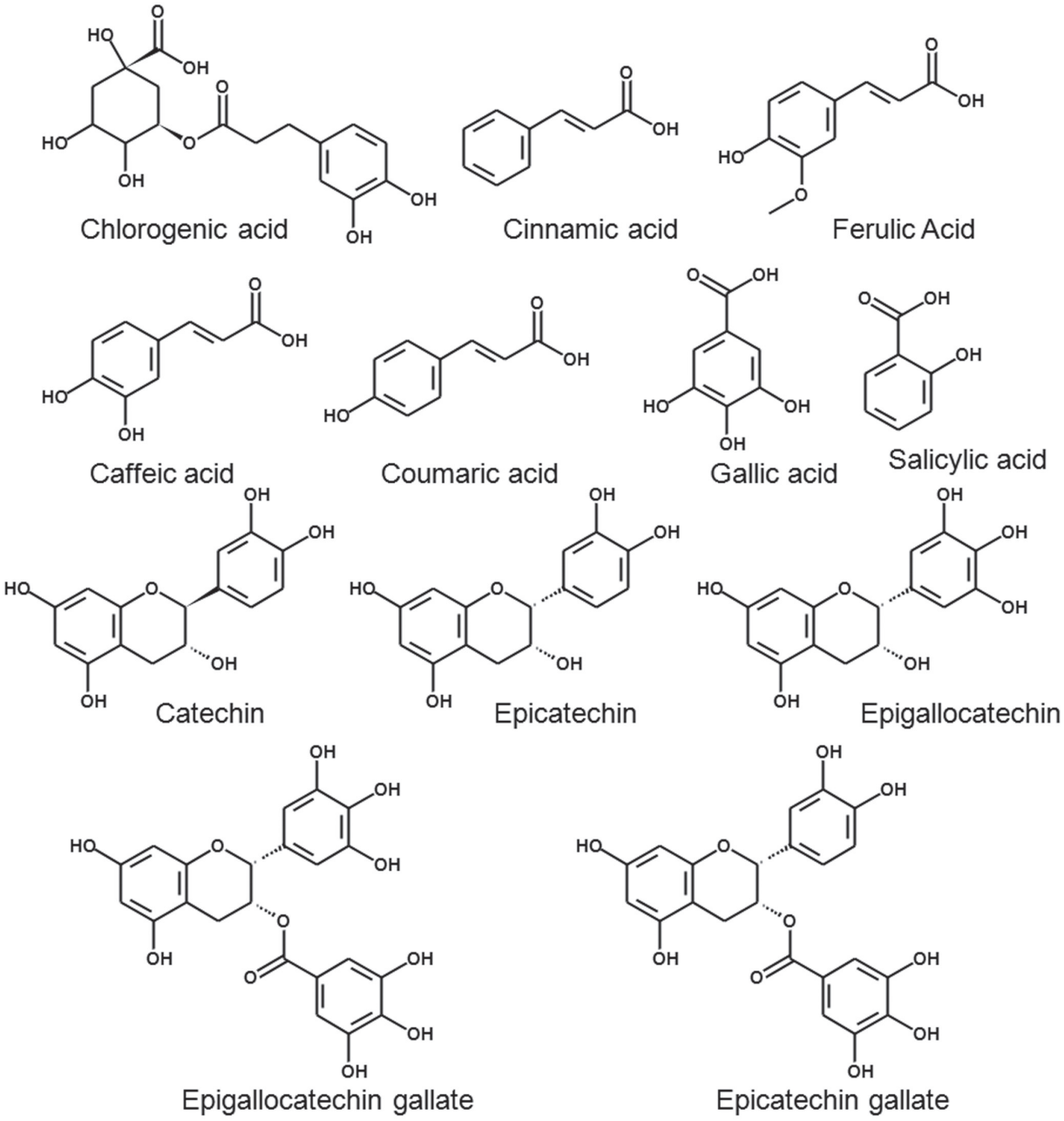


Figure 2.5. Structure of phenolic acids

2.5. Chromatographic Identification of Phenolic Acids from Seaweeds

Phenolic acids constitute a large group of naturally occurring organic compounds with a broad spectrum of pharmacological activities and free radical scavenging activity. These groups of compounds are hydroxylated derivatives of benzoic and cinnamic acids, which often occur in plants as esters, glycosides and bound complexes and are rarely present in free forms (Germano' et al. 2006). Phenolic acids differ according to the number and position of hydroxylation and methoxylation of the aromatic ring. There is a much higher quantity and diversity of hydroxycinnamates than hydroxybenzoates and they consist of *p*-coumaric, caffeic, and ferulic acids. Phenolic acids, which are considered to be a major class of phenolic compounds, were reported to be present in abundance in seaweeds and other marine flora. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids in seaweeds. A number of studies showed that antioxidant phenolic compounds such as phlorotannins were isolated from several brown seaweed families (Jormalainen & Honkanen, 2004, Koivikko et al. 2007). There are also other reports that showed that red and brown seaweeds contain phenolic compounds such as catechins, flavonols and flavonol glycosides (Santoso et al. 2002; Yoshie et al. 2000; Yoshie-Starket al. 2003). Yoshie et al. (2002) identified the presence of catechin, epicatechin, epigallocatechin gallate and gallic acid are reported in seaweed *Halimada*. Reverse-phase liquid chromatographic study to analyse the polyphenols in various red and brown seaweeds collected from the Atlantic coastal region in Galicia (North-Western Spain) revealed the presence of polyphenolic compounds like catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and gallic acid (de Quirós et al. 2010). The brown seaweed *Stypocaulon scoparium* reported to exhibit potential antioxidant activity (López et al. 2011). Food products of the seaweeds belonginging to *Porphyra* and *Undaria pinnatifida* also exhibited the presence of protocatechuic acid, p-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, salicylic acid and cinnamic acid (Onofrejová et al. 2010). Some of the common phenolic acids identified from seaweeds are given by Figure 2.5.

2.6. Antioxidative compounds isolated from seaweeds

Ayyad et al. (2011) isolated fucosterol and fucoxanthin from *Sargassum* sp and identified that fucoxanthin could be used as antioxidant and antitumor compound. Ragubeer et al. (2012) isolated four pure antioxidant compounds *viz,* sargaquinoic acid, sargahydroquinoic acid, sargaquinal and fucoxanthin from marine brown seaweed, *Sargassum elegans* with good antioxidant potential. Reddy & Urban, (2009) isolated three meroditerpenoids fallahydroquinone, fallaquinone and fallachromenoic acid together with sargaquinone, sargahydroquinoic acid, sargaquinoic acid and sargachromenol from southern Australian marine brown seaweed *Sargassum fallax*, from which sargaquinoic acid and sargahydroquinoic acid displayed antitumour activity.

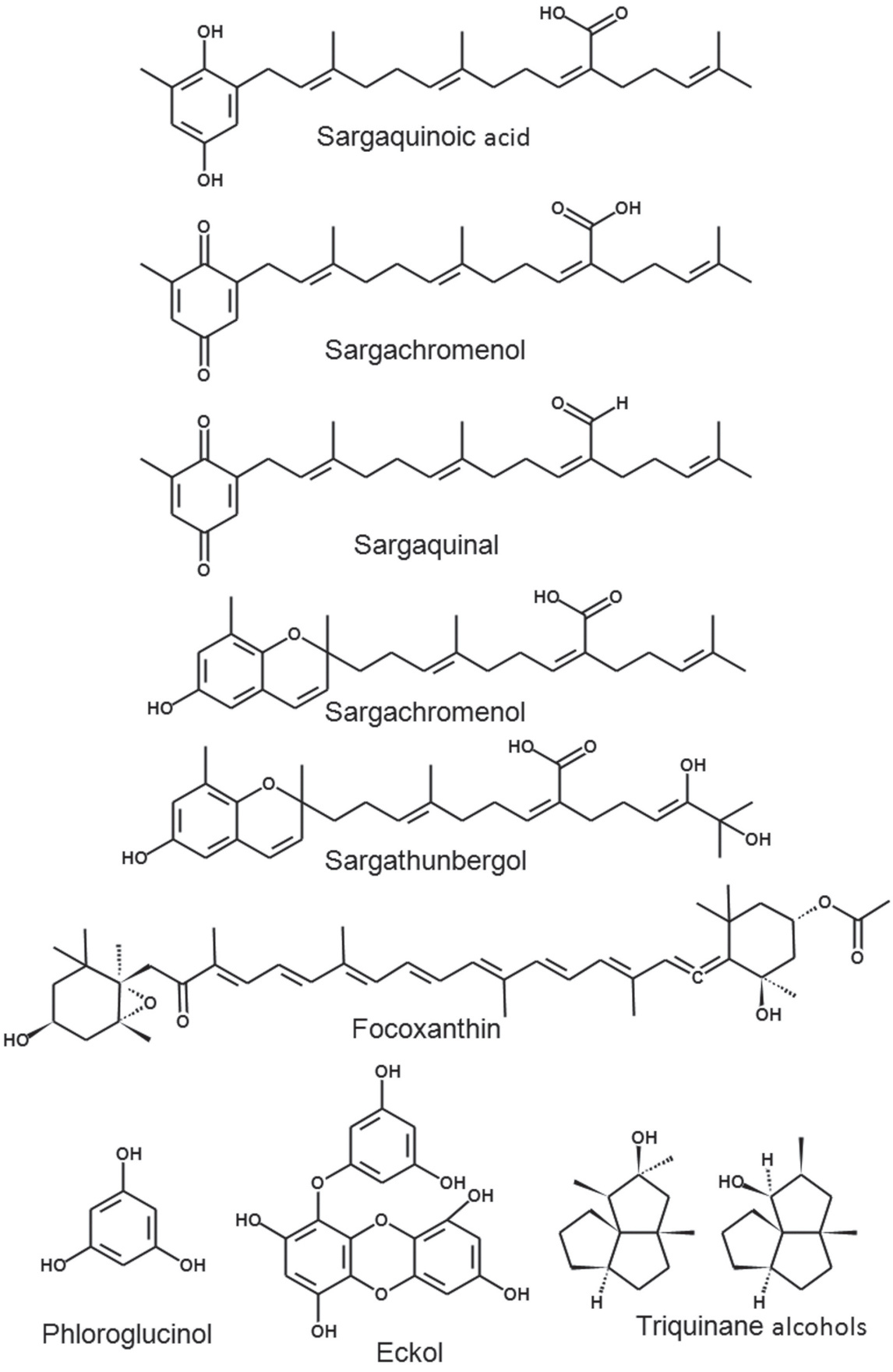


Figure 2.6. Structure of antioxidative compounds isolated from seaweeds

Iwashima et al. (2005) isolated two plastoquinones (known 2-geranylgeranyl-6-methylbenzoquinone and its hydroquinone) from the methanol extract of the brown seaweed *Sargassum micracanthum*. Mori et al. (2005) isolated four plastoquinones from the methanol extract of the brown seaweed, *Sargassum micracanthum* with significant antioxidant activities (inhibitory effect on lipid peroxidation and radical scavenging effect) and some of them also showed cytotoxic activity against cancer cell line. Fisch et al. (2003) isolated six new tetraprenyltoluquinol derivatives, two new triprenyltoluquinol derivatives, and two new tetraprenyltoluquinone derivatives from the brown seaweed *Cystoseira crinita* together with four known tetraprenyltoluquinol derivatives among which hydroquinones were found to have powerful antioxidant activity. Seo et al. 2006 isolated thunbergols, tetraprenyltoluquinols from the brown seaweed *Sargassum thunbergii* and found that both of them exhibited significant scavenging activities on free radical species. Jang et al. (2005) isolated sixteen new antioxidative meroterpenoids of the chromene classes from the brown seaweed *Sargassum siliquastrum* collected from Jaeju Island, Korea. Nahas et al. (2007) isolated metabolites taondiol, isoepitaondiol, stypodiol, stypoldione, sargaquinone and sargaol from *Taonia atomaria* extract, and these compounds were found to possess marked radical-scavenging potential.

Abatis et al. (2005) isolated two novel cyclized meroditerpenoids atomarianones A, and B from the organic extract of the brown seaweed *Taonia atomaria* collected at Serifos Island in the Central Aegean Sea. 2, 7'-phloroglucinol-6,6'-bieckol isolated from *Ecklonia cava* exhibited radical scavenging activities on DPPH, alkyl, hydroxyl, and superoxide radicals, and was effective to inhibit H_2O_2 induced DNA damage (Kang et al. 2012). Kang et al. (2003) reported that the polyphenolic compounds and complex mixtures isolated from brown seaweed species exhibited DPPH radical scavenging activity and ferric reducing antioxidant power.

Fukuyama et al. (1989) isolated Eckol, a novel phlorotannin with a dibenzo-1,4-dioxin skeleton, from the brown seaweed *Ecklonia kurome* Okamura as a potent and specific anti-plasmin inhibitor. Fukuyama et al. (1990) isolated two phlorotannins from the polyphenol powder prepared from the edible marine brown seaweed *Ecklonia kurome* which demonstrated antioxidant potential. Zou et al. (2008) isolated three phlorotannins, including phloroglucinol, diphlorethohydroxycarmalol, and 6,62-bieckol, from *Ishige okamurae* among which diphlorethohydroxycarmalol and 6,62-bieckol which exhibited showed potential radical scavenging activities against the 2,2-diphenyl-1-picrylhydrazyl , hydroxyl, alkyl, and superoxide radicals. Polymeric phlorotannins of the fucol and fucophlorethol classes isolated from that *Fucus spiralis* exhibited antioxidant activity (Ce´rantola et al. 2006). Fucosterol isolated from the marine seaweed *Pelvetia siliquosa* exhibited anti-oxidant potential and also possess the hepatoprotective activities in rats (Lee et al. 2003). Parys et al. (2010) isolated two phloroglucinol derivatives, belonging to the class of fucophlorethols, and the

known fucotriphlorethol A from the ethanolic extract of the brown seaweed *Fucus vesiculosus* L. Trifucodiphlorethol A, trifucotriphlorethol A and fucotriphlorethol A were identified as strong radical scavengers (Parys et al. 2010).

A series of bromophenols and corresponding to the debrominated phenolic compounds was prepared by isolation from red seaweed *Tichocarpus crinitus* and it was observed that the most active free radical scavengers were the debrominated phenolic derivatives 3,4-dihydroxybenzyl alcohol and 3,3',4,4'-tetrahydroxydiphenylmethane (Lee et al. 2007). The seaweeds *Caulerpa* (Costa et al. 2010), *Canistrocarpus cervicornis* (Camara et al. 2011), *Dictyota mertensii* (Costa et al. 2010), *Dictyopteris delicatula* (Magalhães et al. 2011), *Dictyota menstrualis* (Costa et al. 2010), *Fucus vesiculosus* (Rupe´ rez et al. 2002), *Laminaria japonica* (Zhang et al. 2010; Wang et al. 2010; Hou et al. 2012), *Lobophora variegate* (Paiva et al. 2011), *Turbinaria conoides* (Chattopadhyay et al. 2010) and *Turbinaria ornata* (Ananthi et al. 2010) were identified as potent radical scavengers due to the presence of polysaccharides. The extract of *Laurencia undulata* was reported to possess two antioxidative sugars belonging to floridosídeo and D-isofloridosídeo (Yong-Xin et al. 2010). Plastoquinones isolated from the methanol extract of the brown seaweed, *Sargassum micracanthum* exhibited significant antioxidant activities such as an inhibitory effect on lipid peroxidation and radical scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (Mori et al. 2005). Tang et al. (2002) isolated two bioactive steroids from the brown seaweed *Sargassum carpophyllum*. Sheu et al. (1999) isolated antioxidative oxygenated fucosterols from the brown seaweed *Turbinaria conoides*.

Seaweeds are reported to contain tocopherols, eckols, phlorotannins, catechins, mycosporine-like amino acids, carotenoids etc, which are considered to be the reason for its antioxidant potential (Yuan et al. 2005). Prenyl toluquinones isolated from *Cystoseira crinite* (Fisch et al. 2003), eckstolonol isolated from *Ecklonia stolonifera* (Kang et al. 2003), sargothunbergol A and monogalactosyldiacylglycerols isolated from *Sargassum thunbergii* (Seo et al. 2007*,* Kim et al. 2007), fucodiphlorethol G isolated from *Ecklonia cava* (Ham et al. 2007) exhibited good radical scavenging potential. Sesquiterpenes with antioxidant potential were isolated from the essential oil of the red seaweed *Laurencia dendroidea* collected in the Brazilian coast (Gressler et al. 2011).

Rajendran et al. (2013) isolated biologically active compounds fucosterol and hexadec-4-enoic acid from the crude extracts of *Anthophycus longifolius* collected from the Gulf of Mannar. The biogenetic precursor of fucosterol, 24-methylene cholesterol, was found in significant amounts in *Padina vickersiae* (Aknin et al. 1992) and in *Padina gymnospora* collected from the Qatar coast (Al Easa et al. 1995). Cholesterol and fucosterol were identified as the main sterols present in *Padina pavonia* (Kamenarska et al. 2002). Several oxidised sterol derivatives have been found in some *Padina* species, 24-hydroperoxy-24- vinyl-cholesterol in *Padina pavonia* (Ktari & Guyot 1999),

7-ketocholesterol in *Padina tetrastromatica* Hauck. (Parameswaran et al. 1994) and 7α-hydroxyfucosterol in *Padina crassa* (Tan et al. 1992). Few terpenoids have been found in *Padina* species. Halogenated terpenoids were identified in *Padina tetrastromatica* (Parameswaran et al. 1994, 1996). Loliolide was identified in *P. tetrastromatica* (Rao & Pullaiah 1982) and in *Padina crassa* (Tan et al. 1992). Hexahydrofarnesylacetone was found in *P. tetrastromatica* (Rao & Pullaiah 1982). Dimethylsulfide and dimethyl-α-propiothetin were identified in *Padina arborescens* Holmes (Iida et al. 1985). Pigments were investigated in *Padina pavonia* by Hegazi et al. (1998).The characteristic brown algal polysaccharides (alginates and laminarans) were found in *Padina pavonia* (Khafaji 1986) and *P. tetrastromatica* (Rao et al. 1984). In *P. tetrastromatica* two new sulphated heteropolysaccharides, containing sugar and protein residues were also found (Rao et al. 1984). Galactol was identified in *P. tetrastromatica* (Parameswaran et al. 1996). Ktari et al. (1999) isolated oxysterol (1) from the dichloromethane extract of *Padina pavonica.* 18,19-epoxyxenic-19-methoxy-18-hydroxy-4-acetoxy-6,9,13-triene and 18,19-epoxyxenic-18,19-dimethoxy-4-hydroxyl-6,9,13-triene were also isolated from the 80% methanol extract of *Padina pavonia* (Awad et al. 2008).

Compounds like 5α-cholestane-3,6-dione, dipeptides, cholest-5-en-3α-ol, cholest-4-ene-3a,6b-diol (Wahidulla et al. 1986, 1991,19998) cholest-4-ene-3-one (Wahidulla & Kamat 1991), 11a-hydroxy-5a-cholestane-3,6-dione (Prakash et al. 1989) quercetin, (-)- catechin ,acid derivates and tiliroside (Wang et al. 1998, 2003), acanthophorin A and B (Zeng et al. 2001), and antheraxanthin (Aihara and Yamamoto, 1968) were already been identified from *A. spicifera*. Halogenated cyclic ether enynes and related allenes (Erickson et al 1983; Blunt et al 2006), sesquiterpenes, (Blunt et al. 2008), diterpenes (Blunt et al. 2005), C15-acetogenins (Blunt et al. 2004; Wright et al. 1991; Bittner et al. 1985; Howard and Fenical, 1976), calenzanol 6,8-cycloeudesmane sesquiterpenes (Guella et al. 2001, 2002), cuparene sesquiterpenes , (*E*)-2-tridecyl-2-heptadecenal, bromolaurenisol and laurinterol, cyclolaurane sesquiterpenes, (+)-α-isobromocuparene and (-)-α-bromocuparene (Kladi et al. 2005, 2006, 2007), halogenated sesquiterpenoids, diterpenoids, triterpenoids, and C_{15} acetogenins (Erickson 1983; Suzuki & Vairappan 2005) etc were isolated from genus *Laurencia.* Some of the common antioxidative compounds isolated from seaweeds are given by Figure 2.6.

2.7. Role of Seaweeds in Food and Pharmaceutical Industry

Recently, efforts are underway to exploit the antioxidant leads from the natural origin for use as food supplements in the food and pharmaceutical industry. These naturally derived antioxidant leads are the preferred alternatives to the synthetic derivatives due to their safety, sustainability and effectiveness. There are reports that the compounds derived from marine sources are endowed with antioxidant properties and are therefore the potential candidates for use as food supplements in the food industry (Rajauria et al. 2012). The regular consumption of seaweed derived diet can

reduce the risks of cancer, diabetes and heart disease (Yang et al. 2010; Lee et al. 2010; Bocanegra et al. 2008). The long history of the usage seaweeds in the part of their staple diet of the people from Korea, Japan, China etc. demonstrates their nutritional and health supplementing qualities. There are reports that different genera of seaweeds mainly belonging to *Sargassum, Porphyra, Ecklonia, Laminaria* etc have been used in Chinese medicine to treat against different diseases (Dharmananda 2002). Seaweeds, especially *Laminaraia* spp were used to treat against goitre because of its richness in iodine in China and Europe (Kelly 1961). These studies demonstrate the beneficial effect of seaweeds or seaweed derived compounds in food and pharmaceutical industry.

Keeping this facts as background information, the present study has attempted to develop an optimized procedure for extraction and pluralities of *in vitro* reactive oxygen species scavenging assay-guided chromatographic fractionation of seaweed-derived antioxidant lead molecules from the most abundantly available red and brown seaweeds from the Gulf of Mannar region of Mandapam. These seaweed-derived crude extracts have been purified by a series of chromatographic purification steps based upon their antioxidant potential. These compounds so purified have been assayed further for their antioxidative properties and based on the activities of the compounds, the major compounds have been identified on the basis of their ^{1}H NMR and ^{13}C NMR spectra, including 2D NMR (COSY, HSQC, HMBC, NOESY etc.) infra-red, and mass spectra.

CHAPTER 3

Evaluation of the Antioxidant Potential of Seaweeds

3.1. General

In this chapter seaweeds were evaluated for their total phenolic contents (TPC) and antioxidant activities, and the antioxidative properties have been correlated with the phenolic compounds responsible for the target activity. The antioxidant activities have been evaluated using different *in vitro* systems, *viz* 1,1-dipheny1–2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3 ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (H_2O_2) / hydroxyl radical (HO.) radical scavenging, ferrous ion (Fe^{2+}) chelating ability, thiobarbituric acid reactive species (TBARS) formation inhibition assay and reducing potential. A reversed-phase high-performance liquid chromatography method (RP-HPLC) hyphenated to diode-array detection (DAD) were also utilized to characterize the solvent extract fingerprints of phenolic constituents in the seaweed species. In order to understand the antioxidant potential exhibited by the seaweed extracts and fractions in a better way, the seaweeds were grouped into two or three and the results have been described in detail under different sub chapters.

3.1.1. Need for Conducting More than One Antioxidant Assay

It is difficult to predict the antioxidant capacity using one single assay, which may not lead to a valid and correct conclusion, and therefore, different assays were used in this study to evaluate antioxidant capacities of seaweed solvent extracts. We have focused to evaluate antioxidant action based on different mechanisms and variable conditions to get the idea on the functional role of antioxidants to inhibit oxidation process in a model system. The differential bioactivities obtained in various antioxidative assays were reported to be influenced by the nature of oxidation substrate, the components involved, and most importantly the method to measure oxidation (Huang et al. 2005). It is apparent that the antioxidant activity as determined by any specific assay reflects the chemical reactivity of the substrates under the particular conditions of that assay.

It is, therefore, that the antioxidant activity may not be generalized based on the results obtained from a single assay. It is important to evaluate the extracts using different antioxidant assay, which can give a more scientifically valid and correct idea about their antioxidant activities (Huang et al. 2005). These led us to incorporate different antioxidant assays in our present study to evaluate the bioactivities of the solvent extracts from the experimental seaweeds. The assays followed were (1) quantification of phenolic compounds, (2) radical scavenging activities by different mechanisms *viz*., hydrogen transfer, electron transfer, hydroxyl, and peroxide radical scavenging activity, (3) ability to inhibit lipid oxidation in model systems, (4) reducing, and (5) metal ion chelating abilities, in order to

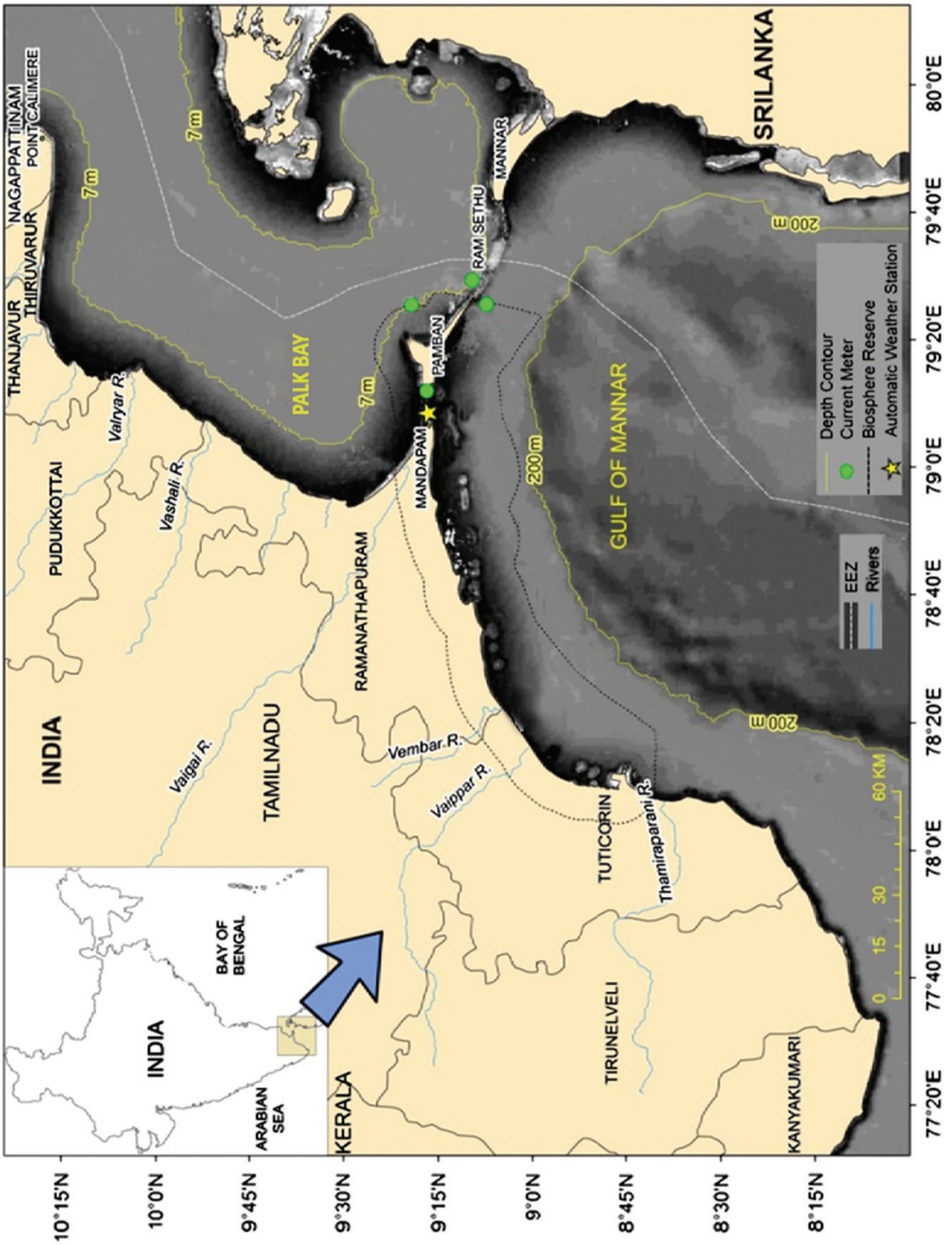


Figure 3.1. Map of Gulf of Mannar

get a comprehensive idea of the antioxidant mechanism operating with the solvent extracts of seaweeds. This enabled us to evaluate the seaweed extracts and fractions in different dimensions so that a better understanding in view of their antioxidative properties may be obtained. In this study methanol extract was selected as the extraction solvent because of the fact that methanol extract of many seaweed species exhibited potential antioxidant activities (Yan et al. 1999).

3.2 Materials and Methods

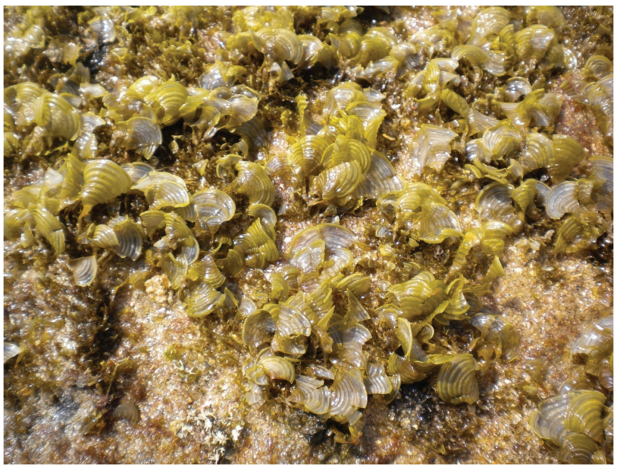
3.2.1. Chemicals

All solvents used for sample preparation were of analytical grade (E-Merck, Darmstadt, Germany). Analytical grade solvents were redistilled in an all-glass system. Doubly distilled water was used throughout this work, while all reagents used were of analytical grade and purchased from E-Merck. The chemicals 1,1-dipheny1–2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), trichloracetic acid (TCA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-42 ,42 -disulfonic acid sodium salt (ferrozine), folin-ciocalteu reagent, and ABTS (2,2'-azino-bis-(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, quercetin, acetyl acetone, ammonium acetate, ascorbic acid, ethylene diamine tetra acetic acid (EDTA), ferrous ammonium sulfate, ferric chloride, potassium ferricyanide and ferrous chloride were purchased from E-Merck and Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). All other unlabeled chemicals and reagents were of analytical, spectroscopic or chromatographic reagent grade and were obtained from E-Merck (Darmstadt, Germany). Water used for HPLC analysis was of Milli-Q quality (Millipore Corp., Cork, Ireland). All solvents used for HPLC were of HPLC grade. Stock solutions of standard were prepared in HPLC grade methanol for HPLC-DAD identification and kept in a refrigerator at 20°C until use. Standards: chlorogenic acid (>99%), syringic acid (≥ 95%), 2, 5 dihydroxy benzoic acid (>99%), 4- hydroxy benzoic acid (≥ 99%), epigallocatechin gallate (EGCG, ≥ 99%) (2000 ppm each) from Sigma-Aldrich. Caffeic acid (≥ 98%), *p*-coumaric acid (≥ 98%), ferulic acid (99%), quercetin, salicylic acid (≥ 99%), gallic acid, (+)-catechin, epicatechin, and epicatechin gallate (ECG, ≥ 98% by HPLC) (1000 ppm each, >99%) from Sigma-Aldrich.Stock solutions of the standards were prepared by dissolving the compounds separately in HPLC methanol, and stored at 15°C until analysis of phenolic compounds by RP-HPLC.

3.2.2. Instrumentation

A benchtop refrigerated high-speed microprocessor controlled centrifuge equipped with asynchronous motor and programmable micro-controlled variable frequency controller (Superspin PlastoCrafts R-V/Fm, Mumbai, India) were used for centrifugation. Crude solvent extracts were

Mandapam



Vethalai

Thonithurai

Puthumadam

Kelaikkarai

Seeniappadurga

Figure 3.2. Seaweed collection sites

concentrated using a rotary vacuum evaporator (Heidolph Instruments GmbH & Co. KG Schwabach, Germany). All spectrophotometric data were acquired using Varian Cary 50 conc UV-visible spectrophotometer (Varian Cary, USA). Glass cuvettes (1 cm × 1 cm × 4.5 cm) were used for visible absorbance measurements. Chromatographic analysis was carried out using high-performance liquid chromatograph (Shimadzu SCL-10A vp, Shimadzu Co., Kyoto, Japan) equipped with a vacuum degasser, a binary pump (LC–20AD), a thermostatted column compartment (CTO–20A) and a diode array detector (SPD–M20A), connected to an LC solution software. Chromatographic separation was carried out at 30°C on a reverse phase Luna C_{18} (250 mm x 4.6 mm, 5µm) phenomenex column.

3.3. Seaweed Material and Description of Study Area

The seaweeds were collected from the Gulf of Mannar of Mandapam region located between 8°48' N, 78°9' E and 9°14' N, 79°14'E on the southeast coast of India (Figure 3.1). The seaweed samples collected from various sites of Gulf of Mannar region (Figure 3.2) were washed with distilled water, to remove salt, epiphytes and other unwanted materials, before being shade dried, powdered and stored in airtight containers at room temperature for further work.

3.4. Preparation of Seaweed Extracts and Fractions

The ground and shade-dried seaweed samples were extracted with methanol and filtered through sodium sulphate (Na_2SO_4). The filtrate thus obtained was evaporated (40°C) using rotary evaporator under vacuum to dryness. This methanol extracts were mixed with an equal volume of distilled water, and partitioned successively with *n*-hexane, dichloromethane (MDC), and ethyl acetate (EtOAc) to furnish corresponding solvent fractions. The extracts were dried over anhydrous sodium sulphate and evaporated under reduced pressure to furnish *n*-hexane, dichloromethane and ethyl acetate fractions, respectively.

3.5. Determination of the Total Phenolic Content (TPC) and Antioxidant Potential of the Methanol Extract and Solvent Fractions of the Seaweeds

3.5.1. Total Phenolic Content (TPC)

The amounts of total phenolics in the seaweed extracts and solvent fractions were determined by the established method with suitable modification (Mcdonald et al. 2001). Gallic acid was used as a standard, and a standard calibration curve was prepared by mixing a methanolic solution of gallic acid (1 mL; 0.025–0.400 mg/mL) with Na_2CO_3 (4 mL, 0.7 M) and folin-ciocalteu reagent (5 mL, diluted ten fold). The absorbance was measured at 765 nm. The methanol extract (1 mL, 5 g/L) was mixed with the reagents as stated earlier and after an incubation period of 2 h the

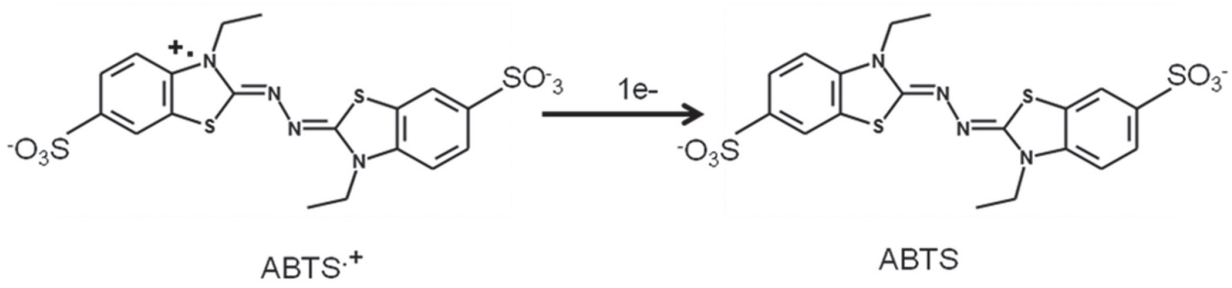


Figure 3.3 The reduction of the blue green ABTS.+ chromophore to colourless ABTS in presence of antioxidants

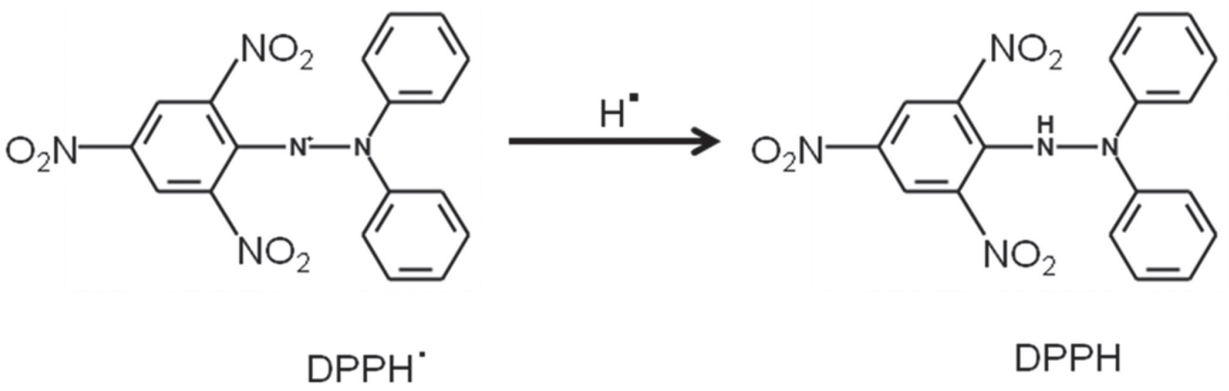


Figure 3.4 The reduction of HPPH. to DPPH in presence of antioxidants

absorbance was measured to determine total phenolic contents. All determinations were carried out in triplicate. The total phenolic content was expressed as gallic acid equivalent (GE) in the mg / g sample, and was calculated by the following formula: T =[C X V]/ M, where the T=total content of phenolic compounds, mg/g plant extract (Gallic acid equivalents, GAE); C=the concentration of galic acid as established from the calibration curve (mg/mL); V= volume of extract (mL); M=the weight of plant extract (g).

3.5.2. 2, 2'-Azino-bis-(3-ethylbenzothiozoline-6-Sulfonic Acid) Diammonium Salt (ABTS) Radical Scavenging Activity (%)

The ABTS.$^+$ assay (Re et al. 1999) was employed to measure the antioxidant activity of the seaweed extracts. Briefly, ABTS.$^+$was dissolved in deionized water to 7 mM concentration, and potassium persulfate ($K_2S_2O_8$) is added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12-16 h) in the dark before use. The resultant intensly-colored ABTS.$^+$ radical cation was diluted with methanol to give an absorbance of ~0.70 at 734 nm. The test materials (seaweed extracts 50 μL) of different concentrations (0.1 – 0.6 μg/mL) was diluted 100 times with the ABTS.$^+$ solution to a total volume of 5 mL. Absorbance was measured spectrophotometrically at different concentrations (0.1-0.6 μg/mL) for each extract results of the assay were expressed as % radical scavenging ability. The assay was performed in triplicates. Fresh stocks of ABTS.$^+$ solution were prepared before analyses.

Chemistry

The blue green ABTS$^{\cdot+}$ chromophore (λ_{max}734nm) is generated by the reaction between ABTS and $K_2S_2O_8$. Antioxidants which are capable of reducing ABTS$^{\cdot+}$ to ABTS by donating an electron will lead to a decrease in absorbance at λ_{max}734nm (Figure 3.3).

3.5.3. 1, 1-Diphenyl-2-Picryl Hydrazil (DPPH•) radical scavenging Activity (%)

The free radical scavenging activity of the crude solvent extracts of the seaweeds was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH.) using established method with suitable modification (Shimada et al. 1992). In brief a solution of DPPH. (80 μg/mL, 2.5 mL) in methanol was added to the equal volume of sample solution (2.5 mL) at different concentrations (0.1-1mg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min, and the absorbance was measured at 517 nm in various time intervals (1-5 h). The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage of the DPPH. radical scavenging effect was calculated using the following equation: DPPH· scavenging effect (%) = $100(A_0 - A_1/A_0)$, where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the standard sample or crude extract.

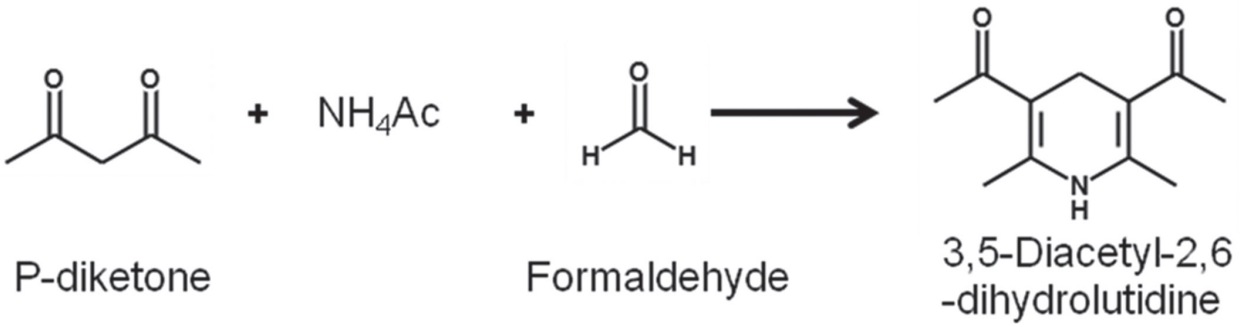


Figure 3.5. Formation of 3,5-diacetyl-2,6-dihydrolutidine with λmax412nm from the reaction between formaldehyde and Nash reagent

Chemistry

DPPH· is a highly reactive free radical with λ_{max}517nm. Antioxidants will readily reduce DPPH· to DPPH by donating H· leading to a decrease in absorbance at λ_{max}517nm (Figure 3.4).

3.5.4. Hydroxyl Radical Scavenging Activity (%)

The hydroxyl radical scavenging activity of the crude solvent extracts of the seaweeds was measured using the established method with modification (Klein et al. 1981**)**. Briefly, methanol extracts of seaweeds (0.1-0.6 mg/mL) were placed in a test tube and evaporated to dryness. Iron-EDTA solution (1 mL, 0.13% Mohr's Salt (ferrous ammonium sulfate and 0.26% w/v ethyline diamine tetra acetic acid), EDTA (0.5 mL, 0.018%), dimethyl sulfoxide (1 mL, 0.85% v/v, in phosphate buffer (0.1 mol/L, pH 7.4), and ascorbic acid (0.5 mL of 0.22%) were added to each tube, which were capped tightly, and heated in a water bath at 80–90°C for 15 min. The reaction was terminated by adding ice-cold trychloro acetic acid (1 mL, 17.5% w/v). Nash reagent (3 mL, 75.0 g amonium acetate 3 mL glacial acetic acid and 2 mL acetyl acetone were mixed and water was added to a total volume of 1 L) was added to each tube and were left at room temperature for 15 min for color development. The intensity of the yellow color formed was measured at 412 nm against a blank of the reagent and expressed as percentage hydroxyl radical scavenging activity.

Chemistry

The OH· radical generated using the ascorbic acid-ion EDTA oxidation reaction, will react with DMSO to produce formaldehyde. The formaldehyde thus formed can further react with Nash reagent which contain a diketone and ammonium acetate to form a condensation product 3,5-diacetyl-2,6-dihydrolutidine with λ_{max}412nm (Figure 3.5) (Compton & Purdy 1980). Antioxidants present with the reaction mixture will deactivate the OH· radical and thus the production of formaldehyde leading to a decrease in absorbance maxima at 412nm.

3.5.5. Hydrogen Peroxide (H_2O_2) Scavenging Activity (%)

The ability of the solvent extracts of the seaweeds to scavenge hydrogen peroxide was determined using established method (Ruch et al. 1989) with suitable modification. In brief, a solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4) and the concentration of H_2O_2 was determined spectrophotometrically from absorption at 230 nm. Seaweed extracts (3 mL, 0.1-1 mg/mL) in distilled water was added to a H_2O_2 solution (3 mL, 40mM) and the absorbance of H_2O_2 at 230 nm was determined after 20 min against a blank solution containing in phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 of seaweed extracts was determined by the following formula: % scavenged (H_2O_2) = $[(A_0 - A_1)/A_0]$ 100, where A_0 was the

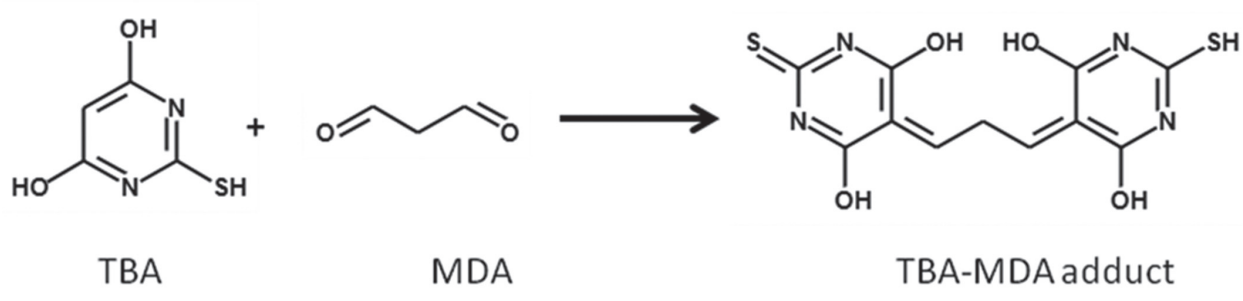


Figure 3.6. Formation of TBA-MDA adduct (thiobarbituric acid reactive species) from the reaction between TBA and MDA

absorbance of the control and A_1 was the absorbance in the presence of the sample of the solvent fractions and standards.

Chemistry

The presence of H_2O_2 in phosphate buffer (pH 7.4) can be determined spectrophotometrically by its characteristic absorbance at λ_{max} 230nm. Compounds with H_2O_2 scavenging potential will accelerate the decomposition of H_2O_2 into water by donating electrons. This will lead to a decrease in absorbance at λ_{max} 230nm.

$$H_2O_2 + 2H^+ + 2e^- \longrightarrow 2H_2O$$

3.5.6. Lipid Peroxidation Inhibition Activity in Model System: Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity (MDAEC/kg)

The ability of the seaweed extracts to arrest lipid peroxidation was assessed by thiobarbituric acid reactive substances assay (TBARS) as described by (Madsen et al. 1997**)** with suitable modification. The model system used for this assay was lyophilized mussel (*Perna viridis* L.) sample as a lipid source. The sample solutions (1mL, 0.1-2 mg/mL) were incubated with of the mussel sample (10 mg), acetic acid (2 mL, 20 % v/v) and an aqueous solution of thiobarbituric acid (TBA, 2 mL, 0.78 % w/v) at 95°C for 45 minutes. The resultant mixture (5 mL) was cooled to room temperature and clarified by centrifugation (8000 rpm, 10 min). The absorbance of the supernatant was recorded at 532 nm and the antioxidant capacity was expressed as equivalent mM of malonaldehyde (MDA)/kg of sample. TBARS concentration was calculated using a standard curve based on MDA.

Chemistry

Fatty acids will break down into aldehydes, particularly malonaldehyde (MDA) as a result of oxidation. This can be measured by its reaction with thiobarbituric acid (TBA) leading to the formation of a condensation product (TBA-MDA adduct) with λ_{max}532nm. Antioxidants present in the reaction mixture will hinder the lipid peroxidation thereby the formation TBA-MDA adduct, leading to a decrease in absorbance at λ_{max}532nm (Figure 3.6).

3.5.7. Evaluation of Reducing Ability (Ab_{700}nm)

Total reduction capabilities of the solvent extracts of seaweeds were estimated by using the method as described earlier (Oyaizu 1986**)** with modifications. The solvent extracts of seaweeds (1 mL) in distilled water were added with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$, 2.5 mL, 1% v/v) to be incubated at 50 °C for 20 min. A portion of trichloroacetic acid (TCA, 2.5 mL, 10% v/v) was added to the mixture to terminate the reaction.

The aliquot was thereafter centrifuged for 10 min at 8000 g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and The absorbance of the reaction mixture after incubation was measured at 700 nm by using a spectrophotometer. The higher absorbance of the reaction mixture indicated greater reducing power.

Chemistry

Antioxidants with reducing power will react with potassium ferricyanide ($K_3Fe(CN)_6$) and ferric chloride ($FeCl_3$) to form potassium ferrocyanide ($K_4Fe(CN)_6$) and ferrous chloride ($FeCl_2$), which then reacts with $FeCl_3$ to form ferric ferrous complex with λ_{max}700 nm (Arulpriya et al. 2010). Higher the antioxidant potential, higher the Fe^{3+}/Fe^{2+} complex formed leading to a higher absorbance.

$$K_3Fe(CN)_6 + FeCl_3 \xrightarrow{\text{Antioxidants}} K_4Fe(CN)_6 + FeCl_2$$

$$K_4Fe(CN)_6 + FeCl_3 \longrightarrow Fe^{3+}\text{-}Fe^{2+}\ \text{Complex}$$

3.5.8. Ferrous Ion (Fe^{2+}) Chelating Activity (%)

The Fe^{2+} ion chelating potential by the seaweed extracts were estimated by the method of (Dinis et al. 1994**)** with suitable modification. Briefly, the samples (0.1-0.6 mg/mL) were added to ferrous chloride solution (0.05 mL, 2mM). The reaction was initiated by the addition of ferrozine (0.2 mL, 5mM) with shaking, and the contents were left at room temperature for 10 min. After the reaction mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given bellow formula: % inhibition = $[(A_0 - A_1)/A_0]$ 100, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of the sample and standards. The control contains ferrous chloride and ferrozine, the complex forming molecules.

Chemistry

Red coloured ferrous-ferrozine complex with a λ_{max}562nm will be formed by the reaction between ferrous chloride and ferrozine. The complex formation will be hindered by the presence of a chelating agent which can be monitored by a decrease in absorbance at 562nm.

$$FeCl_2 + \text{Ferrozine} \longrightarrow Fe^{2+}/\text{Ferrozine complex}$$

3.5.9. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied traits. Analyses were carried out in triplicate and the means of all parameters were examined for

significance (P<0.05) by analysis of variance (ANOVA). The Pearson correlation test was used to assess correlations between means. The experiments were carried out in three different batches of seaweeds. The mean variance in the data set was detected using principal component analysis (PCA). All data were mean centered and scaled to equal unit variance prior to PCA. The selected variables for PCA where the different antioxidant assays and phenolic content, as exhibited by the methanol extract and different solvent fractions of the seaweeds.

3.6. Preparation of the Seaweed Extracts and Solvent Fractions for HPLC Analysis

The pre-purification of methanol extract and ethyl acetate fractions from seaweeds were accomplished using solid-phase extraction (SPE: Supelco LC_{18} mini-columns). These solid-phase extractions were preconditioned for polyphenols by sequentially passing 2 mL of methanol, 2 mL of 50 % aqueous methanol (1:1) and finally 2 mL of water (this last step was repeated 3 times). The sample was dissolved in 1 mL of aqueous methanol (70 %) on the minicolumn conditioned and washed with 2 mL of distilled water. Finally, the sample was eluted with 2 mL of 90% methanol (2 times) to recover the purified extract which was evaporated to dryness and dissolved in water/ methanol (1:1, v/v) for the HPLC identification. The sample was thereafter passed through a 0.25 µm filter (Millipore, Westboro, MA) before being injected into the HPLC. Reverse phase HPLC to determine phenolic compounds was performed using a Shimadzu HPLC (SCL-10A vp, Shimadzu Co., Kyoto, Japan), equipped with a UV (SPD-20A, Shimadzu), a programmable wavelength diode array detector, and manual injector. Separations were achieved on an analytical column packed with modified silicagel (Phenomenex RPC_{18} Luna 150 X 4.6 mm i.d.; 5 µm particle size packing material) fitted with a C_{18} octadecylsilane guard cartridge (4 mm X 3 mm i.d., 5µm) (Phenomenex, Torrance, CA, USA).

3.6.1. Identification of the Phenolic Compounds by HPLC

The sample were dissolved in methanol and chromatographed under gradient conditions, with a flow rate of 0.6 mL/min. An equilibration time of 15 min was maintained before injection. The mobile phase was methanol: water with 0.2% acetic acid (65:35, v/v). The injection volume was 50 µL. The gradient elution was performed as follows: 0–2 min, 5% B isocratic; 2–10 min, linear gradient 5–25% B; 10–20 min, linear gradient 25–40% B; 20–30 min, linear gradient 40–50% B; 30–40 min, linear gradient 50–100% B; 40–45 min, 100% B isocratic and 45–55 min, linear gradient 100–5% B. The time of HPLC run was over 50 min. Simultaneous monitoring of detection wavelength were set at 324 nm for chlorogenic acid, caffeic acid, 2, 5-dihydroxy benzoic acid, coumaric acid, ferulic acid, quercetin, salicylic acid and 277 nm for gallic acid, catechin, epigalocatechin galllate (EGCG), epicatechin (EC), epicatechin gallate (ECG) and syringic acid. Individual phenolic acids of seaweed extracts were identified by comparing their retention times with those of authentic standards using the same HPLC operating conditions. Chlorogenic acid (8.12), caffeic acid (10.49),

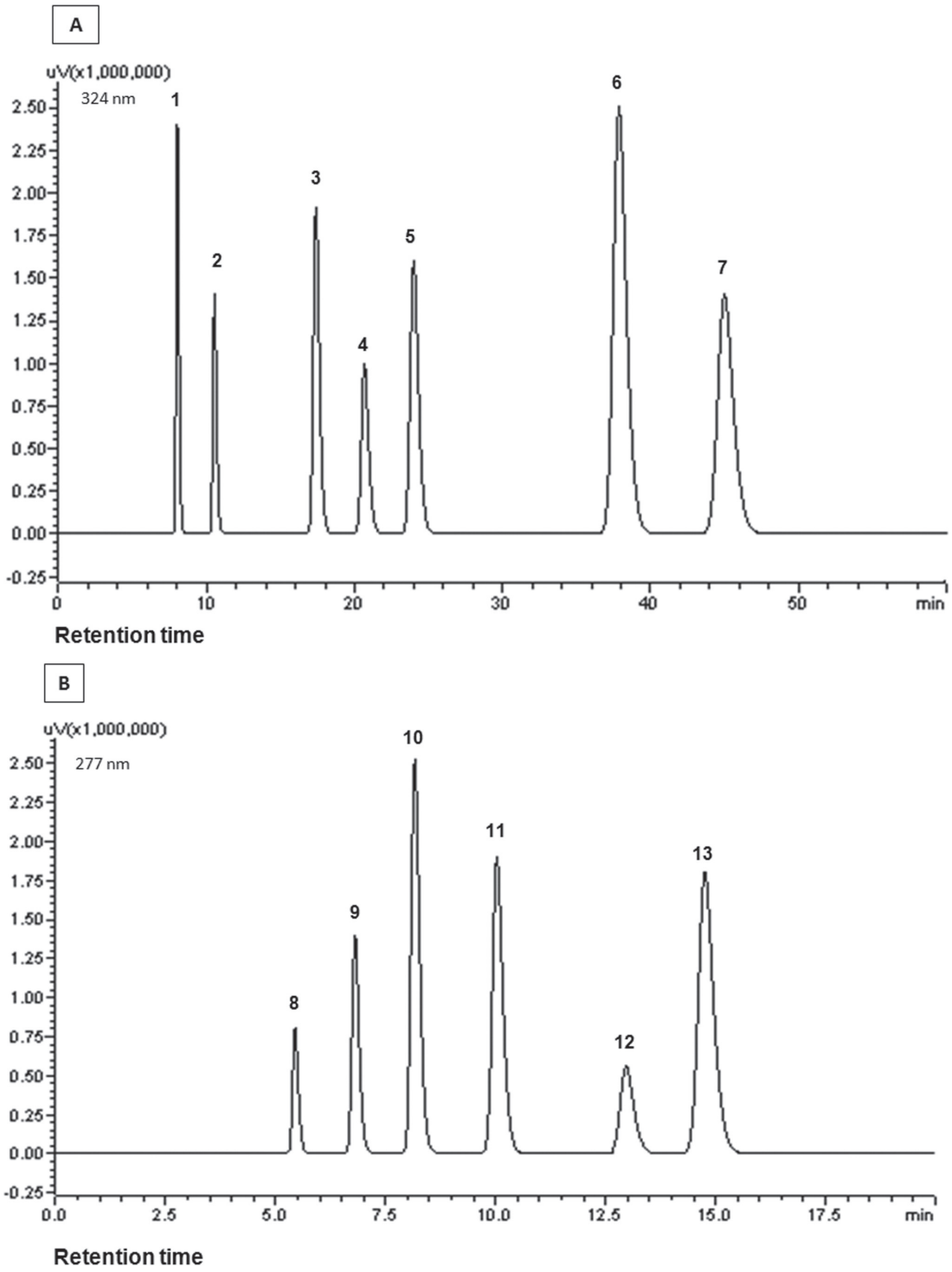


Figure 3.7 (A) HPLC chromatogram of standard polyphenolics detected at 324 nm: (1) chlorogenic acid (8.12); (2) caffeic acid (10.49); (3) 2,5 dihydroxy benzoic acid(17.43); (4) coumaric acid (20.56); (5) ferulic acid (24.19); (6) quercetin (37.9); (7) salicylic acid (44.92).

(B) HPLC chromatogram of standard polyphenolics detected at 277 nm: (8) gallic acid (5.38); (9) catechin (6.82); (10) epigallocatechin gallate (8.13); (11) epicatechin(10.11); (12) epicatechin gallate (13.0); (13) syringic acid (14.78).

2,5 dihydroxy benzoic acid(17.43), coumaric acid (20.56), ferulic acid (24.19), quercetin (37.9) and salicylic acid (44.92) were detected at 324 nm (Figure 3.7). Gallic acid (5.38), catechin (6.82), epigallocatechin gallate (8.13), epicatechin(10.11), epicatechin gallate (13.0) and syringic acid (14.78) were detected at 277 nm (Figure 3.7). The quantification of each compound was determined based on peak area measurements, which were reported to calibration curves of the corresponding standards. Purity of each peak was checked so as to exclude any contribution from interfering peaks. The electronic descriptors *viz*., molecular polar surface area based on fragment contributions (tPSA) and hydrophobic parameter log P as calculated by ChemDraw 12.0 (Chakraborty et al., 2008, 2009) have been taken into consideration to understand the elution behaviour or relative position of the phenolics in the HPLC spectra and their molecular characteristics guiding their separation in the C_{18} HPLC column.

The phenolics with hydroxyl phenyl substituted chroman ring system as in epicatechin with 2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol moiety (tPSA 110.38, log P 1.50), epicatechin gallate with 2-(3,4-dihydroxyphenyl)-5,7-dihydroxychroman-3-yl 3,4,5-trihydroxybenzoate moiety (tPSA 177.14, log P 2.46), epigallocatechin gallate with 5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl 3,4,5-trihydroxybenzoate moiety (tPSA 110.38, log P 1.50), and 4H-chromen-4-one ring system as in quercetin with 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (tPSA 127.45, log P 0.35) appeared to be bonded strongly with the C_{18} matrix and therefore eluted later. Quercetin and epicatechin with chroman ring system were reported to have a hydroxyl group at C-3 of the ring system (Figure 3.7).

3.6.2. Preparation of the Seaweed Samples for HPLC Analysis

The pre-purification of the seaweed crude extract before HPLC injection was accomplished using solid-phase extraction (SPE: Supelco LC_{18} mini-columns). These SPE were preconditioned for polyphenols by sequentially passing 2 mL of methanol, 2 mL of 50 % aqueous methanol (1:1) and finally 2 mL of water (this last step was repeated 3 times). The extract was dissolved in 1 mL of aqueous methanol (70 %) on the mini column conditioned and washed with 2 mL of distilled water. Finally, the extract was eluted with 2 mL of 90% methanol (2 times) to recover the purified extract which was evaporated to dryness and dissolved in water/methanol (1:1, v/v) for the HPLC identification. The extract was thereafter passed through a 0.25 µm filter (Millipore, Westboro, MA) before being injected into the HPLC. Reverse phase HPLC to determine phenolic compounds was performed using a Shimadzu HPLC (SCL-10A vp, Shimadzu Co., Kyoto, Japan), equipped with a UV (SPD-20A, Shimadzu), a programmable wavelength diode array detector, and manual injector. Separations were achieved on an analytical column packed with modified silicagel (Phenomenex RPC_{18} Luna 150 X 4.6 mm i.d.; 5 µm particle size packing material) fitted with a C_{18} octadecylsilane guard cartridge (4 mm X 3 mm i.d., 5µm) (Phenomenex, Torrance, CA, USA).

CHAPTER 3A

Evaluation of the Antioxidant Potential of *Turbinaria conoides* and *Turbinaria ornata*

Figure 3A. *T. ornata* collected from the Gulf of Mannar

Figure 3A. *T. conoides* collected from the Gulf of Mannar

CHAPTER 3A

Evaluation of the Antioxidant Potential of *Turbinaria conoides* and *Turbinaria ornata*

3A.1. Introduction

The genera *Turbinaria* is well-known for its biological activities including antioxidant properties (Zubia 2003), and their chemical defense mechanism is supposed to increase in species from coral reef ecosystems where biodiversity, grazing, and competition for space and food are considerably higher (Hay 1996). Among different seaweeds, the genera *Turbinaria* is well-known for its biological activities including antioxidant and antimicrobial activities (Chakraborty et al. 2013; Zubia et al. 2008). It was reported that the bioactive compounds derived from *Turbinaria* species are endowed with high antioxidant and anti-inflammatory potential and could be considered as a potential antioxidant and anti-inflammatory agent (Ananthi et al. 2010; Chattopadhyay et al. 2010). Earlier reports indicated that the extracts of brown seaweeds belonging to *Turbinaria* sp. were found to have antioxidant and anti-inflammatory activities (Zubia et al. 2009; Vijayabaskar et al. 2012).

The brown seaweeds contain a large assemblage of species that predominate in the coastal shelf areas of the Gulf of Mannar region in the southeastern coast of the Indian subcontinent. Among various seaweeds, *Turbinaria conoides* (J. Agardh) Kuzing (Family: Sargassaceae, Order: Fucales) and *Turbinaria ornata* (Turner) J. Agardh (Family: Sargassaceae, Order: Fucales) are abundantly available in this area throughout different seasons and therefore these species have been shortlisted for the present study to evaluate antioxidant activities and total phenolic contents to understand their beneficial value as human food or additives. In the present study, we have evaluated the antioxidant properties of methanol extract (MeOH) and different solvent fractions(ethyl acetate (EtOAc), dichloromethane (MDC), and *n*-hexane) of *Turbinaria* sp. by 2, 2'-azino-bis-(3ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS) assay, 1, 1-diphenyl-2-picryl-hydrazil (DPPH·) radical scavenging activity, total reduction capability, and Fe^{2+} ion chelating activity. Assays for detection of scavenging of short-lived radicals were determined by HO. radical scavenging activity and scavenging of H_2O_2. The ability to inhibit lipid oxidation in model systems was carried out by thiobarbituric acid-reactive substances assay (TBARS). The content of phenolic compounds considered to have antioxidant activities was also determined. The study was also aimed to quantify the phenolic compounds present in these species by reverse phase HPLC and determine the relationship between antioxidant activity and phenolic principles.

Table 3A.1. Yields obtained for methanol extract (as % w/w of seaweed on dry weight basis) and solvent fractions (as % of total MeOH extract) of *T. ornata* and *T. conoides*.

Seaweed species	Methanol extract (%)	Solvent fractions obtained by partitioning methanol extract with solvents (%)		
		***n*-hexane**	**MDC**	**EtOAc**
T. ornata	8.4 ± 0.36	3.0 ± 1.36	2.5 ± 0.62	1.7 ± 0.39
T. conoides	6.8 ± 0.07	2.5 ± 0.57	2.2 ± 0.44	1.2 ± 0.84

All the values were expressed as mean ± SD; SD: standard deviation. MDC: dichloromethane; EtOAc: ethyl acetate; and MeOH: methanol solvent fraction.

3A.2. Materials and methods

3A.2.1. Preparation of Seaweed Extracts and Fractions

The powdered shade-dried seaweed samples (200g) were extracted with methanol (MeOH, 500 mL x 4) at an elevated temperature (40-45°C) for 3 h. The samples were then filtered with Whatman filter paper no 1 to obtain the clarified filtrates (1.8 L), which were filtered, through Na_2SO_4 (150 g), and evaporated (40°C) using a rotary evaporator under vaccum to dryness to give a dark green viscous oily mass (100 mL) of MeOH extract. This dark green viscous oily mass (100 mL) of MeOH extract was mixed with an equal volume of distilled water (100 mL) and partitioned successively with *n*-hexane (200 mL x 3), MDC (200 mL x 3) and EtOAc (200 mL x 3) to furnish *n*-hexane (600 mL), MDC (600 mL) and EtOAc fractions (600 mL) respectively. The water-free extracts were dried over anhydrous Na_2SO_4 (100 g) and evaporated under reduced pressure to furnish the crude fractions of varying polarity (Table 3A.1). Methanol was selected as the extraction solvent because the methanol extract of many seaweed species exhibited a very good antioxidant activity (Yan et al. 1999).

3A.2.2. Assays for Determination of Phenolic Contents and Antioxidant Potential of Seaweeds

The amount of total phenolics in the samples was determined by the established method described by Mcdonald et al. (2001). The $ABTS.^+$, 1, 1-diphenyl-2-picryl-hydrazil (DPPH.), hydroxyl radical radical assasy, hydrogen peroxide scavenging ability, thiobarbituric acid reactive substances assay (TBARS), reducing ability and Fe^{2+} ion chelating potential were performed by the methods described by Re et al. (1999), Shimada et al. (1992) and Klein et al. (1981), Ruch et al. (1989), Madsen et al. (1997), Oyaizu (1986) and Dinis et al. (1994) respectively, with suitable modifications. HPLC based chromatographic identification of the phenolic acid standards and the samples were done as described by section3.2

3A.2.3. Statistical Analysis

Statistical evaluation was carried out by SPSS software (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied traits. Analyses were carried out in triplicate and the means of all parameters were examined for significance ($P<0.05$) by analysis of variance (ANOVA). A post-hoc test (Scheffe) was carried out when the differences shown by data were significant ($P<0.05$). The Pearson correlation coefficient (r) was calculated ($P<0.05$) to assess the strength of the linear relationship between two variables.

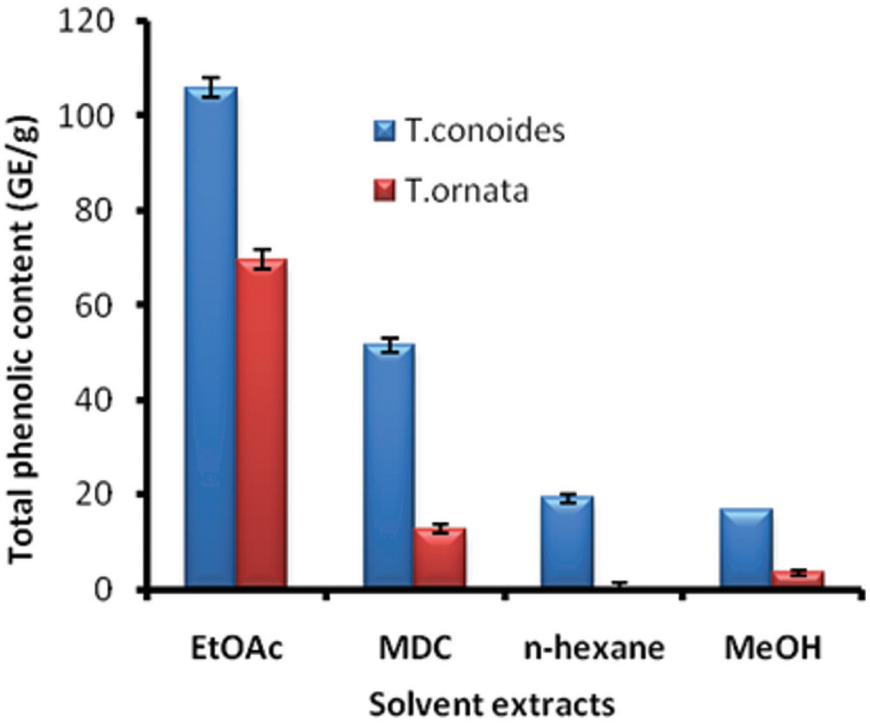


Figure 3A.1. Total phenolic content (mg of GE/g of the sample) of methanol extract and different solvent fractions of *T. conoides* and *T. ornata*. Total phenolic content was expressed as gallic acid equivalent (GE) in mg/g sample.

3A.3. Results and Discussion

3A.3.1. Determination of Total Phenolic Compounds (TPC)

A number of studies have focused on the biological activities of phenolic compounds, which are potent antioxidants and free radical scavengers (Sugihara et al. 1999). Phenolic compounds are considered to protect the seaweed thallus from photo destruction caused by UV radiation (Pavia et al. 1997), and to exhibit radical scavenging properties (Rice-Evans et al. 1997). EtOAc fractions of both *T. conoides* and *T. ornata* registered a significantly higher (P<0.05) TPC (105.97 & 69.63 mg GE/g, respectively) followed by the MDC fractions (51.47 & 12.72 mg GE/g respectively) as compared to other solvent fractions and methanol extract (Figure 3A.1). The methanol extract and all solvent fractions of *T. conoides* exhibited significantly higher TPC than corresponding fractions of *T. ornata* (Table 3A.2). The *n*-hexane extract of *T. ornata* (1.07 GE/g) registered lowest TPC than all other solvent fractions. Phenolic compounds are considered to exhibit radical scavenging properties (Umayaparvathi et al. 2012). Several studies demonstrated a significant correlation between the phenolic content and the antioxidant activity in seaweed extracts (Ganesan et al. 2011). It is evident from the present observations that a higher percentage of TPC was observed in the polar solvent fractions (EtOAc and MDC) of *Turbinaria* sp. indicate their high antioxidant potential. Earlier reports indicated the presence of phenolic compounds viz. catechin and epigallocatechin in EtOAc fraction of brown seaweeds, particularly *Turbinaria* sp. (Kuda et al. 2005; Chandini et al. 2008). It was also reported that the aqueous fraction of *T. conoides* is endowed with a higher phenolic content (Chandini et al. 2008).

3A.3.2. 2,2' -Azino-bis-3-ethylbenzothiozoline-6-Sulfonic Acid Diammonium Salt (ABTS) Radical Scavenging Activity (%)

The assay applied in this study was according to the improved technique described by (Chakraborty & Paulraj 2010) for the generation of ABTS•-, which involves the direct production of the blue/green ABTS•- chromophore though the reaction between ABTS and $K_2S_2O_8$. All the seaweed fractions and MeOH extracts displayed antioxidant activities as they were able to scavenge the ABTS•+ radical cation. The sequence of antioxidant activity of the different solvent fractions of seaweed *T. ornata* (0.6 µg /mL) as determined by ABTS assay was as follows: *n*-hexane (30.84 %) > EtOAc (13.91 %) > MeOH (8.04 %) > MDC fraction (6.38 %) (Table 3A.2). MDC fraction of *T. ornata* was realized with higher ABTS radical scavenging ability (17.57%) than other MeOH extract and other organic fractions. The variation of ABTS radical scavenging activity with concentration (0.1 – 0.6µg/mL) of the tested extract and fractions were described in figure 3A.3. It can be observed from the figure that EtOAc fraction of *T. conoides* and MeOH extract of *T. ornata*

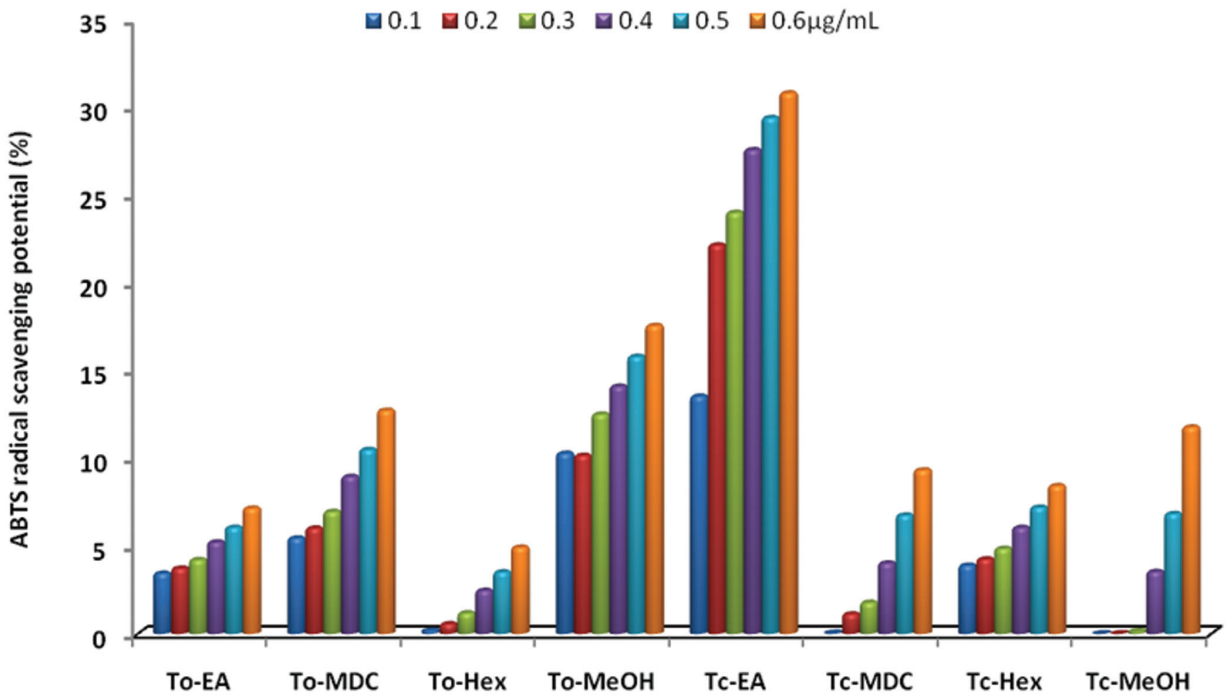


Figure 3A.2. ABTS.+ radical scavenging activities (%) of EtOAc, MDC, *n*-hexane fractions and MeOH extract of *T. conoides* and *T. ornata* with concentration (0.1-0.6 µg/mL).

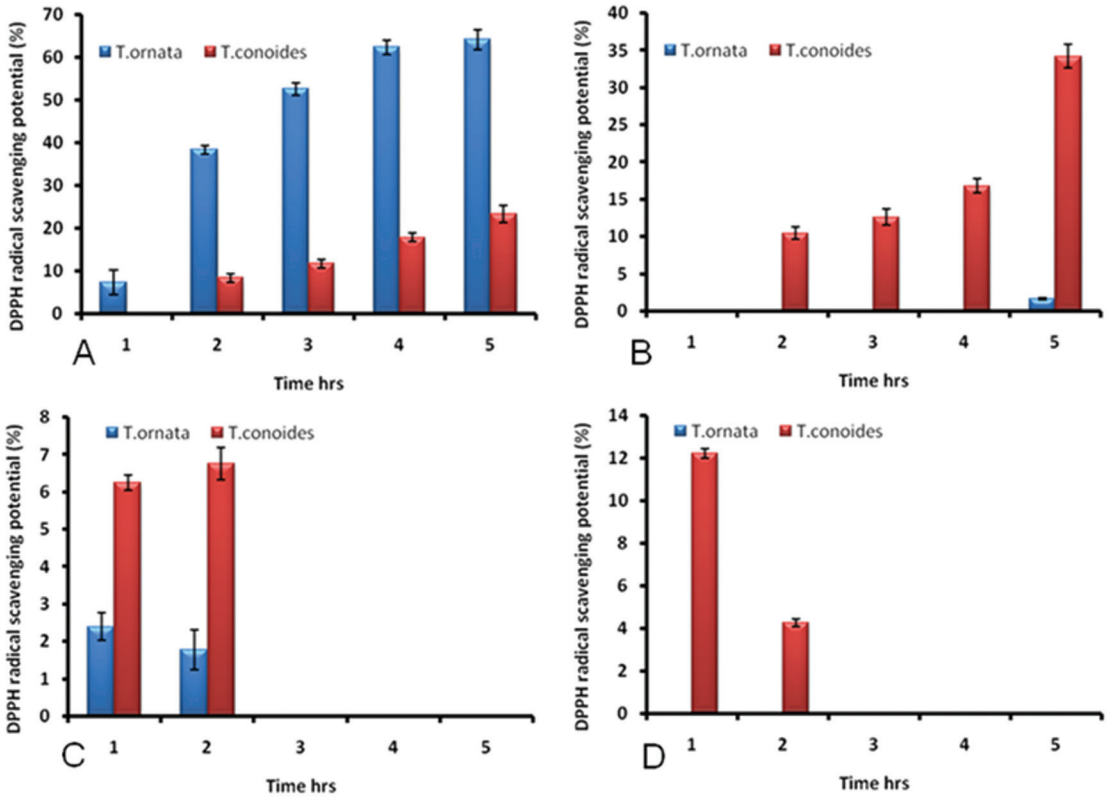


Figure 3A.3. DPPH radical scavenging activities (%) of EtOAc, MDC, *n*-hexane fractions and MeOH extract (A, B, C and D) of *T. conoides* and *T. ornata* with time (1-5hrs)

were more active than other fractions. The higher ABTS radical scavenging ability exhibited by the *n*-hexane fraction may be explained due to the presence of carotenes/other pigments with long hydrocarbon chain (Gupta & Abu-Ghannam 2011). There are reports which showed that hexane, chloroform and methanol extracts of *Porphyra yezoensis* exhibited antioxidant activities (Nakayama et al. 1999) attributed to the presence of β-carotene, chlorophyll analogues (pheophytin) and amino compounds (leucine, phenylalanine and mycosporine- like amino acid, usujirene). There are also other reports claiming that seaweeds contain antioxidant compounds which include some pigments such as fucoxanthin and astaxanthin, polyphenols such as phlorotannins, chlorophyll related compounds, phospholipids, flavonoids, bromophenols and polysaccharides (Gupta & Abu-Ghannam 2011; Umayaparvathi et al. 2012).

3A.3.2. 1, 1-Diphenyl-2-Picryl Hydrazil (DPPH·) radical scavenging Activity (%)

DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle et al. 1996) and is a useful reagent for investigating the free radical scavenging activities of compounds (Duan et al. 2006). The free radical scavenging ability of various solvent extracts from two *Turbinaria* species were evaluated with the change of absorbance caused by the reduction of DPPH radical. EtOAc fractions of both the *Turbinaria* sp. registered significantly higher (P<0.05) DPPH radical scavenging activities than MeOH extract and other fractions (Figure 3A.3). DPPH radical scavenging activity of EtOAc fraction of *T. ornata* registered significantly higher (P<0.05) (64%) than other solvent extracts (<3%, 1 mg/mL). MDC fraction of *T. conoides* (1 mg/mL) exhibited a significantly higher (P<0.05) DPPH radical scavenging activity (34%) followed by EtOAc fraction (23%) at the same dose (Table 3A.2). The potentially higher antioxidant activities of MDC fraction may also be explained due to the presence of carotenes/other pigments with long hydrocarbon chain and aminated compounds (Chew et al. 2008). This was supported by an earlier report, indicating that *n*-hexane and $CHCl_3$ extracts of *Porphyra yezoensis* exhibited antioxidant activities attributed to the presence of mycosporine-like amino acid (Nakayama et al. 1999). Components, such as low molecular weight polysaccharides, pigments, proteins or peptides also reported to influence antioxidant activity (Siriwardhana et al. 2003). It is apparent that the organic polar solvent fractions (EtOAc) of brown seaweeds may contain polyphenolic compounds with multiple –OH groups/centers of unsaturation capable of deactivating DPPH free radical. This hypothesis is reasonable, because of the fact that the radical scavenging capacities by DPPH assay is related to the ability of the substrate (solvent extract) to donate electrons and H^+ ions to DPPH free radical thus neutralizing the latter. The compounds responsible may have polyphenolic group/s with multiple -OH groups and/or center of unsaturation in their structural moieties to enable them to donate H to DPPH (Ruberto et al. 2001). It was reported that active compounds from brown seaweeds with

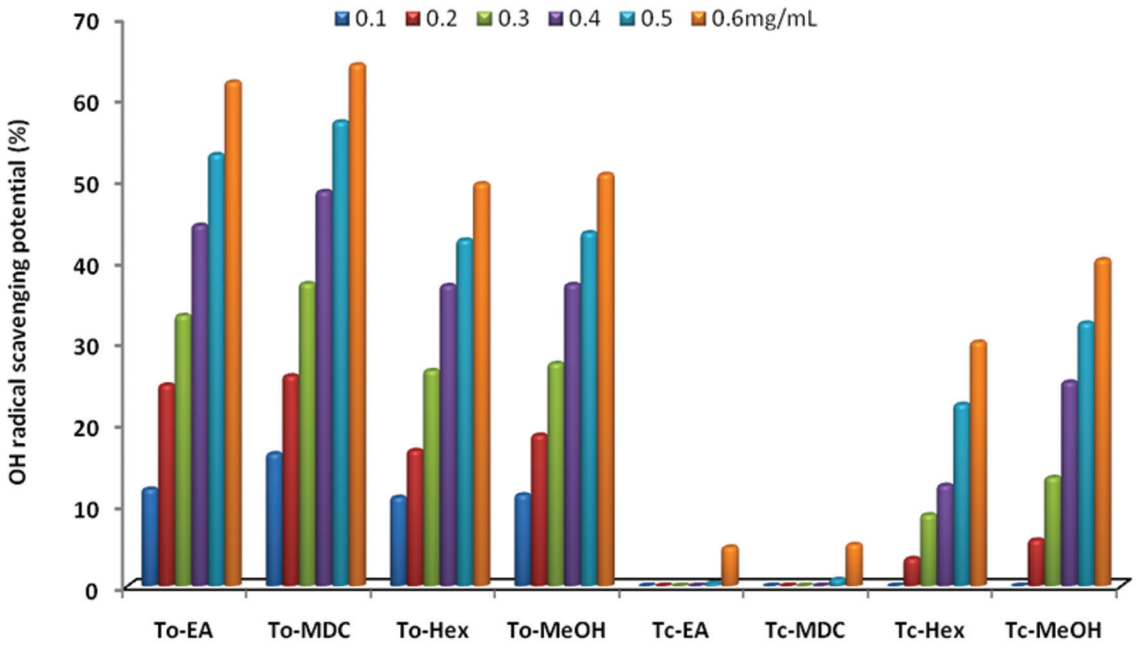


Figure 3A.4. OH radical scavenging activities (%) of EtOAc, MDC, *n*-hexane fractions and MeOH extract of *T. conoides* and *T. ornata* with concentration (0.1-0.6 mg/mL)

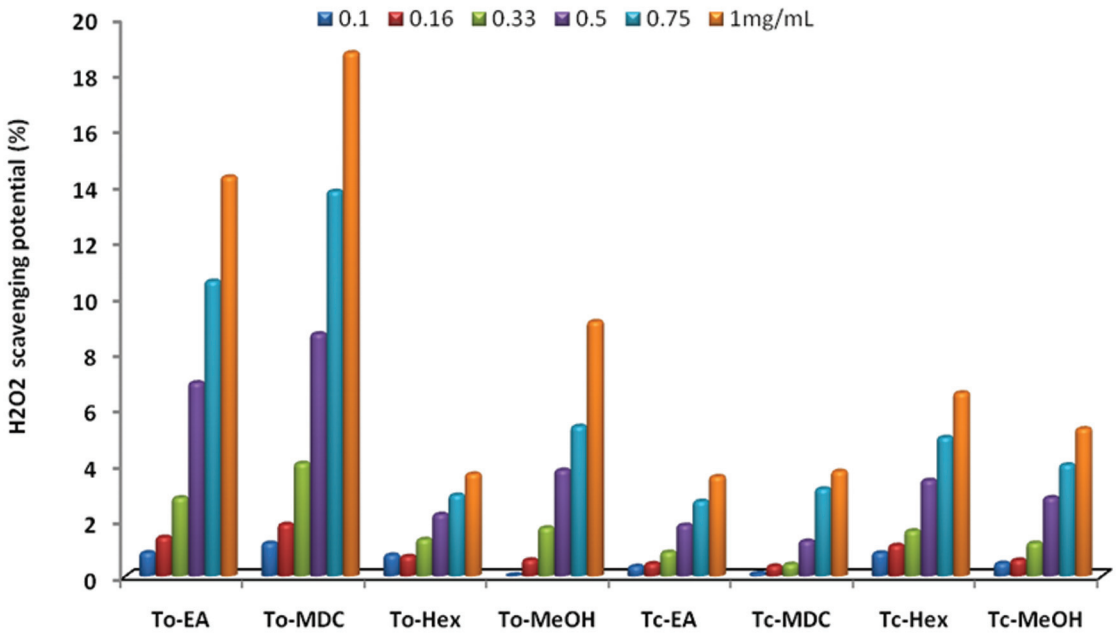


Figure 3A.5. H_2O_2 radical scavenging activities (%) of EtOAc, MDC, *n*-hexane fractions and MeOH extract of *T. conoides* and *T. ornata* with concentration (0.1-1 mg/mL).

antioxidative properties are phlorotannins and fucoxanthin (Yan et al. 1999). The antioxidant property exhibited in the present study may mainly be due to the presence of phlorotannins and fucoxanthin or any other potential antioxidants with center/s of unsaturation present in them.

3A.3.4. Hydroxyl Radical Scavenging Activity (%)

The EtOAc fractions of *T. ornata* (IC_{50} 0.47 mg/mL) and *T. conoides* (IC_{50} 0.44 mg/mL) were found to be highly effective to scavenge HO. radical followed by MDC (IC_{50} 0.59 and 0.58 mg/mL, respectively). The activities were found to be proportionately decreased with concentrations, although EtOAc and MDC fractions of *T. ornata* exhibited significantly higher activities, even at lower concentrations (Figure 3A.4). Also, the EtOAc and MDC fractions obtained from *T. conoides* maintained their potential to inhibit formation of HO. radical at a much lower dose. Hydroxyl radical scavenging activity assay was employed to understand the scavenging potential of methanol extract and different solvent fractions from seaweeds against short-lived radicals, *viz*., HO. radicals. HO. radicals were reported to abstract H- atoms from lipid membranes, and thus bring about peroxide reactions of lipids. The HO. scavenging activities of brown seaweeds were reported to be due to polyphenolic compounds such as phlorotannins which can act as electron traps and are responsible for the multifunctional antioxidant properties such as scavenging of hydroxyl radicals, peroxy radicals or superoxides (Gupta & Abu-Ghannam 2011). Ascorbic acid was also reported to be the principal component responsible for HO. scavenging activities recorded in brown seaweeds (Abe et al. 1992). There are also other reports which showed that seaweed extracts are potential HO. scavengers (Cho et al. 2011). In the present study EtOAc fractions of *Turbinaria* sp. realized higher activities, thereby signifying the importance of using EtOAc to isolate potential antioxidant molecules.

3A.3.5. Hydrogen Peroxide (H_2O_2) Scavenging Activity (%)

H_2O_2 is a non radical compound, and is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells (singlet oxygen and HO. radicals) (Ruch et al. 1989).

Thus, removal of H_2O_2 is essentially important to protect the biological system in general, and food components, in particular. The measurement of H_2O_2 scavenging activity is one of the useful methods, determining the ability of antioxidants to undermine the level of prooxidants *viz*., H_2O_2 (Czochra & Widensk 2002). In the present study the EtOAc fraction of *T. conoides* (IC_{50} 1.49 mg/mL) was found to be highly effective to scavenge H_2O_2 followed by MDC (IC_{50} 2.98 mg/mL). The EtOAc fraction of *T. ornata* realized the lowest IC_{50} value (1.90 mg/mL) followed by MeOH

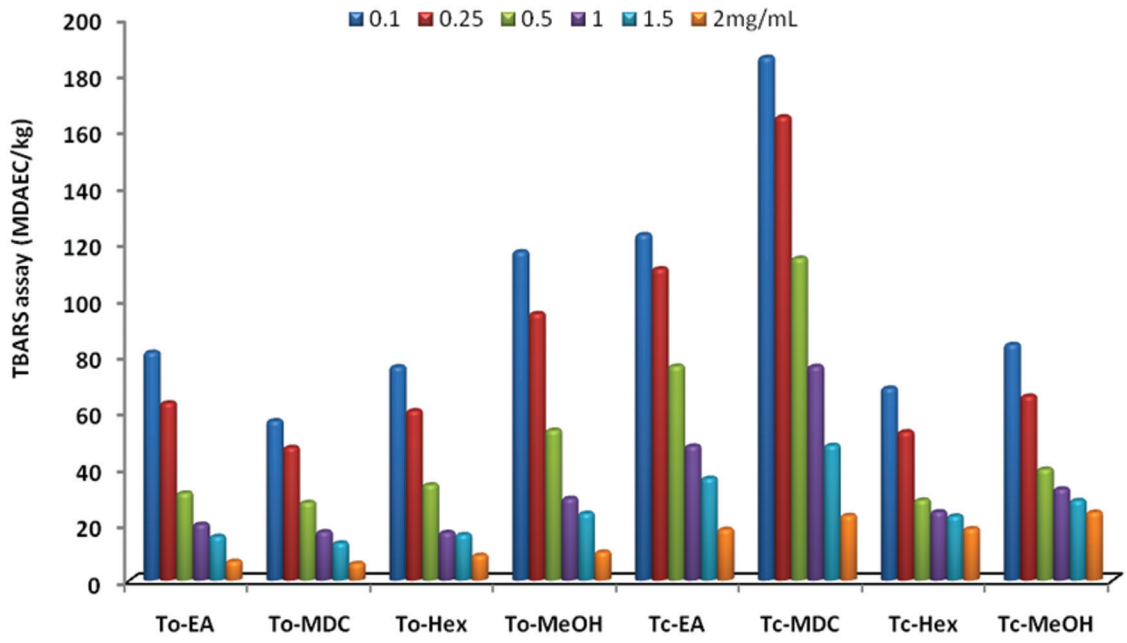


Figure 3A.6. Lipid peroxidation inhibitory assay (MDAEC/kg) of EtOAc, MDC, *n*-hexane fractions and MeOH extract of *T. conoides* and *T. ornata* with concentration (0.1-2 mg/mL).

extract (4.25 mg/mL), *n*-hexane (7.48 mg/mL), and MDC fraction (8 mg/mL) in descending order. The activities of MeOH extract and *n*-hexane fraction were found to be significantly reduced at lower concentrations (0.1-0.3 mg/mL) (Figure 3A.5). Earlier studies showed that seaweeds contained polyphenolic compounds such as phlorotannins which can act as electron traps and are responsible for the multifunctional antioxidant properties such as scavenging of hydroxyl radicals, peroxy radicals or superoxides (Gupta & Abu-Ghannam 2011). It was reported that extracts of some brown seaweeds registered more than 90% H_2O_2 scavenging activity (Heo et al. 2005), thereby supporting the very fact that brown seaweeds are rich source of natural antioxidant compounds, which can scavenge H_2O_2 radical. Many other species of seaweeds were also reported in literature to possess potential H_2O_2 scavenging activity (Gupta & Abu-Ghannam 2011).

3A.3.6. Lipid Peroxidation Inhibition Activity in Model System: Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity (MDAEC/kg)

As a result of oxidation, unsaturated fatty acids (with $\geq$ 2 olefinic double bonds) were reported to break down into low molecular weight aldehydes causing off-flavors (rancid flavor) in oils, and can react with the free amino groups of phospholipid, proteins, and nucleic acids, leading to structural modifications, which induce dysfunction of immune systems (Chakraborty & Paulraj 2007). The fatty acid breakdown products essentially contain malondialdehyde (MDA), which was measured through their reaction with thiobarbituric acid (TBA) (Ganhão et al. 2011). The lower values in mille moles of MDA equivalent compounds formed/kg (MDAEC/kg) indicate a higher lipid peroxidation inhibitory effect. The EtOAc and MDC fractions of *T. ornata* registered significantly higher TBARS inhibition ability (6.78 and 8.91 MDAEC/kg, respectively) (P<0.05) at 2 mg/mL with respect to inhibit lipid peroxidation (Table 3A.2) than MeOH and *n*-hexane fractions (18.36 and 18.14 mM MDAEC/kg, respectively), which were not significantly different (P>0.05). It is evident from Figure 3A.6 that the TBARS inhibition ability is dose dependent and were found to be proportionately decreased with concentrations. Accordingly, the IC_{50} value of the MeOH extract and different organic solvent fractions revealed the order of activity as: EtOAc fraction (0.21 mg/mL) > *n*-hexane fraction (0.24 mg/mL) > MeOH extract (0.26 mg/mL) > MDC fraction (0.43 mg/mL). The lipid peroxidation inhibitory capacities of EtOAc and MDC fractions of *T. conoides* (6.03 & 10.06 MDAEC/kg, respectively at 2 mg/mL) were significantly higher (P<0.05) than that recorded for MeOH extract and *n*-hexane fraction (24.23 and 23.07 mM MDAEC/kg, respectively) (Table 3A.2). The results obtained from TBARS assay indicate the effectiveness of different *Turbinaria* sp. to prevent lipid oxidation *in vitro*. The results obtained from TBARS assay indicate the effectiveness of both *Turbinaria* sp. to prevent lipid oxidation *in vitro*. Earlier studies revealed that EtOAc and MDC fraction are the major fractions of seaweeds harboring the principle

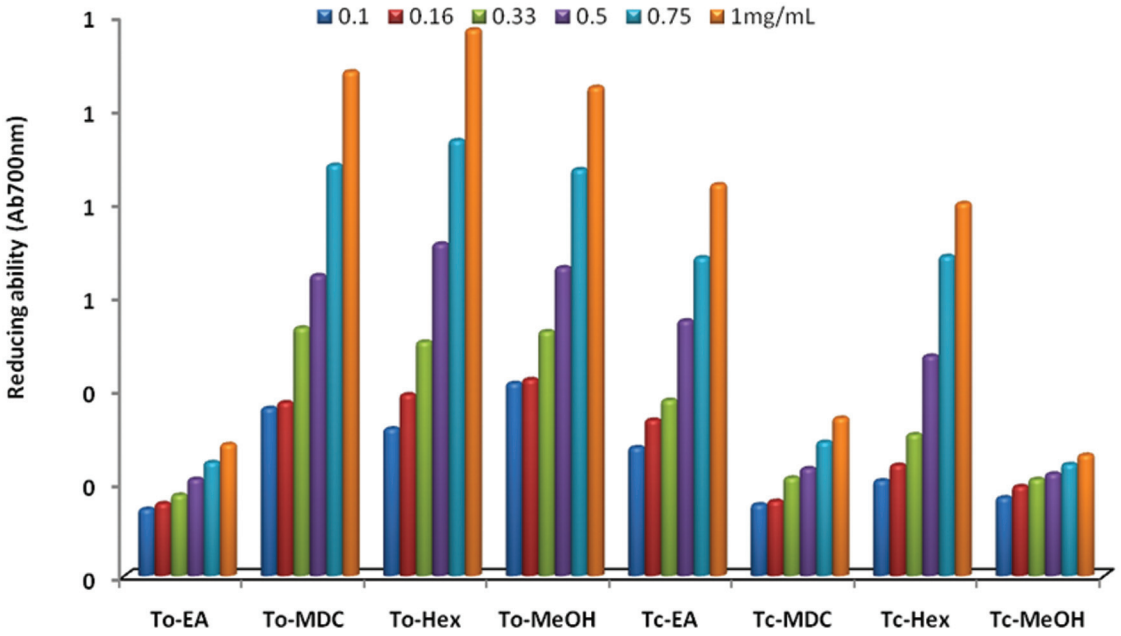


Figure 3A.7. Reducing ability assay (Ab_{700nm}) of EtOAc, MDC, *n*-hexane fractions and MeOH extract of *T. conoides* and *T. ornata* with concentration (0.1-1 mg/mL)

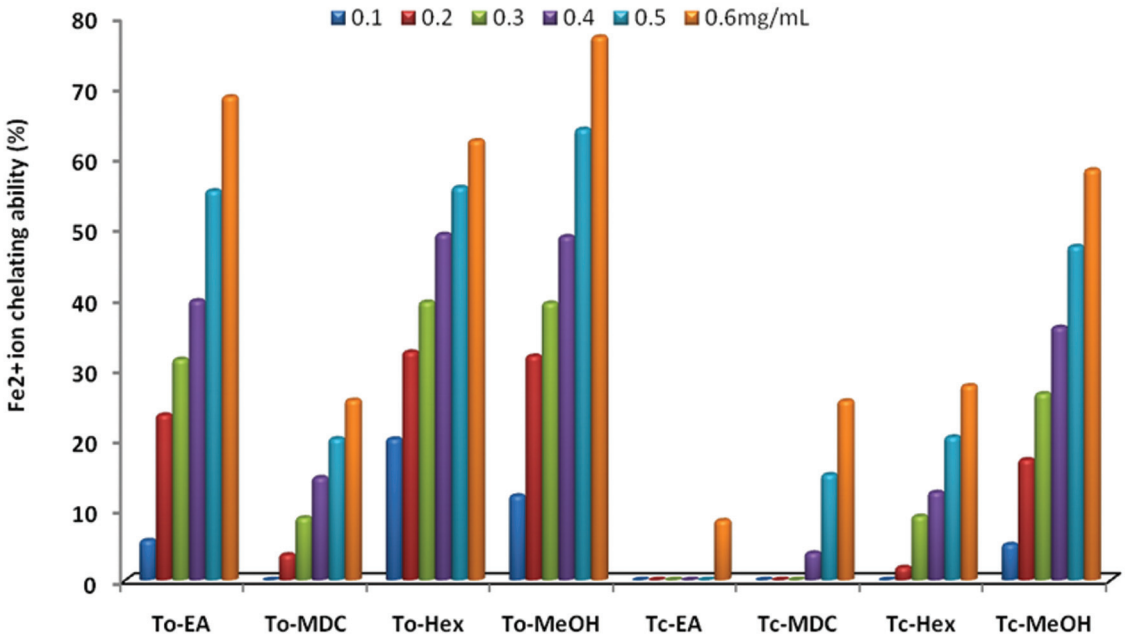


Figure 3A.8. Fe^{2+} ion chelating ability (%) of EtOAc, MDC, *n*-hexane fractions and MeOH extract of *T. conoides* and *T. ornata* with concentration (0.1-0.6 mg/mL)

antioxidative components (Zubia et al. 2009). The inhibition of lipid peroxidation may be due to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions, may have roles to inhibit lipid peroxidation (Gupta & Abu-Ghannam 2011). There are other reports, which suggest that extracts of brown seaweeds belonging to *Turbinaria* sp. are anticipated to be very got inhibitors of lipid peroxidation (Vijayabaskar et al. 2012).

3A.3.7. Evaluation of Reducing Ability (Ab_{700}nm)

The reducing abilities of chemical extracts and/or compounds generally depends on the presence of reductones (Duh, 1998), which have been shown to impart antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). The presence of reductants (i.e. antioxidants) in the solvent fractions apparently reduces the Fe^{3+}/ferricyanide complex to its Fe^{2+} form, which can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Gordon, 1990). A direct correlation was reported between antioxidant activities and reducing power in a series of studies reported in the literature (Duh, 1998). The IC_{50} value of methanol extract and different solvent fractions of *T. conoides* revealed the order of activity as: EtOAc (60.59 mg/mL) > MDC (67.35 mg/mL) > *n*-hexane (236.46 mg/mL) > MeOH (543.98 mg/mL). The same trend was apparent in lower concentrations (0.33-0.75 mg/mL). In *T. ornata* the IC_{50} value of reducing activities of different organic solvent fractions and methanol extract revealed the order of activity as: EtOAc = MDC (52.67 mg/mL) > MeOH extract (71.22 mg/mL) > *n*-hexane (79.70 mg/mL). A dose dependency was observed in all the solvent fractions (Figure 3A.7). Results obtained in the present study are in accordance with the earlier reports suggesting that brown seaweeds collected from different regions were found to be endowed with potential reducing abilities and antioxidant properties (Cho et al. 2011; Ganesan et al. 2011). It was also reported that reducing power exhibited by solvent extracts of seaweeds belonging to *Turbinaria* sp. was comparatively higher than α-tocopherol (Chandini et al. 2008).

3A.3.8. Ferrous Ion (Fe^{2+}) Chelating Activity (%)

The reduced form of iron (Fe^{2+}) can stimulate and accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Costa et al. 2011). As a result chelators of Fe^{2+} ion can be considered as potential inhibitors of lipid peroxidation. In present study different fractions of *Turbinaria* sp. demonstrated a marked capacity for Fe^{2+} binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity. In present study different fractions of *Turbinaria* sp. demonstrated a marked capacity for Fe^{2+} ion binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity. The EtOAc and

Table 3A. 2. Total phenolic content and antioxidant activities of the different crude solvent fractions (MeOH, *n*-hexane, MDC and EtOAc) of brown seaweeds belonging to *Turbinaria* spp

Seaweed species	Solvent fractions of *Turbinaria* spp			
	MeOH	*n*-Hexane	MeOH	EtOAc
Total reduction capability (A_{700nm})				
T. conoides	$0.26^{ap} \pm 0.00$	$0.35^{ap} \pm 0.01$	$1.07^{ap} \pm 0.02$	$1.07^{ap} \pm 0.01$
T. ornata	$0.79^{bp} \pm 0.01$	$0.83^{bp} \pm 0.01$	$1.14^{bp} \pm 0.03$	$0.28^{bp} \pm 0.00$
Total phenolic content (mg of GE)				
T. conoides	$16.64^{ap} \pm 0.10$	$19.26^{ap} \pm 0.68$	$51.47^{aq} \pm 0.99$	$105.97^{ar} \pm 1.47$
T. ornata	$3.42^{bp} \pm 0.35$	$1.07^{bp} \pm 0.05$	$12.72^{bq} \pm 0.62$	$69.63^{br} \pm 1.50$
Scavenging of H_2O_2 (%)				
T. conoides	$5.28^{ap} \pm 0.50$	$3.49^{ap} \pm 0.30$	$9.40^{ap} \pm 0.30$	$18.76^{aq} \pm 0.82$
T. ornata	$6.57^{bp} \pm 0.18$	$3.44^{ap} \pm 0.28$	$3.57^{bp} \pm 0.24$	$14.31^{bq} \pm 0.43$
Fe^{2+} ion chelating activity (%)				
T. conoides	$58.29^{ap} \pm 3.66$	$25.46^{aq} \pm 3.22$	$77.19^{ar} \pm 1.20$	$25.57^{aq} \pm 0.56$
T. ornata	$27.63^{bp} \pm 1.34$	$8.47^{bq} \pm 0.81$	$62.44^{br} \pm 2.49$	$68.67^{bs} \pm 0.56$
Hydroxyl radical scavenging activity (%)				
T. conoides	$40.19^{ap} \pm 2.08$	$5.25^{aq} \pm 0.13$	$52.03^{ar} \pm 1.28$	$64.20^{as} \pm 0.92$
T. ornata	$30.04^{bp} \pm 1.17$	$4.45^{bq} \pm 0.31$	$49.82^{br} \pm 0.40$	$62.08^{bs} \pm 1.48$
Lipid peroxidation inhibitory (TBARS) activity (MDAEC/kg)				
T. conoides	$24.23^{ap} \pm 0.34$	$23.07^{ap} \pm 0.79$	$10.06^{aq} \pm 0.28$	$6.03^{aq} \pm 0.52$
T. ornata	$18.36^{bp} \pm 0.99$	$18.14^{bp} \pm 0.15$	$8.91^{bq} \pm 0.45$	$6.78^{aq} \pm 0.49$
ABTS radical scavenging activity (%)				
T. conoides	$12.20^{ap} \pm 0.59$	$12.94^{ap} \pm 0.56$	$17.57^{ap} \pm 1.51$	$13.06^{ap} \pm 0.08$
T. ornata	$8.04^{bp} \pm 0.55$	$30.84^{bq} \pm 0.68$	$6.38^{bp} \pm 0.35$	$13.91^{ar} \pm 0.42$
DPPH· radical scavenging activity (%)				
T. conoides	$12.22^{bp} \pm 0.17$	$2.16^{ap} \pm 0.15$	$34.23^{aq} \pm 1.16$	$23.32^{ar} \pm 1.41$
T. ornata	ND	$2.40^{aq} \pm 0.27$	$1.68^{bq} \pm 0.08$	$64.14^{br} \pm 1.66$

Data are the mean values of triplicate and expressed as mean ± standard deviation. Row (p-s) and column values (a-c) with different letters are significantly different ($P<0.05$). MDC: dichloromethane, EtOAc: ethyl acetate. The concentration of the crude solvent fractions used were 1 mg/mL for DPPH radical scavenging activity, reducing capacity and H_2O_2 scavenging activity; 0.6 mg/mL for OH radical scavenging activity and Fe^{2+} ion chelating activity; 2 mg/mL for TBARS assay and 0.6 µg/mL for ABTS radical scavenging activity. ND: Non-detectable.

MDC fractions of *T. ornata* (0.6 mg/mL) exhibited significantly higher Fe^{2+} chelating abilities and accordingly, the IC_{50} value*s* exhibited by different solvent fractions of this seaweed followed the order: MDC (0.43 mg/mL) > EtOAc (0.46 mg/mL) > MeOH (1.03 mg/mL) > *n*-hexane (4.37 mg/mL) fractions . The *n*-hexane fraction was found to be ineffective towards Fe^{2+} chelating abilities (<10%, 0.6 mg/mL) (Table 3A.2). At lower concentrations (0.5-0.3 mg/mL), EtOAc and MDC fractions of *T. ornata* realized significantly (P<0.05) higher Fe^{2+} chelating activity than MeOH extract. However, it is interesting to note that the Fe^{2+} chelating abilities of the MDC fraction of *T. conoides* (0.6 mg/mL) as observed in this study, registered significantly higher (P<0.05) (IC_{50} 0.39 mg/mL) than MeOH extract and other solvent fractions. No significant differences are apparent with respect to Fe^{2+} chelating abilities between EtOAc (25.57%), and *n*-hexane fraction (25.46%) (Table 3A.2).The scavenging activity exhibited a dose dependent behavior. Accordingly, lowest IC_{50} was registered by MDC fraction of *T. conoides* (0.39 mg/mL) towards Fe^{2+} chelating abilities followed by the MeOH extract (0.53 mg/mL). It can be observed from the figure that (Figure 3A.8) the MeOH extract and solvent fractions of *T. ornata* showed higher ability to chelate Fe^{2+} ions than that exhibited by the tested samples from *T. conoides.* It was reported that low-molecular compounds in the dried brown seaweed *Scytosiphon lomentaria* with Fe^{2+} ion chelating activity (Kuda et al. 2005). There are other reports that the phlorotannins, which are usually present in the polar solvent fractions of brown seaweeds are strong chelators of heavy metals (Wang et al. 2012; Gupta et al. 2011). The Fe^{2+} chelating abilities of the seaweed fractions were also reported to be due to the presence of non phenolic compounds like different types of polysaccharides present in the seaweed extracts (Hu et al. 2010). A negative correlation observed between TPC and Fe^{2+} chelating abilities proves that in this study chelating ability of algae could be due to the presence of compounds other than phenolics and these seaweeds could be potential rich sources of natural antioxidants. Molecules with hydroxyl, sulfhydryl, carbonyl, and phosphate groups were reported to possess favorable structure–function configuration resulting in Fe^{2+} chelating abilities, and apparently compounds, including phenolic acids, flavonoid quercetin, and phenolic glycosides are noted to chelate transition metal ions like Fe^{2+} ion. These active compounds might have a synergistic effect, playing an important role in antioxidant activity by the inhibition of oxidation and chelating effects.

3A.3.9. Correlations between Phenolic Contents and Different Antioxidant Activity Assays

The positive correlation observed between TPC and radical scavenging activities of seaweed extracts is in agreement with the earlier literature data (Rajauria et al. 2010). Negative correlation realized between TPC and TBARS assay apparently indicate that antioxidant activity did not depend only on total phenol content, but also on other factors as there may be any active metabolites other than phenolics such as polysaccharides capable of inhibiting the TBA-MDA

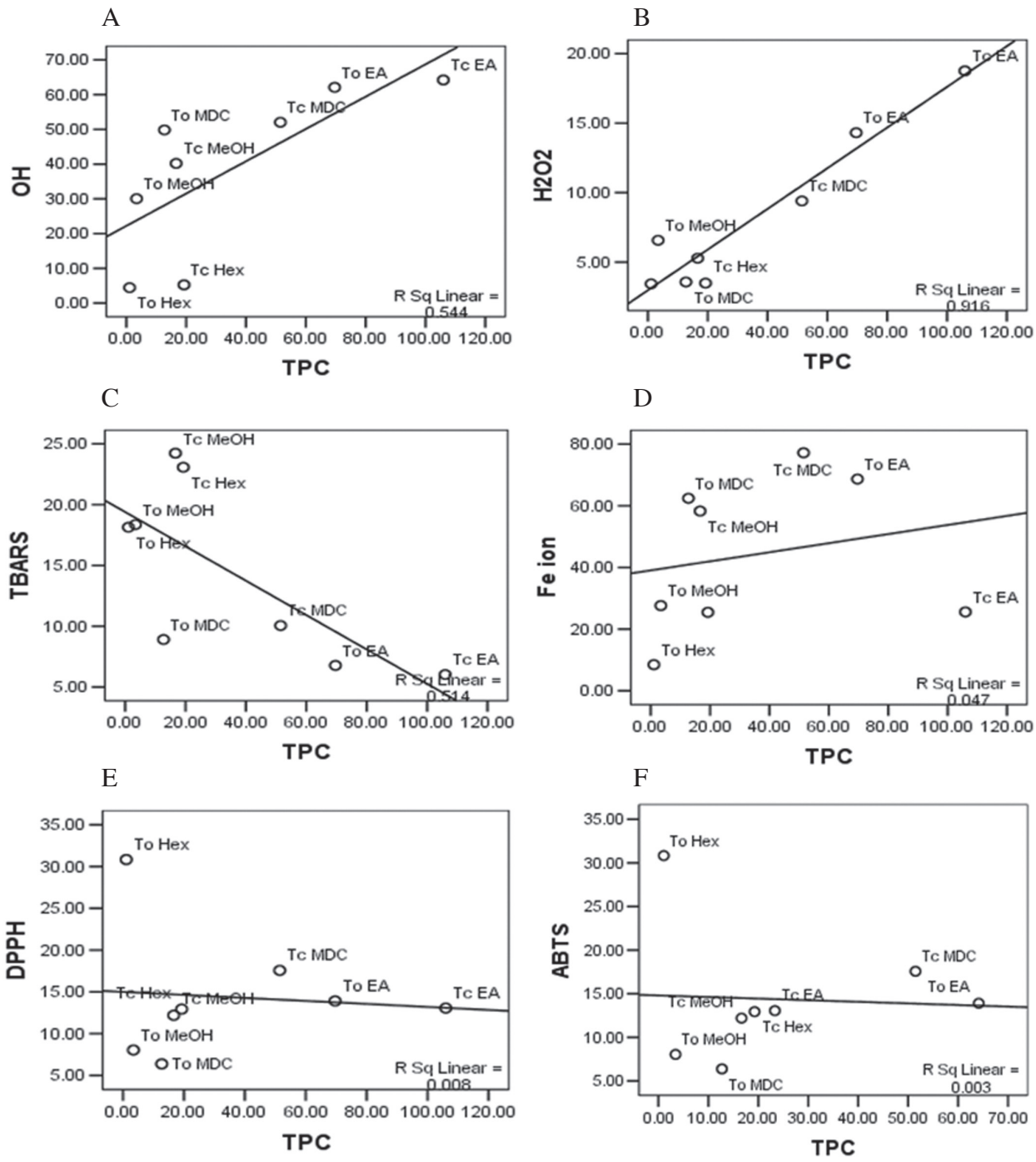


Figure 3A.9. Correlation between antioxidant activity assays vis-à-vis solvent fractions of *T. conoides* (To) and *T. ornata* (Tc) by scatter plot analyses (A-D). Scatter plot diagrams showing the correlation of TPC vis-à-vis (A) hydroxyl radical scavenging assay (n = 8, r = 0.738, P<0.05), (B) H_2O_2 scavenging activity (n = 8, r = 0.957, *P*<0.01), (C) lipid peroxidation (TBARS) inhibitory (n = 8, r = -0.717, *P*<0.05), (D) Fe^{2+} ion chelating activity (n = 8, r^2 = 0.047, *P*<0.01); (E) DPPH. scavenging assay (n=8, r = - 0.054, *P*>0.05); and (F) ABTS radical scavenging assay (n=8, r = - 0.054, *P*>0.05)

adduct formation. Likewise, the total phenolic content and Fe^{2+} ion chelating activity exhibited a negative correlation thus suggesting the presence of some compounds other than phenolics capable of chelating transition metals (Figure 3A.9). Earlier studies conducted by other researchers also showed that polysaccharides (e.g. alginates and fucoidan) and/or phytochelatins were more effective than phlorotannins for the detoxification and resistance to copper accumulation in *Ascophyllum nodosum* (Wang et al. 2012). In addition, some peptides as well as proteins found in seaweed extracts have also been reported to possess the abilities to chelate metal ions (Cian et al. 2012). The results lead to the conclusion that algal polyphenols are probably not strong chelators of transition metals. However, further study is needed to elucidate the mechanism of antioxidant action of different compounds in the seaweed extracts. No significant correlation between phenolic contents and DPPH and ABTS radical scavenging activities in the seaweed extracts also indicated the presence of compounds other than phenolics (small molecular weight polysaccharides, pigments, proteins or peptides) to be involved in the antioxidant activity. Reports of previous studies are also in agreement with our present findings that some seaweed extracts exhibited a lower correlation between TPC and antioxidant activity (Kuda et al. 2009).

3A.3.10. Chromatographic Evaluation of the Phenolic Compounds in the Methanol extract and EtOAc fraction of Seaweeds

The selected groups of phenolics in MeOH and EtOAc solvent extracts of *T. conoides* and *T. ornata* were separated and identified by the RP-HPLC method, are shown in Table 3A.2. A retention time (R_t) library of the standard phenolic compounds was constructed with detection at T_{max} 324 nm and 277 nm (Figure 3.5). The qualitative analysis of the seaweed extracts for phenolic compounds were obtained and the retention time for individual peak was compared to the library of standard phenolic compounds under the same HPLC conditions.The HPLC analysis indicated that salicylic acid, gallic acid, quercetin, and syringic acid were the predominant phenolics in the EtOAc fraction of *Turbinaria conoides*, whilst more polar chlorogenic acid and 2,5-dihydroxybenzoic acid were the major phenolics in MeOH extract of *Turbinaria conoides*. Coumaric acid, ferulic acid, epicatechin and epicatechin gallate were found to be ubiquitous in either of EtOAc fraction and MeOH extract. Chlorogenic acid (R_t 8.12) is one to elute first among other phenolic compounds with a R_t value recorded as 8.12. HPLC profiling of the MeOH extract from *T. conoides* (TC-A) identified four major peaks at T_{max} 324 nm in similar R_t as that of the standard phenolics, which include derivatives of hydroxy benzoic acid (gentisic acid) and hydroxy cinnamic acid (chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid). Similarly, two major peaks were observed at T_{max} 277 nm comprising of flavanols (epigallocatechin gallate and epicatechin gallate). It was observed that gentisic acid (at 324 nm) and epicatechin gallate (at 277 nm) were the predominant

Table 3A. 3. Major phenolic compounds (mg/g extract) identified in the MeOH (A) extract and EA (B) fractions of *T. conoides* (TC) *& T. ornata* (TO) by HPLC.

Sl.No	Phenolic acids	R_t (minute)	Phenolic compounds (mg/g extract) identified in the MeOH extract (A) and EtOAc fraction (B)			
	324nm		**TC-A**	**TC-B**	**TO-A**	**To-B**
1	Chlorogenic acid	8.12	0.3±0.0	-	3.2±0.01	43.0±1.14
2	Caffeic acid	10.49	-	-	4.4±0.11	8.2±0.05
3	2,5 dihydroxy benzoic acid	17.43	14.6±0.14	-	-	-
4	Coumaric acid	20.56	1.3±0.01	0.7±0.01	1.2±0.02	-
5	Ferulic acid	24.19	3.6±0.06	1.6±0.02	13.8±0.14	-
6	Quercetin	37.90	-	2.3±0.01	-	8.6±0.36
7	Salicylic acid	44.92	-	1.3±0.01	-	8.2±0.17
	277nm					
8	Gallic acid	5.39	-	21.4±0.25	9.6±0.08	64.8±1.32
9	Catechin	6.81	-	-	8.6±0.06	5.9±0.05
10	EGCG	8.13	31.2±0.11	-	-	14.3±0.84
11	Epicatechin	10.11	35.7±0.26	12.8±0.32	-	2.7±0.06
12	ECG	13.0	205.2±1.24	17.6±0.31	24.2±1.02	30.9±1.32
13	Syringic acid	14.78	-	73.5±2.11	15.1±0.09	15.3±0.79

Values are mean ± SD (n = 3)

phenolic compounds in TC-A (Figure 3A. 10). The EtOAc fraction of *T. conoides* (TC-B) showed major phenolic compounds at T_{max} 324 nm (Figure 3A.10) as representatives of hydroxy benzoic acid (salicylic acid), hydroxy cinnamic acid (ferulic acid), 4H-chromenone flavonol (quercetin), and 5-caffeoylquinic acid with cyclohexanecarboxylic acid derivative (chlorogenic acid). Four major peaks were observed at T_{max} 277 nm includes hydroxy benzoic acid derivative (gallic acid, syringic acid) and flavanols (epicatechin, epicatechin gallate). Among these, ferulic acid (at 324 nm) and syringic acid (at 277 nm) were the predominant phenolic compound in TC-B. Chlorogenic acid (t_R 8.12), caffeic acid (R_t 10.49), ferulic acid (R_t 24.19), and syringic acid (R_t 14.78) were the major constituents in MeOH fraction of *T. ornata*, whereas the principle components in EtOAc fraction were quercetin (R_t 37.9) and salicylic acid (R_t 44.92). Epicatechin and epicatechin gallate (R_t 13.0 min) with the chroman ring system were found to be ubiquitous in either of EtOAc and MeOH fractions of *Turbinaria ornata.* Four phenolic compounds were identified in the MeOH extract of *T. ornata* (TO-A) (Figure 3A, 11), which include hydroxy cinnamic acid derivatives (chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid) at T_{max} 324 nm. At T_{max} 277 nm, three hydroxy benzoic acid derivatives (gallic acid, syringic acid) and epicatechin gallate were recorded. It was observed that ferulic acid (at 324 nm) and epicatechin gallate (at 277 nm) were the predominant phenolic compounds in TO-A. EtOAc fraction of *T. ornata* (TO-B) realized six major phenolic compounds (Figure 3A. 11) including hydroxy cinnamic acid derivatives (chlorogenic acid, gentisic acid, *p*-coumaric acid, ferulic acid), 4H-chromenone flavonol (quercetin), salicylic acid (at T_{max} 324 nm), chroman flavanols (catechin, epigallocatechin gallate and epicatechin) and hydroxy benzoic acid derivative, syringic acid (at T_{max} 277 nm).

Phenolic acids, which are considered to be a major class of phenolic compounds, were reported to be present in abundance in seaweeds and other marine flora. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids in seaweeds. Seaweeds have previously been reported to be rich in phenolic acids, such as catechin, epicatechin, EGCG, caffeic acid, quercetin and others (de Quirós et al. 2010; López et al. 2011). Phenolic acids constitute a large group of naturally occurring organic compounds with a broad spectrum of pharmacological activities, and free radical scavenging activity in particular. These groups of compounds are hydroxylated derivatives of benzoic and cinnamic acids, which often occur in plants as esters, glycosides and bound complexes and are rarely present in free forms (Germano‘ et al. 2006). Phenolic acids differ according to the number and position of hydroxylation and methoxylation of the aromatic ring. There is a much higher quantity and diversity of hydroxycinnamates than hydroxybenzoates and they consist of *p*-coumaric, caffeic, and ferulic acids either.

The solvent extracts, which exhibited strong antioxidant activities, were found to contain high amounts of total and individual phenolics that may contribute to antioxidative activity. Ethyl

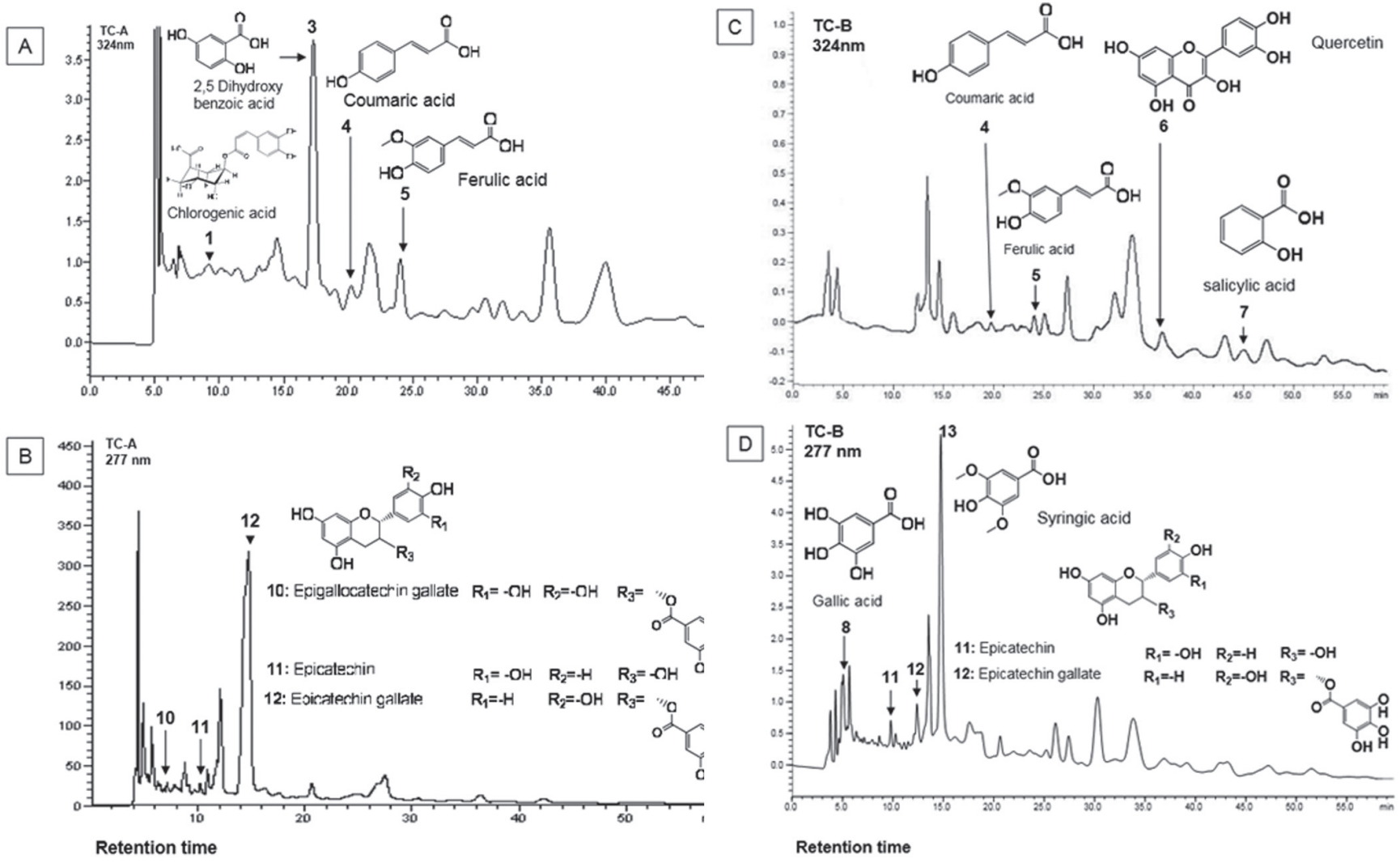


Figure 3A.10. HPLC/DAD chromatogram of phenoilc compounds in MeOH extract (A & B) and EtOAc fraction (C & D) of *T. conoides*. Detection was at 324 nm & 277 nm: Peak: (1) chlorogenic acid, (2) caffeic acid, (3) 2,5 dihydroxy benzoic acid, (4) coumaric acid, (5) ferulic acid, (6) quercetin, (7) salicylic acid, (8) gallic acid, (9) catechin, (10) epigallocatechin gallate, (11) epicatechin, (12) epicatechin gallate, (13) syringic acid.

acetate solvent fraction is an efficient solvent system for extraction of a broad range of phenolic compounds. Epicatechin gallate with 2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxychroman-3-yl 3,4,5-trihydroxybenzoate system recorded highest hydrophobicity (log P 2.46) among the phenolic compounds under consideration and therefore retained strongly by the C_{18} hydrophobic matrix of HPLC (Rt 13.0). Likewise, due to the fact that the hydrophobic parameter of salicylic acid (2-hydroxybenzoic acid) is 2.27, retained strongly in the hydrophobic matrix of HPLC (R_t 44.92). Interestingly, salicylic acid (R_t 44.92) and quercetin (R_t 37.9) appeared in ethyl acetate fraction of *Turbinaria ornata* and *T. conoides*, and not in MeOH fraction. The lower hydrophobicity (log P 0.22) and higher topological polar surface area (tPSA 144.52) of the 3-(3,4-dihydroxyphenyl)acryloyl)oxy)-4,5-dihydroxycyclohexanecarboxylic acid system of chlorogenic acid are implemented to describe the ready elution in HPLC column. Apparently pluralities of phenolic compounds with acrylic acid moiety are ubiquitous in the EtOAc and MeOH extracts of *Turbinaria* sp. Caffeic acid with 3-(3, 4-dihydroxyphenyl)acrylic acid, ferulic acid with 3-(4-hydroxy-3-methoxyphenyl)acrylic acid, and coumaric acid with 3-(4-hydroxyphenyl)acrylic acid are similar type of compounds with various hydroxyl and methoxyl substituents on the phenyl ring system. The close resemblance of their polar (tPSA 58-78) and hydrophobic (log P 1.2-1.5) properties implicated their appearance in both MeOH and EtOAc fractions.

Hydroxyl substituted benzoic acids such as gallic acid (with 3,4,5-trihydroxybenzoic acid system), syringic acid (with 4-hydroxy-3,5-dimethoxybenzoic acid) and 2,5 dihydroxy benzoic acid have close electronic (tPSA 76-98) and lipophilic properties (log P 0.5-1.4) thereby implicating the presence of similar type of compounds in the seaweed MeOH and EtOAc solvent extracts.

The phenolics with hydroxyl phenyl substituted chroman ring system as in epicatechin with 2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol moiety (tPSA 110.38, log P 1.50), epicatechin gallate with 2-(3,4-dihydroxyphenyl)-5,7-dihydroxychroman-3-yl 3,4,5-trihydroxybenzoate moiety (tPSA 177.14, log P 2.46), epigallocatechin gallate with 5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl 3,4,5-trihydroxybenzoate moiety (tPSA 110.38, log P 1.50), and 4H-chromen-4-one ring system as in quercetin with 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (tPSA 127.45, log P 0.35) appeared to be bonded strongly with the C_{18} matrix and therefore eluted later. Quercetin and epicatechin with chroman ring system were reported to have a hydroxyl group at C-3 of the ring system. Kim and Lee (2004) suggested structurally important role of the 3-OH group of the chroman ring responsible for enhancement of antioxidant activity. Phenolic compounds with OCH_3 substituted phenol ring or similar electron withdrawing groups as in hydroxyl substituted benzoic acids or hydroxyl phenyl substituted chroman ring system and α-β-unsaturated carboxylic acid group substituted to an aromatic phenolic ring as in substituted phenyl acrylic acid moiety were found to be ubiquitous in *Turbinaria* sp..

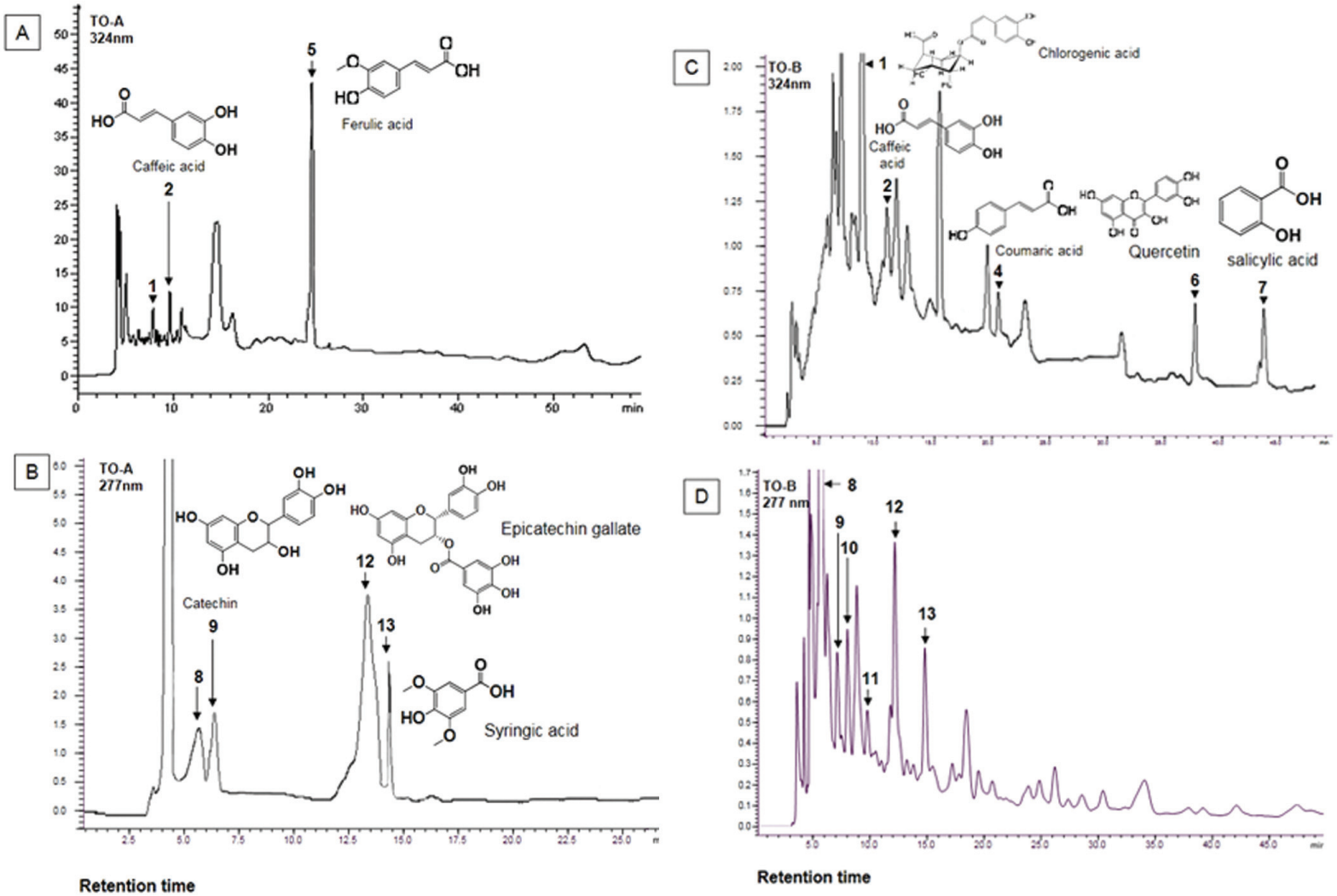


Figure 3A.11. HPLC/DAD chromatogram of phenoilc compounds in MeOH extract (A & B) and EtOAc fraction (C & D) of *T. ornata*. Detection was at 324 nm & 277nm: Peak: (1) chlorogenic acid, (2) caffeic acid, (3) 2,5 dihydroxy benzoic acid, (4) coumaric acid, (5) ferulic acid, (6) quercetin, (7) salicylic acid, (8) gallic acid, (9) catechin, (10) epigallocatechin gallate, (11) epicatechin, (12) epicatechin gallate, (13) syringic acid.

The results obtained in the present study don't imply that the compounds indicated in the standard are necessarily present in the seaweed solvent fractions, but gave a general idea that the type of phenolic compounds belonged to substituted phenyl acrylic acid, hydroxyl substituted benzoic acids, and hydroxyl phenyl substituted chroman ring system, leaving aside the chlorogenic acid with cyclohexanecarboxylic acid moiety. It is apparent that the antioxidant potential of phenolic compounds is dependent on the number and arrangement of the hydroxyl groups as well as the presence of electron-donating substituents in the ring structure (Lapornik et al. 2005).

The retention behavior of the phenolic acids in the bonded phase C_{18} matrix of the stationary phase of HPLC *vis-à-vis* the hydrophobic and electronic also have been described in detail to understand the types of bioactive phenolics in the EtOAc and MeOH fractions of *Turbinaria* sp. Due to the structural diversity of the phenolic acids, it is difficult to characterize every compound by RP-HPLC, but it will provide with the general idea regarding the major groups of phenolic compounds.

3A.4. Conclusions

The ethyl acetate fraction of *Turbinaria conoides* exhibited significantly higher total reduction capability (A_{700nm}1.07, 1 mg/mL), total phenolic content (106 GE/g), scavenging of H_2O_2 (>18%, 1 mg/mL) and hydroxyl radical scavenging activities (64%, 0.6 mg/mL) as compared with *Turbinaria ornata.* The ethyl acetate fraction of *Turbinaria ornata* exhibited higher Fe^{2+} ion chelating (>68%, 0.6 mg/mL), lipid peroxidation inhibitory (7 MDAEC/kg, 2 mg/mL), ABTS radical scavenging (14%, 0.6 µg/mL) and DPPH· radical scavenging activities (64%, 1 mg/mL). The HPLC analysis indicated that gallic acid (21 mg/g) and syringic acid (73 mg/g) were the predominant phenolic acids in the ethyl acetate fraction of *Turbinaria conoides*, whilst epicatechin gallate (205 mg/g) was the major phenolic acid identified in the methanol fraction. Epicatechin gallate (24 mg/g) and syringic acid (15 mg/g) were the major constituents in the methanol fraction of *Turbinaria ornata*, whereas the principle components in ethyl acetate fraction were gallic acid (64 mg/g) and chlorogenic acid (43 mg/g).

CHAPTER 3B

Evaluation of the Antioxidant Potential of *Anthophycus longifolius (= Sargassum longifolium), Sargassum plagiophyllum* and *Sargassum myriocystum*

Figure 3B. *A. longifolius* collected from the Gulf of Mannar

Figure 3B. *S. plagiophyllum* collected from the Gulf of Mannar

Figure 3B. *S. myriocystum* collected from the Gulf of Mannar

CHAPTER 3B

Evaluation of the Antioxidant Potential of *Anthophycus longifolius (= Sargassum longifolium), Sargassum plagiophyllum* and *Sargassum myriocystum*

3B.1. Introduction

Sargassum sp constitute a major share of brown seaweeds, and are potential sources of natural antioxidants, including phenolics *viz.*, phlorotannins, and carotenoid *viz.*, fucoxanthin, and isoprenoids (Swanson & Druehl 2002). Earlier studies reported antioxidant activities of the genus *Sargassum*, and was found that the various solvent extracts obtained from *Sargassum* sp exhibited nutraceutical value as potent antioxidants via alleviations of radical-induced toxicities (Heo et al. 2005; Kim et al. 2010), anti-obesity and blood GSH-Px properties (Matanjun et al. 2010), DPPH radical scavenging ability, reducing power and metal-chelating activity (Prabhasankar et al. 2009**)**. It was reported that polysaccharides from the *Sargassum* genus have antitumor activity (de Sousa et al. 2007**)**. *Sargassum fulvellum* had been reported to inhibit oxidation (Heo et al. 2005**)**.

The brown seaweeds were found to be abundantly available along the Gulf of Mannar off southeastern coast of the Indian subcontinent in all seasons. *Anthophycus longifolius (=Sargassum longifolium)* (Turner) Kützing*, S. plagiophyllum* C. Agardh, and *S. myriocystum J. Agardh* were selected in the present study to evaluate antioxidant activities and total phenolic contents. Although antioxidant properties of seaweeds were proved by numerous studies from the past two decades very few of them have been studied in detail from this very important delta region; findings of antioxidant activity could potentially shortlist candidate species to isolate bioactive fractions/principles to be used as nutraceuticals and/or functional foods, and candidates in combating carcinogenesis and inflammatory diseases. Therefore, the objective of the present study was to characterise the antioxidant properties of methanol (MeOH) extract and different solvent fractions (ethyl acetate (EtOAc), methylene chloride (MDC) and *n*-hexane) of these experimental seaweeds by different *in vitro* systems, to statistically evaluate the role of phenolic compounds responsible for antioxidant activity and to identify and quantify the phenolic compounds present in these species by reverse phase HPLC and determine the relationship between antioxidant activity and phenolic principles.

Table 3B.1. The yields obtained from the MeOH extract (as % w/w of seaweed on dry weight basis) and solvent fractions (as % w/w of total MeOH extract) of *A. longifolius*, *S. plagiophyllum* and *S. myriocystum*

Seaweed species	Methanol extract (%)	Solvent fractions		
		n-hexane	MDC	EtOAc
A. longifolius	6.37 ± 0.21	30.42 ± 0.13	23.57 ± 0.57	26.52 ± 0.35
S. plagiophyllum	8.62 ± 0.52	24.62 ± 0.25	21.39 ± 1.10	29.37 ± 1.36
S. myriocystum	14.53 ± 1.62	33.13 ± 0.74	24.33 ± 2.20	21.36 ± 2.57

Solvent fractions were obtained by partitioning MeOH extract with hexane, MDC, and EtOAc, respectively. All the values were expressed as mean ± SD; SD: standard Deviation. MDC: dichloromethane; EtOAc: ethylacetate

3B.2. Matarials and Methods

3B.2.1. Preparation of Seaweed Extracts and Fractions

The ground seaweed samples (0.5kg) were extracted with MeOH (1 L x 3) at an elevated temperature (40-45^{o}C) for 3 h. The samples were then filtered with to obtain the clarified filtrates (1.35 L), which were filtered, through Na_2SO_4 (100 g), and evaporated (40^{0}C) using a rotary evaporator (Buchii, Switzerland) under vaccum to dryness. This dark green viscous oily mass (150 mL) of MeOH extract was mixed with an equal volume of distilled water (150mL), and partitioned successively with *n*-hexane (150 mL x 3), MDC (150 mL x 3), and EtOAc (150 mL x 3) to furnish *n*-hexane (450 mL), MDC (450 mL), and EtOAc fractions (450 mL), respectively. The fractions were dried over anhydrous Na_2SO_4 (65- 70 g), and evaporated under reduced pressure using a rotary vacuum evaporator to furnish, the different solvent fractions of varying polarity. The yields of each fraction have been illustrated under Table 3B.1.

3B.2.2. Assays for Determination of Phenolic Contents and Antioxidant Potential of Seaweeds

The amount of total phenolics in the samples was determined by the established method described by Mcdonald et al. (2001). The ABTS.$^{+}$, 1, 1-diphenyl-2-picryl-hydrazil (DPPH.), hydroxyl radical radical assasy, hydrogen peroxide scavenging ability, thiobarbituric acid reactive substances (TBARS) formation inhibition assay, reducing ability and Fe^{2+} ion chelating potential were performed by the methods describey by Re et al. (1999), Shimada et al. (1992) and Klein et al. (1981), Ruch et al. (1989), Madsen et al. (1997), Oyaizu (1986) and Dinis et al. (1994) respectively with suitable modifications as described by section 3.5. HPLC based chromatographic identification of the standards and the samples were done as described by section 3.6.

3B.2.3. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied traits. Analysis were carried out in triplicate, and the means of all parameters were examined for significance (P<0.05) by analysis of variance (ANOVA). The Pearson correlation test was used to assess correlations between means. The experiments were carried out in three different batches of seaweeds. The mean variance in the data set was detected using principal component analysis (PCA). All data were mean centered and scaled to equal unit variance prior to PCA. The selected variables for PCA where the different antioxidant assays and phenolic content, as exhibited by the MeOH extract and different solvent fractions (EtOAc, MDC and *n*-hexane) of the seaweeds.

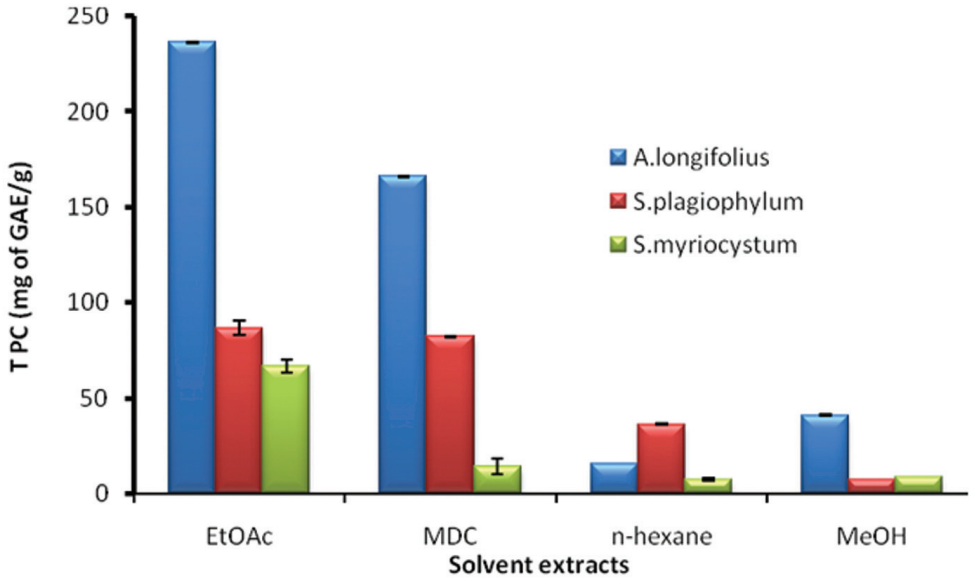


Figure 3B.1. TPC (mg of GE/g of the sample) of methanol extract and different solvent fractions of *A. longifolius*, *S. plagiophyllum* and *S. myriocystum.*

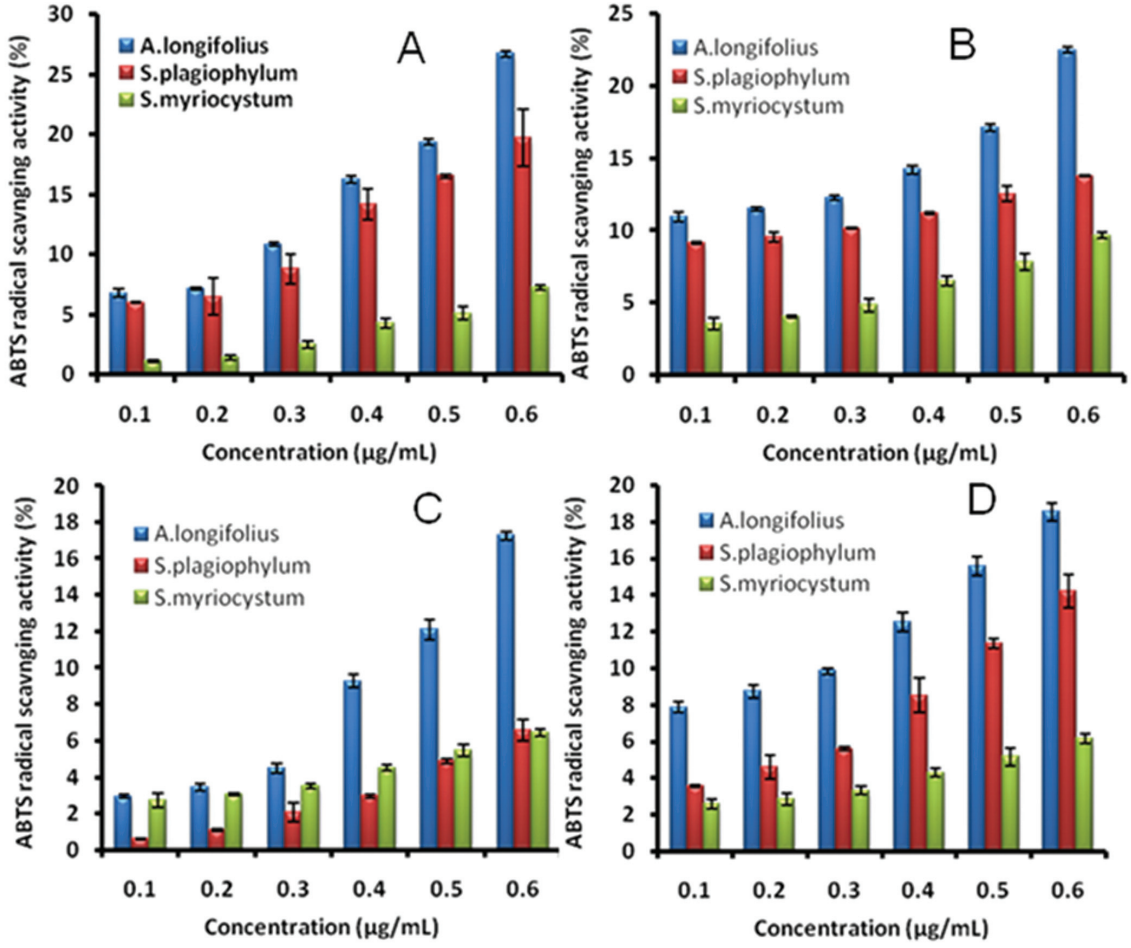


Figure 3B.2. ABTS.+ radical scavenging activities (%) of the EtOAc (A), MDC (B), n- hexane (C) fractions and MeOH extract (D) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation.

3B.3 Results and Discussion

3B.3.1. Determination of Total Phenolic Compounds (TPC)

The EtOAc and MDC fractions of *A. longifolius* registered significantly higher total phenolic contents (TPC) (236.4 and 166.1 mg GE/g, respectively, 5 g / L) than recorded in *S. plagiophyllum* and *S. myriocystum* (<100 mg GE/g) (P<0.05) (Figure 3B.1). Phenolic compounds that were reported to protect seaweed from photoxidation and to exhibit radical scavenging properties are reported in brown seaweeds (Escrig et al. 2001). It has been reported that brown algal phenolic compounds like phlorotannins with potential antioxidative activities are present in *Sargassum* sp. (Yan et al. 1996), suggesting the possibility of such compounds in our present study. Several studies focused on the antioxidative activities of phenolic contents in brown algal extracts and demonstrated their significant correlation (Chandini et al. 2008; Karawita et al. 2005). Phenolic antioxidants transfer H-atom to lipid peroxyl radicals to disrupt free-radical chain reaction to quench the radical process (Ruberto et al. 2001; Arbianti et al. 2007). A significant correlation was apparent between the phenolic content and the antioxidant activities in seaweed extracts in our present study as reported by earlier researchers (Chandini et al. 2008; Karawita et al. 2005).

3B.3.2. 2,2' - Azino-bis-3-ethylbenzothiozoline-6-Sulfonic Acid Diammonium Salt (ABTS) Radical Scavenging Activity (%)

EtOAc, MDC fractions, and MeOH extract of *S. plagiophyllum* contributed significantly (P<0.05) towards $ABTS^{\bullet+}$ radical scavenging activity (19.56, 13.76, and 14.03 %, respectively) than *n*-hexane fraction (6.75% at 0.6 µg/mL) (Figure 3B.2). EtOAc extract of *A. longifolius* was found to exhibit higher $ABTS^{\cdot+}$ radical scavenging activity (18.53%) than its other solvent fractions (Table 3B.2). The MeOH extract and *n*-hexane fractions of *A. longifolius* (0.6 µg/mL) exhibited significantly higher (P<0.05) $ABTS^{\cdot+}$ radical scavenging activities (7.26 and 9.64 %, respectively) than other seaweeds. A sample possessing ABTS. free radical-scavenging activity indicated that its mechanism of action was as a hydrogen donor thereby terminating the oxidation process by converting free radicals to more stable products. From these results it is apparent that EtOAc fraction of *A. longifolius* exhibited highest ability to deactivate $ABTS^{\bullet+}$ radical. Earlier studies reported that algal species including *Sargassum* sp contain potentially active compounds like sargaquinoic acid, sargachromenol or fucosterol capable of deactivating ABTS radical (Wang et al. 2009; Ham et al. 2010). The differences in scavenging activity of the extracts principally depend on the functional -OH groups apparently due to its ability for hydrogen donating ability (Chakraborty & Paulraj, 2010). This result shows that EtOAc fraction of *A. longifolius* could be potential rich sources of natural antioxidants.

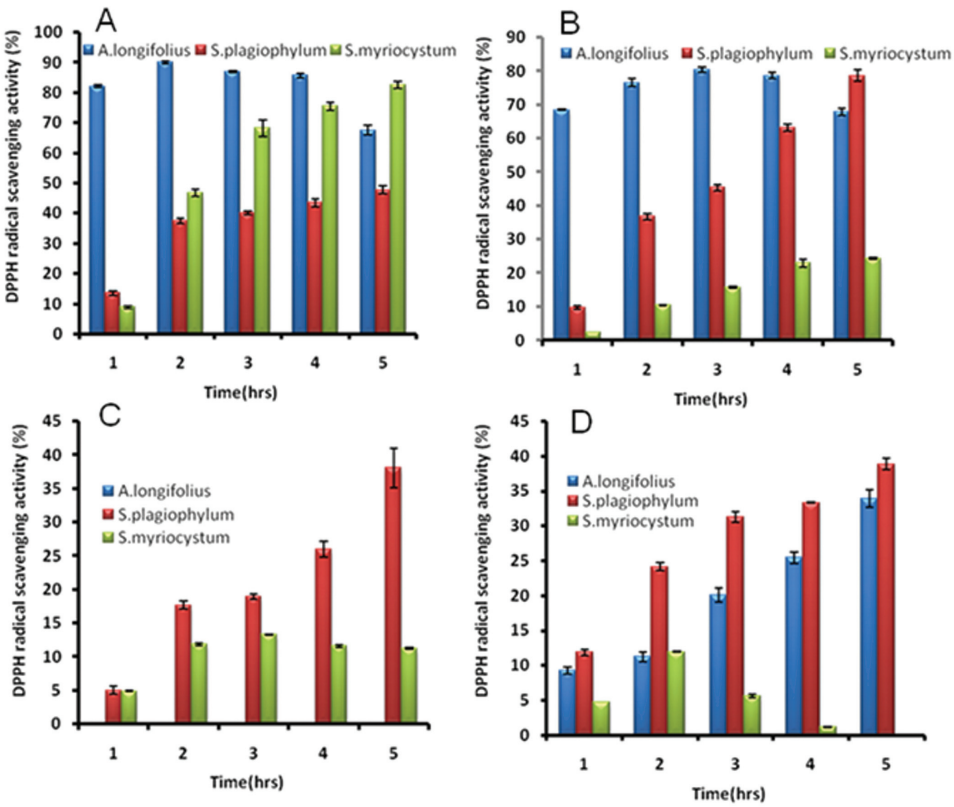


Figure 3B.3. DPPH radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with time (1-5hrs). Values are means of triplicate determinations and expressed with ± standard deviation.

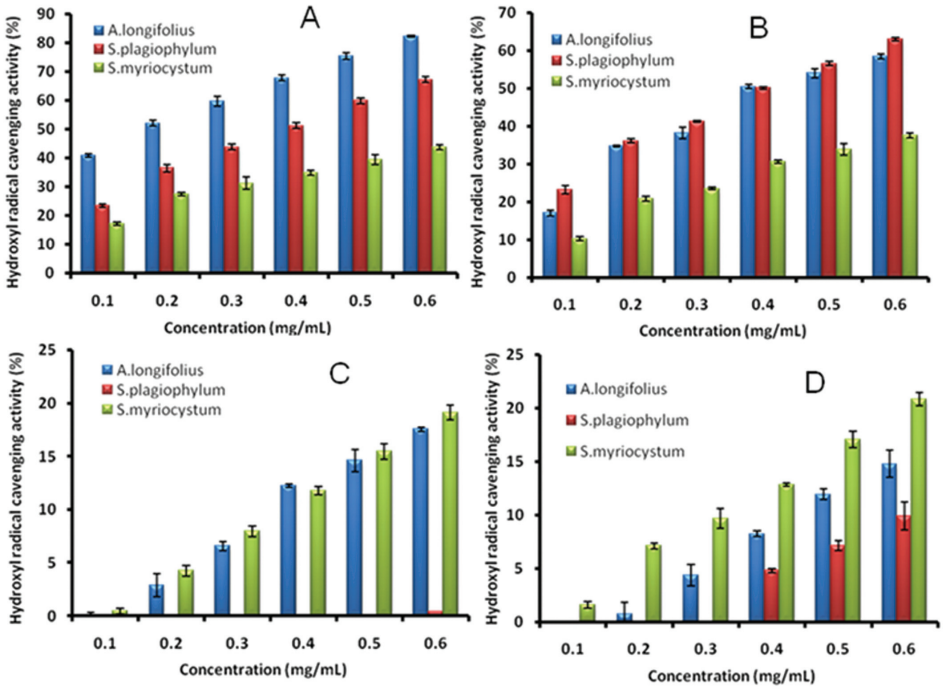


Figure 3B.4. Hydroxyl radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

3B.3.3. 1, 1-Diphenyl-2-Picryl Hydrazil (DPPH•) radical scavenging Activity (%)

EtOAc fraction of *A. longifolius* (1 mg/mL) exhibited a significantly higher (P<0.05) DPPH radical scavenging activity (89%) followed by MDC (79%) fraction (Table 3B.2), and the activities were found to be significantly higher than other seaweeds. EtOAc fraction of *S. myriocystum* exhibited significantly higher (P<0.05) DPPH free radical scavenging activity (82%) than other fractions. The radical scavenging activity of the MeOH extract and the solvent fractions of seaweeds with time are given by Figure 3B.3. In *S. plagiophyllum n*-hexane fraction (1mg/mL) recorded significantly higher DPPH radical scavenging activity (78%) than the other solvent fractions (P<0.05). A positive correlation with total phenolic content and DPPH assay, as realized by PCA analyses infer the presence of phenolic compounds responsible for antioxidant activities. Seaweeds species belonging to *Sargassum* sp. were reported to contain phenolic principles with multiple hydroxyl groups to enable them to donate H to DPPH· radical by hydrogen atom transfer (HAT) to deactivate the free radicals (Wang et al. 2009; Nakamura et al. 1996) and a positive correlation obtained between TPC and DPPH activity of EtOAc extract of *A. longifolius* in our present study revealed the role of phenolics present in the solvent fraction responsible for antioxidant activity.

3B.3.4. Hydroxyl Radical Scavenging Activity (%)

Hydroxyl radical scavenging activity was employed to understand the potential of different seaweed extracts against short-lived radicals, *viz.*, HO. radical. The EtOAc fraction of *A. longifolius* (0.6 mg/mL) exhibited a significantly higher (P<0.05) HO. radical scavenging activity (83.20%) than other seaweeds and solvent fractions (Table.3B 2). A reasonably good activity was recorded for an MDC fraction of *A. longifolius* and *S. plagiophyllum* (57-63%, 0.6 mg/mL). The activities were found to be proportionately decreased with concentrations, although EtOAc and MDC fractions of *A. longifolius* exhibited significantly higher activities even at lower concentrations (Figure 3B.4). The EtOAc and MDC fraction of *S. plagiophyllum* contributed significantly (P<0.05) towards H_2O_2 scavenging activity (IC_{50} 0.39 and 0.41 mg/mL, respectively) than MeOH extract and *n*-hexane fraction (IC_{50} 2.49 & 77.79 mg/mL, respectively). Solvent fractions (0.6 mg/mL) of *S. myriocystum* followed the same trend as exhibited by the solvent fractions of *A. longifolius* with IC_{50} values as: EtOAc (IC_{50} 0.71 mg/mL) > MDC (IC_{50} 0.81 mg/mL) > *n*-hexane fraction (IC_{50} 1.25 mg/mL) > MeOH (IC_{50} 1.39 mg/mL) extract, in increasing order. There are earlier reports showing that the antioxidants from *Sargassum* sp. exhibited higher hydroxyl radical scavenging activity (Nakai et al. 2006). Heo and Jeon, 2009, reported that the intracellular ROS generated by exposure to UV-B radiation, was significantly decreased by addition with various concentrations of fucoxanthin a carotenoid isolated from *Sargassum siliquastrum*. Potential HO. scavenging activities of the brown seaweed *Sargassam fulvellum* solvent extracts were reported in an earlier study (Heo et al. 2005).

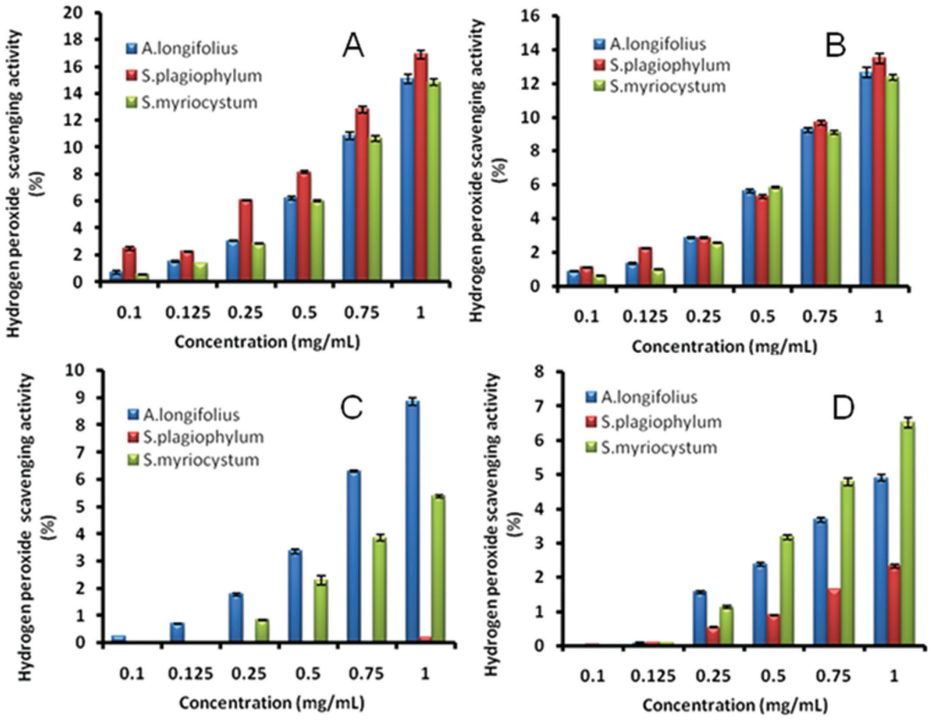


Figure 3B.5. Hydrogen peroxide scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

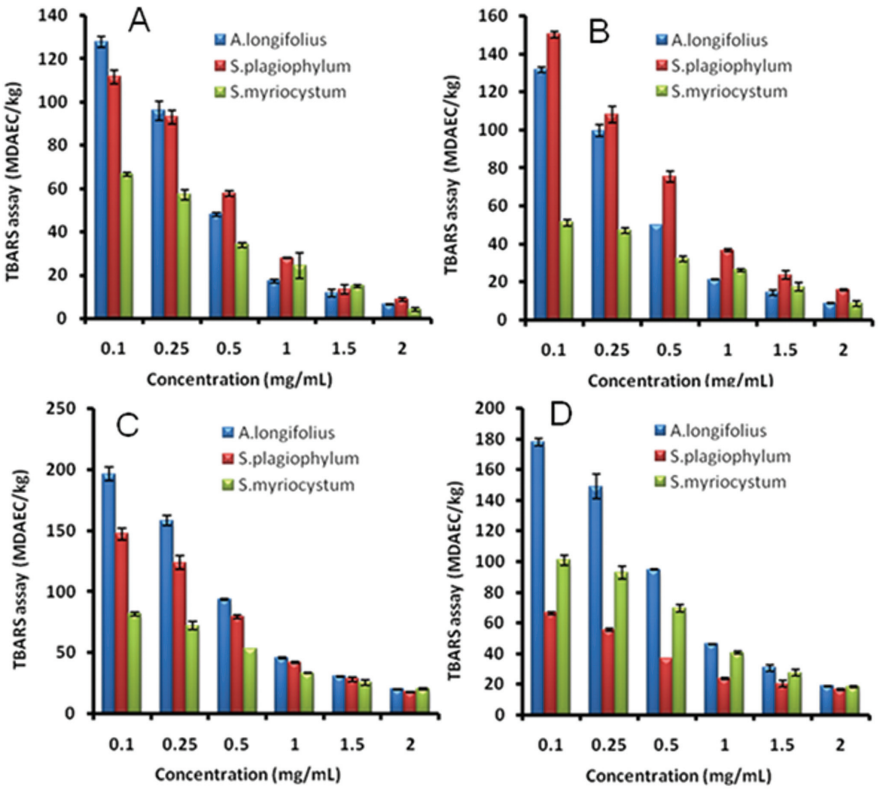


Figure 3B.6. TBARS formation inhibition assay (MDAEC/kg) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with concentration (0.1-2mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation.

3B.3.5. Hydrogen Peroxide (H_2O_2) Scavenging Activity (%)

EtOAc and MDC fractions of *A. longifolius* (1 mg/mL) exhibited significantly higher (P<0.05) H_2O_2 scavenging activities (15.08 and 12.6%, respectively) than other seaweeds (Table 3B.2). EtOAc and MDC fractions of *A. longifolius* and *S. plagiophyllum* found to retain their capacities to scavenge HO. even at lower concentrations (0.25 – 0.75 mg/mL) (Figure 3B.5). The activities of EtOAc and MDC fractions were found to be significantly higher towards H_2O_2 scavenging activity (P<0.05) (14.7 and 12.1%, respectively, 1 mg/mL) than MeOH extract (6.5%) and *n*-hexane fraction (5.3%) of *S. myriocystum*. H_2O_2 is a reactive non radical prooxidant and is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it may convert into more reactive species such as singlet oxygen and HO. radicals. It is obvious that the H_2O_2 activity of seaweeds may possibly be due to phenolic compounds, which are soluble with EtOAc, and, therefore exhibited higher scavenging activity than less polar solvent fractions. Other researchers also observed high H_2O_2 scavenging activity (~96%) in the EtOAc fraction of the brown seaweed *Ishige camera* (Heo et al. 2005**)** thus supporting the fact that the more polar fraction of *Sargassam* sp. harbour rich source of natural antioxidant principles.

3B.3.6. Lipid Peroxidation Inhibition Activity in Model System: Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity (MDAEC/kg)

The TBA reactive substances formation inhibitory capacities of EtOAc and MDC fractions (4.03 & 8.59 MDA equivalent compounds/kg or MDAEC/kg, respectively, 2 mg/mL) were significantly higher (P<0.05) than that of MeOH extract (18.37 MDAEC/kg), and *n*-hexane (20.30 MDAEC/kg) fraction of *S. myriocystum* (Figure 3B. 6)**.** Similarly the EtOAc and MDC fractions of *A. longifolius* were found to possess significantly higher activities (6.54 and 8.83 MDAEC/kg, respectively, 2 mg/mL) (P<0.05) than their solvent fractions (Table 3B.2). It was established that lipid peroxidation is a free radical (peroxides and hydroperoxides) mediated process in biological systems due to the oxidation of unsaturated fatty acids, and is associated with cellular damage as a result of oxidative stress. Antioxidants are able to trap free radicals, and terminate the chain reaction leading to lipid oxidation. It is therefore imperative to determine the degree of lipid peroxidation, and to explore the antioxidant activity of the seaweeds. The fatty acid breakdown products essentially contain malondialdehyde (MDA), which was measured through their reaction with thiobarbituric acid (TBA). The lower values in MDA equivalent compounds formed/kg (MDAEC/kg) indicate a higher lipid peroxidation inhibitory effect. There are no significant differences between the activities between different seaweeds except a few instances. The reduced levels of malonaldehyde, the monitor of lipid peroxidation, and marked antioxidant activity exhibited by EtOAc fraction of seaweeds, exhibited a positive correlation with phenolic contents as realized by PCA analyses. This indicates that the

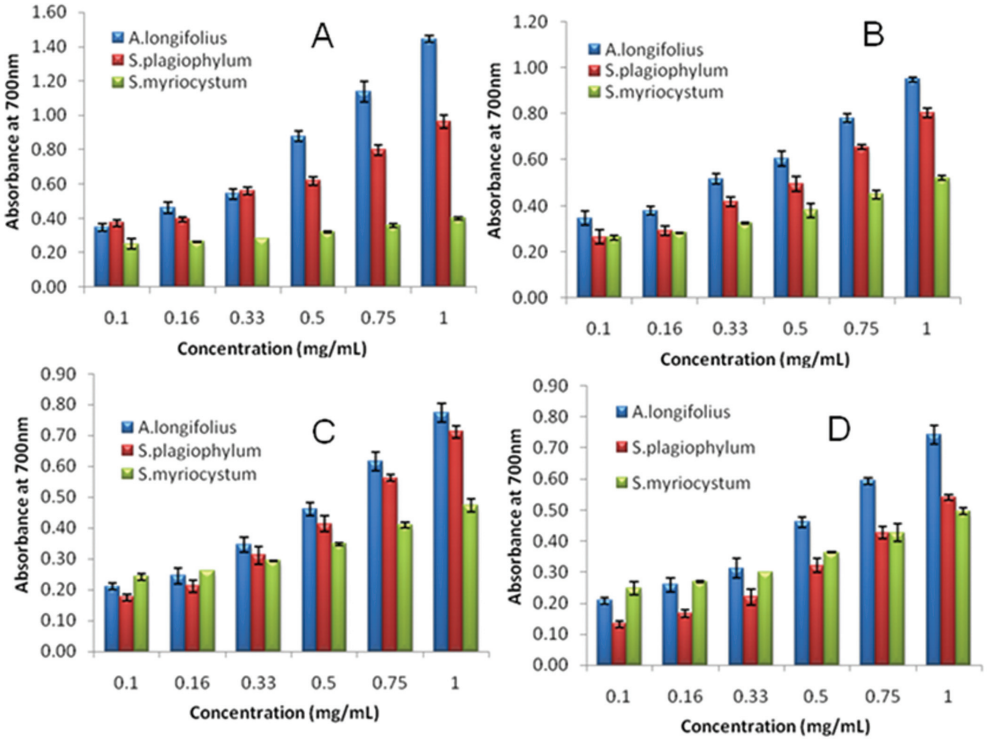


Figure 3B.7. Reducing ability (Ab_{700nm}) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with concentration (0.1-1mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

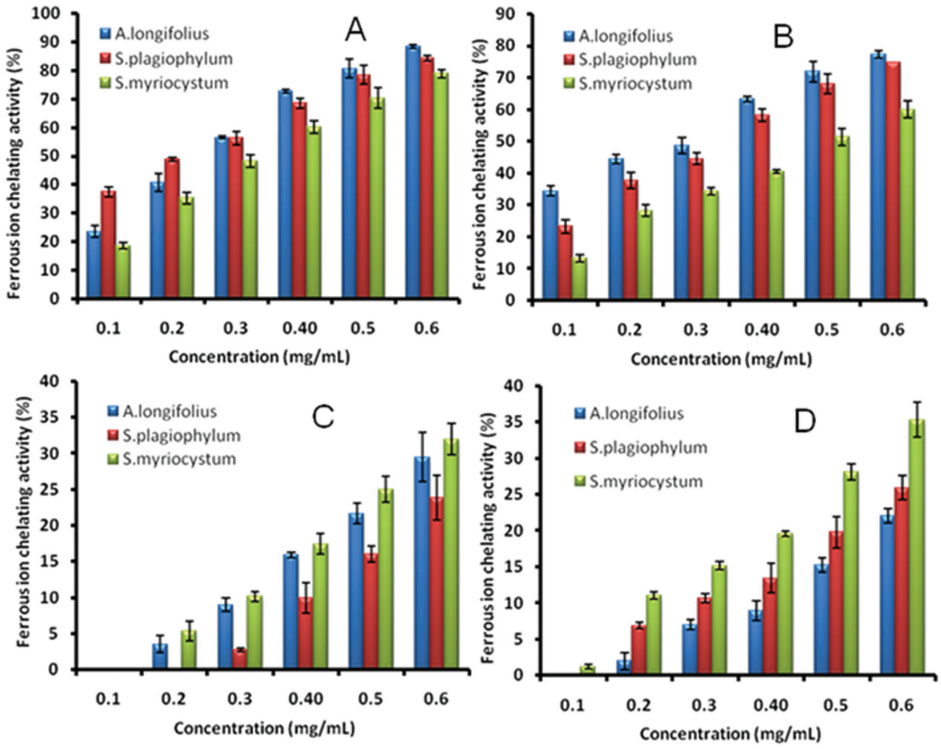


Figure 3B.8. Fe^{2+} chelating ability (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

inhibition of lipid peroxidation may be due to the presence of phenolic antioxidants that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions, may have roles to inhibit lipid peroxidation (Karawita et al. 2005).

3B.3.7. Evaluation of Reducing Ability ($Ab_{700}nm$)

The EtOAc fraction of *A. longifolius* (1 mg/mL) exhibited higher absorbance at 700 nm (Ab_{700nm} 1.4, 1 mg/mL) indicating a higher reducing power (Table 3B.2). The other fractions from the species followed the order CH_2Cl_2 (0.95) > *n*-hexane (0.76) > MeOH (0.73) towards reducing ability (Table 3B 1). Similarly, the reducing capacities of EtOAc and MDC fractions of *S. plagiophyllum* (Ab_{700nm} 0.96 & 0.81, respectively, 1 mg/mL) were found to be higher than that of MeOH extract (Ab_{700nm} 0.53) and *n*-hexane (Ab_{700nm} 0.71) fraction (Figure 3B.7). However, it is interesting to note that the antioxidant activities of MDC fraction, MeOH extract, and *n*-hexanic fraction of *S. myriocystum* were found to be significantly higher (P<0.05) (Ab_{700nm} 0.52, 0.50 and 0.47 respectively) than EtOAc fraction (Ab_{700nm} 0.41). The potential reducing abilities of EtOAc fraction generally depend on the presence of reductones, and a positive correlation between antioxidant activities, total polyphenols and reducing power in this study (Figure 3B. 7), and those reported earlier suggest EtOAc fractions of seaweeds to harbour phenolic compounds as reductones which have been shown to impart antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh 1998; Senevirathne et al. 2006**).** There are other studies reporting the presence of reducing agents in different brown seaweeds including *Sargassum* species collected from different regions (Karawita et al. 2005; Senevirathne et al. 2006**)**. It can also be attributed from these observations that these seaweed species may contain the presence of polyphenols which can act as reducing agents.

3B.3.8. Ferrous Ion (Fe^{2+}) Chelating Activity (%)

In the present study different fractions of *Sargassum* species demonstrated a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the Fe^{2+} binding capacity. EtOAc and MDC fractions of *A. longifolius* (0.6 mg/mL) exhibited significantly high Fe^{2+} chelating ability (88 and 77%, respectively) (P<0.05) than *n*-hexane fraction (30%) and MeOH extract (22%) (Table 3B.2). The EtOAc fraction of *S. plagiophyllum* exhibited a chelating ability of 84% followed by its MDC fraction 74%. Likewise, the EtOAc and MDC fractions of *S. myriocystum* exhibited significantly higher Fe^{2+} chelating ability (78 and 59%, respectively, 0.6 mg/mL) than other solvent fractions (P<0.05). To sum up, among the seaweed species, EtOAc fraction of *A. longifolius* exhibited significantly higher Fe^{2+} chelating activity than others (P<0.05). Comparison of Fe^{2+} chelating activity of different solvent fractions (at 0.6 mg/mL) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* showed that the MDC and EtOAc fractions were more effective

Table 3B.2 Total phenolic content and antioxidant activities of the MeOH extract and crude solvent fractions (*n*-hexane, MDC and EtOAc) of the brown seaweeds

Seaweeds	Solvent extracts			
	MeOH	*n*-hexane	MeOH	EtOAc
Total phenolic content (mg of gallic acid equivalence (GE)/g)				
A. longifolius	$41.11^{ap} \pm 0.51$	$15.86^{aq} \pm 0.02$	$166.09^{ar} \pm 0.14$	$236.35^{as} \pm 0.21$
S. plagiophyllum	$7.48^{bp} \pm 0.02$	$36.33^{bq} \pm 0.18$	$82.16^{br} \pm 0.07$	$86.78^{br} \pm 3.62$
S. myriocystum	$8.71^{cp} \pm 0.17$	$7.50^{cp} \pm 1.00$	$14.20^{cp} \pm 3.99$	$66.75^{cq} \pm 3.38$
ABTS radical scavenging activity (%)				
A. longifolius	$6.91^{ap} \pm 0.14$	$12.50^{aq} \pm 0.05$	$7.26^{ar} \pm 0.08$	$18.53^{aq} \pm 0.17$
S. plagiophyllum	$19.72^{bp} \pm 0.14$	$13.75^{bq} \pm 0.17$	$6.59^{bp} \pm 0.03$	$14.19^{br} \pm 0.15$
S. myriocystum	$7.26^{cp} \pm 0.14$	$9.64^{cp} \pm 0.23$	$6.46^{cp} \pm 0.20$	$6.16^{cp} \pm 0.07$
DPPH· radical scavenging activity (%)				
A. longifolius	$34.04^{ap} \pm 0.20$	ND^{aq}	$79.27^{ar} \pm 0.97$	$88.95^{as} \pm 0.89$
S. plagiophyllum	$38.19^{bp} \pm 0.65$	$38.09^{bp} \pm 0.07$	$77.39^{aq} \pm 1.34$	$46.60^{br} \pm 0.93$
S. myriocystum	$10.84^{cp} \pm 0.95$	$10.92^{cp} \pm 0.31$	$24.34^{bp} \pm 0.20$	$81.95^{cq} \pm 0.60$
Hydroxyl radical scavenging activity (%)				
A. longifolius	$14.87^{ap} \pm 0.19$	$17.95^{ap} \pm 0.38$	$57.05^{aq} \pm 1.64$	$83.20^{ar} \pm 1.01$
S. plagiophyllum	$9.78^{bp} \pm 0.36$	$0.46^{bq} \pm 0.01$	$63.47^{br} \pm 0.66$	$67.82^{br} \pm 0.60$
S. myriocystum	$20.92^{cp} \pm 0.21$	$19.38^{cp} \pm 0.22$	$37.98^{cq} \pm 0.36$	$43.94^{cr} \pm 0.29$
Scavenging capacity of H_2O_2 (%)				
A. longifolius	$4.68^{ap} \pm 0.21$	$8.63^{aq} \pm 0.21$	$12.64^{ar} \pm 0.34$	$15.08^{ar} \pm 0.26$
S. plagiophyllum	$2.30^{bp} \pm 0.03$	$0.23^{bp} \pm 0.02$	$12.94^{bq} \pm 0.72$	$16.28^{bq} \pm 0.76$
S. myriocystum	$6.50^{cp} \pm 0.02$	$5.34^{cp} \pm 0.05$	$12.10^{cp} \pm 0.25$	$14.72^{cp} \pm 0.12$
Total reduction capability (absorbance at 700nm)				
A. longifolius	$0.73^{ap} \pm 0.01$	$0.76^{ap} \pm 0.01$	$0.95^{ap} \pm 0.01$	$1.42^{ap} \pm 0.03$
S. plagiophyllum	$0.53^{bp} \pm 0.01$	$0.71^{bp} \pm 0.01$	$0.81^{bp} \pm 0.01$	$0.96^{bp} \pm 0.01$
S. myriocystum	$0.50^{cp} \pm 0.01$	$0.47^{cp} \pm 0.01$	$0.52^{cp} \pm 0.00$	$0.41^{cp} \pm 0.01$
Lipid peroxidation (TBARS) assay (MDAEC/kg)				
A. longifolius	$18.69^{ap} \pm 0.22$	$19.92^{ap} \pm 0.34$	$8.83^{aq} \pm 0.27$	$6.54^{aq} \pm 0.19$
S. plagiophyllum	$16.65^{bp} \pm 0.51$	$17.86^{bp} \pm 0.24$	$15.82^{bp} \pm 0.07$	$8.62^{bq} \pm 0.59$
S. myriocystum	$18.37^{ap} \pm 0.45$	$20.30^{ap} \pm 0.74$	$8.59^{aq} \pm 1.03$	$4.03^{cq} \pm 0.02$
Fe^{2+} ion chelating activity (%)				
A. longifolius	$22.19^{ap} \pm 0.74$	$29.45^{aq} \pm 2.38$	$77.42^{ar} \pm 0.76$	$88.23^{as} \pm 0.46$
S. plagiophyllum	$25.81^{bp} \pm 1.18$	$24.33^{abp} \pm 2.38$	$74.85^{aq} \pm 0.01$	$84.19^{br} \pm 0.58$
S. myriocystum	$35.60^{cp} \pm 1.79$	$32.16^{acp} \pm 1.58$	$59.74^{aq} \pm 1.89$	$78.65^{cq} \pm 1.11$

Data are the mean values of the triplicate and expressed as mean ± standard deviation. Row (p-s) and column values (a-c) with different letters are significantly different ($P<0.05$). MDC: dichloromethane, EtOAc : ethyl acetate. The concentration of the crude solvent fractions used were 1 mg/mL for DPPH radical scavenging activity, reducing capacity and H_2O_2 scavenging activity; 0.6 mg/mL for OH radical scavenging activity and Fe^{2+} ion chelating activity; 2 mg/mL for TBARS assay and 0.6 µg/mL for ABTS radical scavenging activity.

than methanol extract and *n*-hexane fraction towards Fe^{2+} chelating activity (Figure 3B.8). Ferrozine (disodium salt of 3-(2-pyridyl)-5, 6-bis (4-phenylsulfonic acid)-1, 2, 4-triazine) having –N=C-C=N- moiety can quantitatively form complexes with reduced form of Fe^{2+} to give red colored complex species, and in the presence of chelating agents, the formation of the red colored complex is interrupted, resulting in reduction in intensity of the colour. Fe^{2+} ion is potentially capable to initiate and accelerate lipid peroxidation to form peroxyl and alkoxyl radicals, which further abstract hydrogen to perpetuate the chain reaction. In the present study different fractions of *Sargassum* species demonstrated a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the Fe^{2+} binding capacity. The significantly high Fe^{2+} chelating ability exhibited by EtOAc and MDC fractions of *A. longifolius* suggesting their ability to deter Fe^{2+} dependent oxidation/ lipid peroxidation (Table 3B.2). To sum up, among the seaweed species, EtOAc fraction of *A. longifolius* exhibited significantly higher Fe^{2+} chelating activity than others (P<0.05). Comparison of Fe^{2+} chelating activity of different solvent fractions (at 0.6 mg/mL) of *A. longifolius, S. plagiophyllum* and *S. myriocystum* showed that both MDC and EtOAc fractions are more effective than methanol and *n*-hexane fractions towards Fe^{2+} chelating activity (Figure 3B.8). Significant positive correlations between TPC and Fe^{2+} ion chelating ability, as realized by PCA analyses, indicated the presence of phenolic compounds responsible for metal chelating ability. Earlier studies demonstrated that polyphenols derived from brown seaweeds including *Sargassum* sp. are potential metal chelators due to the presence of phenolics and polysaccharide fractions (Senevirathne et al. 2006; Prabhasankar et al. 2009)**.** Kuda et al. 2005 reported the presence of low-molecular compounds in *Sargassam lomentaria* with Fe^{2+} ion chelating activity. Phenolic phlorotanins were reported to be present in the polar solvent fractions of brown seaweeds, and were demonstrated to be strong chelators of heavy metals (Toth & Pavia, 2000**)**, and in this study the antioxidant activity of *Sargassam* sp. could be due to the presence of these groups of compounds, playing an important role in antioxidant activity by chelating effects.

3B.3.9. Correlations between Phenolic Contents and Different Antioxidant Activity Assays

The similarities and differences between different organic fractions of the three selected *Sargassum* species and the relationships among different antioxidant activity assays were statistically analyzed using PCA. The first two principal components explained 96.40% (PC 1 – 56.41%; PC 2 – 41.36%) of the total variance in the data set (Figure 3B.9B). Total phenolic content of EtOAc fraction was deduced to be significantly correlated with ABTS/DPPH/H_2O_2/OH. scavenging potential, Fe^{2+} ion chelating/reducing activity, and inhibition of TBA-MDA adduct formation. A negative correlation was realized between the total phenolic content of dichloromethane (MDC) fraction and inhibition of TBA-MDA adduct formation/H_2O_2 scavenging potential as evident

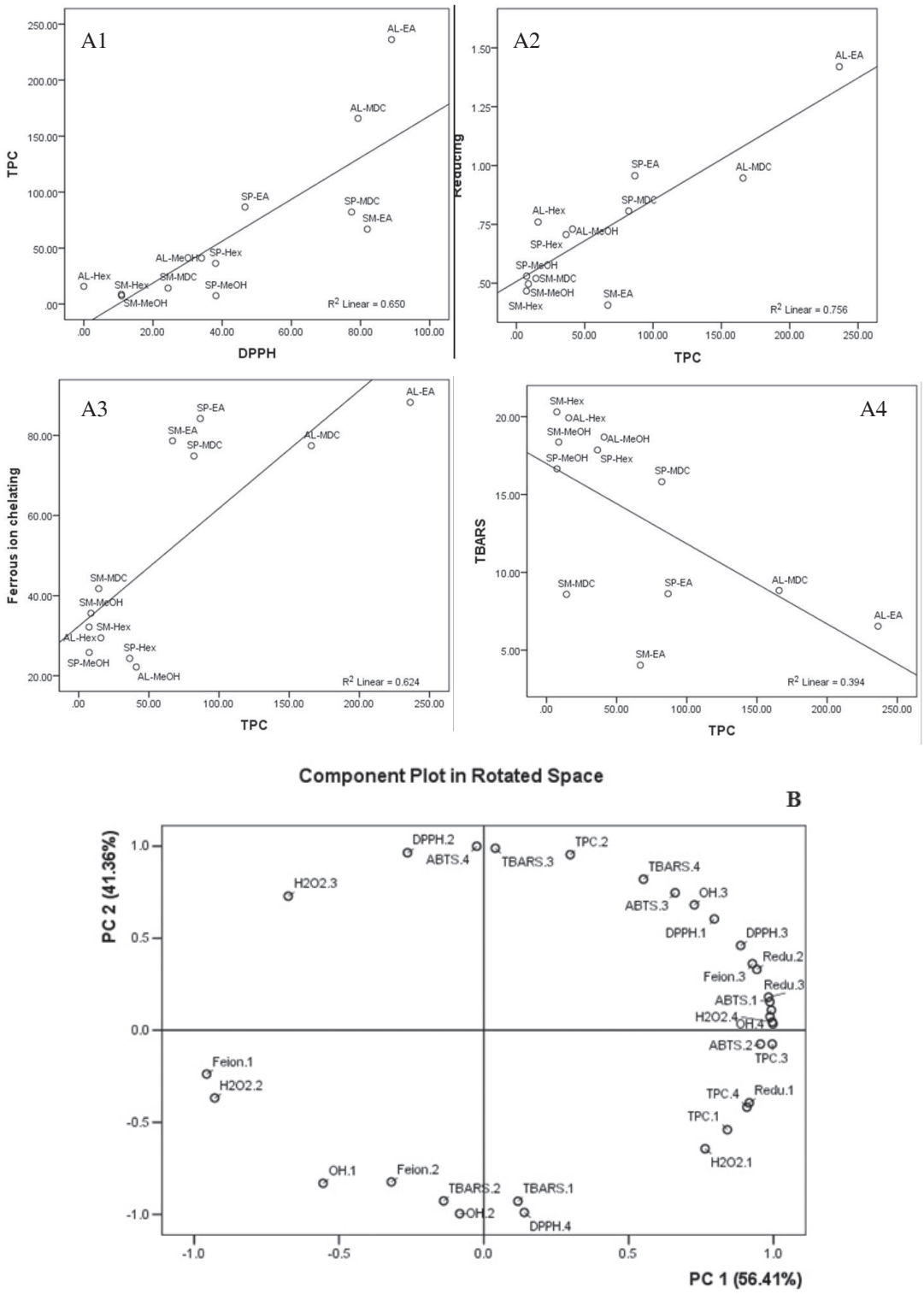


Figure 3B.9 Correlation between antioxidant activities vis-à-vis solvent fractions of experimental seaweeds by the scatter plot (A1-A4), and (B) loading plot diagrams of various components in rotated space. Scatter plot diagrams showing the correlation of TPC vis-à-vis (A1) DPPH. scavenging assay (n = 12, r = 0.806, *P*<0.01); (A2) reducing ability (A700 nm) (n = 12, r = 0.869, P<0.001); (A3) Fe2+ chelating ability (n = 12, r = 0.790, *P*<0.01); and (A4) lipid peroxidation (TBARS) inhibitory assay of different solvent extracts of *A. longifolius* (SL), *S. plagiophyllum* (SP) and *S. myriocystum* (SM) (n = 12, r = -0.628, *P*<0.05); (B) loading plot diagram (various components viz., PC-1 and PC-2 in rotated space) of antioxidant activities of different solvent fractions from experimental seaweeds (1: MeOH extract; 2: *n*-hexane extract; 3: MDC extract; and 4: EtOAc extract).

from the principle component analyses. MDC and EtOAc fractions of *A. longifolius* (SL), *S. plagiophyllum* (SP) and *S. myriocystum* (SM) exhibited high and significant correlation as demonstrated by Pearson correlation analysis (r = 0.916, *P*<0.01) towards total phenolic content and different antioxidant activities. The significant correlation observed between total phenolic content with DPPH/ Fe^{2+} ion chelating and reducing activity, and a negative correlation with inhibition of TBA-MDA adduct formation (Figure 3B.9A-D), indicating the presence of phenolic compounds capable of inhibiting the radical formation and compounds other than phenolics responsible for lipid peroxidation. This finding is in agreement with the earlier reports indicating a positive correlation between total phenolic contents and antioxidant activities of seaweed extracts (Wang et al. 2009). Other studies also observed a positive correlation between phenolic contents and antioxidant activity of different seaweed extracts (Escrig et al. 2001; Karawita et al. 2005). The negative correlation realized between the total phenolic content of dicloromethane (MDC) fraction and inhibition of TBA-MDA adduct formation/H_2O_2 scavenging potential as evident from the principle component analyses plot to demonstrated the role of non-phenolic antioxidants like polysaccharides to inhibit the radical chain reaction and lipid peroxidation. MDC and EtOAc fractions of *A. longifolius* (SL), *S. plagiophyllum* (SP) and *S. myriocystum* (SM) exhibited high and significant correlation as demonstrated by Pearson correlation analysis (r = 0.916, *P*<0.01) towards total phenolic content and different antioxidant activities suggests that the antioxidant activity exhibited by these fractions may mainly be due to the presence of polar compounds. The MDC and EtOAc fractions exhibited no correlation with hexane fraction, which further corroborate the above observation. From this observation it may be inferred that polyphenols present in algal fractions are responsible for its radical scavenging as well as chelating ability.

3B.3.10. Chromatographic Evaluation of the Phenolic Compounds in the Methanol extract and EtOAc fraction of Seaweeds

The selected groups of phenolics in MeOH and EtOAc solvent extracts of *A. longifolius* *S. plagiophyllum*, and *S. myriocystum* were separated and identified by the RP-HPLC method, are shown in Table 3B.2. A retention time (R_t) library of the standard phenolic compounds was constructed with detection at T_{max} 324 nm and 277 nm (Figure 3.5). The qualitative analysis of the seaweed extracts for phenolic compounds were obtained and the retention time for individual peak was compared with the library of standard phenolic compounds under the same HPLC conditions. Chlorogenic acid (R_t 8.12) is one to elute first among other phenolic compounds with a R_t value recorded as 8.12. The HPLC analysis indicated that chlorogenic acid, caffeic acid, 2, 5 dihydroxy benzoic acid, coumaric acid, ferulic acid, gallic acid and syringic acid were the predominant phenolics in the EtOAc fraction of *A. longifolius* whilst chroman flavanols (epicatechin gallate and

Table 3B.3. Major phenolic compounds (mg/g extract) identified in the MeOH (A) extract and EtOAc (B) fraction of *A longifolius* (AL), *S. plagiophyllum* (SP) *& S. myriocystum* (SM) by HPLC.

Sl.No	Phenolic acids	R_t (minute)	Phenolic compounds (mg/g extract) identified in the MeOH extract (A) and EtOAc fraction (B)					
	324nm		**AL-A**	**AL-B**	**SP-A**	**SP-B**	**SM-A**	**SM-B**
1	Chlorogenic acid	8.12	-	0.1±0.00	-	-	-	-
2	Caffeic acid	10.49	12.14±0.14	1.01±0.01	1.25±0.02	1.11±0.01	2.06±0.04	
3	2,5 dihydroxy	-	-	-	-	-	-	-
	benzoic acid	17.43	1.02±0.01	0.5±0.00	4.32±0.05	0.9±0.01	0.3±0.00	-
4	Coumaric acid	20.56	5.11±0.04	0.08±0.00	0.04±0.00	5.27±0.04	0.8±0.00	4.19±0.06
5	Ferulic acid	24.19	2.06±0.01	0.2±0.00	0.01±0.00	2.34±0.01	0.08±0.00	16.27±0.64
6	Quercetin	37.90	-	-	-	4.14±0.08	-	-
7	Salicylic acid	44.92	-	-	-	-	-	6.66±0.11
	277nm							
8	Gallic acid	5.39	9.32±0.03	2.12±0.01	-	-	-	-
9	Catechin	6.81	3.14±0.01	-	-	-	-	-
10	EGCG	8.13	-	-	-	-	3.03±0.01	2±0.01
11	Epicatechin	10.11	-	-	2.27±0.01	21.25±0.84	13.07±0.42	5.31±0.08
12	ECG	13.0	1.22±0.01	-	-	17.41±0.37	-	-
13	Syringic acid	14.78	24.30±0.84	23.31±0.77	26.47±1.01	52.14±1.32	82.71±2.10	0.4±0.01

Values are mean ± SD (n = 3).

catechin) were also found in the MeOH fraction of *A. longifolius.* More over MeOH fraction of *A. longifolius* did not show any traces of chlorogenic acid while it was present with the EtOAc fraction of *A. longifolius.* Caffeic acid, 2,5 dihydroxy benzoic acid, coumaric acid, ferulic acid, chroman flavanols (epicatechin) and syringic acid were found to be ubiquitous in either of EtOAc and MeOH fractions of *S. plagiophyllum.* More than these the EtOAc fraction of *S. plagiophyllum* was also found to contain 4H-chromenone flavonol (quercetin) and ECG. Both the EtOAc and MeOH fractions of *S. myriocystum* were found to contain coumaric acid, ferulic acid, flavanols (epigallocatechin gallate, epicatechin) and syringic acid. Salicylic acid was only present with the EtOAc fraction of *S. myriocystum* where as its MeOH fractions contained the derivatives of hydroxy cinnamic acid (chlorogenic acid and caffeic acid) which were absent with its EtOAc fraction.

The results showed that EtOAc is the best solvent system to extract phenolic acids from the MeOH extract of seaweed species. The EtOAc fractions of *Sargassum* sp., which exhibited strong antioxidant activities (in terms of scavenging $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ free radicals), were found to contain high amounts of total and individual phenolics, thereby validating the fact that phenolic compounds in these seaweeds contributed to the antioxidant activity. It has been reported that free radical-scavenging activity is greatly influenced by the phenolic composition of the sample (Cheung et al. 2003). Earlier results from our laboratory showed that EtOAc fraction of brown seaweeds belonging to *Turbinaria* sp. are efficient scavengers of free radicals (Chakraborty et al. 2013).

There are other reports which showed that HPLC analysis of the crude extracts from a brown seaweed *Stypocaulon scoparium* contained fourteen polyphenols, viz., gallic acid, catechin, epicatechin, rutin, *p*-coumaric acid, myricetin, quercetin, protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids (López et al. 2011). Reverse-phase liquid chromatographic study to analyse polyphenols in various red and brown seaweeds collected from the Atlantic coastal region in Galicia (North-Western Spain) revealed the presence of polyphenolic compounds like catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and gallic acid (de Quirós et al. 2010).

3B.4. Conclusions

EtOAc fraction of *A. longifolius* exhibited higher total phenolic content (mg of gallic acid equivalence (236.35 GE/g), ABTS radical scavenging activity (18.53%), $DPPH\cdot$ radical scavenging activity (88.95%), hydroxyl radical scavenging activity (83.20%), reduction capability (Ab_{700nm} 1.42), Fe^{2+} ion chelating activity (88.23%). EtOAc fraction of *S. plagiophyllum* exhibited higher H_2O_2 scavenging capacity (16.28 %) and that of *S. myriocystum* exhibited higher lipid peroxidation

inhibition ability (4.03 MDAEC/kg). The HPLC analysis indicated that chlorogenic acid, caffeic acid, 2, 5 dihydroxy benzoic acid, coumaric acid, ferulic acid, gallic acid and syringic acid were the predominant phenolics in the EtOAc fraction of *A. longifolius* whilst chroman flavanols (epicatechin gallate and catechin) were also found in the MeOH fraction of *A. longifolius.* Caffeic acid, 2,5 dihydroxy benzoic acid, coumaric acid, ferulic acid, chroman flavanols (epicatechin) and syringic acid were found to be ubiquitous in either of EtOAc and MeOH fractions of *S. plagiophyllum.* Both the of EtOAc and MeOH fractions of *S. myriocystum* were found to contain coumaric acid, ferulic acid, flavanols (epigallocatechin gallate, epicatechin) and syringic acid. The present study provides valuable information regarding the potential of these brown seaweeds especially *A. longifolius* and *S. plagiophyllum* to develop natural alternatives over synthetic antioxidants as oxidative stress induced disease curing remedy.

CHAPTER 3C

Evaluation of the Antioxidant Potential of *Stoechospermum marginatum, Padina tetrastomatica* and *Padina gymnospora*

Figure 3C. *Stoechospermum marginatum* collected from the Gulf of Mannar

Figure 3C. *Padina tetrastomatica* collected from the Gulf of Mannar

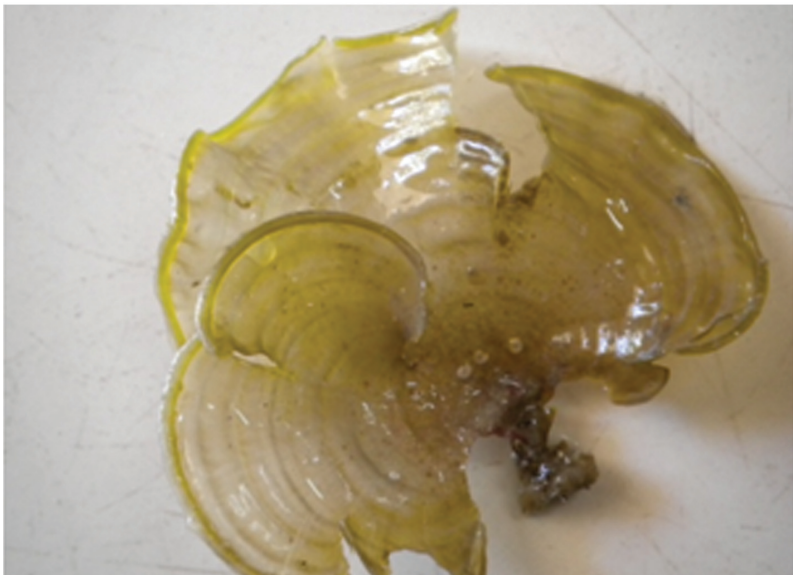


Figure 3C. *Padina gymnospora* collected from the Gulf of Mannar

CHAPTER 3C

Evaluation of the Antioxidant Potential of *Stoechospermum marginatum, Padina tetrastomatica* and *Padina gymnospora*

3C.1. Introduction

Brown seaweeds were reported to possess protective enzymes and antioxidative molecules (phlorotannins, ascorbic acid, tocopherols, carotenoids, phospholipids, chlorophyll related compounds, bromophenols, catechins, mycosporine-like amino acids, polysaccharides, etc.) (Pavia et al. 1986). Solvent extracts from several brown and red seaweeds harvested in Spain (Escrig et al. 2001), China (Yan et al. 1998) and Japan (Yan et al. 1999), have demonstrated potential antioxidant activity *in vitro* thereby indicating the importance of these marine flora as valuable health ingredient. Antioxidant and anticoagulant activity of phenolics and sulfated polysaccharides have been identified from several brown seaweeds especially from *Padina* sp (Matanjun et al. 2008). Alcoholic extracts from *Padina australis* were reported to possess DPPH radical scavenging activity (Gunji et al. 2007). It was already reported that *Stoechospermum marginatum* as an important alginophyte of India for use as food, fodder, and in dairy products and rich in sulfated fucans with spasmogenic and antiherpetic activities (Mrugaiyan & Sivakumar, 2008).

The objective of the present study was to characterise the antioxidant properties of methanol (MeOH) extract and different solvent fractions (ethyl acetate, methylene chloride and *n*-heaxane) of *Stoechospermum marginatum* (C. Agardh) Kützing, *Padina tetrastomatica* Hauck, and *P. gymnospora* (Kützing) Sonder by different *in vitro* systems, to statistically evaluate the role of phenolic compounds responsible for antioxidant activity and to identify and quantify the phenolic compounds present in these species by reverse phase HPLC and determine the relationship between antioxidant activity and phenolic principles.

3C.2. Materials and Methods

3C.2.1. Preparation of Seaweed Extracts and Fractions

The ground and shade-dried seaweed samples (100 g) were extracted with MeOH (500 mL x 3) and partitioned successively with *n*-hexane (100 mL x 2), MDC (100 mL x 2) and EtOAc (100 mL x 2) to furnish *n*-hexane (200 mL), MDC (200 mL) and EtOAc fractions (200 mL), respectively. The extracts were dried over anhydrous Na_2SO_4 (30 g) and evaporated under reduced pressure to furnish *n*-hexane, MDC and EtOAc fractions respectively. The yield obtained for

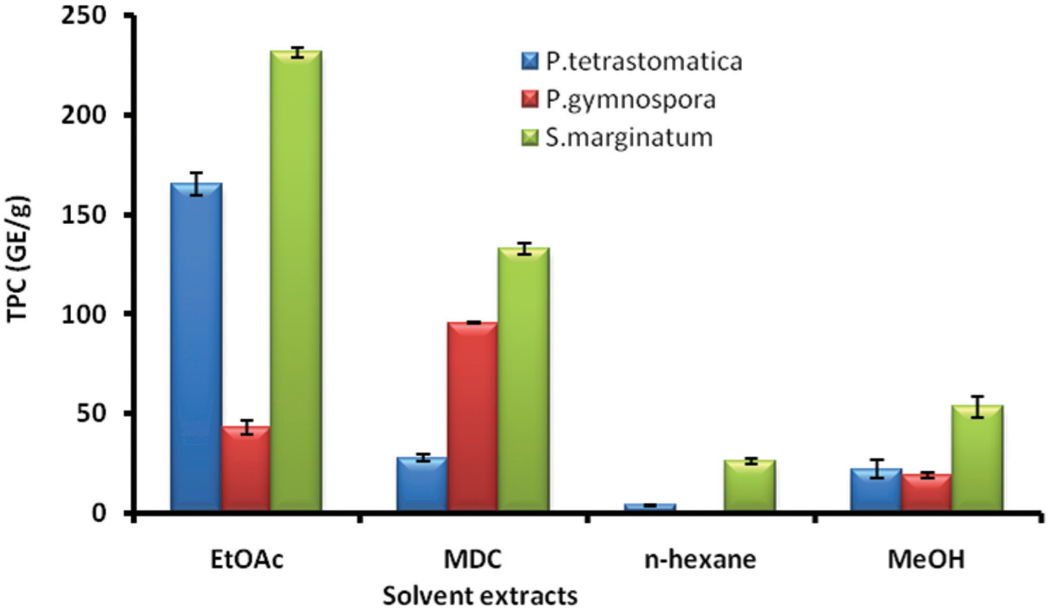


Figure 3C.1. TPC (mg of GE/g of the sample) of methanol extract and different solvent fractions of *S. marginatum, P. tetrastomatica* and *P. gymnospora*

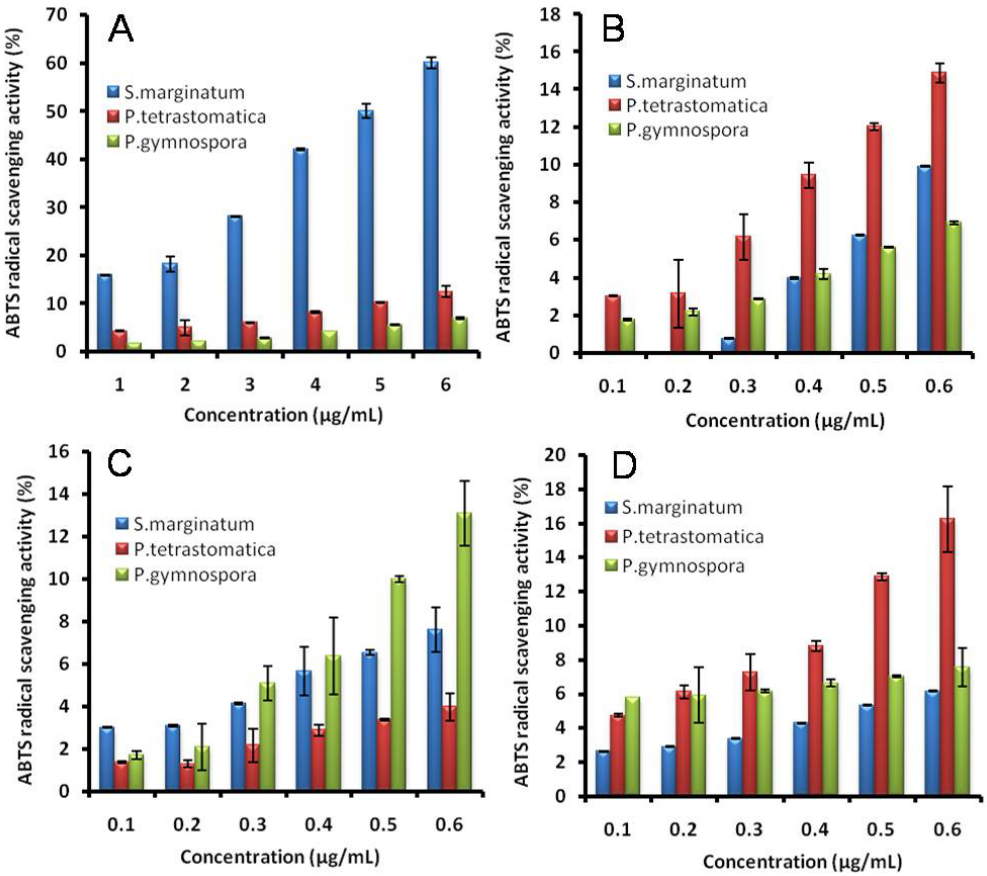


Figure 3C.2. ABTS.+ radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica* and *P. gymnospora* with Concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation.

methanolioc fraction of *P. tetrastomatica* was recorded to be higher (13.52 ± 1.36 % dw) than that obtained for *P. gymnospora* (10.79 ± 0.59 % dw), and *S. marginatum* (7.73 ± 0.72 % dw). On partitioning with *n*-hexane, MDC and EtOAc, the yield obtained for *P. tetrastomatica* was 30.79 ± 0.21%, 27.32 ± 3.27 %, and 16.33 ± 0.57 % (% w/w MeOH extract), respectively. The yields from *P. gymnospora* were registered as 33.02 ± 0.47 %, 24.86 ± 1.36 %, and 20.16 ± 0.92 %, respectively as % w/w of MeOH extract, and from *S. marginatum* as 27.94 ± 1.11 %, 22.36 ± 2.10 %, and 26.55 ± 1.17 % w/w of MeOH extract, in that order.

3C.2.2. Assays for Determination of Phenolic Contents and Antioxidant Potential of Seaweeds

The amount of total phenolics in the samples was determined by the established method described by Mcdonald et al. (2001). The ABTS.$^{+}$, 1, 1-diphenyl-2-picryl-hydrazil (DPPH.), hydroxyl radical radical assasy, hydrogen peroxide scavenging ability, thiobarbituric acid reactive substances (TBARS) formation inhibition assay, reducing ability and Fe^{2+} ion chelating potential were performed by the methods describey by Re et al. (1999), Shimada et al. (1992) and Klein et al. (1981), Ruch et al. (1989), Madsen et al. (1997), Oyaizu (1986) and Dinis et al. (1994) respectively with suitable modifications as described by section 3.5. HPLC based chromatographic identification of the standards and the samples were done as described by section 3.6

3C.2.3. Statistical Analysis

Statistical evaluation was carried out with Statistical Programme for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied traits. Analyses were carried out in triplicate, and the means of all parameters were examined for significance (P<0.05) by analysis of variance (ANOVA). Pearson correlation test was used to assess correlations between means. The experiments were carried out in three different batches of seaweeds. The mean variance in the data set was detected using principal component analysis (PCA). All data were mean centered and scaled to equal unit variance prior to PCA.

3C.3. Results and Discussion

3C.3.1. Determination of Total Phenolic Compounds (TPC)

Phenolic compounds are one of the most effective antioxidants reported in brown seaweeds (Escrig et al. 2001). EtOAc extract of S. marginatum (5 mg/mL) registered significantly higher (P<0.05) total phenolic content (TPC, 231.49 mg GE/g extract) than other solvent extracts (Figure 3C.1) It is apparent that *n*-hexane fractions of the seaweeds do not contain any phenolic compounds. The polar EtOAc and MDC fractions of *Padina* sp and *Stoechospermum marginatum*

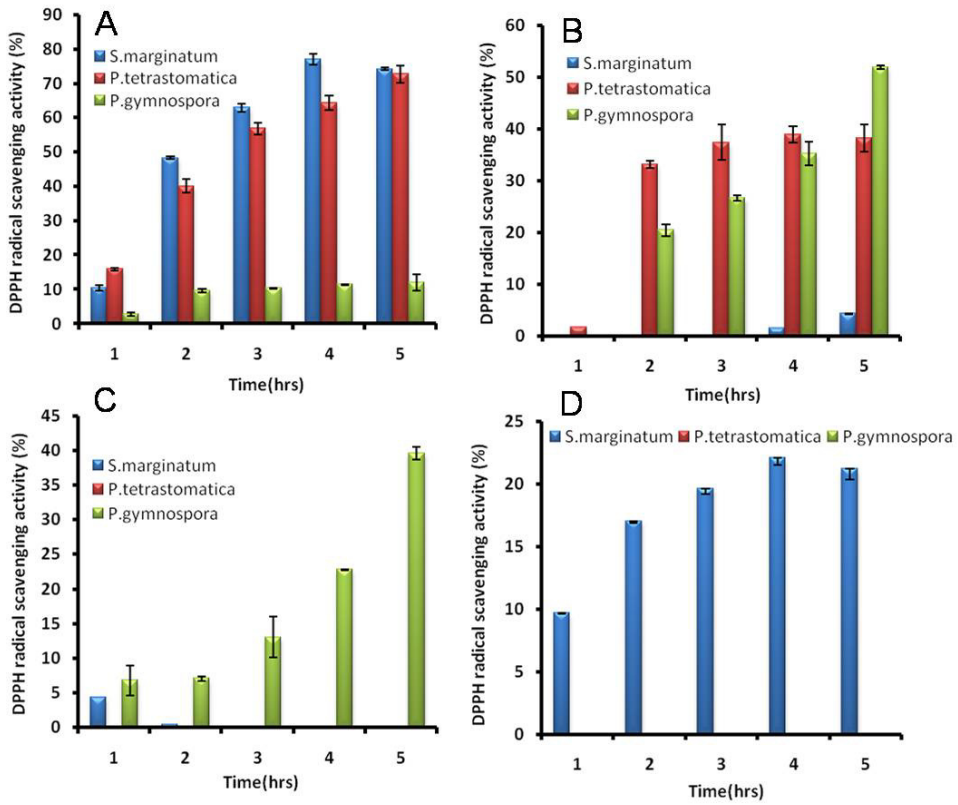


Figure 3C.3. DPPH radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica*, and *P. gymnospora* with time (1-5hrs). Values are means of triplicate determinations and expressed with ± standard.

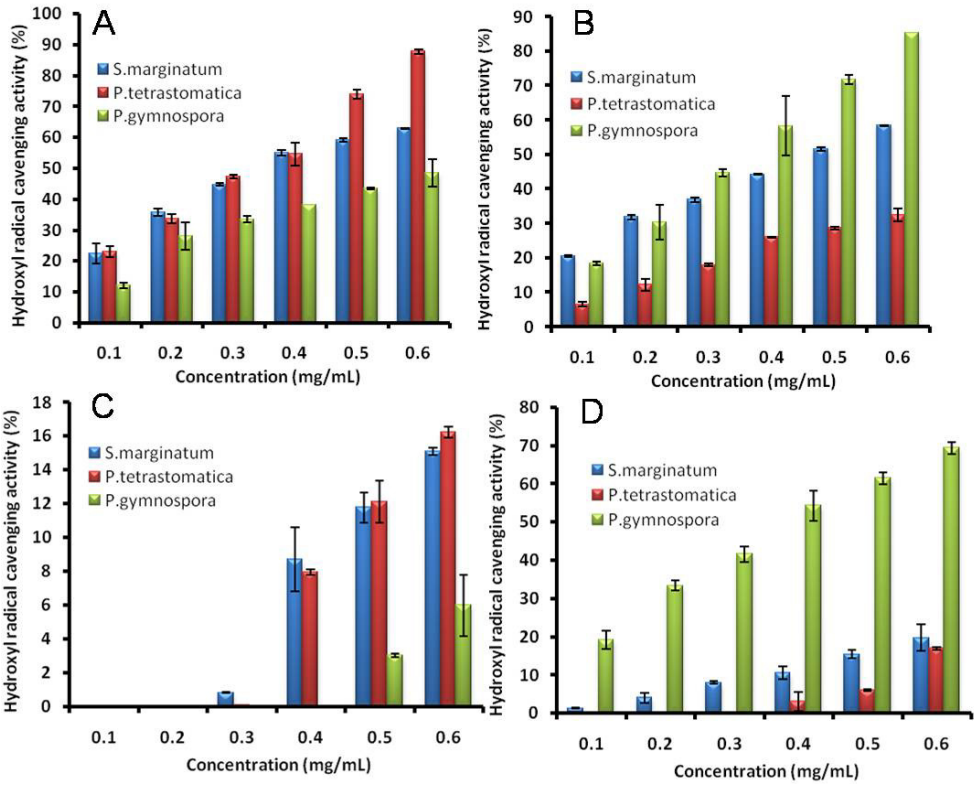


Figure 3C.4. Hydroxyl radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica* and *P. gymnospora* with Concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard.

exhibited higher phenolic content indicating their high antioxidant potential. Earlier studies illustrated the antioxidant activity of phenolic compounds in brown seaweed Sargassum kjellmanianum (Zubia et al. 2007). The phenolic contents of the solvent extracts of the seaweeds selected in this study were found to be significantly higher than those reported in literature (Chew et al. 2008; Escrig et al. 2001). Polyphloroglucinol phenolics (phlorotannins) were reported to be the major antioxidantive phenolic components in brown seaweeds (Pavia & Aberg, 1996).

3C.3.2. 2,2' -Azino-bis-3-ethylbenzothiozoline-6-Sulfonic Acid Diammonium Salt (ABTS) Radical Scavenging Activity (%)

In general, the $ABTS^{\bullet+}$ radical scavenging activity exhibited by the MeOH extract and *n*-hexane fractions were not significantly different ($P>0.05$) from each other, whereas MDC and EtOAc fractions registered significant difference ($P<0.05$) from other solvent extracts. EtOAc fraction of *S. marginatum* (0.6µg/mL) endowed with significantly higher ($P<0.05$) $ABTS^{\cdot+}$ radical scavenging activity (60.08%) than other solvent fractions (Table 3C.1). A significantly higher activities towards scavenging $ABTS^{\cdot+}$ radical (16-60%) ($P<0.05$) were apparent even at lower concentrations (0.1-0.6 µg/mL) than other solvent extracts (Figure 3C.2). The $ABTS^{\cdot+}$ radical scavenging activity of the solvent fractions and their IC_{50} values are shown in Table 2. The differences in scavenging activity of the extracts principally depend on the functional -OH groups apparently due to its ability for H-donating ability (Chakraborty & Paulraj, 2010). However, it is interesting to note that the antioxidant activity of the *n*-hexane fraction of *P. gymnospora* was significantly higher (IC_{50} 2.2 µg/mL). This may be explained due to the presence of carotenes/other pigments with long hydrocarbon chain and aminated compounds. From these results it is apparent that EtOAc fraction of *S. marginatum* exhibited highest ability to deactivate $ABTS^{\bullet+}$ radical, whereas MeOH fraction of *P. tetrastomatica* was most effective. An earlier report indicates that radical-scavenging capacity of MeOH extracts of brown seaweeds might be due to phenolic -OH groups (Ragan & Glombitza, 1986)

3C.3.3. 1, 1-Diphenyl-2-Picryl Hydrazil (DPPH•) radical scavenging Activity (%)

The fundamental principle of the DPPH method is the reduction of the DPPH radical in alcoholic solution by an H-donator antioxidant (AH) to form the non-radical form (DPPH-H). In the present study, the EtOAc fraction of *S. marginatum* registered significantly higher ($P<0.05$) DPPH radical scavenging activity (77.51%) followed by MeOH (21.98%). The EtOAc fraction of *P. tetrastomatica* endowed with significantly higher ($P<0.05$) DPPHð scavenging activity (72.13%), followed by MDC (50.26%) (Figure 3C.3). An earlier report indicated that sulphated polysaccharides in brown seaweed *P. tetrastromatica*, contributed to the antioxidant activity (Chew et al. 2008). Interestingly, EtOAc fraction of *P. gymnospora* exhibited lower antioxidant capacity (11.66%),

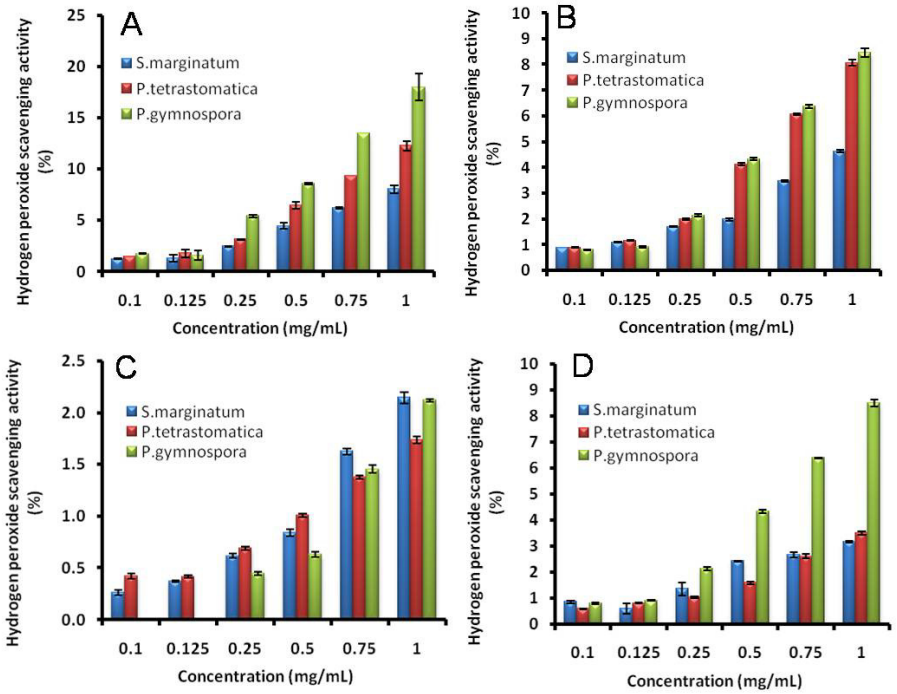


Figure 3C.5. Hydrogen peroxide scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica* and *P. gymnospora* with Concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard.

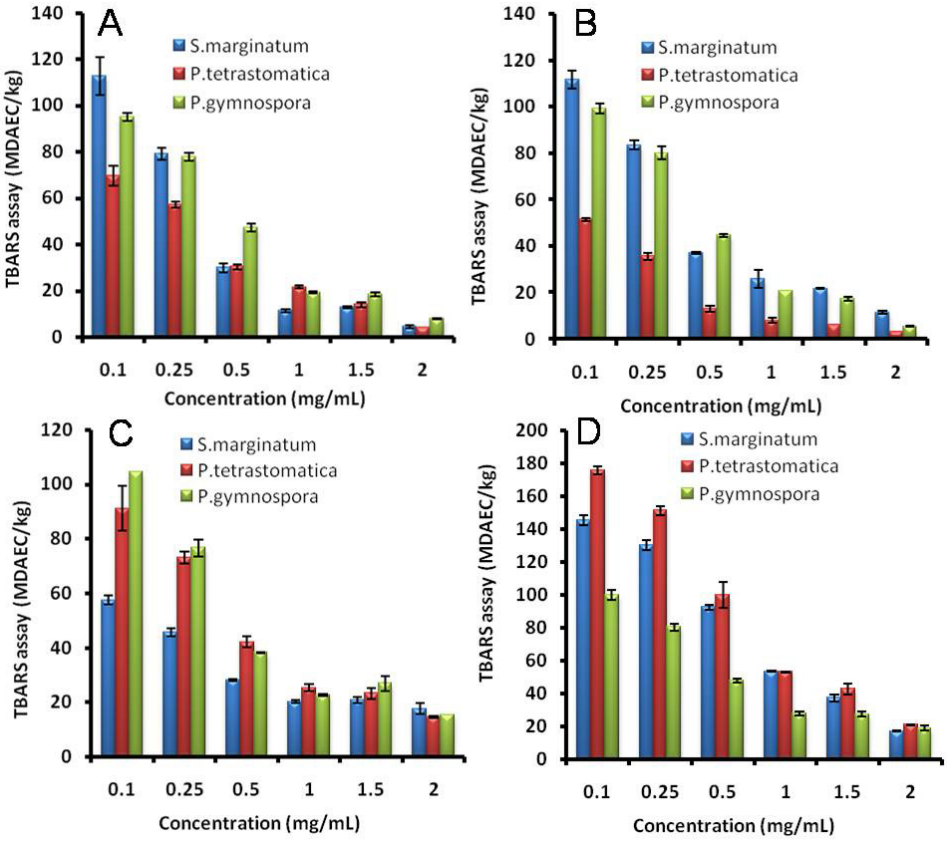


Figure 3C.6. TBARS formation inhibition assay (MDAEC/kg) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica* and *P. gymnospora* with Concentration (0.1-2mg/mL). Values are means of triplicate determinations and expressed with ± standard.

whereas MeOH extract was found to be ineffective to scavenge DPPH· (Table 3C.1). The activities were found to reduce with decrease in concentration. It was reported that the DPPH· scavenging capacities are significantly higher in EtOAc fraction of brown seaweed *Ecklonia cava* (Senevirathne et al. 2006). It is apparent that EtOAc and MDC fractions of brown seaweeds contain polyphenolic compounds with multiple –OH groups/centers of unsaturation capable of deactivating DPPH· radical.

3C.3.4. Hydroxyl Radical Scavenging Activity (%)

The scavenging activity of seaweed solvent fractions against the hydroxyl radical was investigated using Fenton's reaction (Table 3C.1). EtOAc fraction of *S. marginatum* (0.6 mg/mL) exhibited significantly higher (P<0.05) highest HO. scavenging activity (62.91%) than other solvent fractions (Table 3C.1). Accordingly, EtOAc fraction of *S. marginatum* registered the lowest IC_{50} value (0.39 mg/mL) followed by MDC (0.48 mg/mL), MeOH (1.65 mg/mL) and *n*-hexane fraction (1.47 mg/mL), in descending order (Table 3C.2). EtOAc fraction of *P. tetrastomatica* (0.6 mg/mL) exhibited significantly higher (P<0.05) HO. scavenging activity (87.87%) than other solvent fractions (Figure 3C.4). The HO. scavenging activities of brown seaweeds were found to be due to different compounds, including ascorbic acid-like structure and phenolic compounds mainly phlorotannins (Ahn et al. 2007).

3C.3.5. Hydrogen Peroxide (H_2O_2) Scavenging Activity (%)

The EtOAc fraction of *P. gymnospora* (1 mg/mL) exhibited a significantly higher (P<0.05) H_2O_2 scavenging activity (18.02%) than other solvent fractions (Figure 3C.5). No significant differences are apparent in H_2O_2 scavenging activity between MeOH and MDC fractions (10.43 and 8.47%, respectively at 1 mg/mL (Table 3C.1). The EtOAc and MDC fraction of *S. marginatum* contributed significantly towards H_2O_2 scavenging activity (IC_{50} 3.5 & 6.74 mg/mL, respectively) than MeOH fraction (IC_{50} 9.30 mg/mL), thereby signifying the importance of using EtOAc to isolate potential antioxidant molecules. Solvent fractions (1 mg/mL) of *P. tetrastomatica* followed the same trend as exhibited by the solvent fractions of *P. gymnospora*. These results indicated the presence of polar compounds in EtOAc fraction capable to scavenge H_2O_2. Similar results were apparent in the earlier studies reporting the significantly higher H_2O_2 scavenging activities (IC_{50} 0.009 mg/mL) of EtOAc fraction from *E. cava* indicating the potential of hydrophilic total phenolics to impart H_2O_2 scavenging activity (Senevirathne et al. 2006).

3C.3.6. Lipid Peroxidation Inhibition Activity in Model System: Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity (MDAEC/kg)

In *S. marginatum* the different solvent fractions recorded an IC_{50} value of 0.72, 0.86, 0.96, and 1.33 mg/mL for EtOAc, MDC, *n*-hexane, and MeOH fractions, respectively, thereby

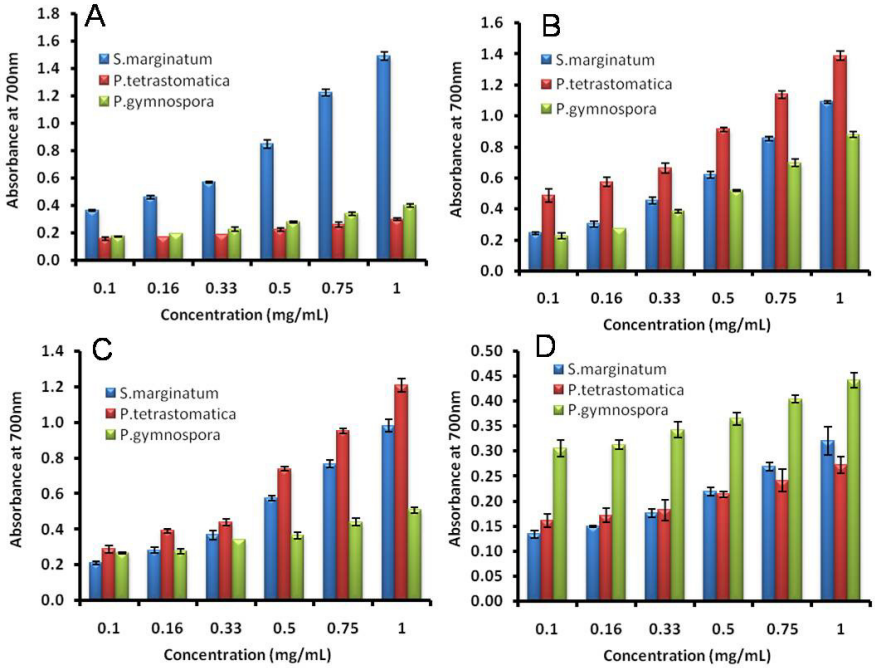


Figure 3C.7. Reducing ability (Ab_{700nm}) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica* and *P. gymnospora* with Concentration (0.1-1mg/mL). Values are means of triplicate determinations and expressed with ± standard.

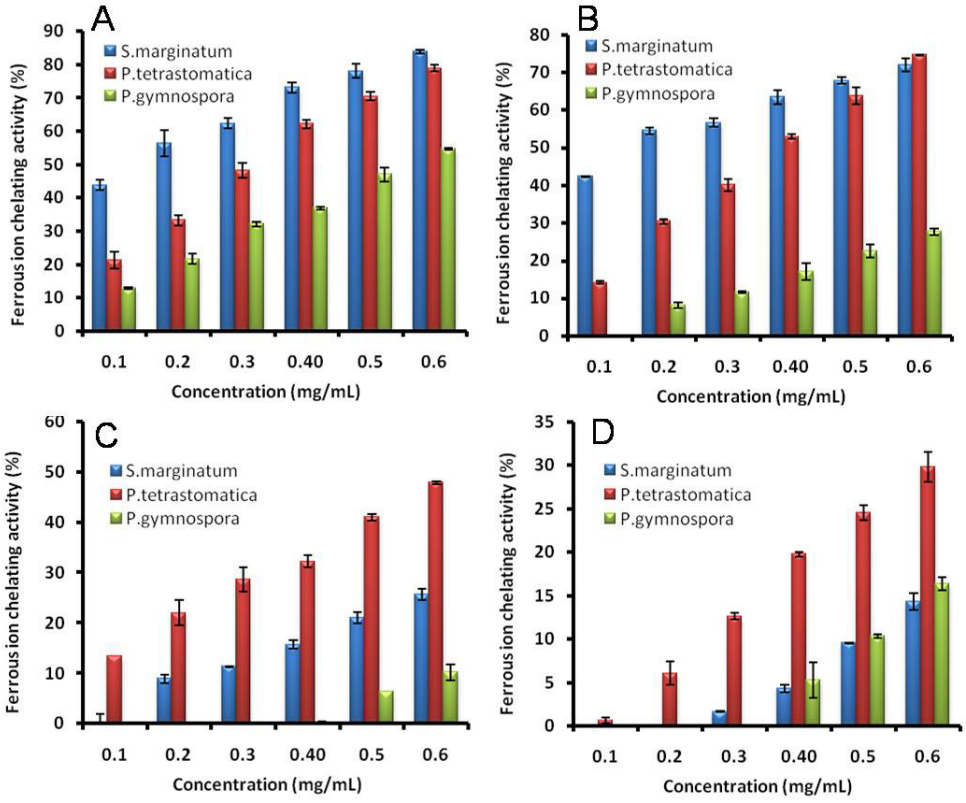


Figure 3C.8. Fe^{2+} chelating ability (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *S. marginatum, P. tetrastomatica* and *P. gymnospora* with Concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard.

indicating the potentially higher activity of EtOAc fraction. Different solvent extracts of *P. gymnospora* followed the same trend as shown by *P. tetrastomatica* thereby indicating the potentially higher activity of EtOAc and MDC fractions to inhibit lipid peroxidation (Figure 3C.6). The inhibition in lipid peroxidation may be due to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating proton to fatty acid radicals to terminate chain reactions, may have roles to inhibit lipid peroxidation (Karawita et al. 2005).

3C.3.7. Evaluation of Reducing Ability ($Ab_{700}nm$)

The reducing capacities of EtOAc fraction of *S. marginatum* as exhibited by absorbance at 700 nm (Ab_{700nm}1.48, 1 mg/mL) were found to be higher than that of MDC (Ab_{700nm} 1.11), *n*-hexane (Ab_{700nm} 0.96) and MeOH (Ab_{700nm} 0.34) extracts (Figure 3C.7). However, it is interesting to note that the antioxidant activities of *n*-hexane and MDC fractions of *P. gymnospora* were found to be significantly higher (P<0.05) (IC_{50} 101.23 & 28.85 mg/mL, respectively) than EtOAc (0.30 ± 0.01) and MeOH fractions (0.29 ± 0.01). This may be explained due to the presence of carotenes/ other pigments with long hydrocarbon chain and aminated compounds (Chew et al. 2008). The MDC fraction of *P. gymnospora* contributed significantly towards $ABTS^{\bullet+}$ radical scavenging activity (IC_{50} 28.85 mg/mL) than other solvent fractions (IC_{50} >100 mg/mL) (Table 3C.2). It was reported that *Padina antillarum* collected from a coral reef in Teluk Kumang, Malaysia exhibited 15.7 mg GE/g for reducing Fe (III) (Chew et al. 2008).

3C.3.8. Ferrous Ion (Fe^{2+}) Chelating Activity (%)

The Fe^{2+} ion can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Dinis et al. 1994). EtOAc and MDC fractions of *S. marginatum* realized significantly higher Fe^{2+} binding capacities (82.06 & 72.59%, respectively at 0.6 mg/mL) (P<0.05) than MeOH (14.68%) and *n*-hexane (25.94%) fractions (Figure 3C.8). EtOAc and MDC fractions of *P. tetrastomatica* (0.6 mg/mL) exhibited significantly high Fe^{2+} chelating ability (77 & 75%, respectively) (P>0.05) (Figure 3C.8). The EtOAc and MDC fraction of *P. gymnospora* contributed significantly towards $ABTS^{\bullet+}$ radical scavenging activity (IC_{50} 0.54 & 0.86 mg/mL, respectively) than MeOH and *n*-hexane fraction (IC_{50} 1.7-2.8 mg/mL), thereby signifying the potential presence of antioxidant molecules in EtOAc and MDC fractions (Table 3C.1). It was reported that *Padina antillarum* collected from a coral reef in Teluk Kumang, Port Dickson, Malaysia possessed potentially high Fe^{2+} chelating ability (Chew et al. 2008). The activities appeared to be due to the presence of compounds *viz*., phlorotanins, carrageenan, and alginate, which are usually present in brown seaweeds, and are strong chelators of heavy metals *viz*., Fe^{2+} (Toth & Pavia, 2000).

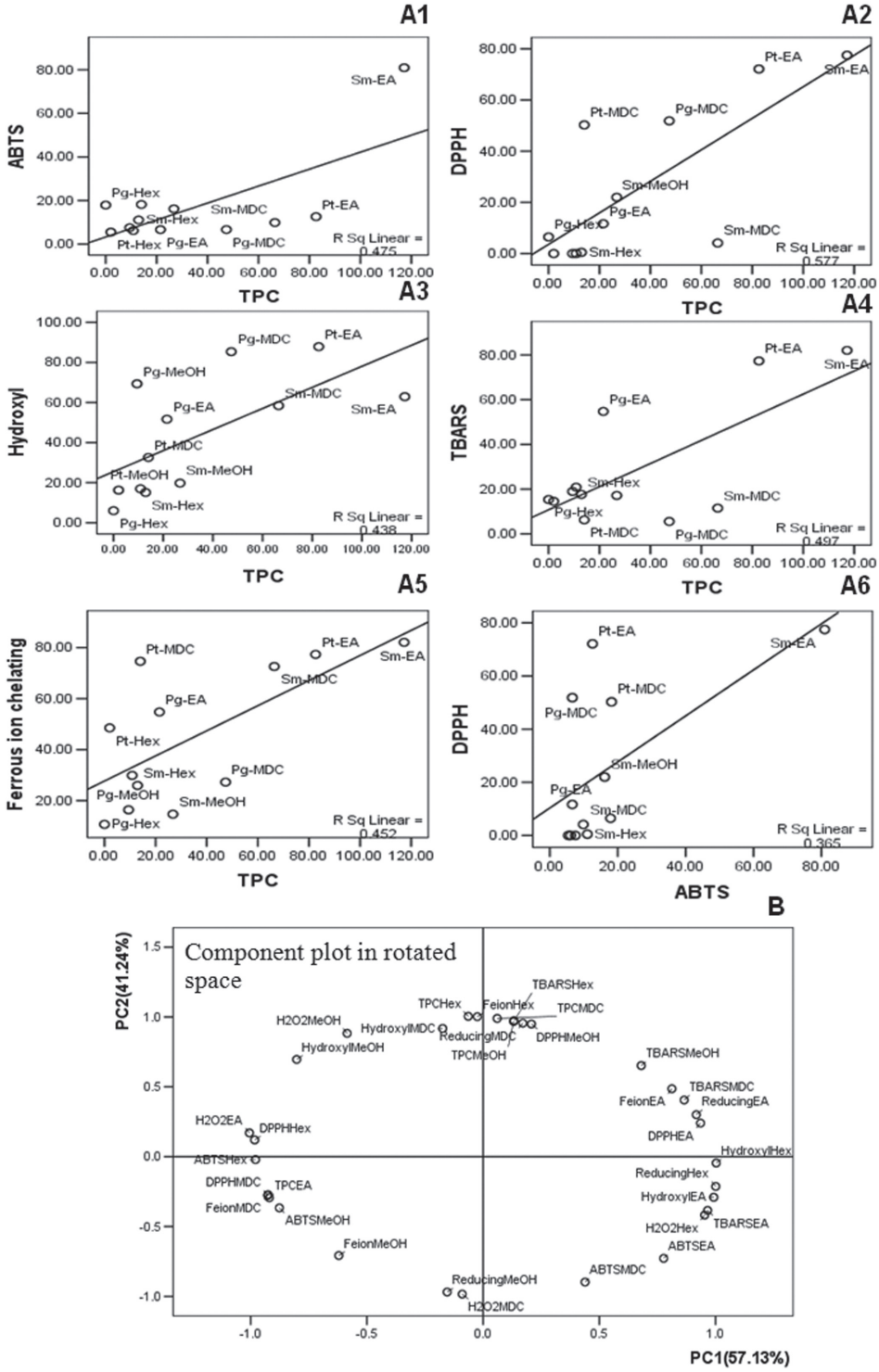


Figure 3C. 9. Correlation between antioxidant activity assays of different antioxidant assays and between solvent fractions of experimental seaweeds by scatter plot (A1-A6), and (B) loading plot diagrams of various components in rotated space. Scatter plot diagrams showing the correlation of TPC vis-à-vis (A1) ABTS.+ (n = 12, r = 0.689, *P*<0.05), (A2) DPPH (n = 12, r = 0.760, *P*<0.01), (A3) HO. radical scavenging assays (n = 12, r = 0.662, *P*<0.05), (A4) lipid peroxidation (TBARS) inhibitory (n = 12, r = 0.705, *P*<0.01), & (A5) Fe2+ ion chelating activities (n = 12, r = 0.672, *P*<0.05). (A6) Scatter plot showing correlation between DPPH and ABTS radical scavenging activity assay (n = 12, r = 0.604, *P*<0.05). (B) loading plot diagram (various components viz., PC-1 and PC-2 in rotated space) of antioxidant activities of different solvent fractions from *P. tetrastomatica, P. gymnospora* and *S. marginatum*.

3C.3.9. Correlations between Phenolic Contents and Different Antioxidant Activity Assays

The similarities and differences among different organic fractions of *P.tetrastomatica*, *P. gymnospora* and *S. marginatum* and the relationships among different antioxidant activity assays were statistically analyzed using PCA. The first two principal components explained 98.37% (PC 1– 57.13%; PC 2 – 41.24%) of the total variance in the data set (Figure 3C.10). Total phenolic content assay registered significant correlation with ABTS, DPPH, and HO. scavenging activities; TBARS assay, and Fe^{2+} chelating ability. A significant correlation was realized between the DPPH and ABTS radical scavenging assays (Figure 3C. 9). From this observation it may be inferred that polyphenols present in algal extracts are responsible for its radical scavenging, lipid peroxidation, and metal ion chelating abilities. Earlier reports showed that (Toth and Pavia, 2000) phlorotanins (brown seaweed polyphenolics), are strong chelators of heavy metals, which are believed to be responsible for the chelating ability. Earlier studies indicated that seaweeds with high phenolic contents are also good chelators of Fe^{2+} (Chew et al. 2008). It is believed that polyphenolic antioxidants disrupt the free-radical chain reaction by donating their H atom to fatty acid radicals to terminate chain reactions (Karawita et al. 2005). The marked antioxidant activity exhibited by EtOAc and MDC fractions were found to be correlated with their high contents in phenolic compounds as realized by PCA analyses. Phenolic compounds are thought to protect the seaweed thallus against photodestruction by UV radiation (Escrig et al. 2001; Pavia & Aberg, 1996), and to exhibit radical scavenging properties of *Halidrys siliquosa* extracts, suggesting that hydroquinols with oxygenated diterpene side-chains (tocopherol-like compounds) could act as antiradical or antioxidant molecules (Ragan & Glombitza 1986).

3C.3.10 Chromatographic Evaluation of the Phenolic Compounds in the Methanol extract and EtOAc fraction of Seaweeds

The selected groups of phenolics in MeOH and EtOAc solvent extracts of *S. marginatum*, *Padina tetrastomatica* and *P. gymnospora* were separated and identified by the RP-HPLC method, are shown in Table 3C.2. A retention time (R_t) library of the standard phenolic compounds was constructed with detection at T_{max} 324 nm and 277 nm (Figure 3C.5). The qualitative analysis of the seaweed extracts for phenolic compounds were obtained and the retention time for individual peak was compared with the library of standard phenolic compounds under the same HPLC conditions. It was found that all these seaweeds contain caffeic acid, 2, 5 dihydroxy benzoic acid, coumaric acid, ferulic acid, and syringic acid as phenolic acids in either of their MeOH or EtOAc extracts. Chlorogenic acid was found to be present only with the EtOAc fraction of *P. tetrastomatica*. Similarly quercetin was found only with the EtOAc fraction of *S. marginatum* and catechin was detected only in the EtOAc fraction of *P. gymnospora*. Both the MeOH and EtOAc fraction of

Table 3C.1. Total phenolic content and antioxidant activities of the different crude solvent fractions (MeOH, *n*-hexane, MDC and EtOAc) of the brown seaweeds *P. tetrastomatica, P. gymnospora,* and *S. marginatum*

Seaweeds	Solvent extracts			
	MeOH	*n*-hexane	MDC	EtOAc
Total phenolic content (mg of GE)				
P. tetrastomatica	$22.18^{ap} \pm 4.52$	$4.17^{aq} \pm 0.32$	$27.77^{ar} \pm 1.57$	$165.39^{as} \pm 0.85$
P. gymnospora	$19.07^{ap} \pm 1.21$	ND	$95.168^{bq} \pm 0.23$	$43.25^{br} \pm 3.41$
S. marginatum	$53.39^{bp} \pm 5.10$	$26.12^{bq} \pm 1.39$	$132.85^{cr} \pm 3.08$	$231.49^{cs} \pm 5.64$
ABTS assay (%)				
P. tetrasatomatica	$6.16^{ap} \pm 0.00$	$5.47^{ap} \pm 1.05$	$18.17^{aq} \pm 1.03$	$12.58^{ar} \pm 0.18$
P. gymnospora	$7.49^{bp} \pm 0.06$	$17.88^{bq} \pm 2.16$	$6.64^{bp} \pm 0.40$	$6.62^{bp} \pm 0.28$
S.marginatum	$16.15^{cp} \pm 0.10$	$11.05^{cpq} \pm 0.81$	$9.85^{cq} \pm 0.08$	$28.94^{cr} \pm 0.53$
DPPH· radical scavenging activity (%)				
P. tetrastomatica	ND	ND	$50.26^{a} \pm 0.62$	$72.13^{a} \pm 1.74$
P. gymnospora	ND	$6.47^{ap} \pm 0.31$	$51.85^{aq} \pm 0.58$	$11.66^{bp} \pm 1.17$
S. marginatum	$21.98^{p} \pm 0.23$	$0.46^{bq} \pm 0.02$	$4.12^{bq} \pm 0.15$	$77.51^{cr} \pm 1.36$
Hydroxyl radical scavenging activity (%)				
P. tetrastomatica	$16.94^{ap} \pm 0.22$	$16.24^{ap} \pm 0.23$	$32.57^{aq} \pm 1.34$	$87.87^{ar} \pm 0.55$
P. gymnospora	$69.34^{bp} \pm 1.15$	$6.00^{bq} \pm 1.27$	$85.36^{br} \pm 0.05$	$51.67^{bs} \pm 3.05$
S. marginatum	$19.76^{ap} \pm 2.48$	$15.09^{ap} \pm 0.15$	$58.44^{cq} \pm 0.14$	$62.91^{cq} \pm 0.03$
Scavenging of H_2O_2 (%)				
P. tetrastomatica	$3.50^{apq} \pm 0.05$	$1.74^{ap} \pm 0.03$	$8.07^{aq} \pm 0.08$	$12.29^{ar} \pm 0.34$
P. gymnospora	$10.83^{bp} \pm 0.88$	$2.12^{bq} \pm 0.01$	$8.47^{bp} \pm 0.12$	$18.02^{br} \pm 0.95$
S.marginatum	$3.18^{ap} \pm 0.02$	$2.15^{bp} \pm 0.04$	$4.64^{cp} \pm 0.04$	$8.04^{cp} \pm 0.26$
Total reduction capability (A_{700nm})				
P. tetrastomatica	$0.29^{ap} \pm 0.01$	$1.23^{ap} \pm 0.03$	$1.37^{ap} \pm 0.02$	$0.30^{ap} \pm 0.01$
P. gymnospora	$0.45^{bp} \pm 0.01$	$0.52^{bp} \pm 0.01$	$0.87^{bp} \pm 0.01$	$0.41^{bp} \pm 0.01$
S. marginatum	$0.34^{cp} \pm 0.02$	$0.96^{cp} \pm 0.03$	$1.11^{cp} \pm 0.02$	$1.48^{cp} \pm 0.02$
TBARS activity (mM of MDA equivalent compounds /kg)				
P. tetrastomatica	$20.87^{ap} \pm 0.04$	$14.54^{aq} \pm 0.24$	$3.25^{ar} \pm 0.08$	$4.08^{ar} \pm 0.09$
P. gymnospora	$18.95^{bp} \pm 1.13$	$15.34^{ap} \pm 0.02$	$5.53^{aq} \pm 0.16$	$7.79^{bq} \pm 0.13$
S. marginatum	$17.17^{cp} \pm 0.31$	$17.64^{bp} \pm 1.36$	$11.52^{bp} \pm 0.53$	$4.38^{aq} \pm 0.36$
Fe^{2+} ion chelating activity (%)				
P. tetrastomatica	$29.88^{ap} \pm 0.27$	$48.52^{aq} \pm 0.77$	$74.62^{ar} \pm 1.00$	$77.33^{ar} \pm 1.83$
P. gymnospora	$16.39^{bp} \pm 1.05$	$10.70^{bq} \pm 0.82$	$27.25^{br} \pm 0.68$	$54.75^{bs} \pm 0.47$
S. marginatum	$14.68^{bp} \pm 0.59$	$25.94^{cq} \pm 0.48$	$72.59^{ar} \pm 0.93$	$82.06^{cs} \pm 1.86$

Data are the mean values of triplicate and expressed as mean ± standard deviation. Row (p-s) and column values (a-c) with different letters are significantly different ($P<0.05$). MDC: dichloromethane, EtOAc: ethyl acetate. The concentration of the crude solvent fractions used were 1 mg/mL for DPPH radical scavenging activity, reducing capacity and H_2O_2 scavenging activity; 0.6 mg/mL for OH radical scavenging activity and Fe^{2+} ion chelating activity; 2 mg/mL for TBARS assay and 0.6 µg/mL for ABTS radical scavenging activity. ND: Non-detectable.

P. tetrastomatica were found to contain caffeic acid, 2,5 dihydroxy benzoic acid and epicatechin whereas coumaric acid, ferulic acid, ECG and syringic acid were only present in the MeOH fraction and chlorogenic acid and EGCG were detected only in the EtOAc fraction of *P. tetrastomatica*. In *P. gymnospora* Both the MeOH and EtOAc fraction were found to contain 2,5 dihydroxy benzoic acid, coumaric acid and syringic acid whereas caffeic acid, ferulic acid and epicatechin were only present in the MeOH fraction and catechin and EGCG were detected only in the EtOAc fraction of *P. gymnospora*. Similarly both the MeOH and EtOAc fraction of *S. marginatum* were found to contain caffeic acid, 2,5 dihydroxy benzoic acid, coumaric acid, ferulic acid and epicatechin whereas ECG and syringic acid were only present in its MeOH fraction and quercetin and EGCG were detected only in the EtOAc fraction of *S. marginatum.*

The selected groups of phenolics in MeOH and EtOAc solvent extracts of *S. marginatum*, *Padina tetrastomatica*, and *P. gymnospora* were separated and identified by the RP-HPLC method, are shown in Table 3C.2. A retention time (R_t) library of the standard phenolic compounds was constructed with detection at T_{max} 324 nm and 277 nm (Figure 3.5). The qualitative analysis of the seaweed extracts for phenolic compounds were obtained and the retention time for individual peak was compared with the library of standard phenolic compounds under the same HPLC conditions. It was found that all these seaweeds contain caffeic acid, 2, 5 dihydroxy benzoic acid, coumaric acid, ferulic acid, and syringic acid as phenolic acids in either of their MeOH or EtOAc extracts. Chlorogenic acid was found to be present only with the EtOAc fraction of *P. tetrastomatica*. Similarly quercetin was found only with the EtOAc fraction of *S. marginatum* and catechin was detected only in the EtOAc fraction of *P. gymnospora*. Both the MeOH and EtOAc fraction of *P. tetrastomatica* were found to contain caffeic acid, 2,5 dihydroxy benzoic acid and epicatechin whereas coumaric acid, ferulic acid, ECG and syringic acid were only present in the MeOH fraction and chlorogenic acid and EGCG were detected only in the EtOAc fraction of *P. tetrastomatica*. In *P. gymnospora.* Both the MeOH and EtOAc fraction were found to contain 2,5 dihydroxy benzoic acid, coumaric acid and syringic acid whereas caffeic acid, ferulic acid and epicatechin were only present in the MeOH fraction and catechin and EGCG were detected only in the EtOAc fraction of *P. gymnospora*. Similarly both the MeOH and EtOAc fractions of *S. marginatum* were found to contain caffeic acid, 2,5 dihydroxy benzoic acid, coumaric acid, ferulic acid and epicatechin whereas ECG and syringic acid were only present in its MeOH fraction and quercetin and EGCG were detected only in the EtOAc fraction of *S. marginatum.*

Seaweeds have previously been reported to be rich in phenolic acids, such as catechin, epicatechin, EGCG, caffeic acid, quercetin and others (de Quirós et al. 2010; López et al. 2011). Phenolic acids constitute a large group of naturally occurring organic compounds with a broad

Table3C.2. Major phenolic compounds (mg/g extract) identified in the MeOH extract (A) and EtOAc fractions (B) of *P. tetrastomatica* (PT), *P. gymnospora* (PG) *& S. marginatum* (SM) by HPLC.

Sl.No	Phenolic acids	R_t (minute)	Phenolic compounds (mg/g extract) identified in the MeOH extract (A) and EtOAc fraction (B)					
	324nm		**PT-A**	**PT-B**	**PG-A**	**PG-B**	**SM-A**	**SM-B**
1	Chlorogenic acid	8.12	-	32.57 ±2.06	-	-	-	-
2	Caffeic acid	10.49	6.05±0.25	26.34 ±2.97	6.04 ±0.85	-	4.36 ±0.36	12.15 ±1.27
3	2,5 dihydroxy benzoic acid	17.43	2.13 ±0.13	12.22 ±1.32	2.20 ±0.06	163 ±7.89	6.22 ±1.09	10.54 ±0.85
4	Coumaric acid	20.56	1.07 ±0.01	-	0.61 ±0.01	21 ±2.14	0.61 ±0.01	43.35 ±3.43
5	Ferulic acid	24.19	1.13 ±0.01	-	0.53 ±0.01	-	0.09 ±0.01	27.23 ±2.06
6	Quercetin	37.90	-	-	-	-	-	32.56 ±3.55
7	Salicylic acid	44.92	-	-	-	-	-	-
	277nm							
8	Gallic acid	5.39	-	-	-	-	-	-
9	Catechin	6.81	-	-	-	13 ±1.33	-	-
10	EGCG	8.13	-	84.14 ±5.87	-	6 ±0.69	-	4.08 ±0.11
11	Epicatechin	10.11	94.28 ±5.62	112.65 ±8.13	43.23 ±2.47	-	2.10 ±0.08	6.11 ±0.18
12	ECG	13.0	19.17 ±2.11	-	-	-	1.08 ±0.03	
13	Syringic acid	14.78	30.63 ±2.85	-	132.34 ±11.55	11 ±0.32	36.80 ±2.22	

Values are mean ± SD (n = 3)

spectrum of pharmacological activities and free radical scavenging activity in particular. These group of compounds are hydroxylated derivatives of benzoic and cinnamic acids, which often occur in plants as esters, glycosides and bound complexes and are rarely present in free forms (Germano‘ et al. 2006). Phenolic acids differ according to the number and position of hydroxylation and methoxylation of the aromatic ring. There is a much higher quantity and diversity of hydroxycinnamates than hydroxybenzoates and they consist of *p*-coumaric, caffeic, and ferulic acids either.

3C.4. Conclusions

EtOAc fraction of *P. tetrastomatica* exhibited higher total phenolic content (165.39 GE/g) hydroxyl radical scavenging activity (87.87%) and TBARS formation inhibition activity (4.08 MDAEC /kg). Higher ABTS radical scavenging ability (28.94%), DPPH· radical scavenging activity (77.51%), reduction capability (Ab_{700nm}1.48) and Fe^{2+} ion chelating activity (82.06%) was observed with its EtOAc fraction of *S. marginatum*. MDC fraction of *P. gymnospora* exhibited higher hydroxyl radical scavenging activity (85.36%) and its EtOAc fraction exhibited higher H_2O_2 scavenging potential (18.02%). It was found that all these seaweeds contain caffeic acid, 2, 5 dihydroxy benzoic acid, coumaric acid, ferulic acid and syringic acid as phenolic acids in either of their MeOH or EtOAc extracts. Both the MeOH and EtOAc fraction of *P. tetrastomatica* were found to contain caffeic acid, 2,5 dihydroxy benzoic acid and epicatechin whereas coumaric acid, ferulic acid, ECG and syringic acid were only present in the MeOH fraction and chlorogenic acid and EGCG were detected only in the EtOAc fraction of *P. tetrastomatica*. In *P. gymnospora.* Both the MeOH and EtOAc fraction were found to contain 2,5 dihydroxy benzoic acid, coumaric acid and syringic acid whereas caffeic acid, ferulic acid and epicatechin were only present in the MeOH fraction and catechin and EGCG were detected only in the EtOAc fraction of *P. gymnospora*. Similarly both the MeOH and EtOAc fraction of *S. marginatum* were found to contain caffeic acid, 2,5 dihydroxy benzoic acid, coumaric acid, ferulic acid and epicatechin whereas ECG and syringic acid were only present in its MeOH fraction and quercetin and EGCG were detected only in the EtOAc fraction of *S. marginatum.*

CHAPTER 3D

Evaluation of the Antioxidant Potential of *Laurencia papillosa, Gelidiella acerosa* and *Acanthophora spicifera*

Figure 3D. *Laurencia papillosa* collected from the Gulf of Mannar

Figure 3D. *Gelidiella acerosa* collected from the Gulf of Mannar

Figure 3D. *Acanthophora spicifera* collected from the Gulf of Mannar

CHAPTER 3D

Evaluation of the Antioxidant Potential of *Laurencia papillosa Gelidiella acerosa,* and *Acanthophora spicifera*

3D.1. Introduction

Red seaweeds (division: Rhodophyta) (division: Phaeophyta) contain a large assemblage of species that predominate in the coastal and continental shelf areas of tropical, temperate and cold-water regions. Phytochemical studies of the red seaweed *Acanthophora spicifera* revealed the presence of potentially high anticancerous and antioxidant activities (Wang et al. 1998). The members of *Gelidiaceae*, the red seaweeds are of superior quality and widely used in a number of preparations in biomedical, food, cosmetics and pharmaceutical industries (Armisen 1995). *Gelidiella acerosa* is warm water tropical red seaweed occurring in the Indian, Pacific and Atlantic Oceans (Rao 1972) and is the starting material to manufacture agar (Armisen 1995). *A. spicifera* and *G. acerosa* are warm water tropical seaweeds occurring in the Indian and Pacific Rao Oceans (Rao 1972) and are the major sources of raw materials for the manufacture of agar in several countries, and widely used in a number of preparations in biomedical, food, cosmetics and pharmaceutical industries (Armisen 1995). The ethanol extract and solvent fractions of *L. papillosa* was also reported to have potential antifungal, nematicidal and hypolipidaemic activity and some of these activities are attributed due to the presence of various fatty acid esters (Ara et al. 2005).

The red and brown seaweeds from a major share of the seaweed population around the eastern coast of Indian Peninsula, and along the Gulf of Mannar area in particular and were available in all seasons. Among different seaweed species, *Laurencia papillosa (C.Agardh) Greville* (division: Phaeophyta, order: Ceramiales, family: Rhodomelaceae), *Acanthophora spicifera* (Vahl) Børgesen (division: Rhodophyta, order: Ceramiales, family: Rhodomelaceae), and *Gelidiella acerosa* (Forsskal) Feldmann & Hamel (division: Rhodophyta, order: Gelidiales, family: Gelidiellaceae) were selected in the present study to evaluate antioxidant activities and total phenolic contents in an attempt to understand their beneficial value as human food or additives. Findings of antioxidant activity could potentially shortlist candidate species to isolate potent antioxidant molecules to be used for increasing the shelf-life of food industry and as nutraceuticals and expand their dietary market. Based on this background, the objective of the present study was to characterise the antioxidant properties of methanol (MeOH) extract and different solvent fractions (ethyl acetate, dichloromethane and *n*-hexane) of these experimental seaweeds by different *in vitro* systems, to statistically evaluate the role of phenolic compounds responsible for antioxidant activity and to

Table 3D.1. Yields obtained for methanol extract (as % w/w of seaweed on dry weight basis) and solvent fractions (as % of total methanol extract) of *L. papillosa, G. acerosa* and *A. spicifera*.

Seaweed species	Methanol extract (%)	Fractions obtained by partitioning methanol		
		n-hexane	MDC	EtOAc
L. papillosa	32.12^{a}±1.32	33.36^{a}±2.14	23.17^{a}±0.49	19.29^{a}±0.32
G. acerosa	29.06^{b} ±0.89	29.27^{b}±1.27	19.33^{b}±0.96	16.45^{b}±0.79
A. spicifera	22.38^{c}±1.17	30.16^{b}±2.06	26.64^{c}±1.84	17.81^{ab}±1.10

All the values are mean ± SD (n =3); SD standard deviation. a,b column wise values with different superscripts are significantly different (P<0.05). MeOH methanol, MDC dichloromethane, EtOAc ethyl acetate.

Table 3D.2. Total phenolic content and antioxidant activities of the methanol extract and different solvent fractions (*n*-hexane, MDC and EtOAc) of the seaweeds *L. papillosa , G. acerosa,* and *A. spicifera.*

Scientific name	**Solvent extracts**			
Total phenolic content. (mg GE/g)	**methanol**	***n*-hexane**	**MDC**	**EtOAc**
L. papillosa	30.32^{ap} ± 0.68	28.62^{ap} ± 0.71	47.70^{aq} ± 3.58	283.00^{ar} ± 1.68
G. acerosa	10.60^{bp} ± 0.55	8.45^{bp} ± 0.66	105.05^{bq} ± 2.28	147.02^{br} ± 3.49
A. spicifera	6.72^{cp} ± 1.03	27.89^{aq} ± 0.98	82.01^{cr} ± 0.64	119.28^{cs} ± 3.84
ABTS assay (%)				
L. papillosa	28.31^{ap} ± 1.04	9.25^{aq} ± 1.11	38.83^{ar} ± 0.91	97.24^{as} ± 0.21
G. acerosa	11.95^{bp} ± 1.51	18.08^{bpq} ± 1.53	22.52^{bq} ± 0.61	42.23^{br} ± 0.29
A. spicifera	8.06^{cp} ± 1.76	8.94^{ap} ± 0.60	14.45^{cp} ± 0.67	37.80^{cq} ± 0.57
DPPH· radical scavenging activity (%)				
L. papillosa	76.45^{ap} ± 0.31	34.96^{aq} ± 1.58	36.28^{aq} ± 2.85	97.10^{ar} ± 0.16
G. acerosa	44.66^{bp} ± 1.90	0.00	68.11^{bq} ± 1.36	77.12^{br} ± 0.49
A. spicifera	51.73^{cp} ± 1.95	0.00	72.31^{bq} ± 0.42	81.99^{cr} ± 0.56
Hydroxyl radical scavenging activity (%)				
L. papillosa	39.19^{ap} ± 0.54	26.99^{aq} ± 0.82	42.62^{ap} ± 0.41	75.42^{ar} ± 0.25
G. acerosa	26.81^{bp} ± 0.22	11.35^{bq} ± 1.08	66.09^{br} ± 0.35	78.87^{bs} ± 0.78
A. spicifera	26.23^{bp} ± 0.34	24.65^{ap} ± 0.28	65.47^{cq} ± 0.32	66.60^{cq} ± 1.19
Scavenging of H_2O_2 (%)				
L. papillosa	7.18^{ap} ± 0.06	1.87^{ap} ± 0.12	0.94^{ap} ± 0.12	15.60^{aq} ± 0.08
G. acerosa	1.35^{bp} ± 0.02	5.48^{bp} ± 0.02	7.16^{bp} ± 0.04	16.09^{aq} ± 0.68
A. spicifera	0.53^{cp} ± 0.00	2.43^{cp} ± 0.20	18.42^{cq} ± 1.10	14.49^{aq} ± 0.51
Total Reduction Capability (Ab_{700nm})				
L. papillosa	0.53^{ap} ± 0.01	0.81^{ap} ± 0.01	0.48^{ap} ± 0.01	1.14^{aq} ± 0.01
G. acerosa	0.45^{bp} ± 0.00	0.81^{ap} ± 0.01	0.18^{bp} ± 0.01	1.31^{abp} ± 0.03
A. spicifera	0.54^{ap} ± 0.01	1.12^{bp} ± 0.06	1.28^{cp} ± 0.08	1.46^{bp} ± 0.13
TBARS assay (MDAEC /kg of the compound)				
L. papillosa	6.43^{ap} ± 0.50	20.04^{aq} ± 0.43	5.05^{ap} ± 0.21	3.35^{ap} ± 0.13
G. acerosa	35.17^{bp} ± 0.74	20.69^{aq} ± 0.32	12.21^{br} ± 0.31	3.26^{as} ± 0.22
A. spicifera	22.76^{cp} ± 0.13	33.16^{bq} ± 0.21	11.26^{cr} ± 0.35	4.21^{bs} ± 0.22
Ferrous Metal Ions Chelating Activity (%)				
L. papillosa	32.40^{ap} ± 1.07	22.64^{aq} ± 1.54	44.13^{ar} ± 0.43	47.60^{ar} ± 1.25
G. acerosa	18.07^{bp} ± 2.01	12.08^{bp} ± 1.71	63.48^{bq} ± 2.80	36.07^{br} ± 1.27
A. spicifera	17.83^{bp} ± 0.87	26.25^{aq} ± 0.60	31.08^{cq} ± 1.44	61.58^{cr} ± 2.87

Data are the mean values of triplicate and expressed as mean ± standard deviation. Row (p-s) and column values (a-c) with different letters are significantly different (P<0.05). MDC: dichloromethane, EA: ethyl acetate. The concentration of the crude solvent fractions used were 1 mg/mL for DPPH radical scavenging activity, reducing capacity and H_2O_2 scavenging activity; 0.6 mg/mL for OH radical scavenging activity and Fe^{2+} ion chelating activity; 2 mg/mL for TBARS assay and 0.6 µg/mL for ABTS radical scavenging activity.

identify and quantify the phenolic compounds present in these species by reverse phase HPLC and determine the relationship between antioxidant activity and phenolic principles.

3D.2. Materials and Methods

3D.2.1. Preparation of Seaweed Extracts and Fractions

The ground and shade-dried seaweed samples (0.75 kg) were extracted with MeOH (2 L x 3) at an elevated temperature (40-45^{o}C) for 3.5 h. The samples were then filtered with Whatman filter paper no 1 to obtain the clarified filtrates (2.50 L), which were filtered, through Na_2SO_4 (200 g), and evaporated (40^{0}C) using a rotary evaporator under vaccum to dryness. This dark green viscous oily mass (200 mL) of MeOH extract was mixed with an equal volume of distilled water (250 mL), and partitioned successively with *n*-hexane (200 mL x 3), MDC (200 mL x 3), and EtOAc (200 mL x 3) to furnish *n*-hexane (500 mL), MDC (450 mL), and EtOAc fractions (450 mL), respectively. The extracts were dried over anhydrous Na_2SO_4 (100 g), and evaporated under reduced pressure using a rotary vaccum evaporator to furnish the different solvent fractions of varying polarity. The yields of each fraction have been illustrated under Table 3D.1.

3D.2.2. Assays for Determination of Phenolic Contents and Antioxidant Potential of Seaweeds

The amount of total phenolics in the samples was determined by the established method described by Mcdonald et al. (2001). The ABTS.$^{+}$, 1, 1-diphenyl-2-picryl-hydrazil (DPPH.), hydroxyl radical radical assasy, hydrogen peroxide scavenging ability, thiobarbituric acid reactive substances (TBARS) formation inhibition assay, reducing ability and Fe^{2+} ion chelating potential were performed by the methods describey by Re et al. (1999), Shimada et al. (1992) and Klein et al. (1981), Ruch et al. (1989), Madsen et al. (1997), Oyaizu (1986) and Dinis et al. (1994) respectively with suitable modifications as described by section 3.5. HPLC based chromatographic identification of the standards and the samples were done as described by section 3.6

3D.2.3. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied traits. Analyses were carried out in triplicate, and the means of all parameters were examined for significance (P<0.05) by analysis of variance (ANOVA). The Pearson correlation test was used to assess correlations between means. The experiments were carried out in three different batches of seaweeds. The mean variance in the data set was detected using principal component analysis (PCA). All data were mean centered and scaled to equal unit variance prior to PCA. The selected

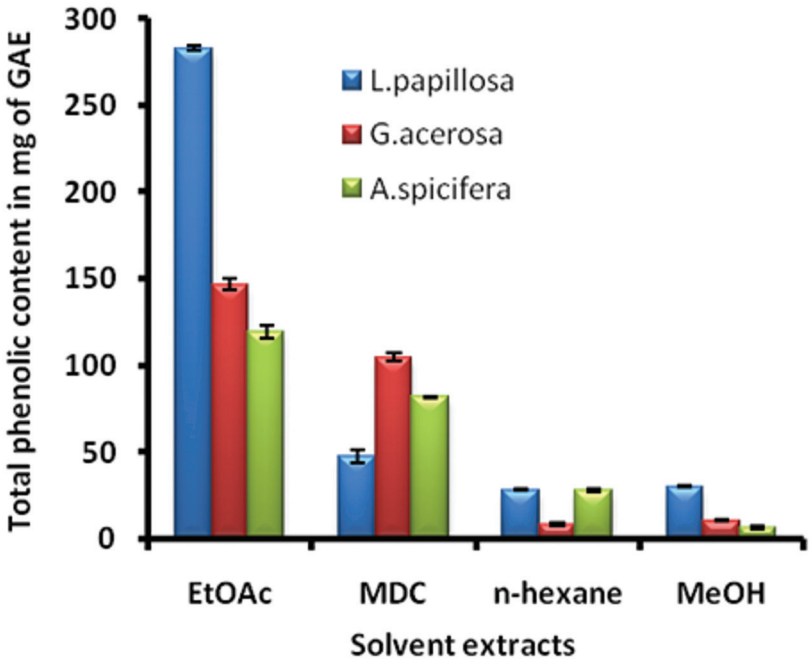


Figure 3D.1. TPC (mg of GE/g of the sample) of methanol extract and different solvent fractions of *L. papillosa, G. acerosa* and *A. spicifera*

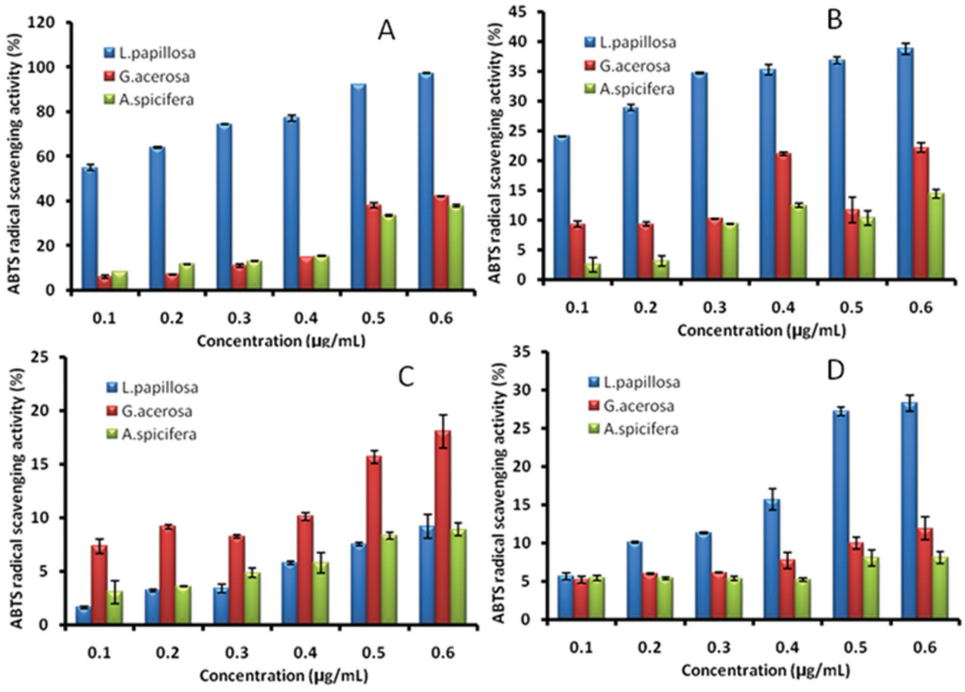


Figure 3D.2. ABTS.+ radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with concentration (0.1-0.6µg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

variables for PCA were the different antioxident assays *viz*., $ABTS^{\cdot+}$, $DPPH^{\cdot}$ radical scavenging activity, total reduction capability, Fe^{2+} ion chelating activity, HO. radical scavenging activity, scavenging of H_2O_2, thiobarbituric acid-reactive substances (TBARS) and phenolic content, as exhibited by EtOAc, MDC, *n*-hexane fractions, and MeOH crude extracts of the seaweeds.

3D.3. Results and Discussion

3D.3.1. Determination of Total Phenolic Compounds (TPC)

The EtOAc extract of *L. papillosa* exhibited significantly higher (P<0.05) TPC (283mg of gallic acid equivalence (GE)/g) than its MDC and MeOH extract (Table 3D.2). Both EtOAc and MDC fractions of *G. acerosa* exhibited significantly higher (P<0.05) TPC (147.0 & 105.1 mg GE/g, respectively) than its other solvent fractions. Similarly, EtOAc extract of *A. spicifera* recorded significantly higher (P<0.05) phenolic content (119.2 mg GE/g) than its MDC, *n*-hexane and MeOH extracts (Figure 3D.1). Apparently, EtOAc extract of *L. papillosa* exhibited a significantly higher value in phenolic content than the corresponding solvent extracts of both *G. acerosa* and *A. spicifera*, which signify the presence of rich phenolic compounds in the former. Among all the three seaweed species EtOAc extract exhibited significantly (P<0.05) higher phenolic content than all other solvent fractions indicating the presence of polar phenolic compounds in these seaweeds. Other researchers also identified the presence phenolic compounds in red and brown seaweeds (Zubia et al. 2007, Duan et al. 2006). A statistical correlation obtained between the content of phenolic compounds and antioxidant activity in our present study infers that these seaweeds are rich natural resources of potent phenolic antioxidant compounds (Wangensteen et al. 2004).

3D.3.2. 2,2' -Azino-bis-3-ethylbenzothiozoline-6-Sulfonic Acid Diammonium Salt (ABTS) Radical Scavenging Activity (%)

EtOAc extract of *L. papillosa* (0.6 µg/mL) exhibited significantly higher (P<0.05) $ABTS^{\cdot+}$ radical scavenging activity (97.2 %) compared with its other solvent fractions. Solvent fractions of *G. acerosa* followed the order EtOAc extract (42.2 %) > MDC extract (22.2 %) > *n*-hexane extract (18.1 %) > MeOH extract (11.9 %) respectively towards scavenging $ABTS^{\cdot+}$ radical (Table 3D.2). In *A. spicifera* $ABTS^{\cdot+}$ radical scavenging activity of the different solvent fractions followed the order EtOAc (37.8 %) > MDC (13.2 %) > *n*-hexane fraction (8.9 %) > MeOH (5.6 %) (Figure 3D.2). Radical-scavenging capacity of seaweed extracts might be mostly related to their phenolic hydroxyl group (Ragan et al. 1986). Statistical studies had shown a high correlation of TPC with $ABTS^{\cdot+}$ radical scavenging activity. This may be due to the high amount of polyphenolic constituents present in the seaweeds (Pavia & Aberg 1996) which were capable of functioning as free radical scavengers.

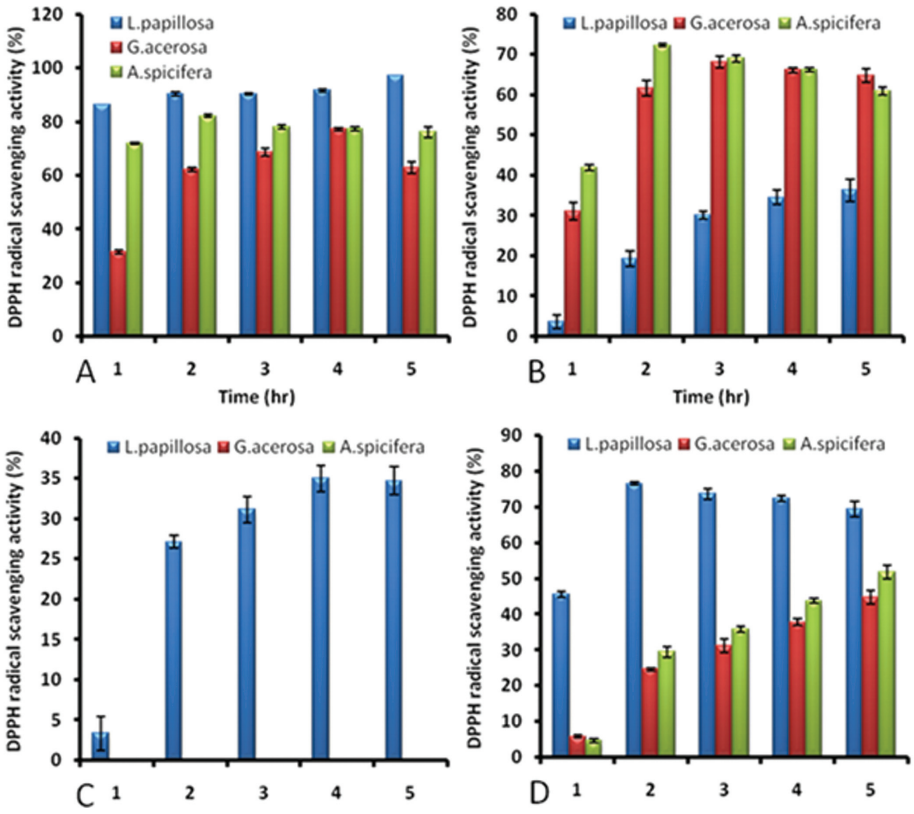


Figure 3D.3. DPPH radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with time (1-5hrs). Values are means of triplicate determinations and expressed with ± standard deviation

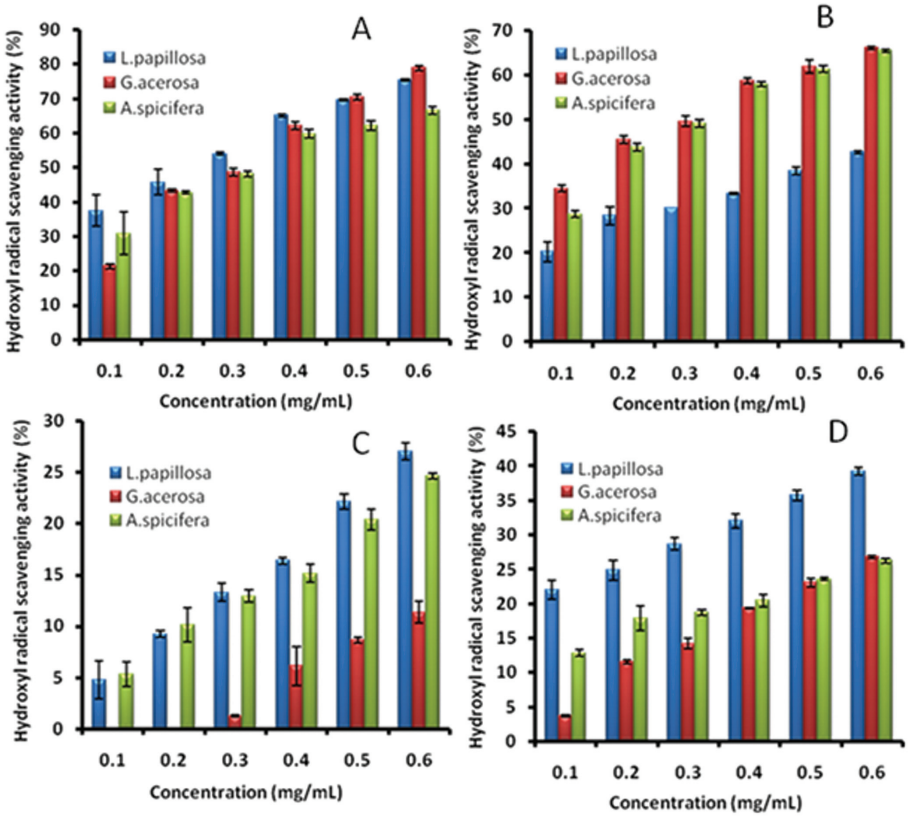


Figure 3D.4. Hydroxyl radical scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

3D.3.3. 1, 1-Diphenyl-2-Picryl Hydrazil (DPPH•) radical scavenging Activity (%)

EtOAc extract of *L. papillosa* exhibited a significantly higher (P<0.05) DPPH radical scavenging activity (97.1 %) followed by its MeOH extract at the same dose (Table 2). EtOAc fraction and MeOH extract of *L. papillosa* exhibited an increase in DPPH radical scavenging activities with time (Figure 3). Similarly, the EtOAc extract of *A. spicifera* exhibited significantly higher (P<0.05) DPPH free radical scavenging activity (81.9 %) than its other fractions (Table 2). MeOH extract of *A. spicifera* with the same dose had shown lesser value (51.7 %) than EtOAc and MDC fractions whereas *n*-hexane extract was not at all effective in this concentration (1 mg/mL) to scavenge DPPH free radical (Figure 3D.3). In *G. acerosa* higher polar organic fraction (EtOAc extract- 77.1%), exhibited a significantly higher (P<0.05) DPPH free radical scavenging activity followed by the next lower polar organic solvent extract (MDC extract - 68.1 %) and MeOH extract (44.6 %). A high correlation between DPPH radical-scavenging activities and total polyphenolics were already reported by other researchers (Siriwardhana et al. 2003) as we also obtained from our statistical correlation studies.

3D.3.4. Hydroxyl Radical Scavenging Activity (%).

Hydroxyl radical scavenging activity was employed to understand the potential of different seaweed extracts against short-lived radicals, *viz*., HO. radical. The EtOAc fraction of *G. acerosa* exhibited a significantly higher (P<0.05) HO. radical scavenging activity (78.8 %) than its other solvent fractions (Table 3D.2). A reasonably good activity was recorded for the MDC fraction followed by the MeOH extract and *n*-hexane fraction in *G. acerosa* (Figure 3D.4). The EtOAc and MDC extract of *L. papillosa* also contributed significantly (P<0.05) towards HO. scavenging activity than its MDC, MeOH extract and *n*-hexane fractions thereby signifying the importance of using EtOAc to isolate potential antioxidant molecules (Table 3D.2). The solvent fractions of *A. spicifera* followed the trend as: EtOAc > MDC > *n*-hexane > MeOH extract (Figure 3D4) in increasing order towards scavenging OH. radical, thereby signifying the highest activity of EtOAc fraction (Table 2). Earlier reports are there to show the hydroxyl radical scavenging activity (%) of different solvent extracts obtained from red and brown seaweeds. For example the HO. radical scavenging activity (%) of three red seaweeds (*Acanthophora spicifera*, *Gracilaria edulis*, and *Euchema kappaphycus* (1000 µg) were more than 90% in MDC, butanol and aqueous fractions and a lower inhibition rate of 65.81% was observed in solvent fraction of *E. kappaphycus* (Ganesan et al. 2008). Another study reported the enzymatic extract of *Sargassum fullvelum* (a brown seaweed) possessed little effect on scavenging the HO. radical (Heo et al. 2005). But the results of our study indicated a higher activity in some cases could be due to the fact that most of the enzymatic extraction is aqueous based and may not be as effective in extracting the active principles like in the case of solvent extraction.

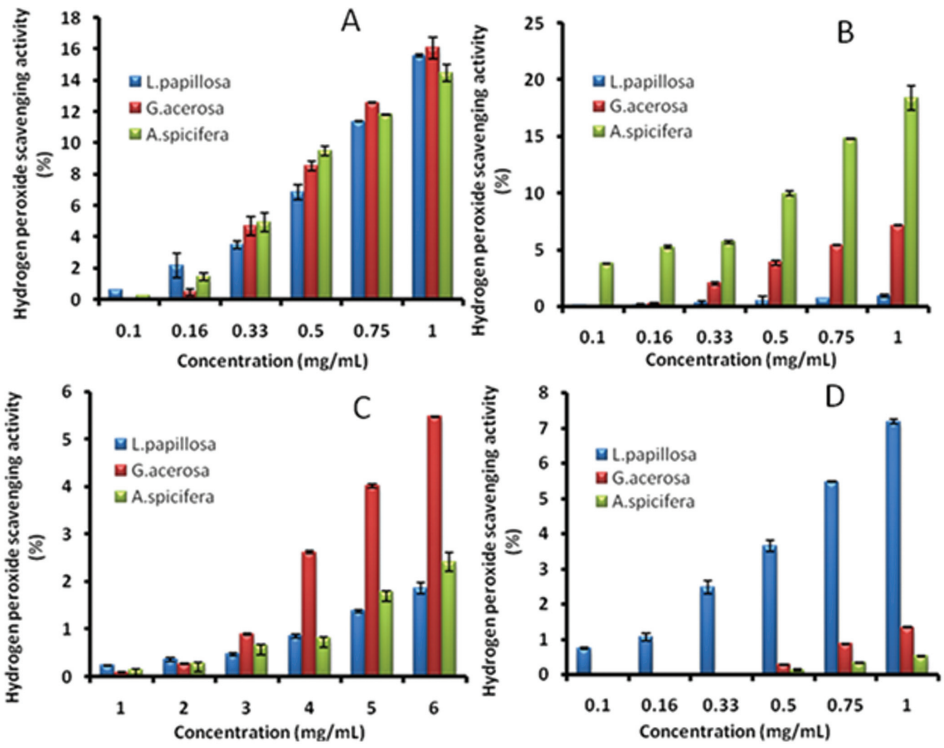


Figure 3D.5. Hydrogen peroxide scavenging activities (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

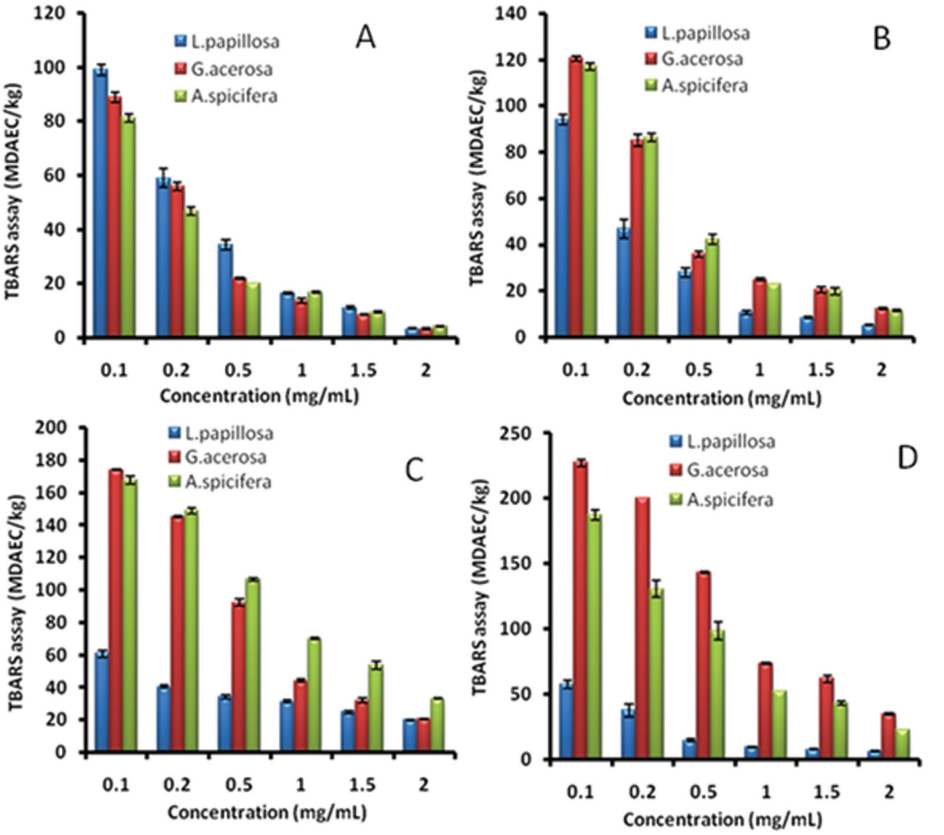


Figure 3D.6. TBARS formation inhibition assay (MDAEC/kg) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with concentration (0.1-2mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

3D.3.5. Hydrogen Peroxide (H_2O_2) Scavenging Activity (%)

H_2O_2 is a reactive non radical compound and is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it may convert into more reactive species such as singlet oxygen and HO. radicals. MDC (18.4 %) and EtOAc (14.4 %) fractions of *A. spicifera* exhibited significantly higher (P>0.05) H_2O_2 scavenging activity than its *n*-hexane fraction and MeOH extract (Table 3D.2). MDC and EtOAc fractions of *A. spicifera* found to retain their capacities to scavenge H_2O_2 even at lower concentrations (Figure 3D.5). EtOAc fraction of *G. acerosa* exhibited significantly higher (P<0.05) H_2O_2 scavenging activity (16.1 %) than its MDC > *n*-hexane fraction> MeOH extract (Table 3D.2). The EtOAc fraction of *L. papillosa* exhibited significantly higher (P<0.05) hydrogen peroxide scavenging activity (15.6 %) than its all other solvent fractions. In general the EtOAc fraction of all the seaweeds were effective towards scavenging H_2O_2, followed by the MDC fraction and MeOH extract indicating the presence of polar compounds capable of scavenging H_2O_2. The lower values exhibited by the *n* – hexane fractions in all the seaweed species further supports the fact polar groups present with the compound may be the reason for their radical scavenging activity. Earlier studies conducted by other researchers (Heo et al. 2006) also showed that the EtOAc fraction of seaweeds are good scavengers of H_2O_2, which is similar to our present findings.

3D.3.6. Lipid Peroxidation Inhibition Activity in Model System: Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity (MDAEC/kg)

The TBARS formation inhibitory capacities of EtOAc and MDC fractions (3.26 & 12.21MDAEC/kg respectively) were significantly higher (P<0.05) than that of *n*-hexane fraction and MeOH extract of *G. acerosa* (Table 3D.2). The EtOAc and MDC fractions of *L. papillosa* were found to possess significantly higher activities (3.35 and 5.05 MDAEC/kg, respectively) (P<0.05) at 2 mg/mL with respect to inhibit lipid peroxidation. Different solvent extracts of *A. spicifera* followed the same trend as shown by the different solvent extracts of *L. papillosa* as EtOAc fraction > MDC fraction > MeOH extract > *n*- hexane fraction (4.2, 11.3, 22.8 and 33.2 MDAEC/kg respectively) towards TBARS inhibition (Figure 3D.6). Earlier reports show that marine macroalgae are a rich source of various natural antioxidants, which play an important role in preventing lipid peroxidation (Wang et al. 2009; Senevirathne et al. 2006). A negative correlation was observed between the phenolic compounds and TBATRS assay indicated the presence of compounds other than phenolics like poly saccharides or poly unsaturated fatty acids etc as responsible for its lipid peroxidation inhibition activity.

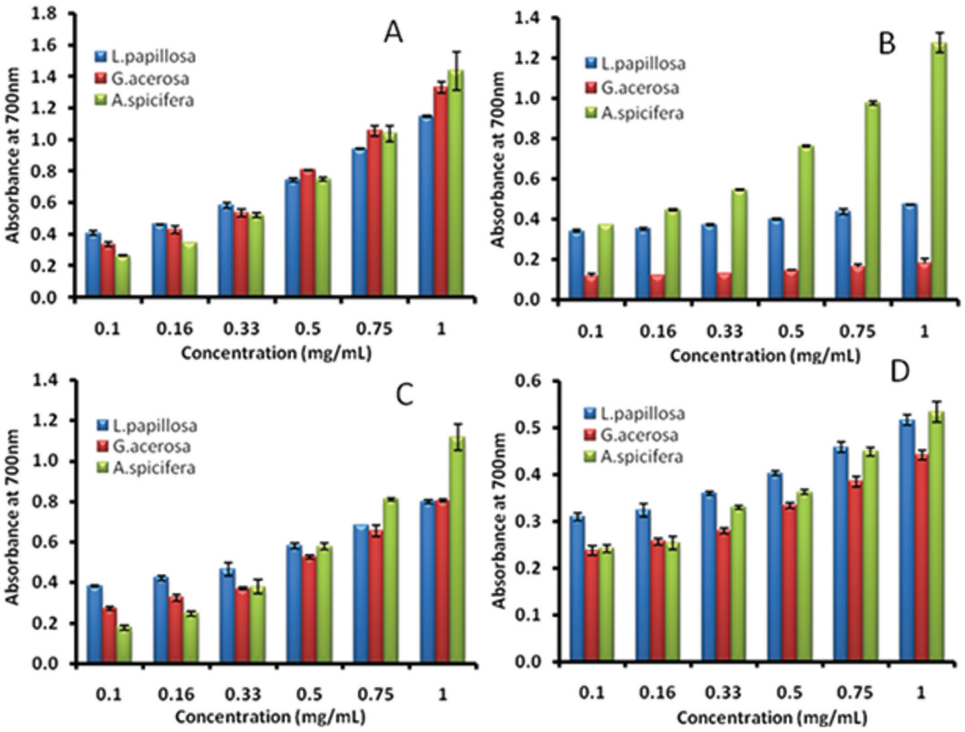


Figure 3D.7. Reducing ability (Ab_{700nm}) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with concentration (0.1-1mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

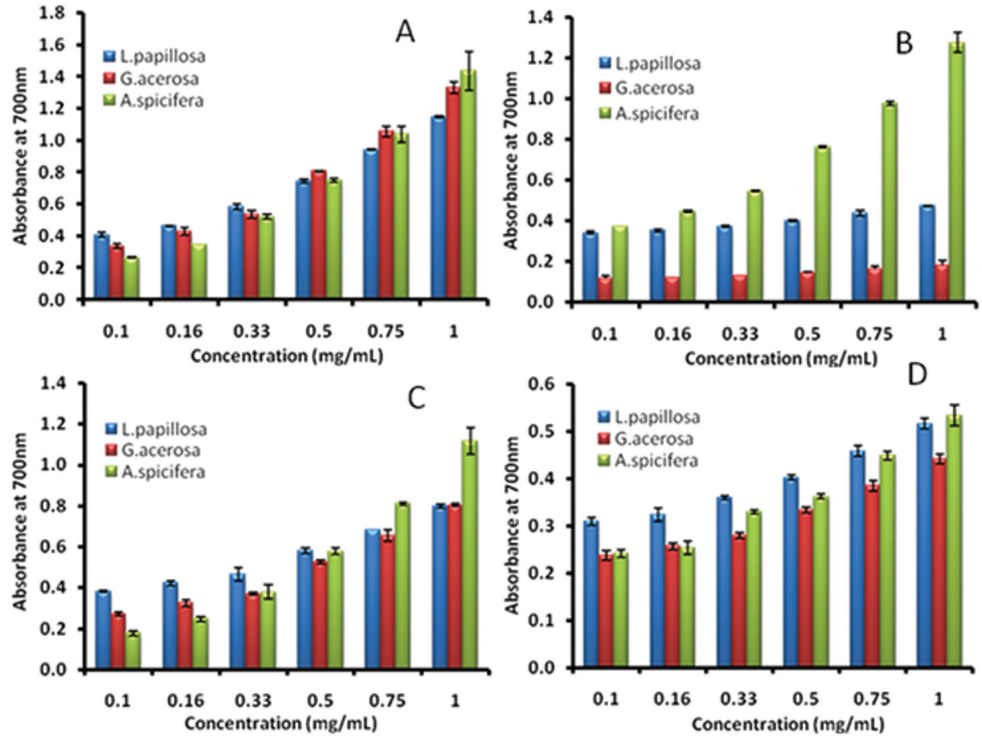


Figure 3D.8. Fe^{2+} chelating ability (%) of the EtOAc (A), MDC (B), *n*-hexane (C) fractions and MeOH extract (D) of *L. papillosa, G. acerosa* and *A. spicifera* with concentration (0.1-0.6mg/mL). Values are means of triplicate determinations and expressed with ± standard deviation

3D.3.7. Evaluation of Reducing Ability (Ab_{700}nm)

The EtOAc fractions of *A. spicifera* exhibited higher absorbance at 700 nm (Ab_{700}), (1.46) indicating a higher reducing power (Table 3D.2) and its other fractions of the same dose followed the order MDC> *n*-hexane > MeOH towards reducing ability (Table 3D.2). The reducing capacities of EtOAc and *n*- hexane fractions of *G. acerosa* as exhibited by absorbance at 700 nm (Ab_{700nm} 1.31 & 0.81, respectively) were found to be higher than that of its MeOH extract (Ab_{700nm} 0.45) and MDC (Ab_{700nm} 0.18) fraction (Figure 3D.7). EtOAc fraction of *L. papillosa* registered a significantly higher (P<0.05) reducing ability (Ab_{700nm} 1.14) followed by the *n*-hexane fraction (Ab_{700nm} 0.81). It is believed that antioxidant activity and reducing power are related as reductones can inhibit lipid peroxidation by donating a hydrogen atom and thereby terminating the free radical chain reaction (Shon et al. 2003). Earlier research works also showed that the MeOH extracts of eight seaweeds obtained from Sabah waters exhibited reducing activity (Matanjun et al. 2008).

3D.3.8. Ferrous Ion (Fe^{2+}) Chelating Activity (%)

Ferrous ion binding capacities of MDC fraction (63.48 %) were significantly higher (P<0.05) than that exhibited by the EtOAc fraction of *G. acerosa*. EtOAc fractions of *A. spicifera* exhibited a significantly higher (P <0.05) ferrous ion chelating ability (61.58 %). The other fractions of the same dose followed the order MDC> *n*- hexane > MeOH extract towards ferrous ion chelating ability (Table 3D.2). In *L. papillosa* the EtOAc fraction (47.60 %) and MDC fraction (44.13 %) were shown no significant difference(P>0.05) with each other (Figure 3D.8). Some studies have demonstrated that polyphenols derived from seaweeds are potent ferrous ion chelators (Senevirathne et al. 2006; Toth & Pavia 2000; Chew et al. 2008) and metal chelating potency of phenolic compounds is dependent upon their unique phenolic structure and the number and location of the hydroxyl groups (Santoso et al. 2004). In our present study same results were emerged by getting a high correlation between TPC and ferrous ion chelating activity.

3D.3.9. Correlations between Phenolic Contents and Different Antioxidant Activity Assays

The similarities and differences between different organic fractions of *L. papillosa* , *G. acerosa* and *A. spicifera* and the relationships among different antioxidant activity assays were statistically analyzed using PCA. The first two principal components explained 99.78 % (PC 1 – 94.04 %; PC 2 – 5.74 %) of the total variance in the data set (Figure 3D.10). TPC assay showed significant correlation with DPPH radical scavenging activity, $ABTS^{\cdot+}$ radical scavenging activity, hydroxyl radical scavenging activity, H_2O_2 scavenging activity and ferrous ion chelating ability (Figure 3D.10). From this observation it may be inferred that polyphenols present in algal extracts are responsible for its radical scavenging as well as chelating ability. It was reported earlier that,

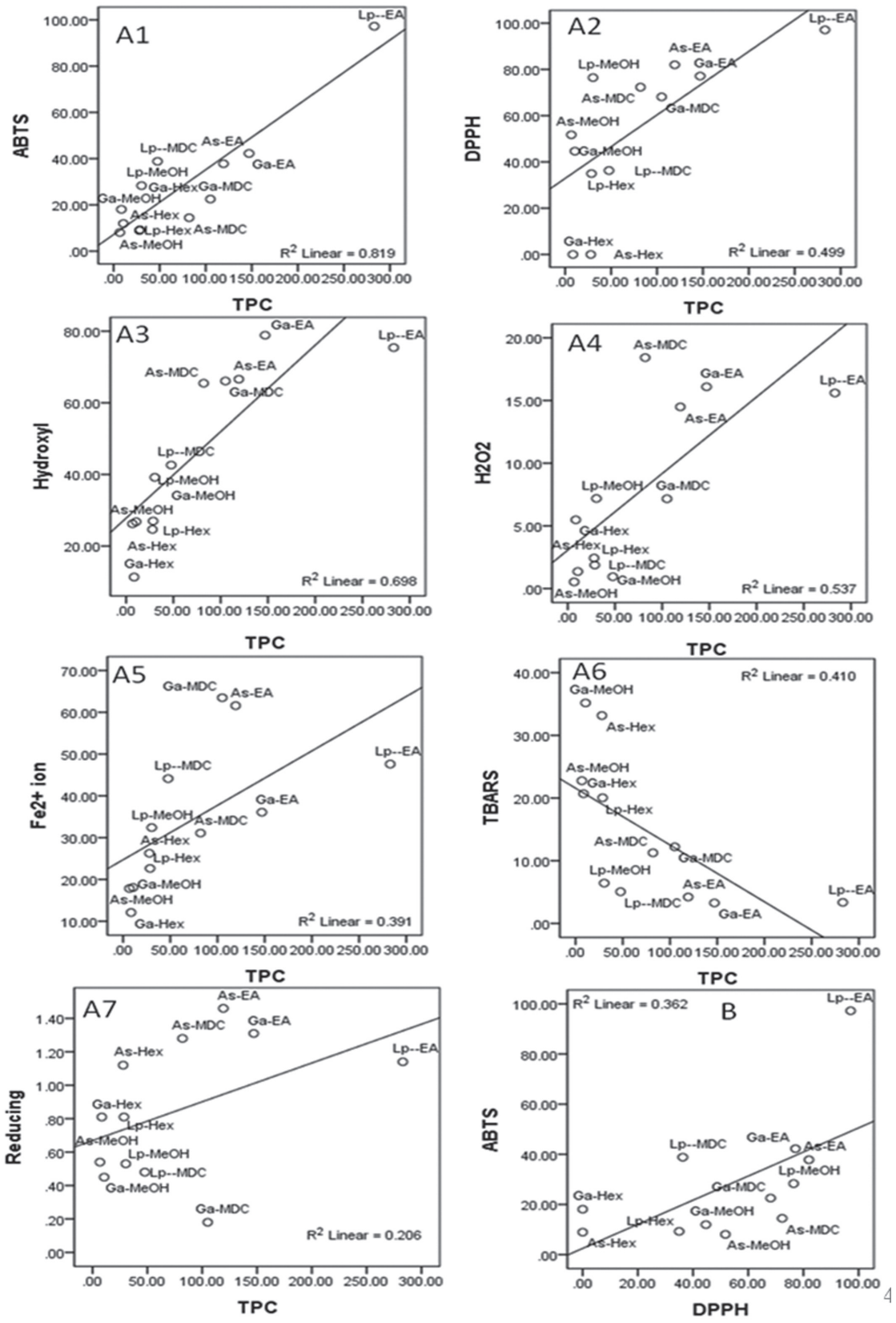


Figure 3D.9. Scatter plot showing the correlation of TPC with A1) ABTS.+ radical scavenging activity assay (n = 12, r = 0.905, *P*<0.01), A2) DPPH radical scavenging activity assay (n = 12, r = 0.707, *P*<0.05), A3) hydroxyl radical scavenging assay (n = 12, r = 0.836, P<0.01), A4) H2O2 scavenging assay (n = 12, r = 0.733, *P*<0.01), A5), ferrous ion chelating activity (n = 12, r = 0.625, *P*<0.05), A6) TBARS formation inhibition assay (n = 12, r = 0.640, *P*<0.01) and B) scatter plot showing the correlation between DPPH and ABTS.+ radical scavenging activity assay (n = 12, r = 0.602, *P*<0.05)

seaweeds with highest TPC are also good chelators of ferrous ion (Toth & Pavia 2000). This observation was in contradiction with the study of Wang et al. (2009) in which it was reported that the algal polyphenols are probably not strong chelators of transition metals. From a negative correlation observed between TPC and TBARS assay (Figure 3D.9), it can be speculated that the antioxidant activity did not depend only on the total phenol concentration, but also on their polarity and molecular structure (Hernández et al. 2009) or there may be some active metabolites other than phenolics such as polysaccharides capable of inhibiting the TBA-MDA adduct formation (Muzzarelli 1997).

3D.3.10. Chromatographic Evaluation of the Phenolic Compounds in the Methanol Extract and EtOAc Fraction of Seaweeds

The selected groups of phenolics in MeOH and EtOAc solvent extracts of *L. papillosa*, *G. acerosa* and *A. spicifera* were separated and identified by the RP-HPLC method, are shown in Table 3D.2. A retention time (R_t) library of the standard phenolic compounds was constructed with detection at T_{max} 324 nm and 277 nm (Figure 3D.5). The qualitative analysis of the seaweed extracts for phenolic compounds were obtained and the retention time for individual peak was compared with the library of standard phenolic compounds under the same HPLC conditions. A higher amount of syringic acid (23.6mg) was observed with the methanol extract of *L. papillosa* where as its EtOAC fraction registered higher amount of ECG (33.17mg) than other tested phenolic acids. Ferulic acid, epicatechin and ECG were found to be common with both these extracts. The HPLC analysis indicated that caffeic acid was the predominant phenolics in the EtOAc fraction of *G. acerosa*, whilst more polar chlorogenic acid was the major phenolics in its MeOH fraction. Catechin and epicatechin gallate (at 277 nm) were also present copiously among both these extracts. In *A. spicifera* both MeOH and EtOAc extracts contain phenolic acids epicatechin gallic acid, caffeic acid and 5-caffeoylquinic acid with cyclohexanecarboxylic acid derivative (chlorogenic acid R_t 8.12). A high amount of epicatechin was found with the MeOH fraction of *A. spicifera* where as hydroxy benzoic acid derivative (gallic acid) and hydroxy cinnamic acid (caffeic acid) was dominant with its EtOAc fraction.

The EtOAc fractions of both *G. acerosa* and *A. spicifera* which exhibited strong antioxidant activities (in terms of scavenging $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ free radicals), were found to contain high amounts of total and individual phenolics, thereby validating the fact that phenolic compounds in these seaweeds contributed to the antioxidant activity. It has been reported that free radical-scavenging activity is greatly influenced by the phenolic composition of the sample (Cheung et al. 2003). There are other reports which showed that HPLC analysis of the crude extracts from red and brown seaweeds contained fourteen polyphenols, *viz.*, gallic acid, catechin, epicatechin, rutin,

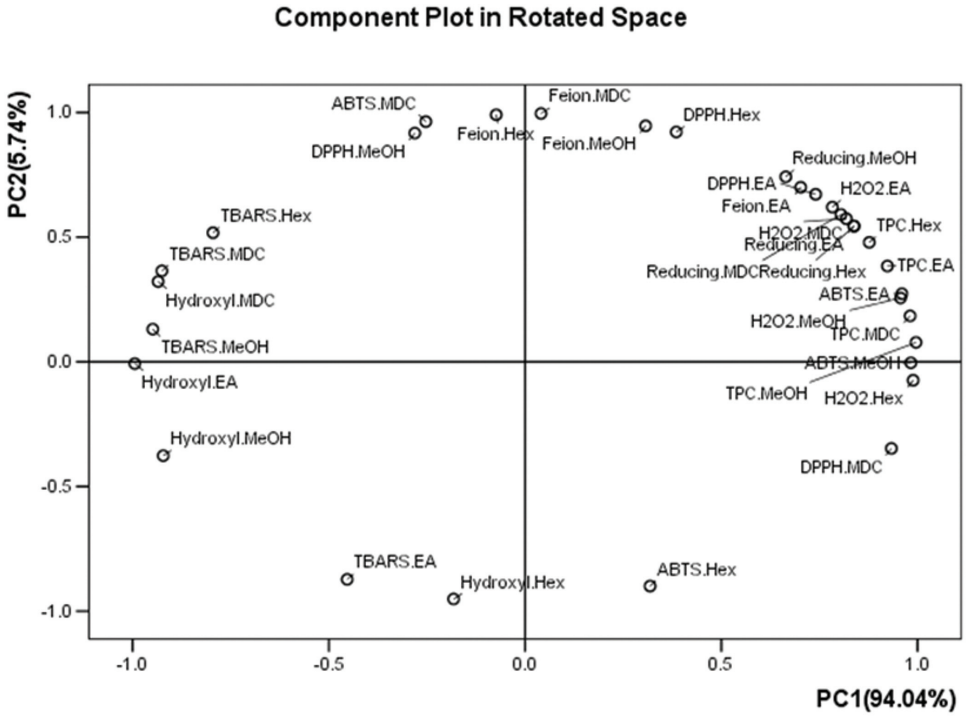


Figure 3D.10. Loading plot of different antioxidant activity assays of different solvent fractions from the *L. papillosa* (Lp), *G. acerosa* (GA) and *A. spicifera* (AS) (MeOH - Methanol extract; Hex - n-hexane fraction; MDC- dichloromethane fraction; EA - ethylacetate fraction)

Table 3D.3. Major phenolic compounds (mg/g extract) identified in the MeOH (A) extract and EtOAc (B) fractions of *L. papillosa* (LP), *G. acerosa* (GA) & *A. spicifera* (AS) by HPLC

Sl.No	Phenolic acids	R_t (minute)	phenolic compounds (mg/g extract) identified in the MeOH extract (A) and EtOAc fraction (B)					
	324nm		LP-A	LP-B	GA-A	GA-B	AS-A	AS-B
1	Chlorogenic acid	8.12	-	0.1±0.0	27.08±0.16		5.03± 0.14	0.4± 0.01
2	Caffeic acid	10.49	-		8.11± 0.23	11.40± 0.36	3.11± 0.06	2.22± 0.02
3	2,5 dihydroxy benzoic acid	17.43	-	10.2±0.10	-	-	-	-
4	Coumaric acid	20.56	-	0.3±0.01	-	-	-	-
5	Ferulic acid	24.19	1.2±0.01	0.6±0.01	-	-	-	-
6	Quercetin	37.90	0.3±0.01	-	-	-	-	-
7	Salicylic acid	44.92	1.4±0.01	-	-	-	-	-
	277n m							
8	Gallic acid	5.39	11.4±0.51	-	-	-	7.33± 0.17	3.17± 0.04
9	Catechin	6.81	-	-	-	21.36± 1.25	9.25± 0.35	-
10	EGCG	8.13	-	23.2±1.06	28.19± 2.64	8.26± 1.03	-	-
11	Epicatechin	10.11	2.8±0.33	24.7±2.34	8.21± 0.27	1.02±0.02	14.13± 1.10	0.6±0.01
12	ECG	13.0	14.6±0.33	33.17±1.57	-	-	-	-
13	Syringic acid	14.78	23.6±2.18	-	-	-	-	-

Values are mean ± SD (n = 3).

p-coumaric acid, myricetin, quercetin, protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids (de Quirós et al. 2010, López et al. 2011, Namvar et al. 2012 ,Wang et al. 2013).

3D.4. Conclusions

EtOAc fraction of *L. papillosa* exhibited higher total phenolic content (283GE/g), ABTS (97.24 %) as well as DPPH· radical scavenging activities (97.10%). Higher hydroxyl radical scavenging activity (78.87%) and TBARS inhibition ablity (3.26 MDAEC /kg) was observed with the EtOAc fraction of *G. acerosa* whereas higher ferrous metal Ions chelating activity (63.48%) was observed with its MDC fraction. MDC fraction of *A. spicifera* exhibited higher H_2O_2 scavenging ability (18.42%) and a higher reducing capability (1.46 Ab_{700nm}) was observed with its EtOAc fraction. A higher amount of syringic acid (23.6mg) was observed with the methanol extract of *L. Papillosa* where as its EtOAC fraction registered higher amount of ECG (33.17mg) than other tested phenolic acids. Ferulic acid, epicatechin and ECG were found to be common with both these extracts. The HPLC analysis indicated that caffeic acid was the predominant phenolics in the EtOAc fraction of *G. acerosa*, whilst more polar chlorogenic acid was the major phenolics in its MeOH fraction. In *A. spicifera* both MeOH and EtOAc extracts contain phenolic acids epicatechin gallic acid, caffeic acid and 5-caffeoylquinic acid with cyclohexanecarboxylic acid derivative (chlorogenic acid R_t 8.12). These results indicated a significant correlation of TPC in EtOAc fractions of seaweeds with $ABTS^{\cdot+}$, DPPH, HO. scavenging activity, and reducing ability, apparently indicating that polyphenols present in seaweed extracts, particularly in EtOAc fraction are responsible for radical scavenging ability. The present study provides valuable information regarding the potential of these seaweeds especially *L. papillosa* and *G. acerosa* as candidate species to develop natural sources of antioxidant compounds to include in food supplements and as nutraceuticals for disease curing remedy.

CHAPTER 4

Isolation and Characterization of Antioxidant Secondary Metabolites from Seaweeds

CHAPTER 4

Isolation and Characterization of Antioxidant Secondary Metabolites from Seaweeds

The isolation was carried out as bioassay guided and pure compounds were obtained by repeated purification done with the aid of various chromatographic techniques. The structural characterization was done with the aid of various spectroscopic techniques. The pure compounds were further assayed to document its antioxidant and ion chelating potential. Among the different seaweeds, *Anthophycus longifolius* (Turner) Kützing (*Sargassum longifolium*), *Acanthophora spicifera* (M. Vahl) Børgesen, *Padina gymnospora* (Kützing) Sonder, and *Laurencia papillosa* (C. Agardh) Greville were found to be abundantly available and were demonstrated to possess potential antioxidant activity. Some of the seaweeds exhibited potentially high radical scavenging activity as discussed in chapter 3. However on purification the column fractions were found to lose their activity. It is therefore the seaweeds as mentioned above have been reported for isolation and characterization of antioxidant molecules. These seaweeds also found to be abundantly available as the availability was found to be independent of seasonal variations. The representative bioactive compounds separated from these species have been described below.

4.1. Materials and Methods

4.1.1. General Experimental Procedures

Fourier Transform Infra Red spectrometer (FTIR) spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted in to mid IR range, i.e., between 4000-400cm^{-1}. UV spectra were obtained on a Varian Cary 50 UV-VIS spectrometer (Varian Cary, USA). Thin layer chromatography was carried out on the precoated silica gel 60F_{254} plates (E-Merck, Germany). A flash column chromatograph (Biotage SP Flash Purification System, SP1-B1A, Biotage AB, Sweden) was used with a collection UV wavelength at 236 nm and monitoring wavelength at 227 nm using the flow rate of 12 mL/min. The fractionation on the flash chromatograph has been performed using the flash silica gel cartridge (Biotage No. 25+M 0489-1). The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in $CDCl_3$ as aprotic solvent at ambient temperature with TMS as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ^{13}C NMR signals was determined from DEPT experiments. Standard pulse sequences were used for DEPT, ^{1}H–^{1}H COSY, two-dimensional NOESY, HSQC, and HMBC experiments. Liquid chromatography–mass spectrometry experiments were performed

Figure 4.1. *Anthophycus longifolius* collected from Kelaikkarai of Gulf of Mannar

on an Applied Biosystems QTrap 2000 (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) using a Luna 5 μ C_{18} column (100 A, 100 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) or a Luna 3 μ C_{18} column (100 A, 50 × 1.0 mm, Phenomenex, Aschaffenburg, Germany) and a gradient of solvents A (0.1% HCOOH) and B (CH_3CN + 0.1% HCOOH; gradient 0% B to 100% B in 10 min) with a flow rate of 1.5 mL/min or 60 μL/min, respectively. The GC-MS analyses were performed in electronic impact (EI) ionization mode in a Varian GC (CP-3800) interfaced with a Varian 1200L single quadruple Mass Spectrometer. ESI-MS spectra were acquired in the positive and negative modes with a turboionspray voltage, curtain gas, turbo temperature, and nebulizer gas of -4500 V, 30 psi, 500 °C, and 50 psi (positive mode) at a flow rate of 1.5 mL/min. Elemental analysis of the compounds was carried out using a Euro Vector elemental analyzer (model no. EA3011). All compounds were of analytical, spectroscopic or chromatographic reagent grade, and were obtained from E-Merck (Darmstadt, Germany). All reagents and chemical solvents used for products isolation were of analytical grade or higher.

4.2. Isolation and Characterization of Antioxidant Secondary Metabolites from *Anthophycus longifolius* (Turner) Kützing

4.2.1. Bioassay Guided Chromatographic Purification of the Methanol Extract of *Anthophycus longifolius*

The MeOH extract of *A. longifolius* (1260 mg) collected from Gulf of Mannar (Figure 4.1) was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*-hexane, *n*- hexane:EtOAc and finally EtOAc:MeOH. Collected fraction were concentrated under vacuum and TLC evaluated and fractions with same TLC profile were pooled together to furnish eight fractions (Al_{1-8}). The column fractions obtained from Al/MeOH extract was evaluated for its antioxidant activity by measuring its ability to scavenge DPPH (0.1 mg/mL) and ABTS (0.1 μg/mL) radicals and to chelate Fe^{2+} ion (0.1mg/mL). The sub fractions Al_5 which exhibited high radical scavenging ability as obtained from its DPPH radical scavenging ability (57.04%), ABTS radical scavenging ability (24.17%) and Fe^{2+} ion chelating ability (16.92%) was further chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*- hexane, *n*- hexane:EtOAc and finally EtOAc:MeOH. Collected fractions were concentrated under vacuum and TLC evaluated and fractions with same TLC profile were pooled together to furnish six sub fractions ($Al_{5,\ 1-6}$).

The sub fraction $Al_{5,4}$ which exhibited 60.27% DPPH, 21.37% ABTS radical scavenging potential and 16.74% ion chelating ability was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*- hexane, *n*- hexane: EtOAc and finally EtOAc : MeOH to collect another six sub fractions ($Al_{5,4,1-6}$) of which $Al_{5,4,1}$ and $Al_{5,4,3}$ exhibited higher radical scavenging and ion chelating potential and selected for further purification (Figure 4.2).

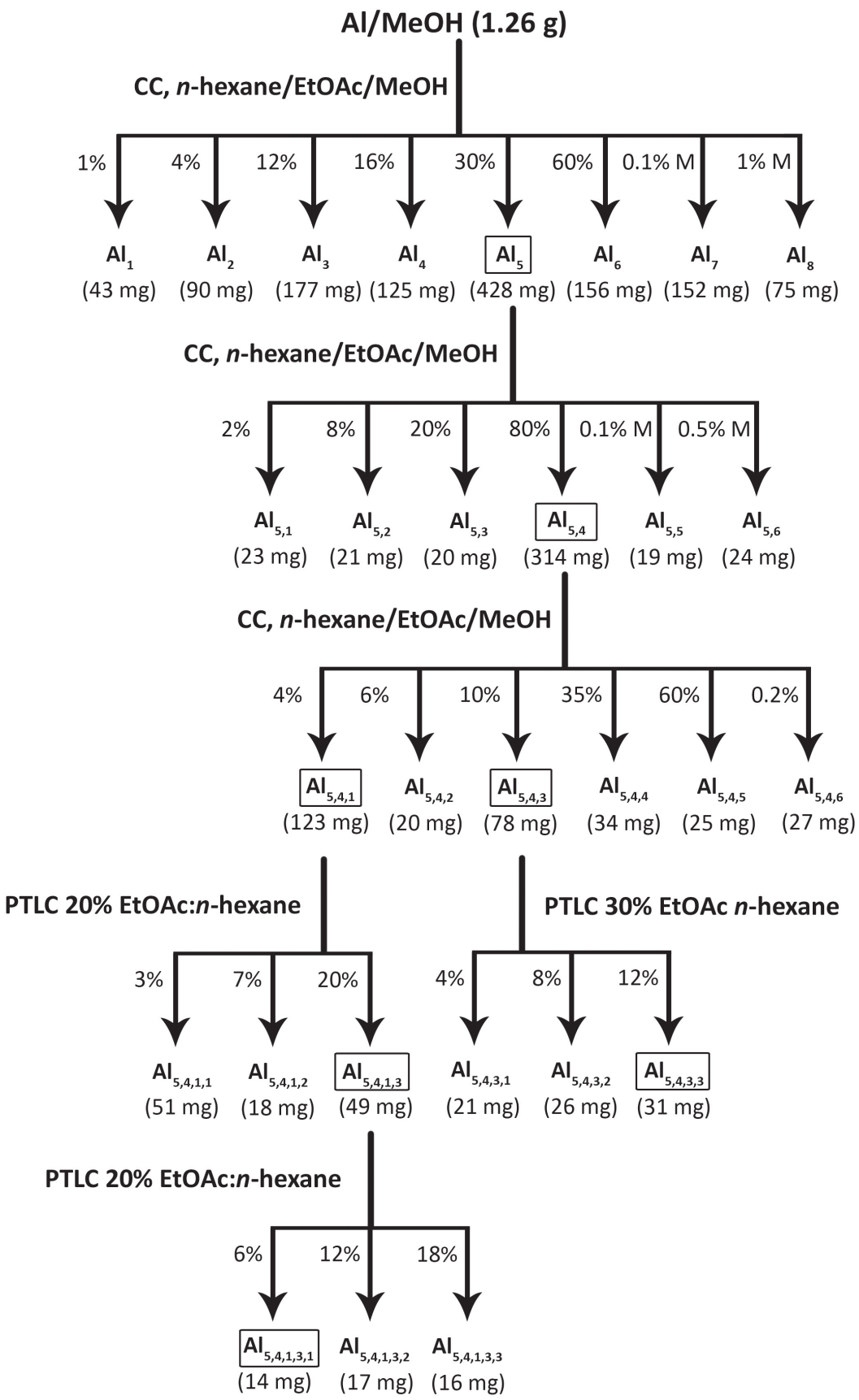


Figure 4.2. Schematic diagram representing the chromatographic purification of the MeOH extract of *A. longifolius* (Al). CC: column chromatography, PTLC: preparative thin layer chromatography

The sub fraction $Al_{5,4,1}$ which exhibited good DPPH (63.35%), ABTS (26.17%) radical scavenging potential and Fe^{2+} ion chelating ability (18.41%) was re-purified using preparative thin layer chromatography over Si gel GF_{254} (particle size 15 µm) (25% EtOAc: *n*- hexane) to obtain three sub fractions of which the sub fraction $Al_{5,4,1,3}$ exhibited higher antioxidative potential. The pure and active compound $Al_{5,4,1,3,1}$ ((9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate) (65.50% and 23.48% for DPPH and ABTS radical scavenging ability respectively, and 20.01% ion chelating potential) was obtained by the preparative thin layer chromatography purification of $Al_{5,4,1,3}$ over Si gel GF_{254} (particle size 15 µm) (30% EtOAc: *n*- hexane).

The crude sub fraction $Al_{5,4,3}$ which showed high radical scavenging (66.56% and 26.73% for DPPH and ABTS radical scavenging ability respectively) and ion chelating potential (19.75%) upon preparative thin layer chromatography purification over Si gel GF_{254} (particle size 15 µm) (30% EtOAc: *n*- hexane) resulted in three sub fractions of which $Al_{5,4,3,3}$ (3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid) was pure and active (76.40% and 30.00% for DPPH and ABTS radical scavenging ability respectively, and 26.02% ion chelating potential).

The structural characterization were carried out be detailed spectroscopic techniques and are discussed in following sections (4.2.2 and 4.2.3)

4.2.2. Structural Characterization of ((9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate

(9H-Fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate: Amorphous yellow solid; UV (MeOH) λ_{max} (log ε): 247 nm (3.91); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 10:90, v/ v) R_f: 0.55; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) R_t: 7.20 min.; Elemental analysis found: C,72.42;H,7.13;N,3.67;O,16.78; IR (KBr, cm^{-1}) ν_{max} 721.40cm^{-1} γ_r(C-H alkanes), 1034.84 cm^{-1} ν(C-N), 1168.90 1313.57 1377.22 cm^{-1} γ_r(C-H), 1464.02 cm^{-1} δ(C-H of alkanes), 1600.97 1665.59 δ(N-H), 1741.78 cm^{-1} ν(C=O), 2728.40, 2852.81, 2924.18, 2956.01 cm^{-1} ν(C-H alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.2; EIMS *m/e* (rel. int. %): 382 $[M+1]^+$ (24), 336(70), 264(48), 203(28), 196(34), 166 (12), 97(72), 64(96), 55(100); HRMS (ESI) *m/e*: calcd. for $C_{23}H_{27}NO_4$ 381.4814; found 381.4891 $[M+H]^+$.

(9H-Fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate (Figure 4.3), a new derivative of the fluorens was isolated as yellowish amorphous solid upon chromatography over silica columns. The IR absorption bands (in MeOH) have been detailed in the methodology section. Its mass spectrum exhibited a molecular ion peak at *m/e* 382, which in combination with its ^{1}H and

Table: 4.1. Antioxidant and Fe^{2+} ion chelating potential (%) of the column fractions obtaines by the chromatographic purification of the methanol extract of *A. longifolius*.

Sample ID	ABTS Radical Scavenging Activity (%)	DPPH Radical Scavenging Activity (%)	Fe^{2+} ion Chelating Ability (%)
Al/MeOH			
Al_1	18.81±0.25	45.54±0.61	13.75±0.18
Al_2	21.67±0.29	54.40±0.73	16.19±0.21
Al_3	15.81±0.21	39.36±0.53	11.34±0.15
Al_4	17.11±0.23	42.28±0.57	12.73±0.17
Al_5	24.17±0.32	57.04±0.77	16.92±0.22
Al_6	14.81±0.20	35.54±0.48	9.874±0.13
Al_5			
$Al_{5,1}$	7.445±0.10	17.71±0.24	5.033±0.06
$Al_{5,2}$	21.33±0.28	50.34±0.68	13.98±0.18
$Al_{5,3}$	14.30±0.19	36.61±0.49	10.89±0.14
$Al_{5,4}$	21.37±0.28	60.27±0.81	16.74±0.22
$Al_{5,5}$	9.841±0.13	27.75±0.37	7.708±0.10
$Al_{5,6}$	8.577±0.11	21.18±0.28	6.195±0.08
$Al_{5,4}$			
$Al_{5,4,1}$	26.17±0.35	63.35±0.85	18.41±0.24
$Al_{5,4,2}$	14.11±0.19	35.43±0.48	10.21±0.13
$Al_{5,4,3}$	26.73±0.36	66.56±0.90	19.75±0.26
$Al_{5,4,4}$	18.79±0.25	46.43±0.62	13.81±0.18
$Al_{5,4,5}$	20.04±0.27	47.30±0.64	14.29±0.19
$Al_{5,4,6}$	15.08±0.20	36.20±0.49	10.90±0.14
$Al_{5,4,1}$			
$Al_{5,4,1,1}$	6.977±0.09	16.60±0.22	4.613±0.06
$Al_{5,4,1,2}$	9.504±0.12	22.42±0.30	6.501±0.08
$Al_{5,4,1,3}$	31.50±0.42	74.98±1.01	21.12±0.28
$Al_{5,4,1,3}$			
$Al_{5,4,1,3,1}$	23.48±0.35	65.50±0.84	20.01±0.24
$Al_{5,4,1,3,2}$	5.059±0.06	12.49±0.16	3.601±0.04
$Al_{5,4,1,3,3}$	14.95±0.20	35.29±0.47	10.26±0.13
$Al_{5,4,3}$			
$Al_{5,4,3,1}$	6.787±0.09	17.30±0.23	5.151±0.06
$Al_{5,4,3,2}$	17.22±0.23	42.55±0.57	11.82±0.16
$Al_{5,4,3,3}$	30.00±0.27	76.40±0.76	26.02±0.21

Data are the mean values of the triplicate and expressed as mean ± standard deviation. The concentration of the solvent fractions used were 0.1 mg/mL for DPPH radical scavenging activity, and Fe^{2+} ion chelating activity; and 0.1 μg/mL for ABTS radical scavenging activity.

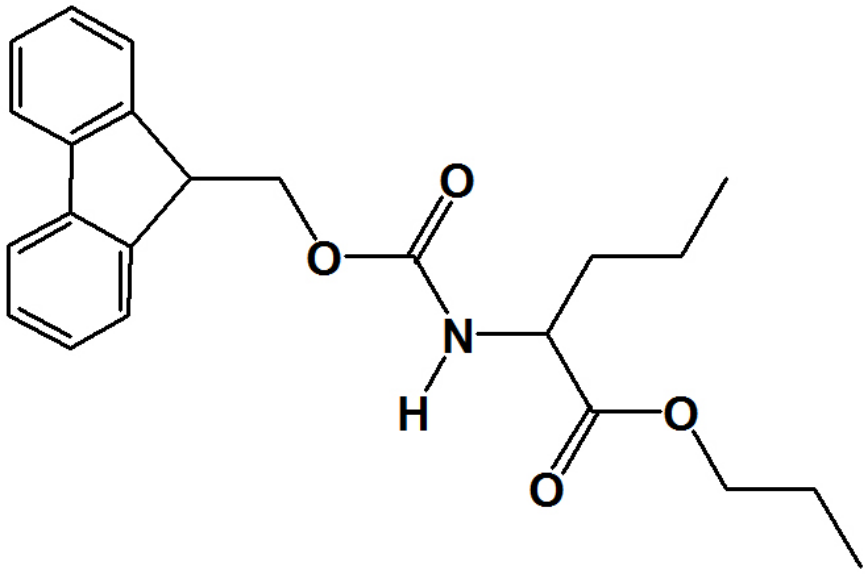


Figure 4.3. (9H-Fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate

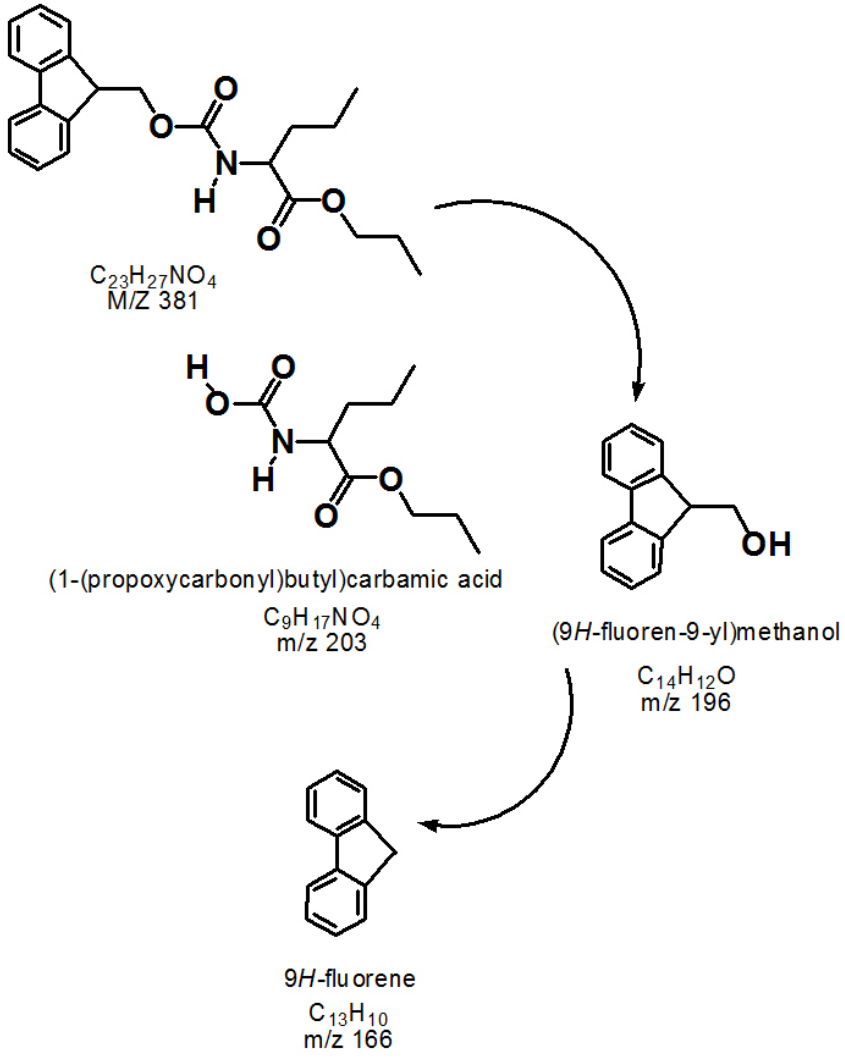


Figure 4.4. Mass fragmentation pattern of (9H-fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate

Table 4.2. NMR spectroscopic data of (9H-fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR(int., mult., *J* in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	86.9	H-1	1.60(t)	H-11	C-5,C-6a
1a	139				
1b	131				
2	130	H-2	7.72(m)		C-3
3	128	H-3	7.72(m)	H-4	C-1b
4	129	H-4	7.52(m)	H-3	
5	129	H-5	7.52(m)		C-1a,C-1
6a	139.38				
6b	131				
7	130	H-7	7.72(m)	H-8	C-10
8	128	H-8	7.72(m)	H-9	
9	129	H-9	7.25(m)	H-8	C-10,C-11
10	129	H-10	7.52(m)	H-9	
11	47.9	H^a-11,H^b-11	H^a-11at2.04(d), H^b -11at2.96(d)		C-12
12	163.83				
	N	H	4.31(s)		
13	33.9	H-13	2.3 (t)	H-13a	C-14,13a
13a	24.7	H-13a	1.58(q)	H-13,13b	C-14,13b
13b	22.7	H-13b	1.30(m)	H-13a,H-13c	C-14,13c
13c	14.2	H-13c	0.91(t)	H-13b	
14	177.28				
15	65.7	H^a-15,H^b-15	H^a -15at3.3(t), H^b-15, 4.1(t)	H-16	C-14,
16	19.69	H-16	3.2(q)	H-15,H-17	C-15,17
17	14.1	H-17	0.88(t)	H-16	C-15

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments.

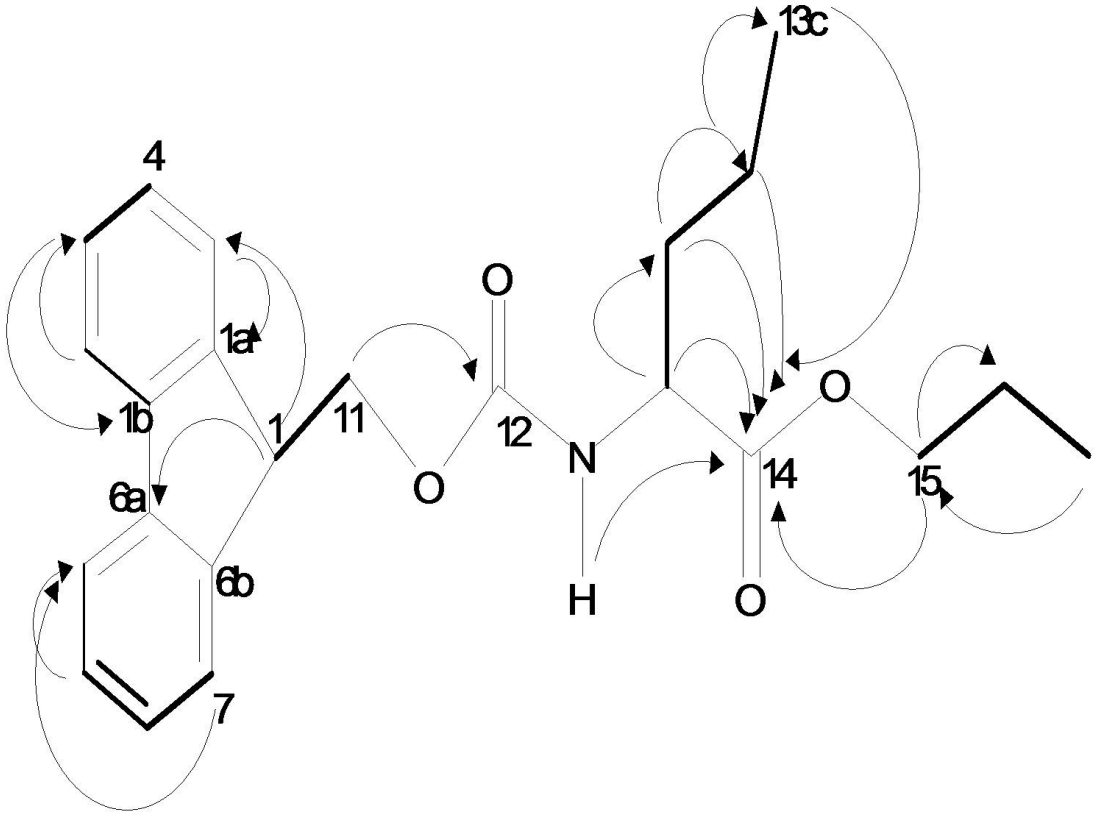


Figure 4.5. 2D NMR correlations as observed in (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

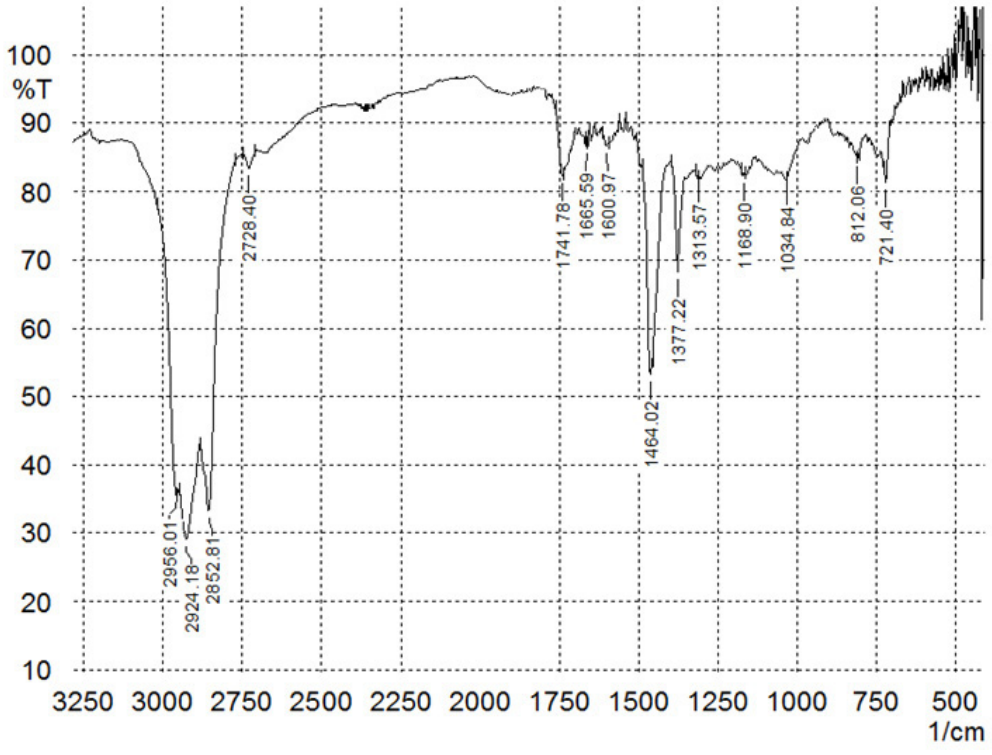


Figure 4.6. IR spectrum of (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate

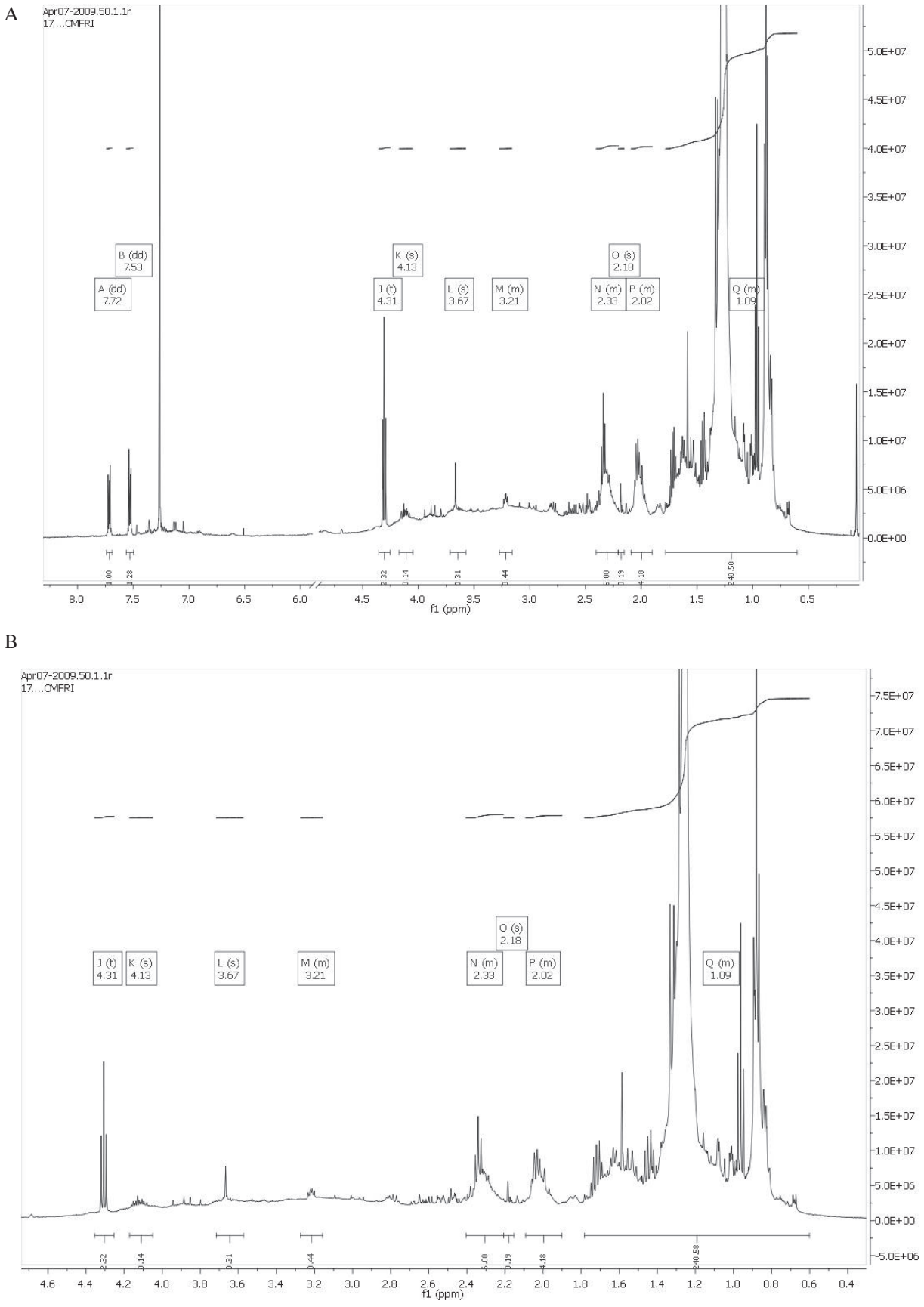
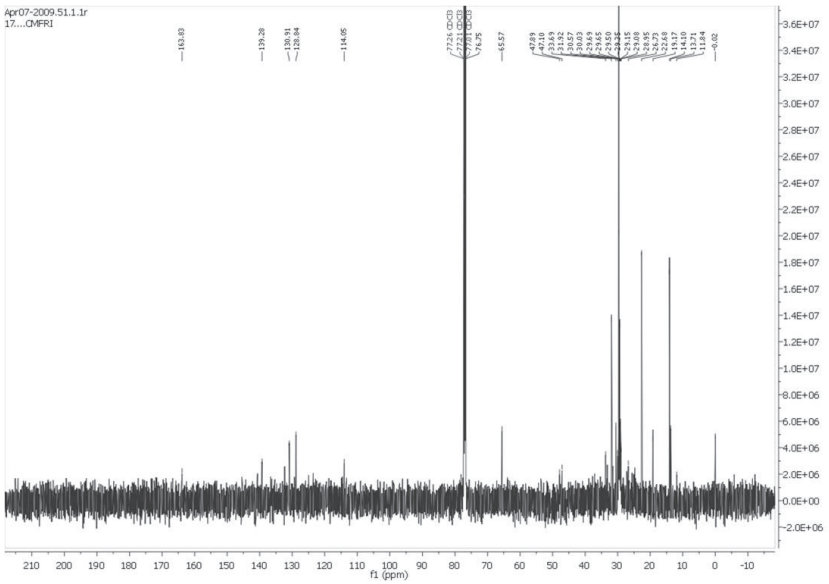
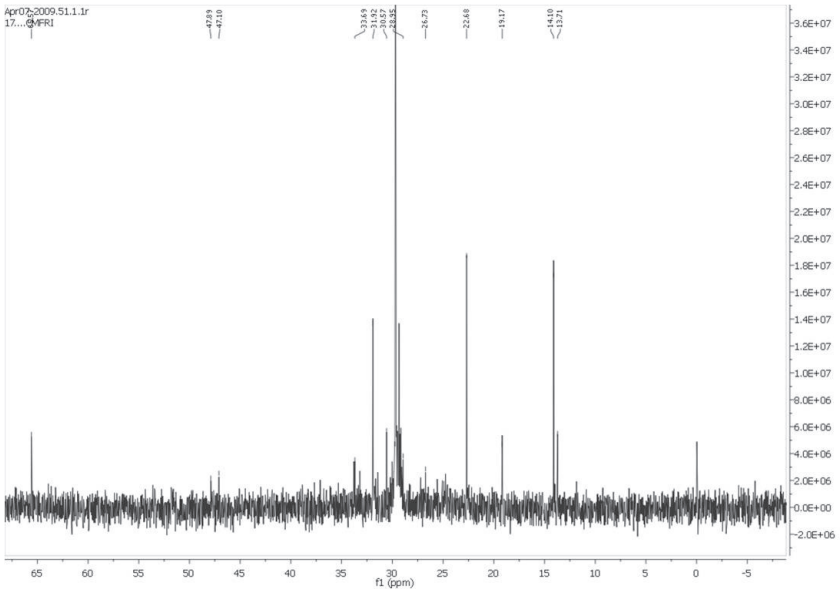


Figure 4.7. Proton NMR spectra of (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate (A - full view, B - expanded view)

A



B



C

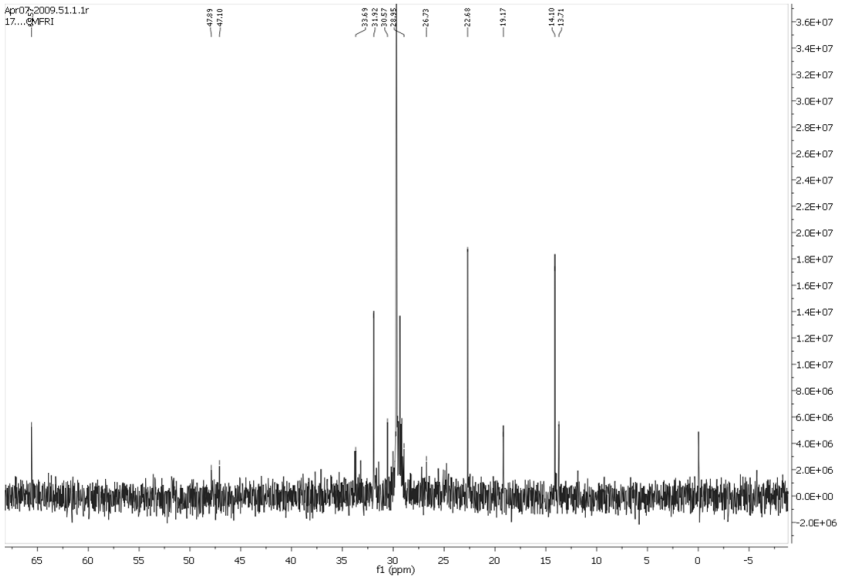


Figure 4.8. ^{13}C spectra of (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate (A - full view, B & C - expanded view)

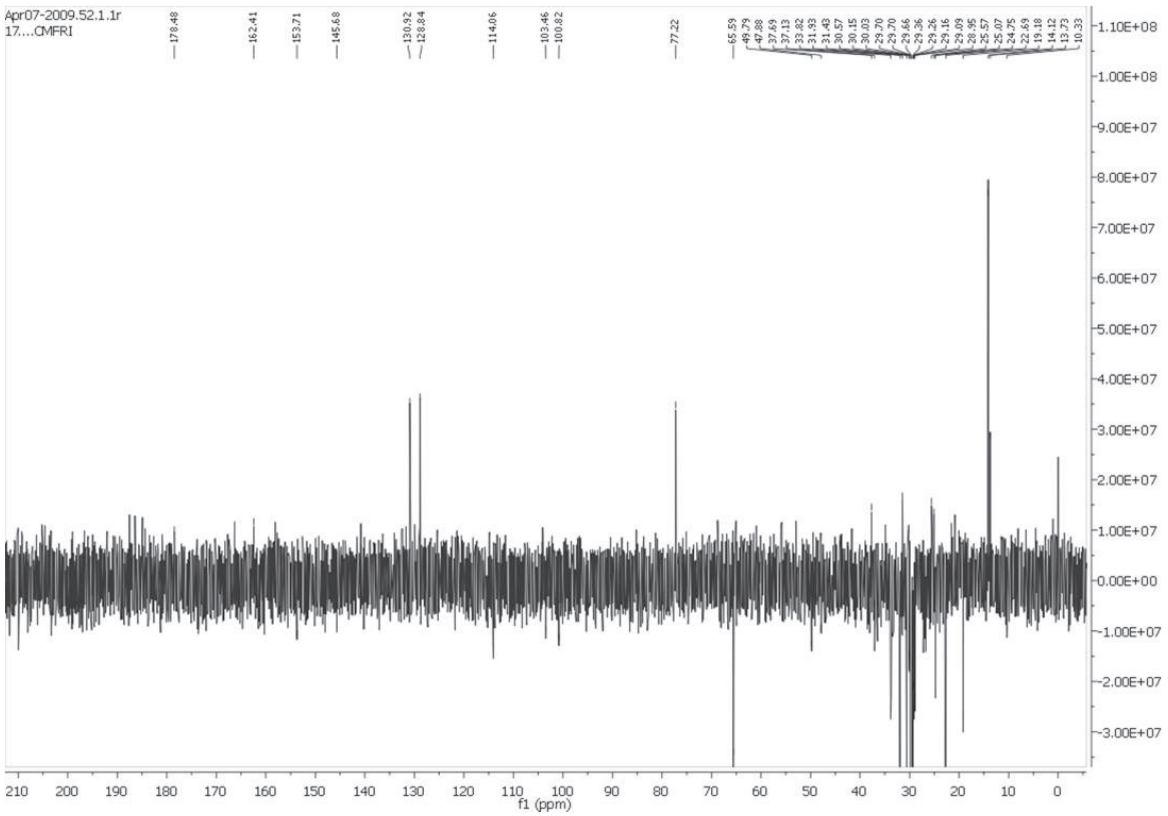


Figure 4.9. DEPT spectrum of (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate

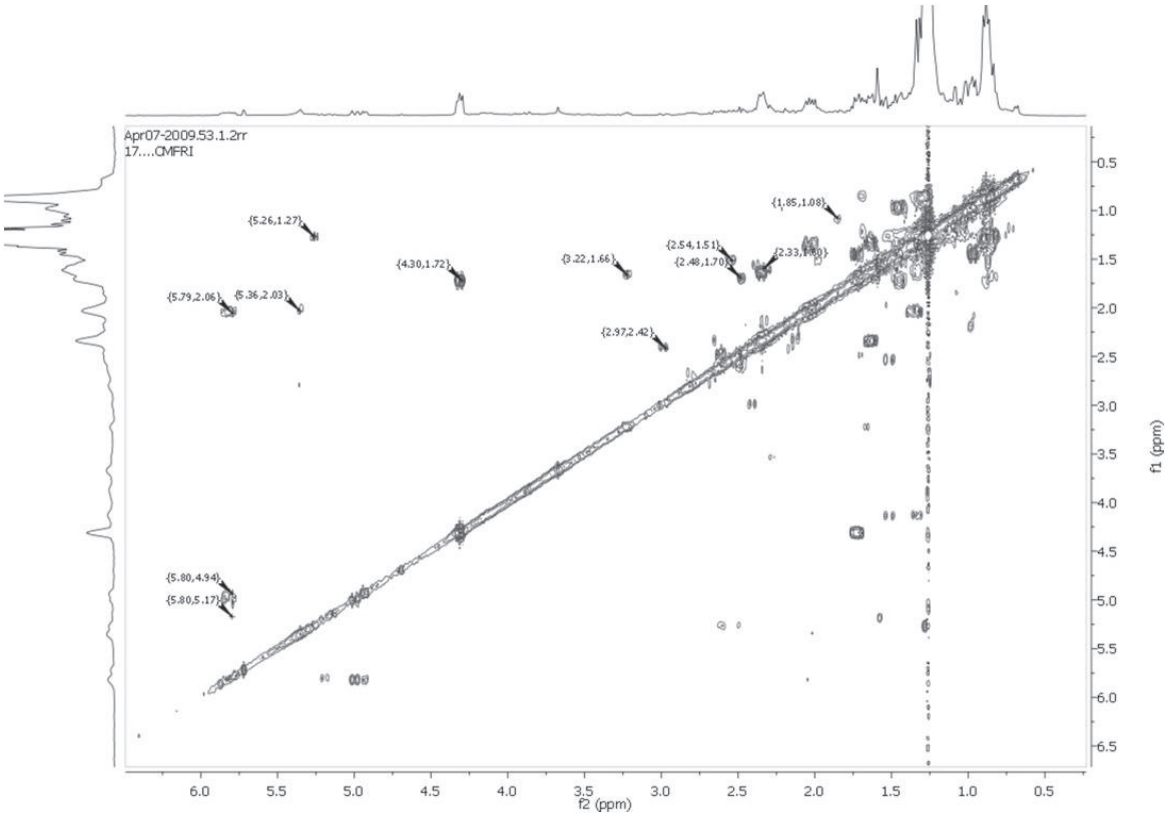


Figure 4.10. ^{1}H-^{1}H-COSY spectrum of (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate

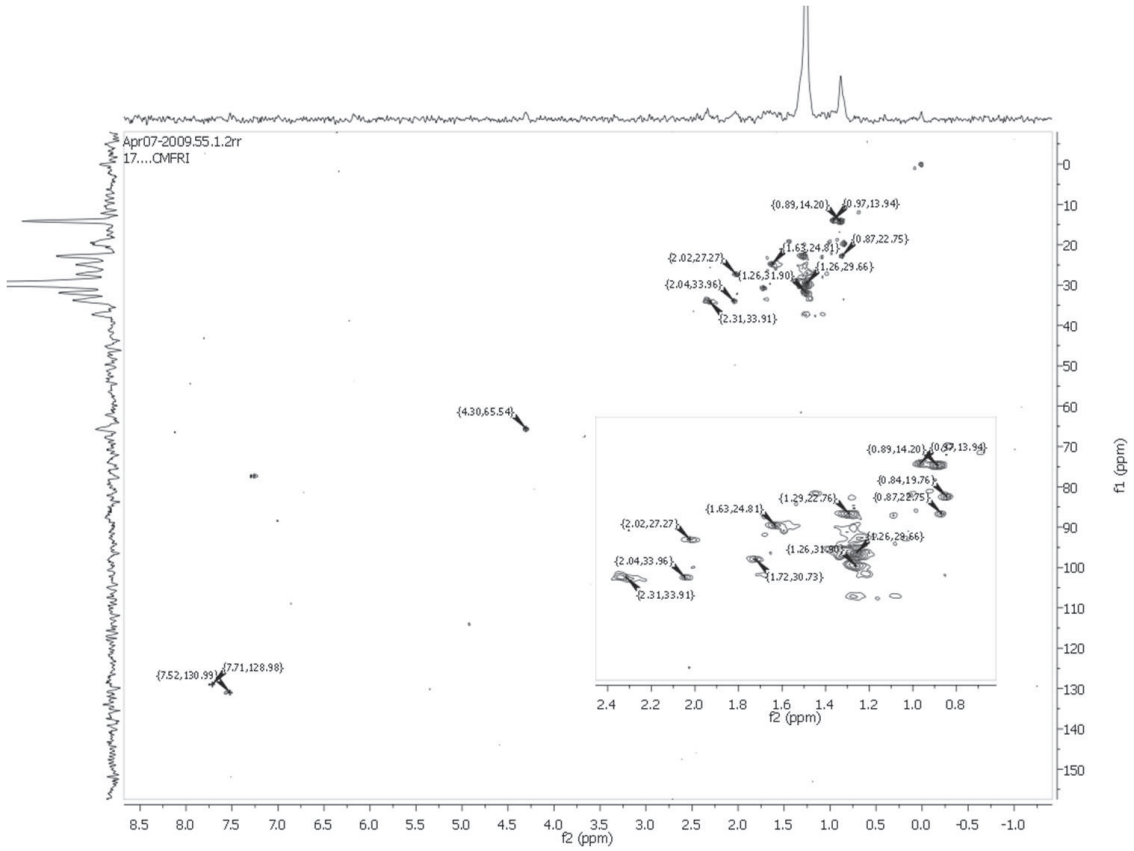


Figure 4.11. HMBC spectrum of (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate

^{13}C NMR data (Table 3) indicated the elemental composition of $C_{23}H_{27}NO_4$. The molecular ion peak at *m/e* 382 appeared to undergo elimination of C_3H_9+ (45) to yield *m/e* 336 ($C_6H_{12}O_2$), which undergoes fragmentation to afford the fragments with *m/e* 264, 201 etc (Figure 4.4). These signature peaks established the presence of the (9H-fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate moiety.

The ν(C-H) vibrations were assigned by the signals at around 2800-2900 cm^{-1}. Absorbance around 1700 cm^{-1} is assigned due to ν(C=O) vibrations. Bands of about 1420 cm^{-1} is due to ν_s(C=O) vibrations. ν(C-N) vibrations were observed at 1034.84 cm^{-1} and bands around 1600.97 1665.59 cm^{-1} are due to δ(N-H) vibrations (Figure 4.6).

The ^{1}H NMR in conjugation with ^{13}C-NMR recorded the presence of the methine groups CH δ 7.3-7.7, which are assigned to be due to fluoren (Figure 4.7), whereas the methine group attached to the side chain appeared at δ 2.30 is a part of the methyl 1-(propoxycarbonyl) butylcarbamate system, and the downfield shift (about δ 0.6 ppm) of the –CH group is due to the presence of beta -O-C=O from the methane group (Figure 4.8 & 4.9). The methylene groups at δ 3.3 and 4.1 ppm appeared significantly downfield due to the presence of alpha -OC(=O)N and alpha -OC(=O)-C moieties in the methyl 1-(propoxycarbonyl)butylcarbamate side chain. The

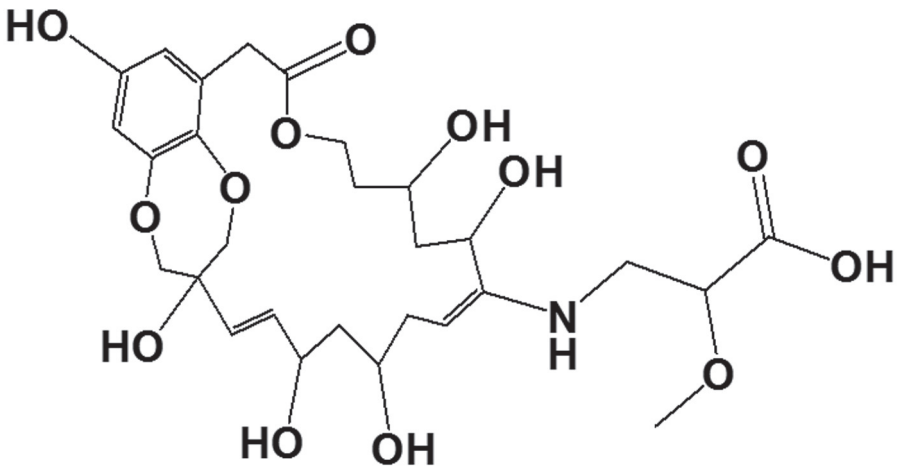


Figure 4.12. 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

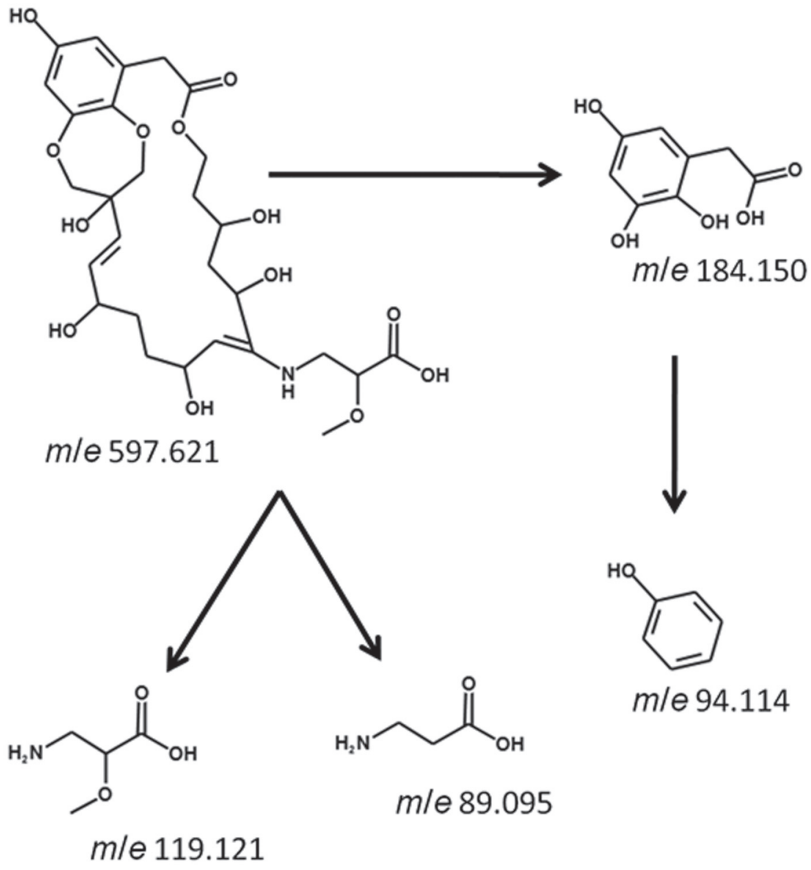


Figure 4.13. Mass fragmentation pattern of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

methylene protons at δ 2.04, 2.96 ppm flanked between the fluoren group and carbamate group of the side chain, whereas the methylene protons appeared at δ 1.58 and 1.30 ppm is a part of the methyl butylcarbamate moiety of the side chain. The other methylene groups at δ 3.3 and 4.1 ppm also corroborated with the deduced structure and part of the side chain attached with the fluoren group. The peak at δ 4.31 ppm is due to the secondary amide group. The methine group at appeared downfield at δ 2.3 ppm deduced to be due to the electron withdrawing groups alpha -N-C=O and alpha -C(=O)OR in the carbamate and propyl hydrogen carbonate moieties. The two methyl signals at 0.88 and 0.91 ppm are due to the terminal methyl groups in (1-(propoxycarbonyl)butyl)carbamate acid moiety in the side chain. Peaks at C-14 (δ 177.28) and C-12 (δ 163.83) indicate two carbonyl groups and C13 peaks with high intensity at δ 139,131,130,129, and 128 showed the presence of the aromatic carbons giving the HSQC correlation with H-2,3 (δ 7.72), H-4,5(δ 7.52), and H-9(δ 7.25), whilst all are coupled with the neighboring protons forming the multiplets to give the proton integration value as 8H (Figure 4.10).

The protons at H-4, and H-8 showed HMBC correlation with C-1, which indicate that C-1 is situated between two aromatic rings (Figure 4.11). C-1 gives HSQC correlation with H-1(δ 1.60) to demonstrate a triplet. The H-1 showed ^{1}H-^{1}H COSY correlation with H-11 (δ 2.04), which showed HMBC correlation with C-12(δ 163.83). The carbon at C-15(δ 65.7) exhibited DEPT signal for methylene group, and its downfield shift demonstrates the presence of highly electronegative group at its close proximity. The bunches of carbons at C-13, 13a, 13b, 13c are linearly aligned as established by ^{1}H-^{1}H COSY correlation results. The ^{1}H-^{1}H COSY correlations at H-13(δ 2.3) with H-13a (δ 1.58), H-13b (δ 1.30), and H-13c (δ 0.91) are the prominent ones. These protons at H-13a, 13b and H-13 showed HMBC correlation with C-14. One singlet proton at δ 4.31 showed HMBC correlation with C-14 and C-12, and therefore, is situated to middle of these two. The proton doesn't show any HSQC correlation with any C13 peak, and that it connected to N confirmed by the CHNS analysis result. The ^{1}H-NMR spectrum showed four exchangeable hydroxyl protons, which disappeared upon addition of D_2O. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 23 carbon atoms in the molecule. The position of the hydroxyl groups was further confirmed from the ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY spectra. In the ^{1}H–^{1}H COSY spectrum, couplings were apparent as described in the figure support the presence of the established skeleton.

Earlier studies reported the isolation of a new compound, 1,4,5-trihydroxy-7-methoxy-9H-fluoren-9-one, together with two known fluorenones, dendroflorin and denchrysan A, from the whole plant of *Dendrobium* genus, used as a health-food (Chen et al. 2008). There are other reports that dimeric diazofluorenes known as the lomaiviticins are produced by the marine bacterium *Salinispora pacifica* DPJ-0019 (Woo et al 2013).

Table 4.3. NMR spectroscopic data of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR(int., mult., *J* in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	139.36				
2	129.72				
3	130.1				
4	132.29	4-H	7.53(m)	6-H	C-5,3,28
5	131.2				
6	129.72	6-H	7.71(m)	4-H	C-1,7,8,29
7	31.92	7-H^{a}at 1.727-H^{b} at 1.61	1.72(s)		
8	167.73				
9	33.91	9-H^{a}at 2.34,9-H^{b} at 2.22	2.34(m)	10-H	C-8,11
10	29.36	10-H	1.63(q)	11-H	C-9,11
11	33.9	11-H,-OH	2.02(m),3.4(s)	10-H	
12	31.92	12-H	1.29(t)		C-13,15
13	29.36	13-H^{a},13-OH	13-H^{a}at1.85(t) 13-OH at 4.28(s)	12-H	C-15
14	130.2				
15	N		4.25(t)		
16	34.3	16-H	1.61(d)	17-H	C-17,18,
17	33.5	17-H	2.3(t)	16-H	
18	178.65	-OH	10.2(s)		
19	51.44	19-H	3.66(s)		C-18,17,16
20	114	20-H	4.99(t)	21-H^{b}	
21	37.23	21-H^{a},21-H^{b}	21-H^{a}at1.8421-H^{b} at2.01(dt)		C-15,20,23
22	29.36	22-H,-OH	1.86(m),5.3(s)		C-24
23	31.92	23-H	1.24(m)	22-H	
24	25.62	24-H^{a},24-H^{b}	24-H^{a}at2.81,24-H^{b} at2.06(m)	23-H	C-25,26,22
25	131.1	25-H	5.82(q)	24-H^{b}	C-26,29
26	128.83	26-H	5.79(d)	25-H	C-27,25
27	37.23				
28	68.16	28-H	4.22(s)		
29	65.53	29-H	4.30(s)		C-8,7,2

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500)

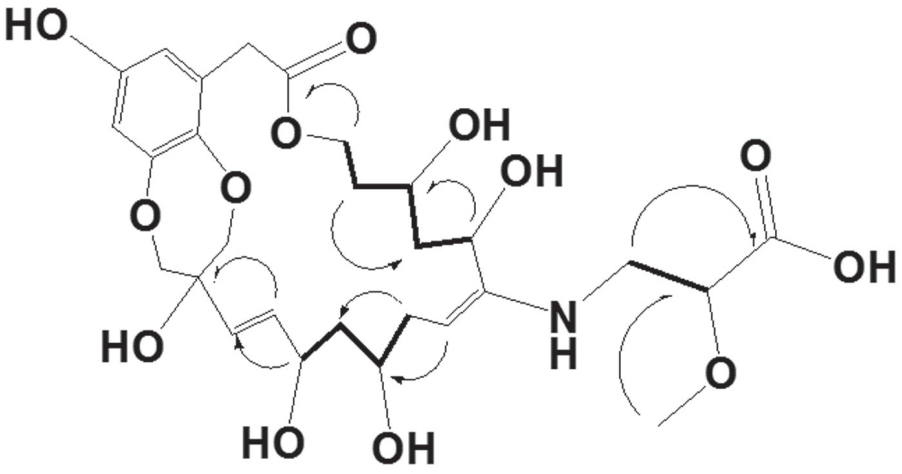


Figure 4.14. 2D NMR correlations as observed in 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

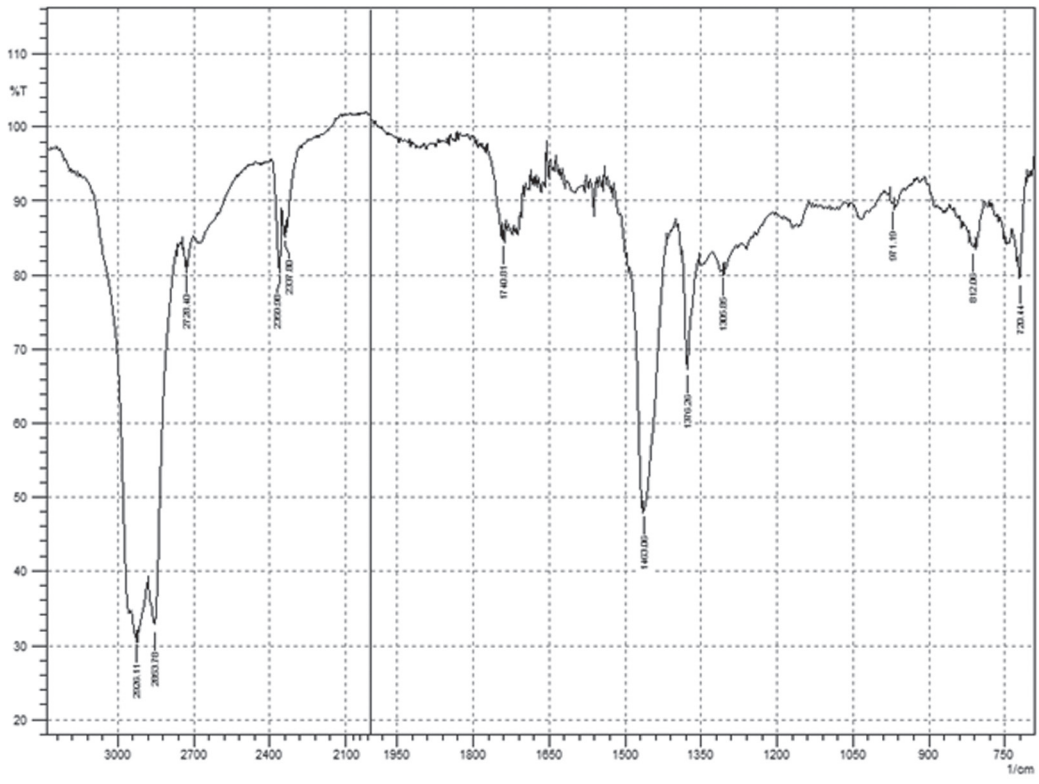
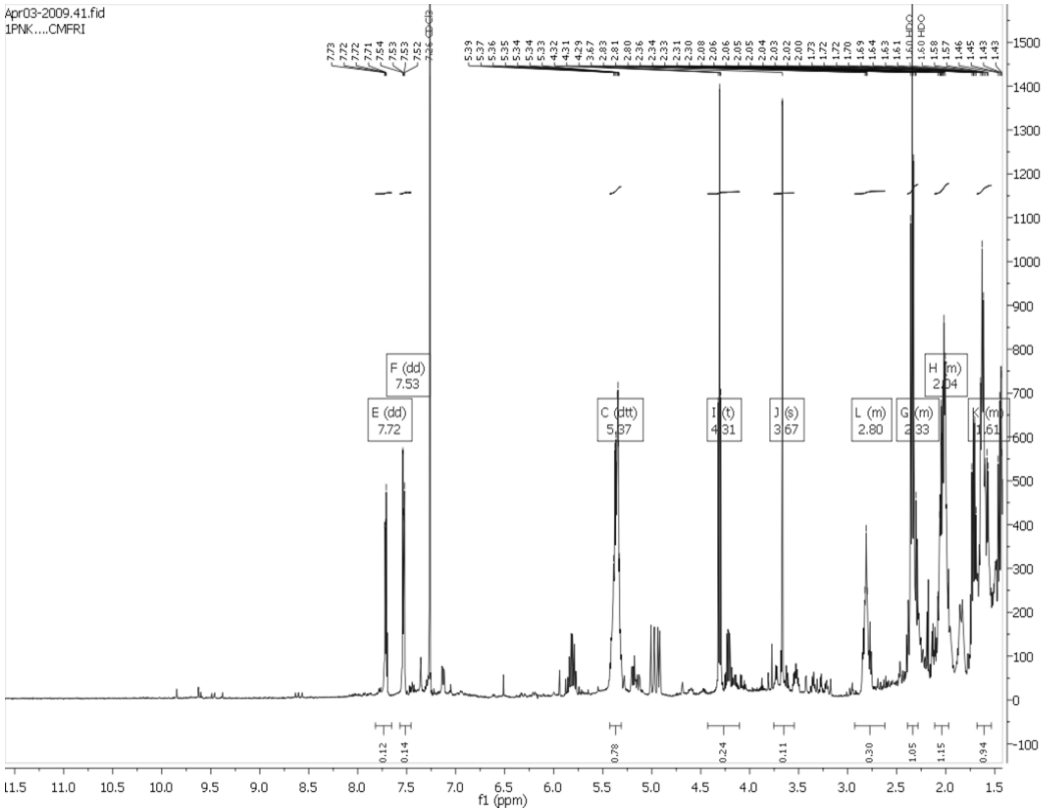


Figure 4.15. IR spectrum of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

A



B

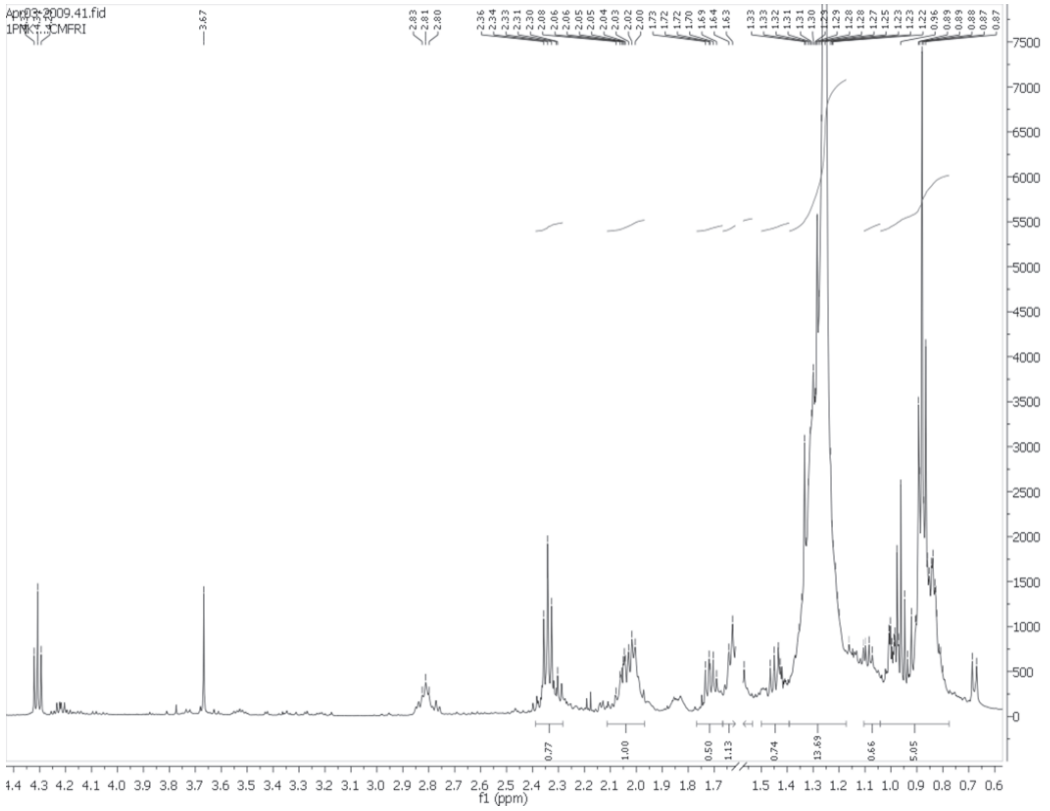
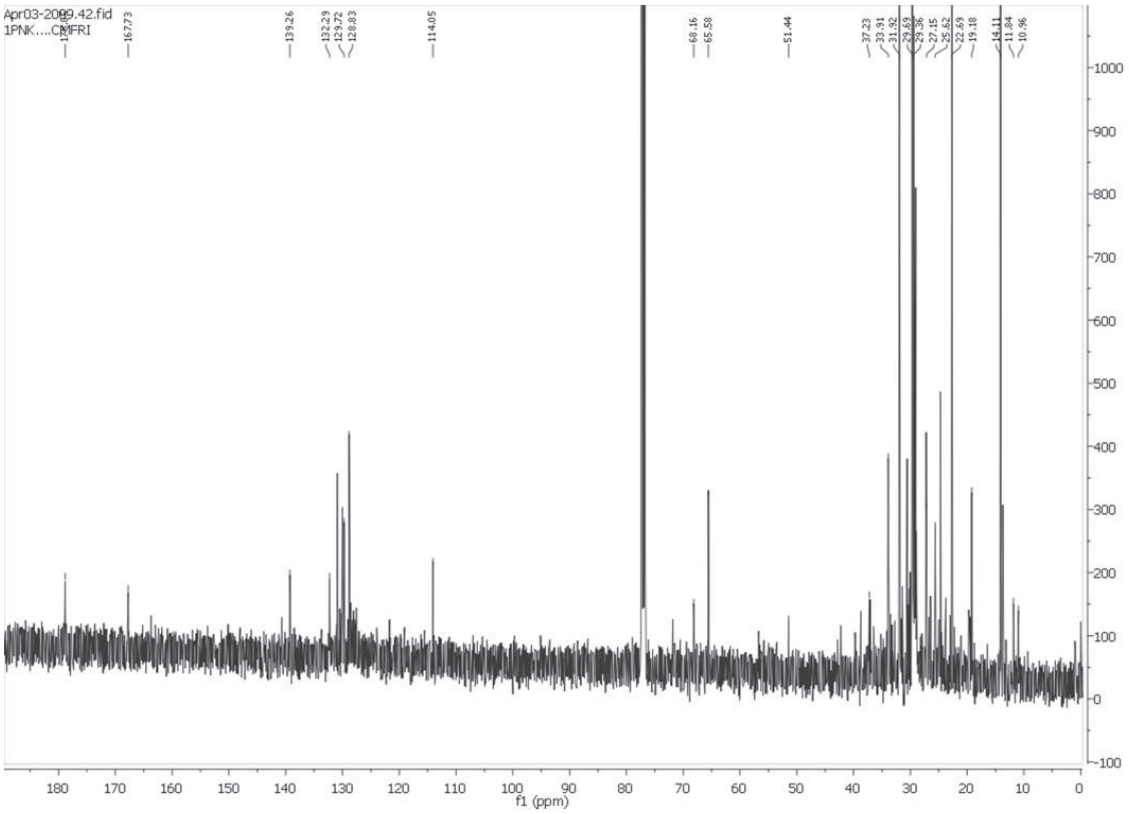


Figure 4.16. Proton NMR spectra of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid (A - full view, B - expanded view)

A



B

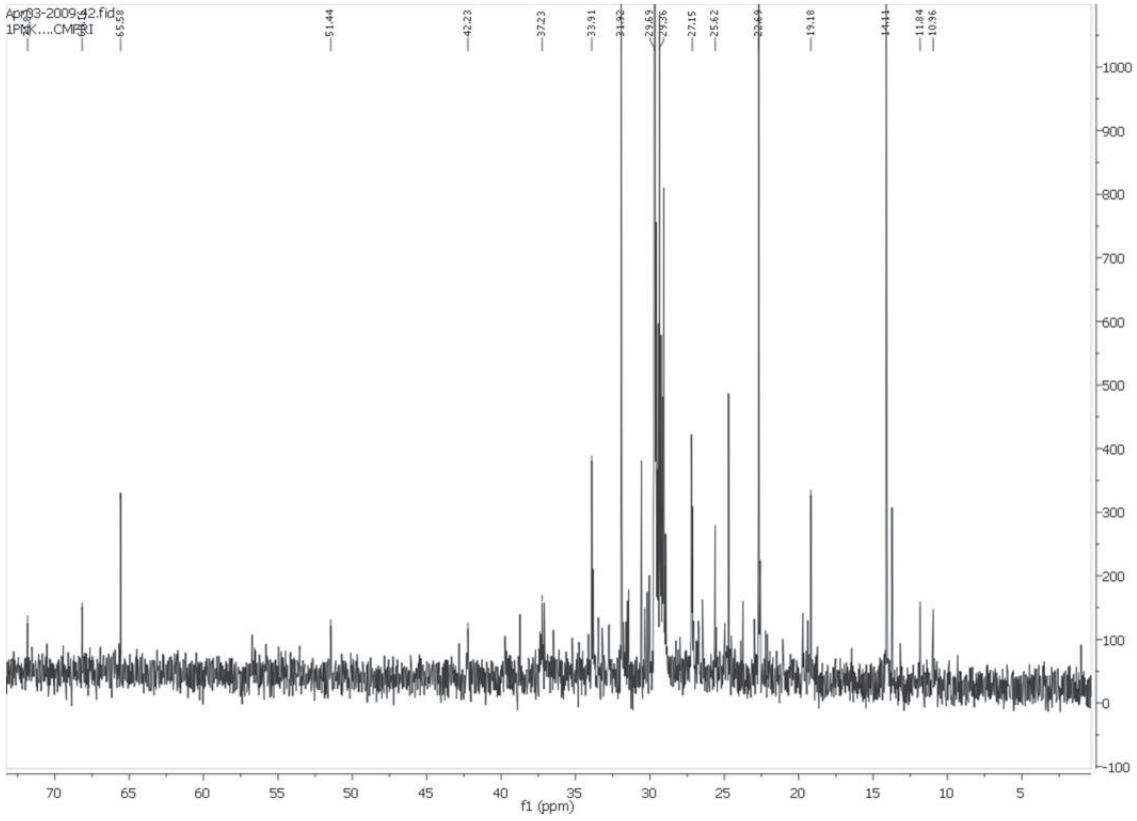


Figure 4.17. ^{13}C NMR spectra of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa bicyclo [17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid (A - full view, B - expanded view)

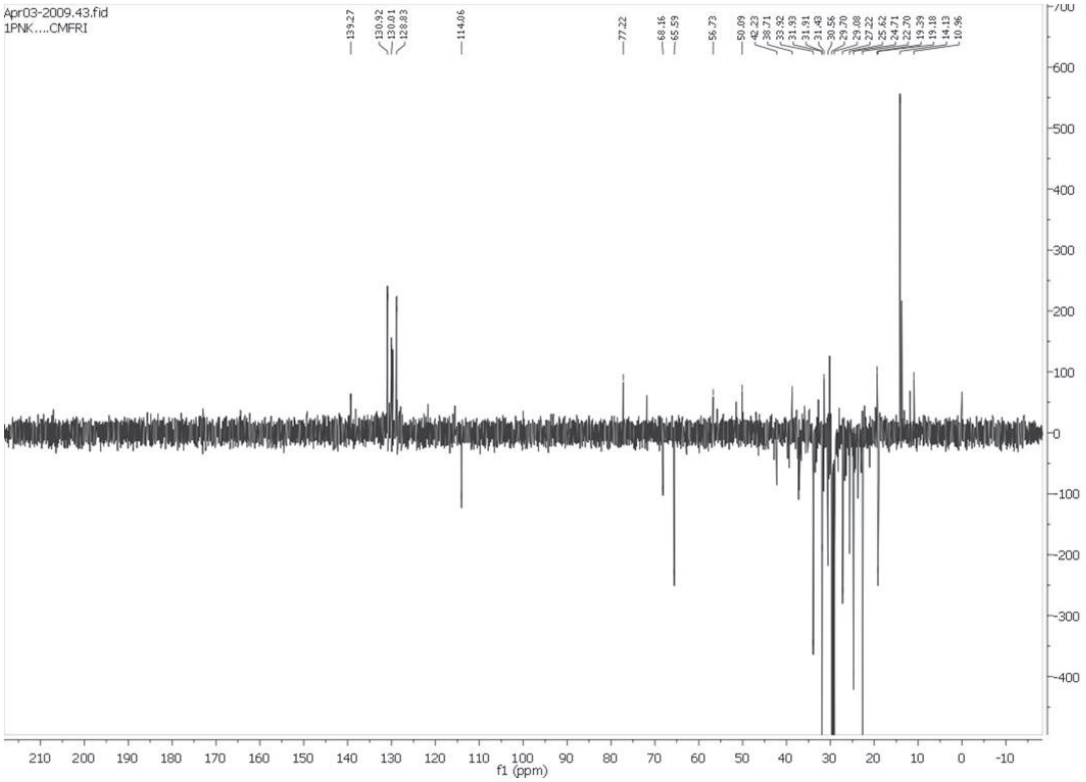


Figure 4.18. DEPT spectrum of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2] tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

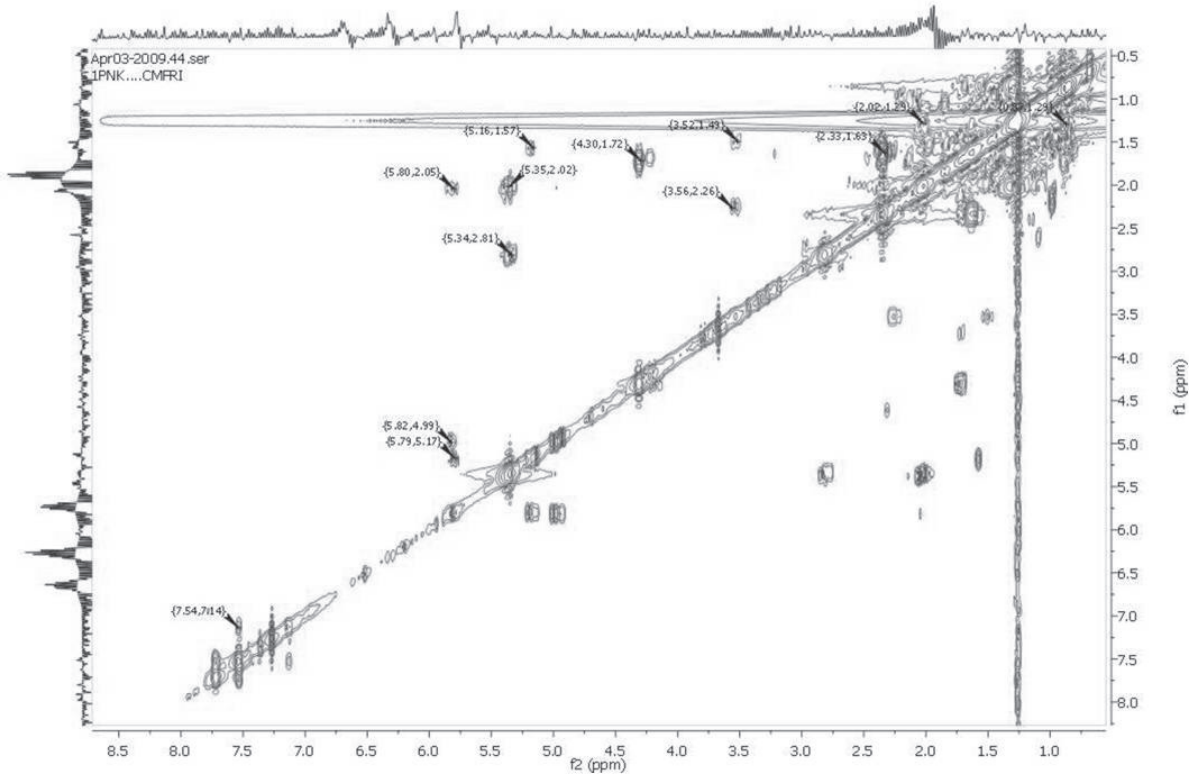


Figure 4.19. ^{1}H-^{1}H COSY spectrum of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

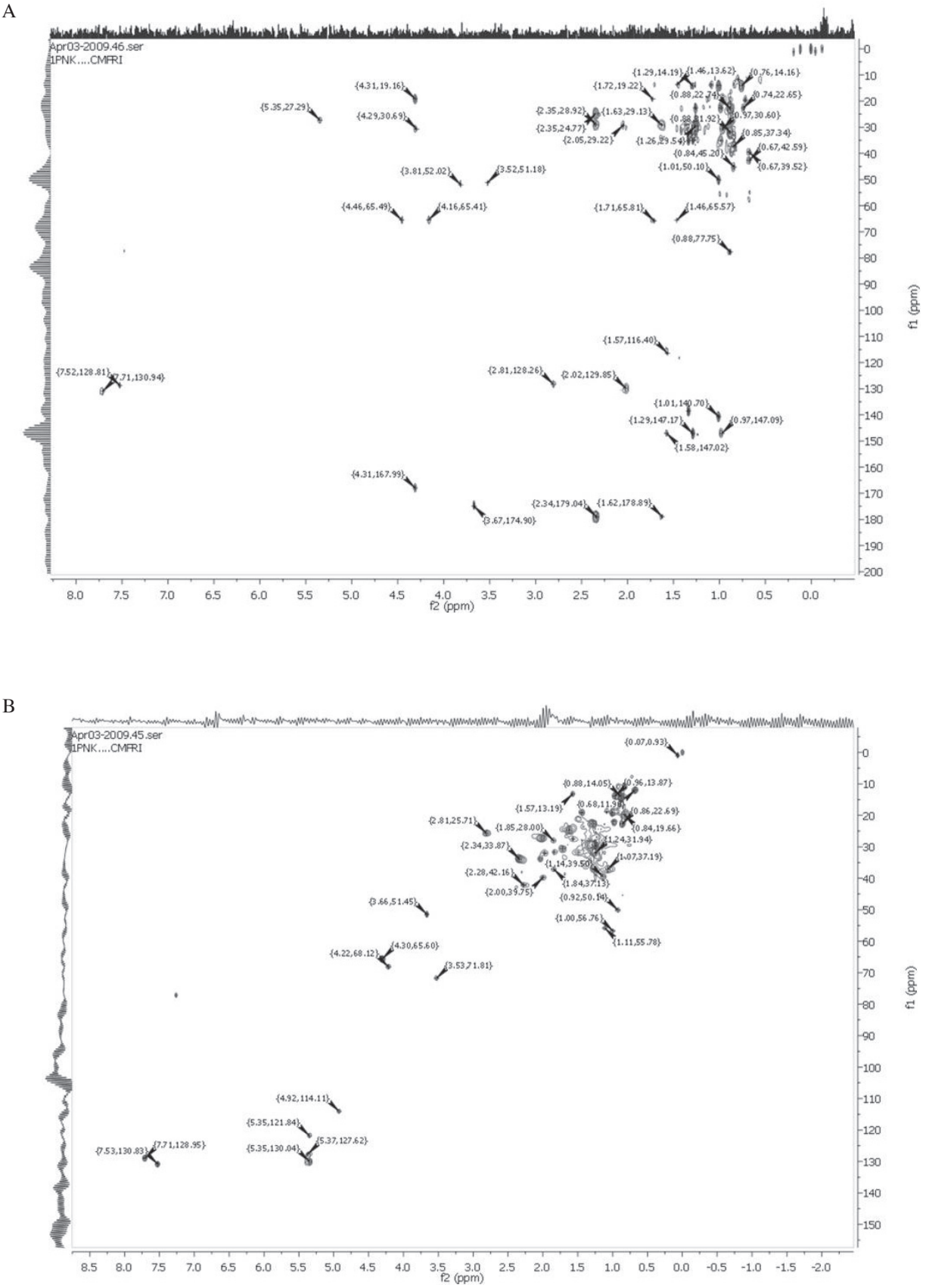


Figure 4.20. (A) HSQC and (B) HMBC spectra of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2] tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

4.2.3. Structural Characterization of 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid

3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid : Light yellow semisolid; UV (MeOH) λ_{max} (log ε): 256 nm (4.81); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.50; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) R_t: 8.20 min.; Elemental analysis found:C,56.28;H,6.58;N,2.34;O,34.80; IR (KBr, cm^{-1}) ν_{max} 720.44 cm^{-1} γ_w (N-H), 812.06 971.19 1305.85 1376.26 cm^{-1} γr(C-H alkanes), 1463.06 cm^{-1}, ν(C=C aromatic), 1740.81 cm^{-1} ν(C=O), 2337.80 cm^{-1} ν(C-N), 2359.98 cm^{-1} ν(C-N), 2853.78, 2926.11 cm^{-1} ν(C=O carboxylic acids); ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.3; EIMS *m/e* (rel. int. %): 598 $[M+1]^+$ (15), 567(31), 480(6), 260(12), 184(52),142 (3), 119.121(65), 94 (100), 90 (22), 89(28); HRMS (ESI) *m/e*: calcd. for $C_{28}H_{39}NO_{13}$ 597.6214; found 597.6265 $[M+H]^+$.

The mass spectral pattern showed the molecular ion peak at 597. The molecular ion loses the methoxy group (*m/e*=31) to furnish the peak at *m/e*=567. The other signature mass peaks appeared at *m/e*= 119 ($C_4H_9NO_3$), $C_8H_8O_5$ (*m/e*=184), *m/e*=94, 90, and 89, which supports the molecular structure (Figure 4.13).The peak around 1740cm^{-1} is due to ν_{as} (C=O) vibrations of -COOH group and also the bands around 2800-2900 cm^{-1} are due to ν(C=O carboxylic acids). A ν(C-N) band was observed around 2337.80cm^{-1} and γ_w (N-H) vibrations were observed at 720.44cm^{-1}. An aromatic ν(C=C) vibration was observed at 1463.06cm^{-1}.Bands at 812.06 971.19 1305.85 1376.26 cm^{-1} are mainly assigned due to C-H vibrations of alkane groups (Figure 4.15). The aromatic signals appeared at δ 4.5-7.7 ppm (Figure 4.16). Among the five methine signals, two appeared downfield at δ 1.85 and 2.02 ppm due to the electron withdrawing effects of alpha -C=C and alpha –O groups in the heptenediol moiety of the compound. The other two methine groups at δ 1.86 and 2.81 ppm are due to the deshielding effects of alpha O- and beta C=C groups in the octa-1,7-diene-3,5-diol moiety. The –NH and hydroxyl groups appeared as broad singlets at about 4-5 ppm. The phenolic proton appeared at 5 ppm due to the deshielding effect of the aryl ring system. The carboxylic –OH appeared downfield at δ 10.2 ppm. This carboxyl group is at the terminal position of 3-amino-2-methoxypropanoic acid side chain system of the compound. Three olefinic protons appeared at δ 4.99-5.82 ppm as established by integrating the number of protons. The methylene group protons at δ 2.34 ppm are due to the presence of 2-(3-hydroxyphenyl) acetate group. The downfield shift is due to aryl ring system and alpha -C(=O)O-C group. The –CH_2 group at δ 2.34 appeared downfield due to the presence of alpha -OC(=O)-C moiety in the 3-

Figure 4.21. *Padina gymnospora* collected from Thonithurai of Gulf of Mannar

hydroxybutyl acetate part of the compound. Two magnetically equivalent $–CH_2$ groups appeared at δ 4.2, 4.3 ppm, and demonstrated to be a part of (*Z*)-6, 7-dihydro-5H-1, 4-dioxepine ring system of the 3, 4-dihydro-2H-benzo[b] [1, 4] dioxepine-3, 7-diol moiety of the compound. The $–CH_2$ signals at δ 1.61 ppm is at the side chain 3-amino-2-methoxypropanoate group, and is deshielded due to the presence of alpha –N and beta -C(=O)O moieties. The methyl signal at 3.66 ppm is apparently deshielded due to the close proximity of an electronegative group such as oxygen, and is demonstrated to be a part of 3-amino-2-methoxypropanoate moiety. The four methylene groups appeared at δ 1.7-2.3 ppm are the part of the 19-hydroxy-5, 21, 23-trioxa-bicyclo [17.3.2] tetracosa-1(22), 11, 17-trien-4-one ring system of the compound. The ^{13}C spectra displayed signal at δ 167.7 ppm and 178.6 ppm showed two carbonyl compounds. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 28 carbon atoms in the molecule (Figure 4.17 & 4.18). The position of the hydroxyl groups was further confirmed from the $^{1}H–^{1}H$ COSY, HSQC, HMBC (Figure 4.19 & 4.20), and NOESY spectra. In the $^{1}H–^{1}H$ COSY spectrum, couplings were apparent as described in the figure support the presence of the established skeleton.

A novel trioxa-bicyclo phenolic compound, 4-(2,4,7-trioxa-bicyclo[4.1.0]heptan-3-yl) phenol with potent antibacterial and antifungal activity was isolated from an endophytic fungus *Pestalotiopsis mangiferae*, (Subban et al. 2012). Another novel bicyclic diterpenoid, (-)-3α-acetoxy-6β-hydroxy-15,16-dinorlabd-8(9)-ene-13-yne-7-one with coagulant activity by shortening the activated partial thromboplastin time, prothrombin time, and thrombin time, and increasing the fibrinogen levels was isolated from biennial herbaceous plant *Leonurus japonicus* Houtt. (Lamiaceae) widely distributed and cultivated in China (Peng et al. 2013). Compounds with bi-bicyclic and bi-tricyclic ring systems were also reported to be isolated from stems of *Dendrobium thyrsiûorum* (Zhang et al. 2005).

4.3. Isolation and Characterization of Antioxidant Secondary Metabolites from *Padina gymnospora* (Kützing) Sonder

4.3.1. Bioassay Guided Chromatographic Purification of the Methanol Extract of *P. gymnospora*

The MeOH extract of *P. gymnospora* (1.2 g) collected from the Gulf of Mannar (Figure 4.21) was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*-hexane, *n*-hexane: EtOAc and finally EtOAc: MeOH and the collected fractions were concentrated under vacuum and TLC evaluated and fractions with same TLC profile were pooled together to furnish nine fractions (Pg_{1-9}). The column fractions obtained from Pg/MeOH extracts were evaluated for its antioxidant activity by measuring its ability to scavenge DPPH (0.1 mg/mL) and ABTS (0.1 µg/mL) free radicals and to chelate Fe^{2+} ion (0.1mg/mL). The fractions Pg_{4}, Pg_{7}

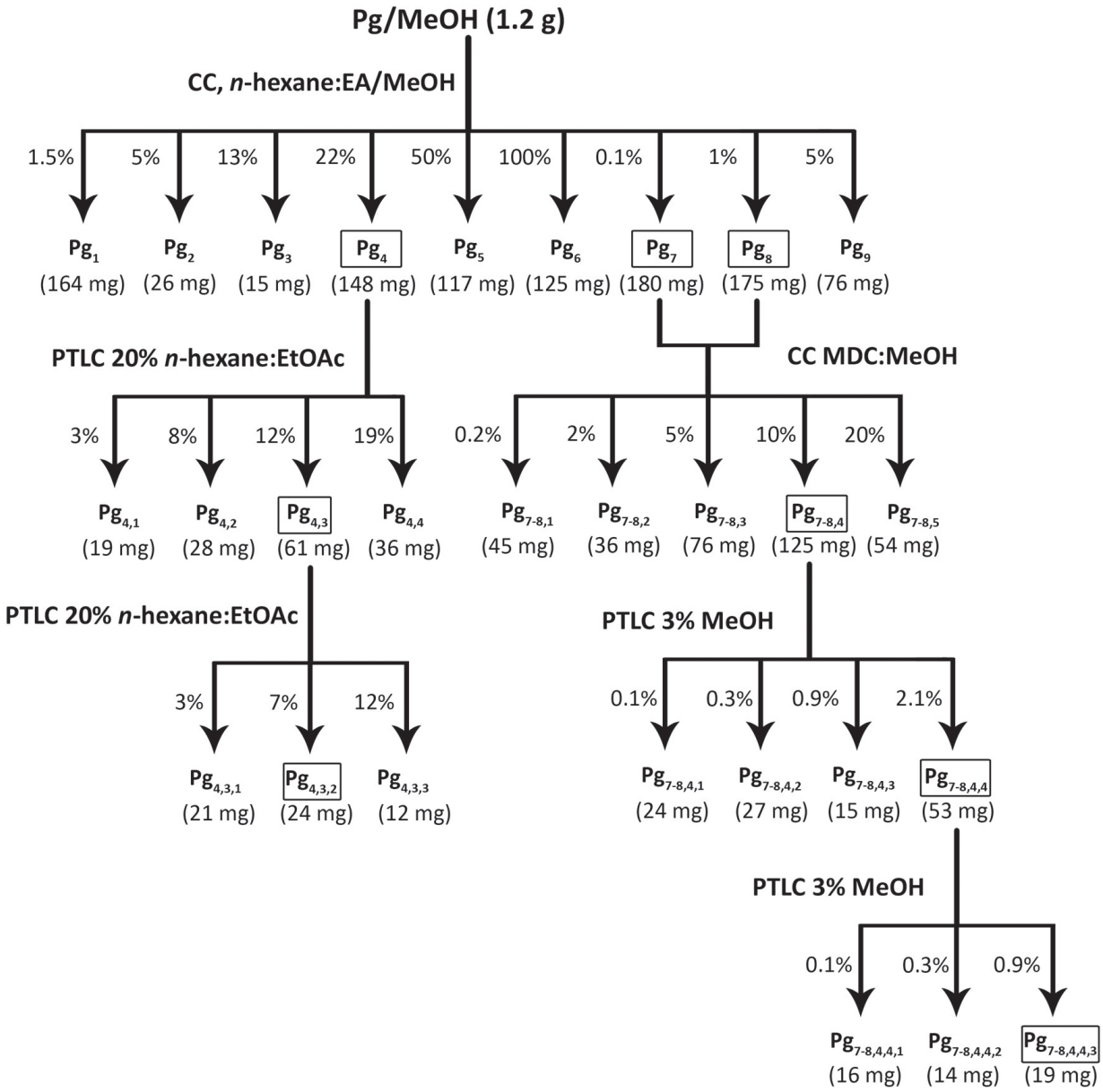


Figure 4.22. Schematic diagram showing the purification of the MeOH extract of *P. gymnospora* (Pg). CC: column chromatography, PTLC: preparative thin layer chromatography

Table: 4.4. Antioxidant and Fe^{2+} ion chelating potential (%) of the different fractions obtained by the chromatographic purification of the MeOH extract of *P. gymnospora*.

Sl NO	Sample ID	Solvent System	Yield (g)	ABTS Radical Scavenging Activity (%)	DPPH Radical Scavenging Activity (%)	Fe^{2+} ion Chelating Ability (%)
Pg						
1	Pg_1	1.5	164	20.74±0.28	50.40±0.68	14.60±0.19
2	Pg_2	5	26	17.63±0.23	44.26±0.60	13.17±0.17
3	Pg_3	13	15	13.13±0.17	30.98±0.42	9.19±0.12
4	Pg_4	22	148	25.21±0.34	62.27±0.84	18.75±0.25
5	Pg_5	50	117	11.48±0.15	28.59±0.38	8.24±0.11
6	Pg_6	100	125	7.69±0.10	18.46±0.25	5.12±0.06
7	Pg_7	0.1	180	22.88±0.31	55.39±0.75	16.73±0.22
8	Pg_8	1	175	25.04±0.33	59.60±0.80	16.93±0.22
9	Pg_9	5	76	15.40±0.20	36.36±0.49	10.10±0.13
Pg_4						
1	$Pg_{4,1}$	4	18	7.91±0.10	19.32±0.26	5.44±0.07
2	$Pg_{4,2}$	10	95	10.76±0.14	26.58±0.36	7.77±0.10
3	$Pg_{4,3}$	18	43	23.11±0.31	65.18±0.88	18.10±0.24
4	$Pg_{4,4}$	13	31	20.95±0.28	51.75±0.70	15.40±0.20
$Pg_{4,3}$						
1	$Pg_{4,3,1}$	1	24	22.56±0.44	48.80±1.06	16.90±0.31
2	$Pg_{4,3,2}$	5	20	28.45±0.25	69.32±0.62	24.34±0.18
3	$Pg_{4,3,3}$	9	17	24.19±0.49	51.42±1.15	20.80±0.34
Pg_{7-8}						
1	$Pg_{7-8,1}$	0.2	45	22.28±0.30	52.59±0.71	15.37±0.20
2	$Pg_{7-8,2}$	2	36	21.11±0.39	54.53±1.01	16.17±0.28
3	$Pg_{7-8,3}$	5	76	14.63±0.19	35.70±0.48	10.38±0.14
4	$Pg_{7-8,4}$	10	125	21.38±0.28	60.30±0.81	17.37±0.23
5	$Pg_{7-8,5}$	20	54	11.23±0.15	27.75±0.37	8.38±0.11
$Pg_{7-8,4}$						
1	$Pg_{7-8,4,1}$	0.1	24	10.15±0.13	24.68±0.33	7.11±0.09
2	$Pg_{7-8,4,2}$	0.3	27	14.88±0.20	42.56±0.57	12.82±0.17
3	$Pg_{7-8,4,3}$	0.9	15	15.63±0.21	36.90±0.50	10.63±0.14
4	$Pg_{7-8,4,4}$	2.1	53	21.64±0.29	55.42±0.75	15.39±0.20
$Pg_{7-8,4,4}$						
1	$Pg_{7-8,4,4,1}$	0.1	16	21.54±0.29	53.20±0.72	15.46±0.20
2	$Pg_{7-8,4,4,2}$	0.3	14	19.55±0.40	43.35±1.13	13.68±0.32
3	$Pg_{7-8,4,4,3}$	0.9	19	23.30±0.31	70.14±0.95	20.81±0.28

Data are the mean values of the triplicate and expressed as mean ± standard deviation. The concentration of the solvent fractions used were 0.1 mg/mL for DPPH radical scavenging activity, and Fe^{2+} ion chelating activity; and 0.1 µg/mL for ABTS radical scavenging activity.

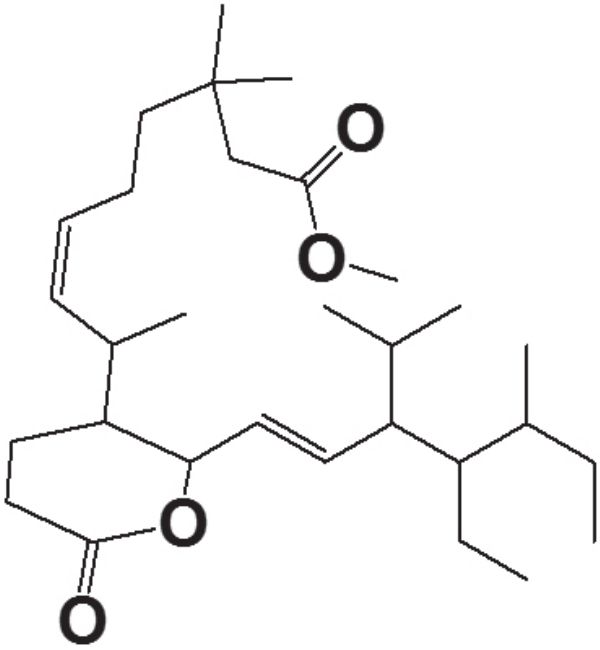


Figure 4.23. (6Z)-methyl 8-(2-(*E*)-4-ethyl-3-isopropyl-5- methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate

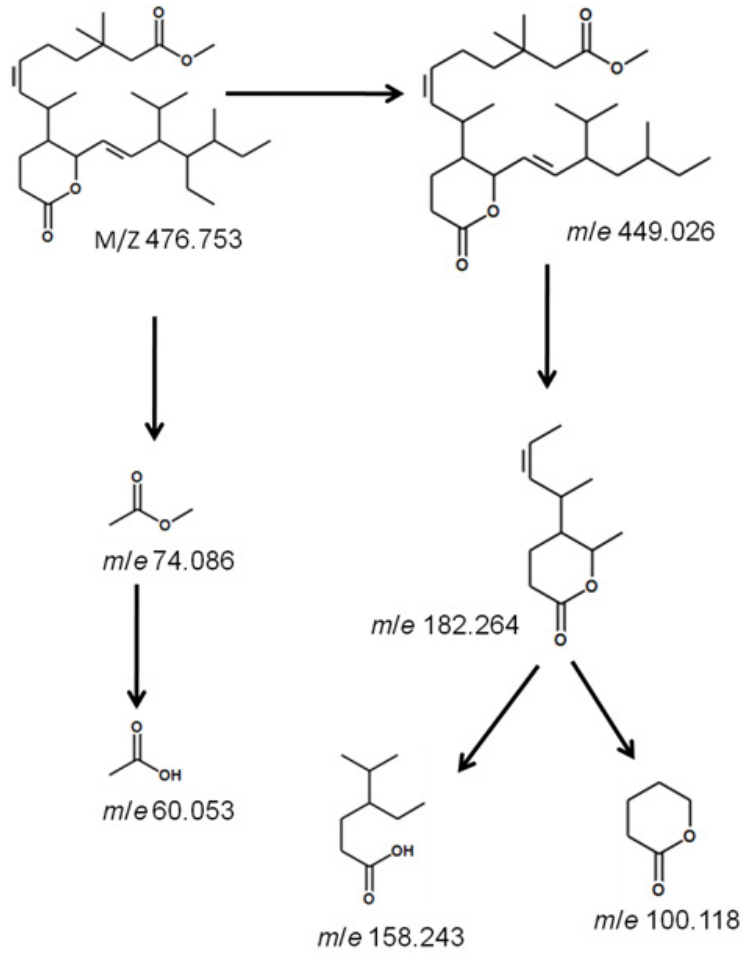


Figure 4.24. Mass fragmentation pattern of (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5- methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate

Table 4.5. NMR spectroscopic data of (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR(int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	61.02	1-H	4.5(t)		C-1'
1'	130.20	1'-H	5.26(dt)	1-H	C-2
2	33.5	2-H	2.24(m)		C-3,4
2a	25.54	2a-H	2.73(ddt)		C-6,7,8
2'	129.72	2'-H	5.29(m)	1'-H, 3'-H	
3	33.81	3-H	2.28(t)		C-5,4
3a	24.51	3a-H	1.43(m)	3'-H	C-3b,3c
3b	13.98	3b-H	0.80(m)	3a-H	
3c	19.51	3c-H	0.77(m)		
3'	27.19	3'-H	3.46(m)	2'-H	
4	25.6	4-H	1.6(dt)		C-5,7,8
4'	32.79	4'-H	2.21(dd)	3'-H, 5'-H, 4a-H	C-4a,3'
4a	22.71	4a-H	0.91(m)	4b-H	
4b	14.10	4b-H	0.80(m)		
5	179.41				
5'	24.79	5'-H	1.54(m)	5''-H, 6'-H	C-5'',6'
5''	24.68	5''-H	0.94(m)	5'-H	
6	37.02	6-H	0.97(m)		
6'	39.22	6'-H	1.05(m)	7'-H, 5'-H	
7	130.01	7-H	5.16(t)	8-H, 2a-H	C-2,3,8
7'	20.54	7'-H	0.94(t)	6'-H	C-6',5'
8	128.23	8-H	5.08(t)	9-H	C-7
9	39.69	9-H	1.94(m)	10-H	C-7,8,10
10	29.06	10-H	1.25(m)		C-8,9
11	24.51				
11a	27.97	11a-H	1.56(s)		
11b	29.06	11b-H	1.71(d)		
12	37.71	12-H	4.01(s)		C-13,11,14
13	179.37				
14	51.31	13-H	3.6(s)		C-13

[a] NMR spectra recorded using spectrometers. Bruker AVANCE III 500 MHz (AV 500)
[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments.

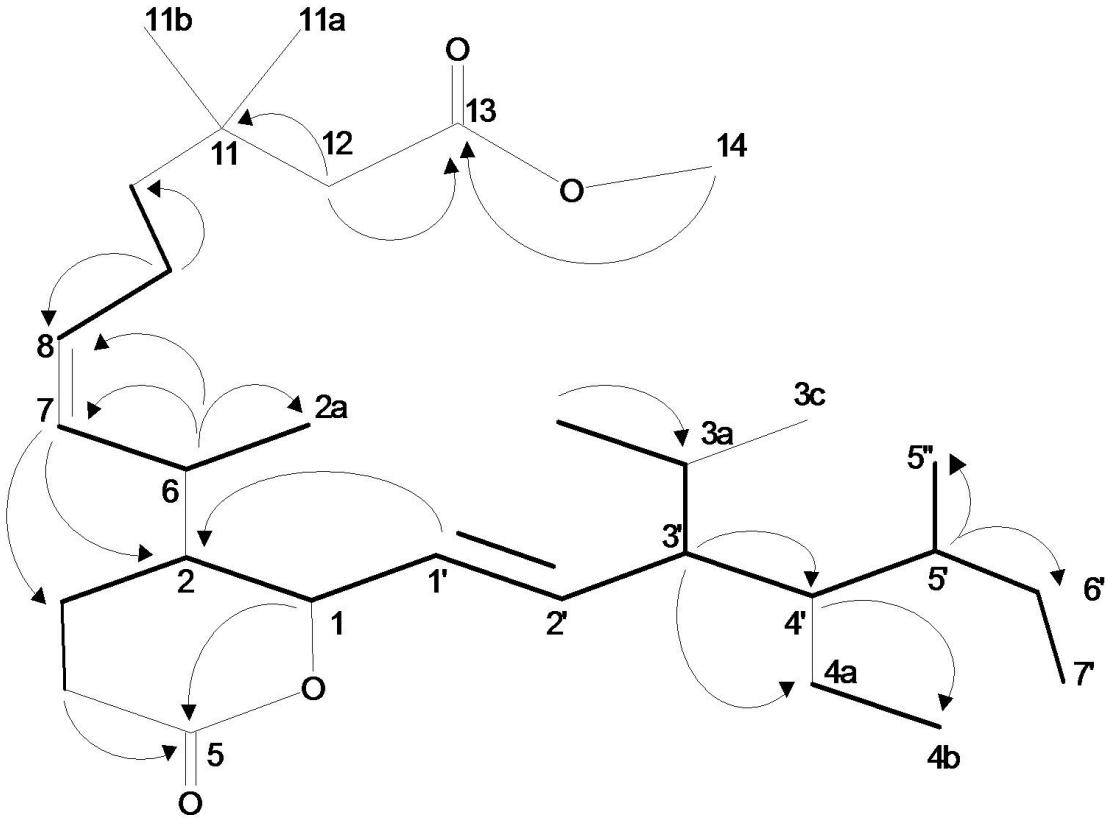


Figure 4.25. 2D NMR correlations as observed in (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

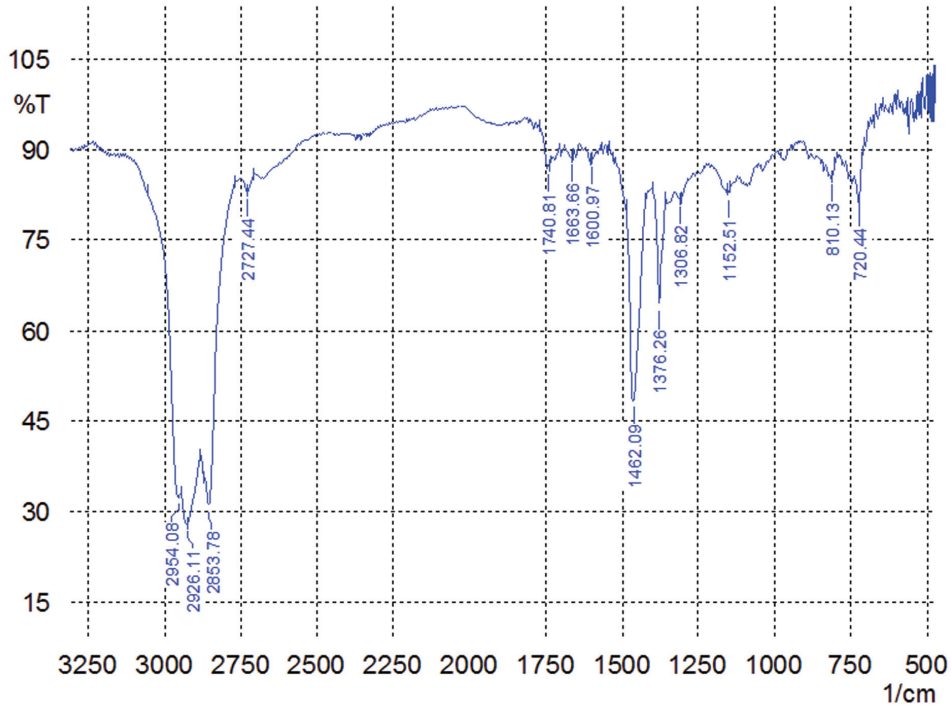
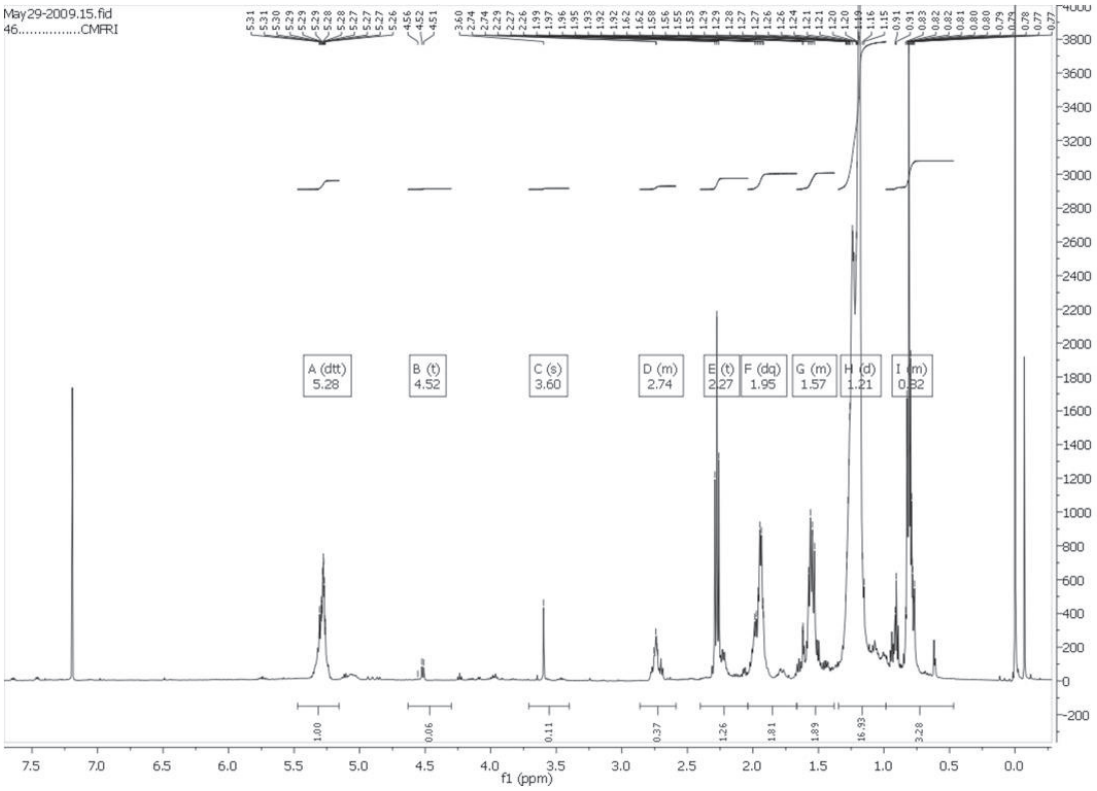


Figure 4.26. IR spectrum of (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate

A



B

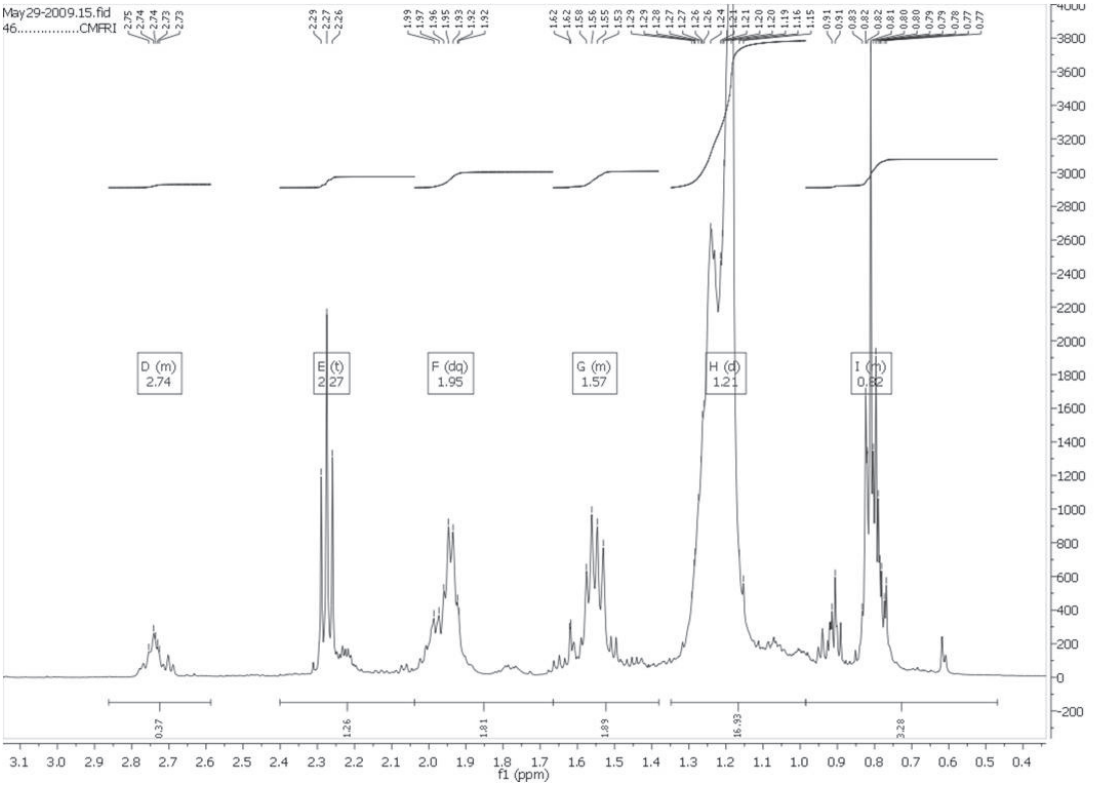


Figure 4.27. Proton NMR spectra of (6*Z*)-methyl 8-(2-((E)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate) (A - full view, B - expanded view)

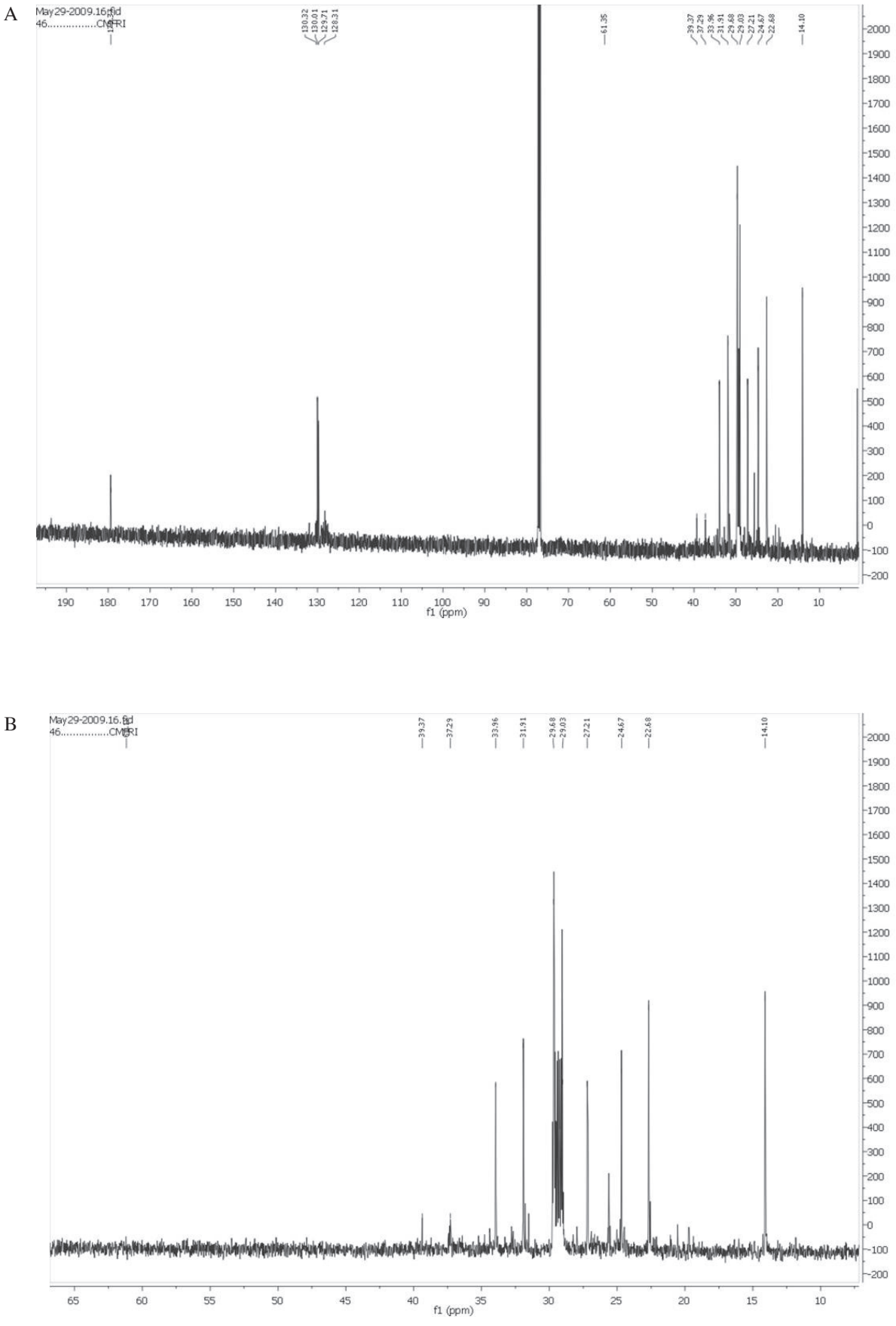


Figure 4.28. ^{13}C NMR spectra of (6*Z*)-methyl 8-(2-(*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate) (A - full view, B - expanded view)

A

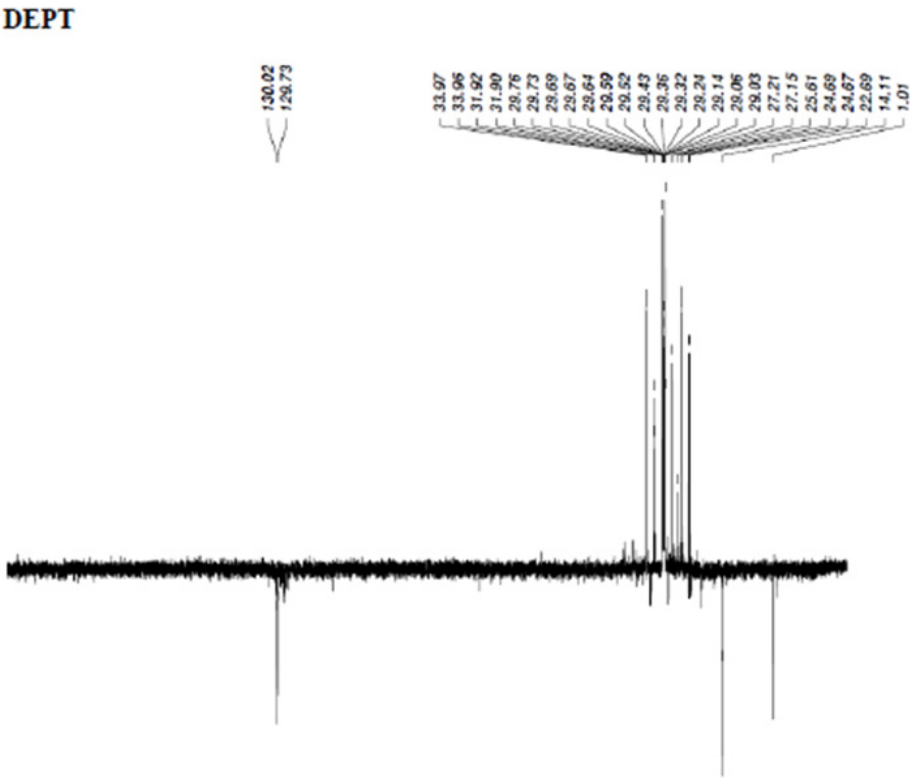


Figure 4.29. DEPT spectrum of (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate)

B

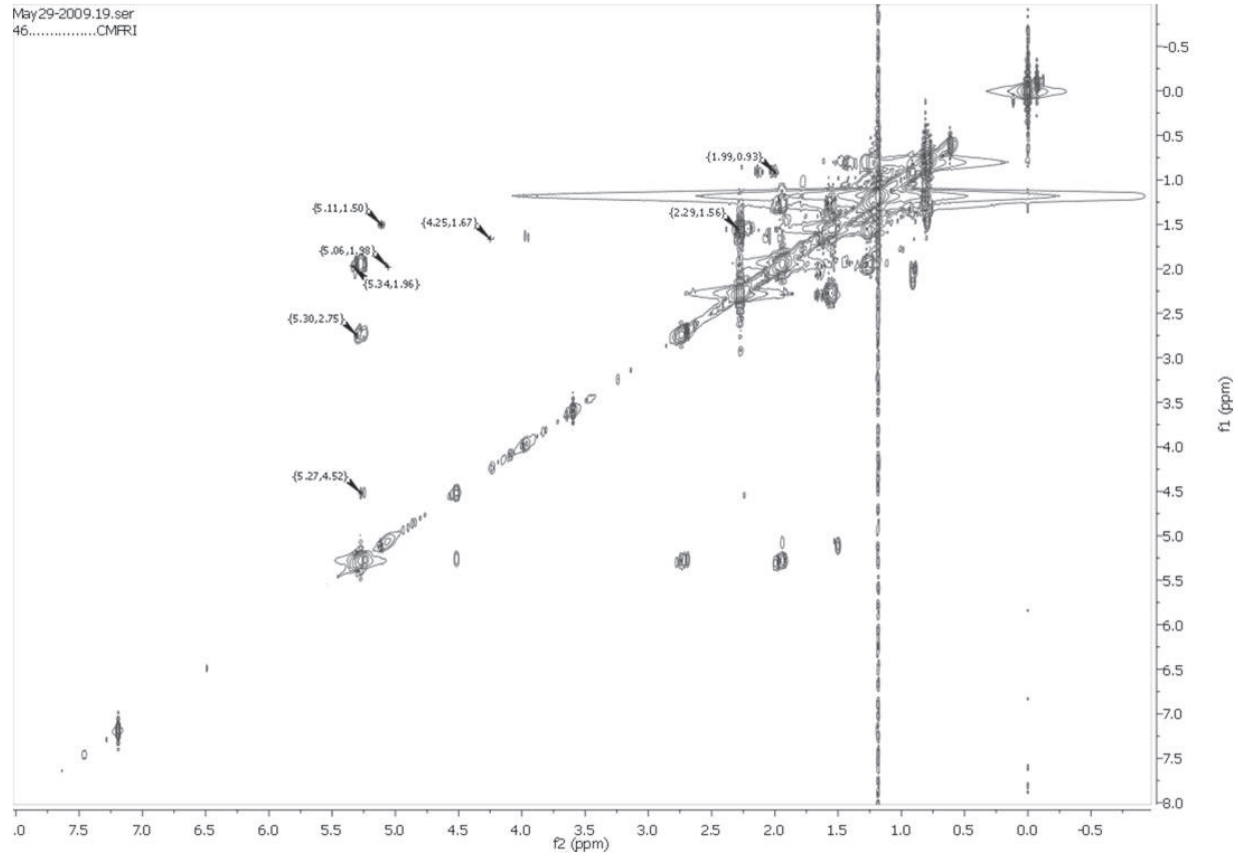


Figure 4.30. ^{1}H-^{1}H COSY spectrum of (6*Z*)-methyl 8-(2-(*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate) (A - full view, B - expanded view)

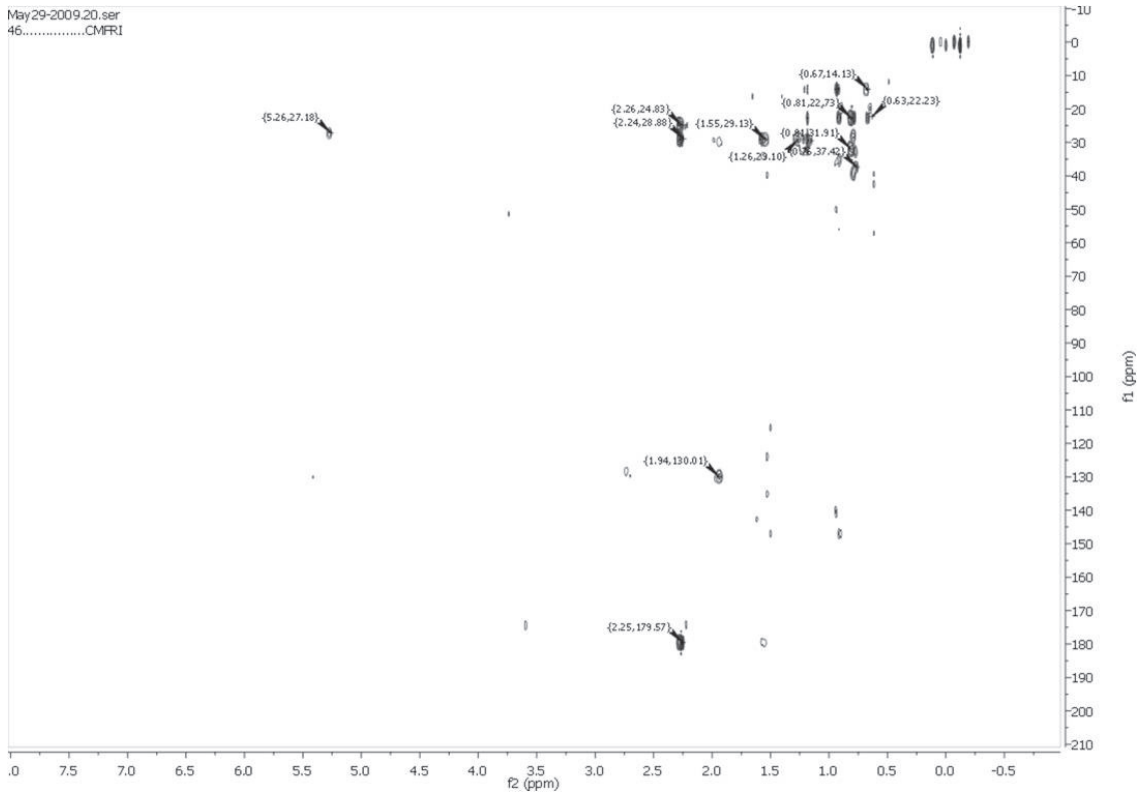


Figure 4.31. HMBC spectrum of (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate)

and Pg_{8} exhibited good radical scavenging and chelating ability so that they were selected for further purification. These fractions showed higher DPPH and ABTS radical scavenging ability (> 50% and > 20% respectively) and Fe^{2+} ion chelating ability (> 14 %) than other corresponding fractions.

Repurification of Pg_4 (Figure 4.22) using preparative thin layer chromatography over silica gel GF_{254} (particle size 15 µm) (20 % EA: *n*-hexane) resulted in four sub fractions $Pg_{(4,1\text{-}4,4)}$. The sub fraction $Pg_{4,3}$ which exhibited good DPPH (65.18%), ABTS (23.11%) radical scavenging potential and Fe^{2+} ion chelating ability (18.10%) was re purified again using preparative thin layer chromatography over Si gel GF_{254} (particle size 15 µm) (20 % EA: *n*-hexane) to get the pure and active compound $Pg_{4,3,2}$(69.32% and 28.45% for DPPH and ABTS radical scavenging ability respectively, and 24.34% ion chelating potential).

The two sub fractions with almost similar TLC profile and radical scavenging potential $Pg_{7\text{-}8}$ were pooled together and purified over silica column (60-120 mesh, 3.5x15cm) with a stepwise gradient of solvents from $CHCl_3$:MeOH and pooling up of similar fractions with same TLC profile to get five sub fractions $Pg_{7\text{-}8,1\text{-}5}$. The sub fraction $Pg_{7\text{-}8,4}$ which exhibited good DPPH (60.30%), ABTS (21.38%) radical scavenging potential and Fe^{2+} ion chelating ability (17.37%) was again purified using preparative thin layer chromatography (3% MDC: MeOH) to get another four fractions ($Pg_{7\text{-}8,4,1\text{-}4}$). The repeated purification of $Pg_{7\text{-}8,4,4}$ (55.42%, 21.64%, 15.39% for DPPH, ABTS radical scavenging ability and ion chelating potential respectively) by preparative thin layer chromatography method (3% MDC: MeOH) to get the pure and active compound $Pg_{7\text{-}8,4,4,1}$ (70.14% and 23.30% for DPPH and ABTS radical scavenging ability respectively, and 20.81% ion chelating potential).

The structural characterization were carried out be detailed spectroscopic techniques and are discussed in following sections (4.3.2 and 4.3.3)

4.3.2. Structural Characterization of (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate

(6*Z*)-Methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5- methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate: Yellow amorphous solid, UV(MeOH) λ_{max} 246(3.14), 276(2.81)nm TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.65; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) R_t: 7.42 min.; Elemental analysis found:C,75.58;H,10.99;O,13.42; IR (KBr, cm^{-1}) ν_{max} 720.44 cm^{-1} γr(C-H alkanes), 810.13 cm^{-1} δ(C-H), 1152.51 cm^{-1} δ(C-H), 1306.82 1376.26 cm^{-1} γr(C-H), 1462.09 cm^{-1} δ(C-H alkanes), 1600.97 1663.66 1740.81 cm^{-1} ν(C=O), 2727.44 cm^{-1}, ν(C-H alkanes), 2853.78 cm^{-1}, ν(C-H alkanes), 2926.11 cm^{-1}, ν(C-H alkanes), 2954.08 cm^{-}

^{1} ν(C-H alkanes). It has degree of unsaturation is 5; ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.5; EIMS *m/e* (rel. int. %): 478 $[M+1]^+$ (12), 449(11), 310 (26), 226 (52), 182 (34), 170(19), 158(73), 100(100), 74(62), 60 (51); HRMS (ESI) *m/e*: calcd. for $C_{30}H_{52}O_4$ 476.7545; found 476.7593 $[M+H]^+$.

The molecular ion peak was observed at *m/e*=477. The peak at *m/e*=449 is due to the elimination of methoxy group (*m/e*=28) or by the elimination of a C_2H_5 (*m/e*=28) side chain. Peak at *m/e* 182 and *m/e* 100 are due to the fragments $C_{11}H_{18}O_2$ (*m/e*=182) and $C_5H_8O_2$ (*m/e*=100). The mass fragments at *m/e*=158, 74, and 60 are the characteristic signature peaks of the proposed structure (Figure 4.24). The vibration bands around 1152.51, 2727.44, 2853.78, 2926.11 and 2954.08cm^{-1} are assigned due to ν(C-H alkane chain). A strong δ(C-H alkane chain) was observed at1462.09 cm^{-1}. ν(C=O) were observed at around 1600.97 1663.66 and 1740.81 cm^{-1} (Figure 4.26). The methylene group protons appeared at δ 1.6 and 2.28 ppm are assigned to be the part of valerolactone (Figure 4.27). The other methine protons at the valerolactone moiety appeared at δ 4.5 and 2.24 ppm, and are present at the alpha and beta position to the -C=C of the valerolactone group. The signature peaks of ^{13}C NMR at δ 179.41 and 174.37 indicate the presence of two carbonyl compounds (Figure 4.28 & 4.29). The signals in olefinic protons appeared at δ 130.20, 130.01, 129.71 and 128.23 ppm, which give HSQC correlation with the protons at 5.26, 5.16, 5.29 and 5.08 ppm, respectively. The linear ^{1}H-^{1}H COSY correlations were apparent between the protons at δ 1.6 ppm (C-4), 2.28 ppm (C-3), 2.24 ppm (C-2) and 4.5 ppm (C-1) that supports the valerolactone moiety (Figure 4.30). These protons showed the HMBC correlation with the carbonyl carbon at C-5 (δ 179.41 ppm), which also support the cyclic valerolactone structure. The proton at C-1 (δ 4.5 ppm) shows ^{1}H-^{1}H COSY correlation with the olefinic proton at δ 5.26 ppm (C-1'), which, in turn realized the ^{1}H-^{1}H COSY correlation with the olefinic proton at δ 5.29 ppm (C-2'). This established the tetrahydro-6-(prop-1-enyl) pyran-2-one moiety of the compound. The proton at C-1' (δ 5.26 ppm) gives triplet, which established the presence of one proton each at its either end. The *J* values of 14Hz, 7Hz of olefinic carbons C1'-C2' conformed the olefinic bond as '*E*'-type. The C-2' proton (2'-H, δ 5.29 ppm) correlates with the signal at δ 3.46 ppm (3'-H) as established by the ^{1}H-^{1}H COSY experiment. The 3'-H proton (δ 3.46 ppm) further showed linear ^{1}H-^{1}H COSY correlations with 3a-H (δ 1.43 ppm) and 4'-H (δ 2.21 ppm), which established the presence of the tetrahydro-6-((*E*)-3-methylbut-1-enyl)pyran-2-one moiety. The proton at 4'-H (δ 2.21 ppm, dd) exhibited ^{1}H-^{1}H COSY correlations with the ethyl group of 4a-H (δ 0.91 ppm) and 4b-H (δ 0.80 ppm), isopropyl group at C5' (δ 1.54 ppm), C5" (δ 0.94 ppm), C6' (δ 1.05 ppm) and C7' (δ 0.94 ppm). The proton at 2-H (δ 2.24 ppm) showed ^{1}H-^{1}H COSY correlation to 6-H (δ 0.97 ppm, m), which showed linear ^{1}H-^{1}H COSY correlation to 7-H (δ 5.16 ppm). The triplet with *J* value of 5 Hz indicates '*Z*' type of olefinic bond, whereas the ^{1}H-^{1}H COSY correlation continued with 8-H (δ 5.08 ppm). The proton at

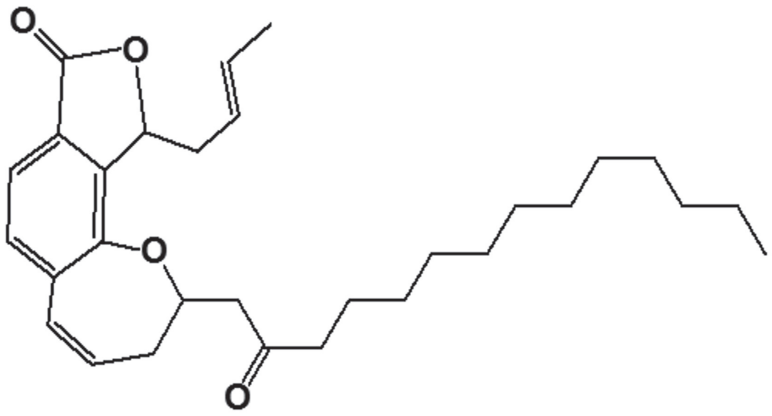


Figure 4.32. 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

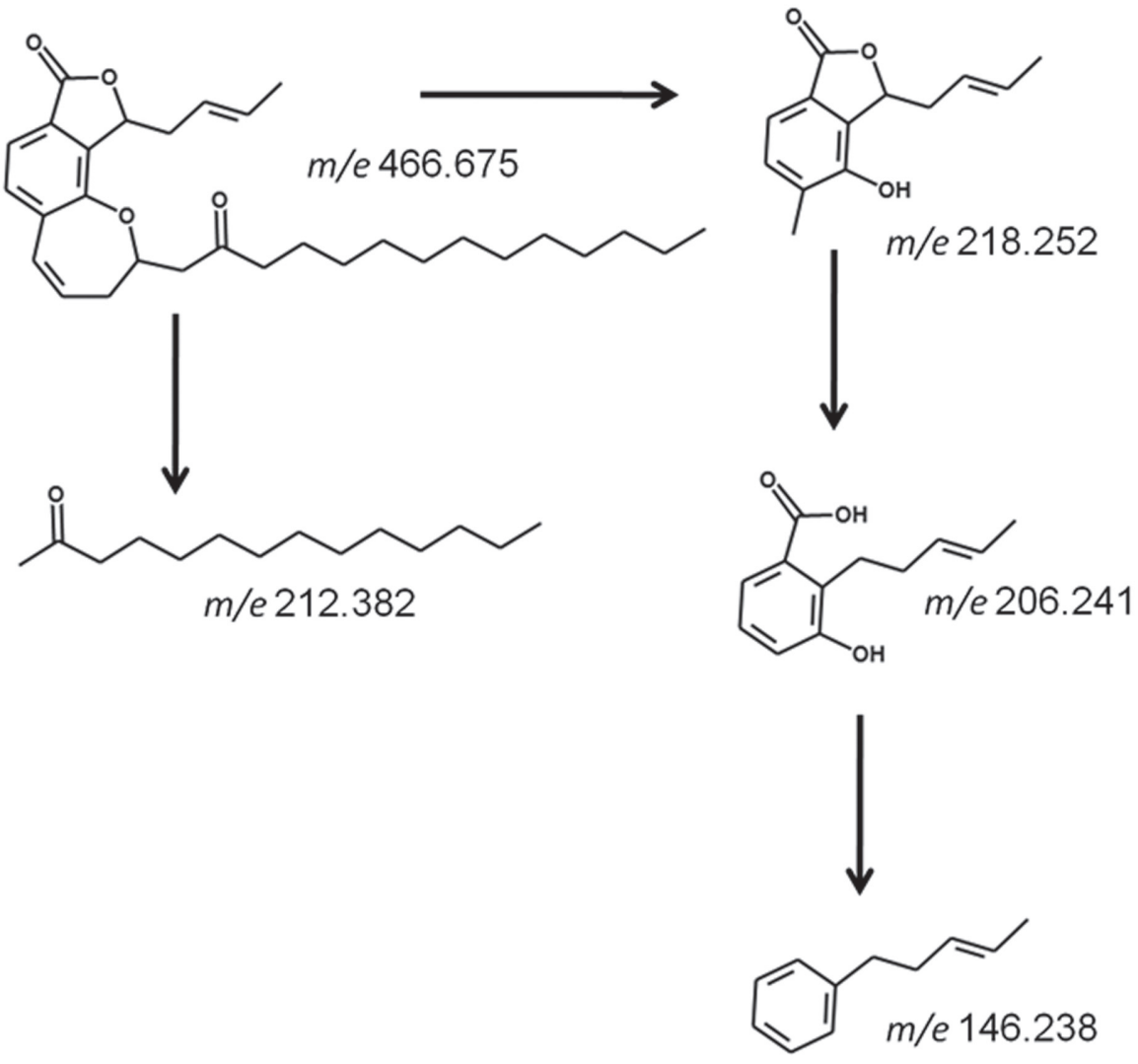


Figure 4.33. Mass fragmentation pattern of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

8-H exhibited ^{1}H-^{1}H COSY correlation it with 9-H (δ 1.94 ppm) and 10-H (δ 1.25 ppm), and HMBC correlation with C-11 carbon (δ 24.51 ppm) (Figure 4.31). This established the presence of 5-((*Z*)-hept-3-en-2-yl)-tetrahydropyran-2-one moiety of the compound. The carbon at C-12 showed HSQC correlation with 11-H (δ 4.01 ppm) and HMBC correlation with C-13 (δ 179.31 ppm), which was established to be connected with the methyl acetate group at C-14 (δ 179.37ppm).

4.3.3. Structural Characterization of 1-((4*Z*)-2,3,7,8-tetrahydrobenzo [b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one: White semisolid; UV (MeOH) λ_{max} (log ε): 246 nm (3.71); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.45; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/ $CHCl_3$) R_t: 9.20 min.; Elemental analysis found: C: 77.21, H 9.07, O: 13.7; IR (KBr, cm^{-1}) ν_{max} 723.33 cm^{-1} γ*r*(=C-H), 812.06 cm^{-1} (2 neighbouring aromatic C-H), 1377.26 cm^{-1} γ*r*(C-H), 1464.02 cm^{-1} ν(C=C), 1564.91 cm^{-1} δ(C-H alkenes), 1683.21 cm^{-1} ν(α, β unsaturated), 1742.96 cm^{-1} ν(C=O 5-membered ring), 2873.74 cm^{-1}, ν(C-H), ν(C-H alkanes), 2957.04 cm^{-1}, ν(C-H alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.6; EIMS *m/e* (rel. int. %): 468 $[M+1]^+$ (16), 266(13), 230(8), 218(16), 206(29), 212(85), 170(100), 146(74), 100(68); HRMS (ESI) *m/e*: calcd. for $C_{30}H_{42}O_4$ 466.6422; found 466.6483 $[M+H]^+$.

The strong ν(C-H) vibrations observed at 2873.74 and 2957.04cm^{-1} are due to long alkane chain. The band at1464.02 cm^{-1} arise due to ν(C=C) . A ν(C=O 5-membered ring) was observed at 1742.96 cm^{-1}. Band observed around 812.06 cm^{-1} is assigned due to 2 neighboring aromatic C-H vibrations (Figure 4.35).

The molecular ion peak was observed at *m/e*=467. The peak at *m/e*=146 is due to the fragment $C_{11}H_{15}$. Peak at *m/e* 212 and *m/e* 72 are due to the fragments $C_{14}H_{26}O$ (*m/e*=212) and the alkane fragment C_5H_{12} (*m/e*=72). The mass fragments at *m/e*=218, 206, and 100 are the characteristic signature peaks of the proposed structure (Figure 4.33). The methine protons appeared at 3.6 ppm, connected to the tertiary carbon (δ 51.38 ppm), and is a part of the substituted dihydrofuran-2(3H)-one ring system (Figure 4.36). The downfield shift of the proton is due to the electronegative -O-C=O group as established by their HMBC connectivities (Figure 4.40).

The presence of a beta -C=C to the carbon at δ 3.6 ppm is established by the linear ^{1}H-^{1}H COSY connectivity (Figure 4.39). The results have been recorded in the following table. The protons at δ 7.71 and 7.53 are typical of the aryl ring system. The proton connectivity with carbon as established by the HSQC spectrum (^{13}C at δ 128.7 and 130.7 ppm) established the aromatic

Table 4.6. NMR spectroscopic data of 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR(int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	147.75				
2	139.23				
3	132.25				
4	130.91				
5	130.73	5-H	7.53(dd)	6-H	C-7,1,8
6	128.70	6-H	7.71(dd)	5-H	C-13
7	213.08				
8	51.38	8-H	3.6(t)	9-H[a]	C-9,
9	36.80	9-H[a]9-H[b]	1.28(dt)1.62(dt)		C-1,2,3
10	114.04	10-H	4.98(q)	9-H	
11	129.83	11-H	5.14(m)	12-H	
12	22.65	12-H	2.27(d)	11-H	C-11
13	114.16	13-H	5.53(dd)	14-H	C-14
14	125.06	14-H	5.81(m)	15-H[b]	
15	30.67	15-H[a]15-H[b]	1.72(m)2.02(m)	14-H	C-17,14
16	65.53	16-H	4.33(m)	15-H[a],17-H	C-17,18
17	34.02	17-H	2.33(d)	16-H	C-18,19
18	176.16				
19	33.16	19-H	2.07(t)	20-H	C-18,17
20	31.89	20-H	1.36(m)	21-H	C-22
21	31.4	21-H	1.26(m)	22-H	C-23
22	30.17	22-H	2.75(p)		
23	29.66	23-H	2.14(m)	22-H	
24	28.92	24-H	2.03(m)	25-H	C-25,26
25	26.44	25-H	1.9(m)	26-H	C-26,28
26	24.73	26-H	1.69	27-H	C-28
27	22.66	27-H	1.30(m)		
28	20.85	28-H	1.45(m)	29-H	C-29
29	19.9	29-H	0.97(m)	30-H	
30	14.09	30-H	0.88(m)	29-H	C-27,29

[a] NMR spectra recorded using spectrometers. Bruker AVANCE III 500 MHz (AV 500)

[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments.

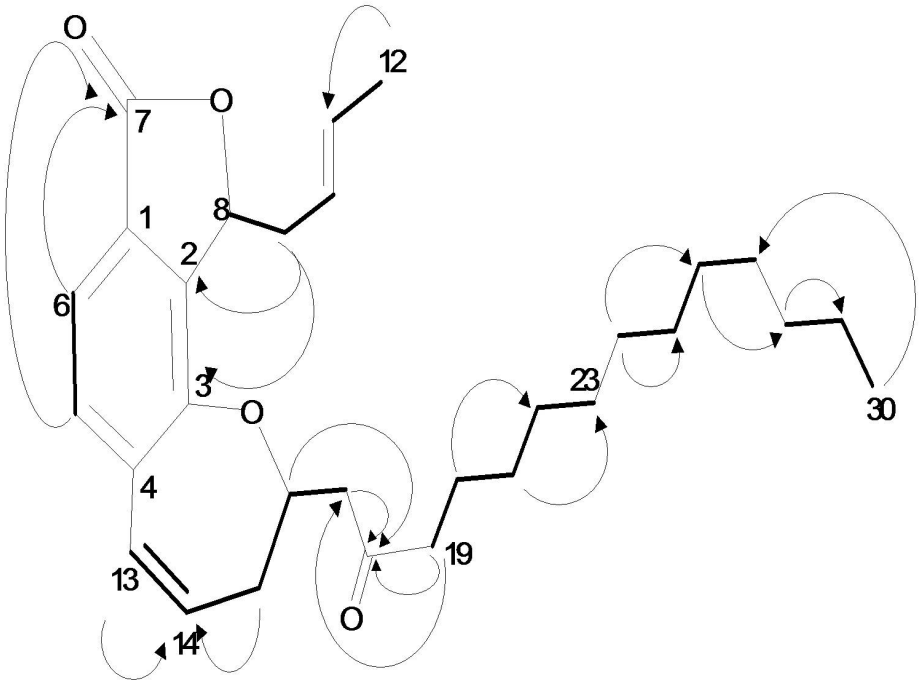


Figure 4.34. 2D NMR correlations as observed in 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

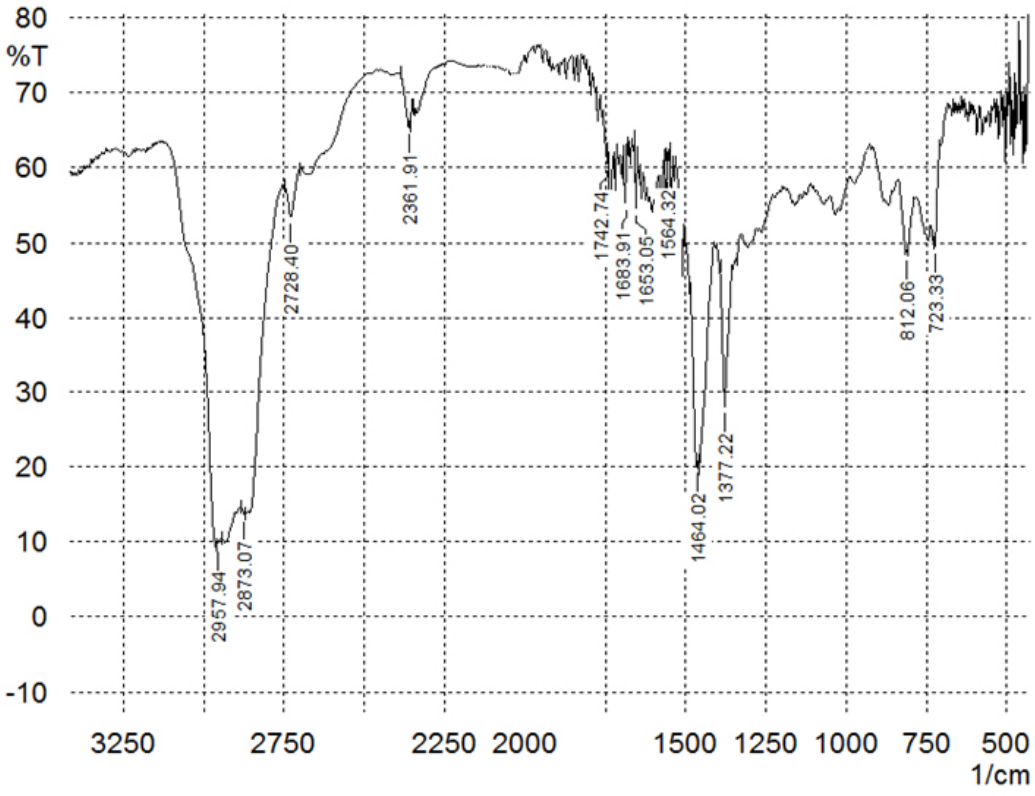
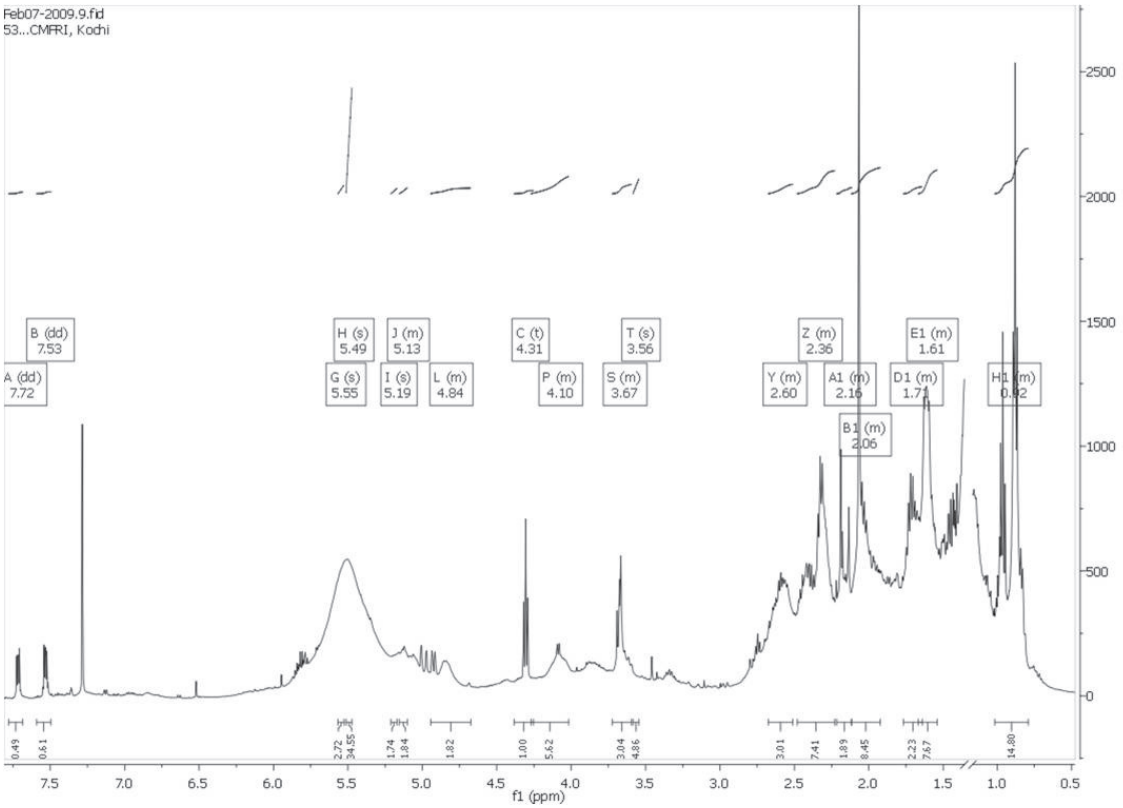


Figure 4.35. IR spectroscopic data of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

A



B

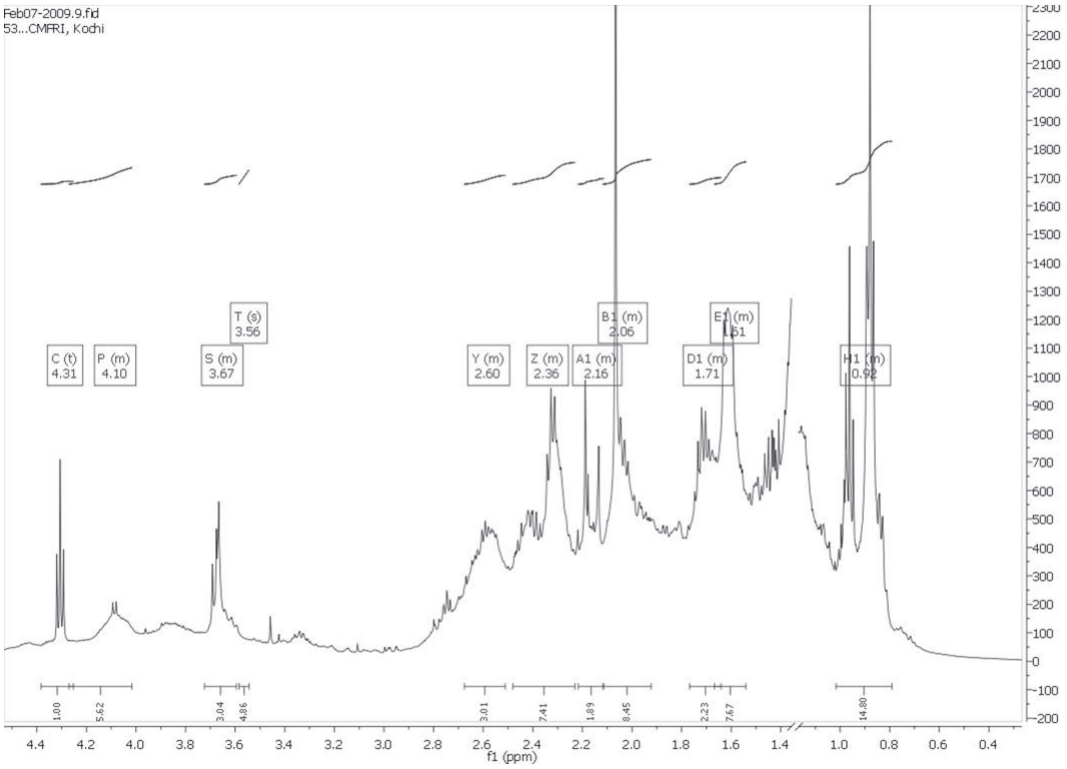
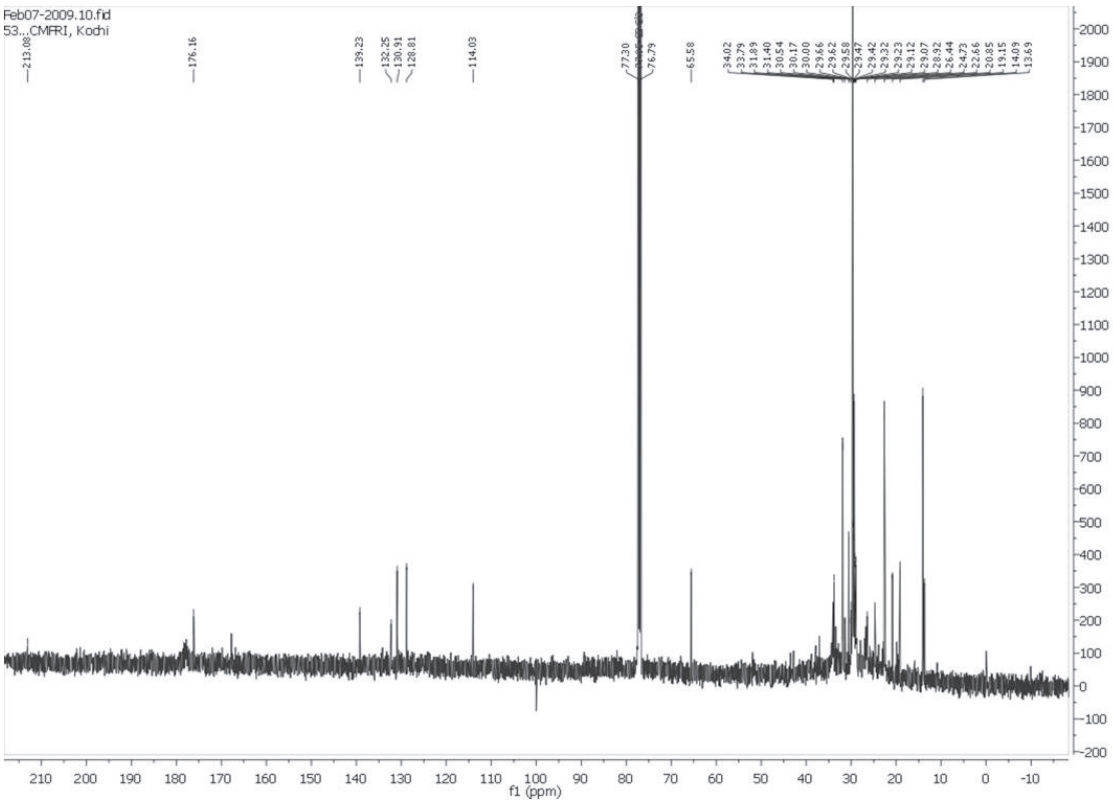


Figure 4.36. Proton NMR spectra of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one (A - full view, B - expanded view)

A



B

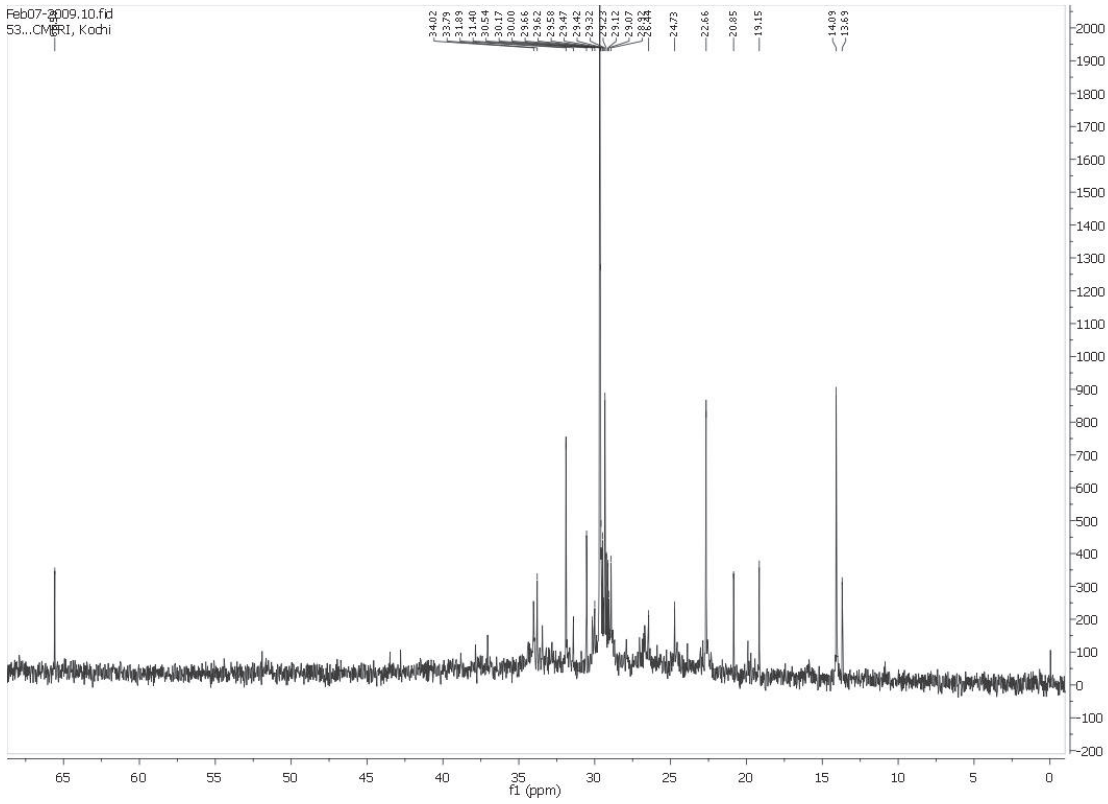


Figure 4.37. ^{13}C NMR spectra of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one (A - full view, B - expanded view)

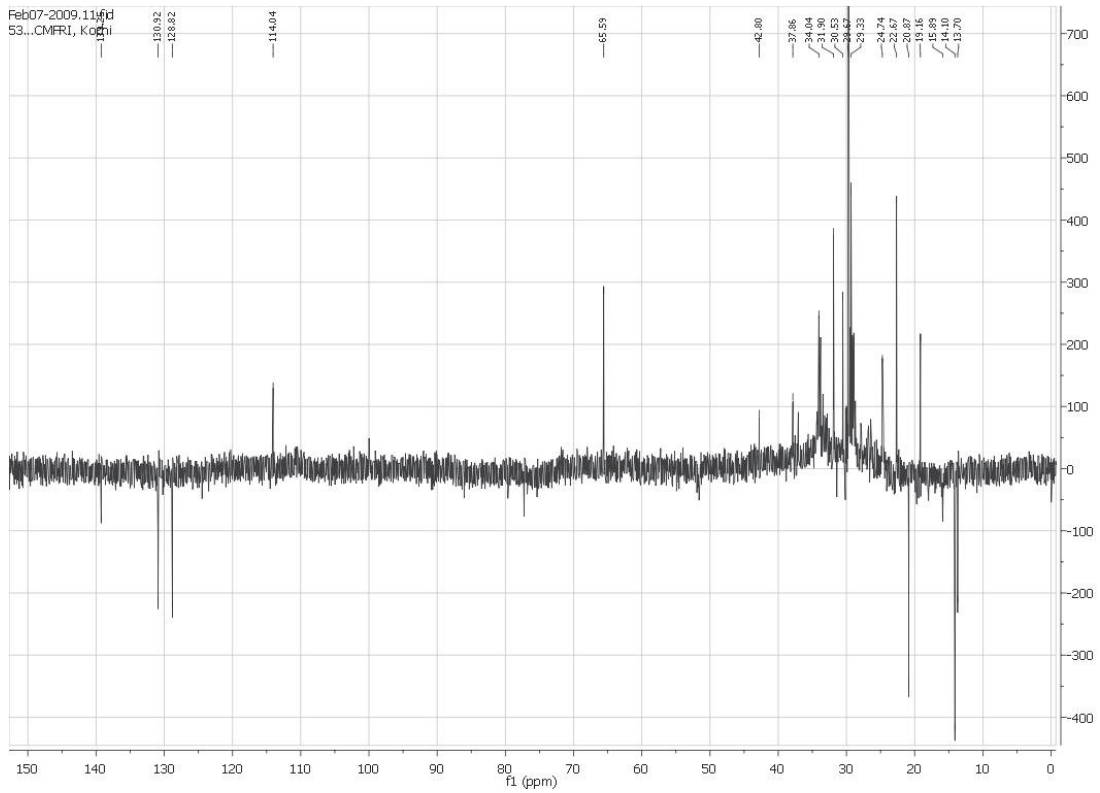


Figure 4.38. DEPT spectrum of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

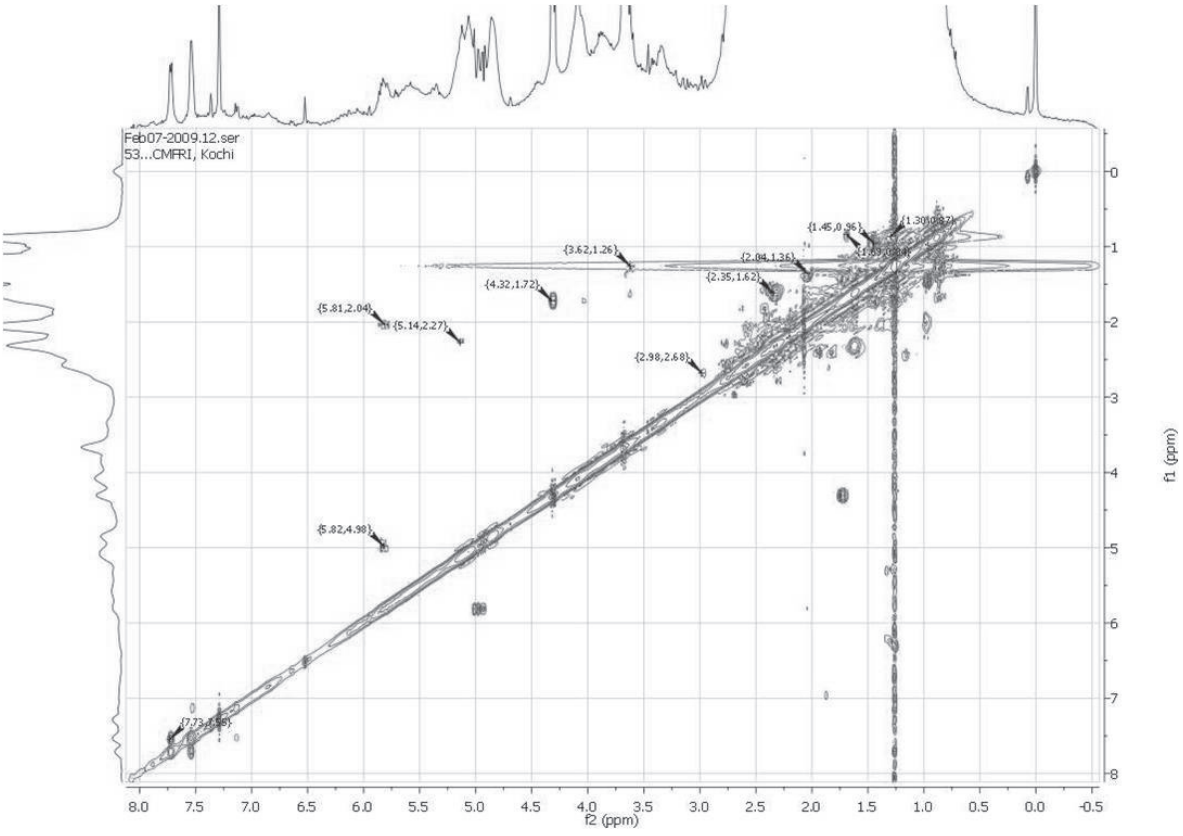


Figure 4.39. ^{1}H-^{1}H COSY spectrum of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

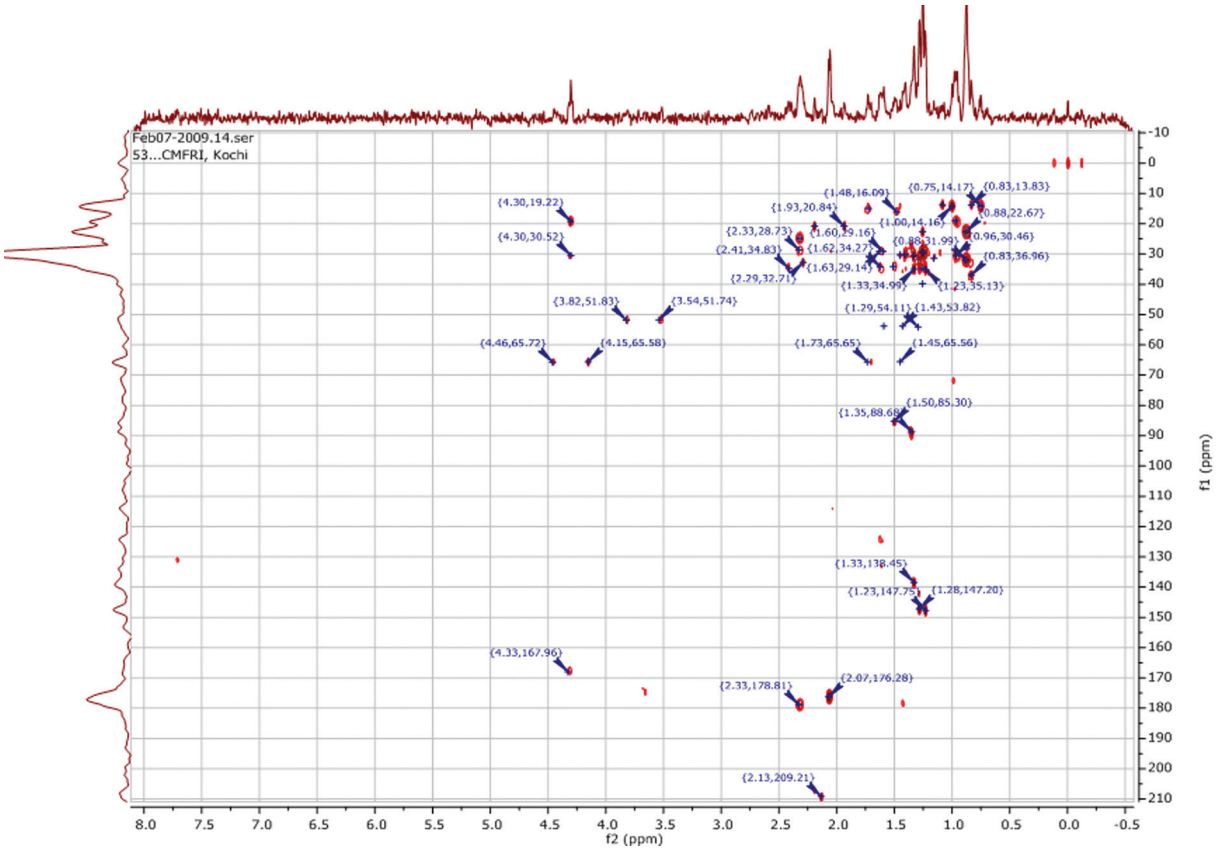


Figure 4.40. HMBC spectrum of 1-(4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one

system in the compound. The structure of the 3-methylisobenzofuran-1(3H)-one system has been established by the HMBC correlation analyses. The structure of (Z)-2,3-dihydrobenzo[b]oxepine has been established by the ^{1}H-^{1}H COSY and HMBC analyses and calculation of the coupling constants (J) of the protons at δ 5.53 and 5.81 ppm, which confirm the Z-configuration of the olefinic system. The characteristic olefinic signals were present at δ 114.04 and 129.83 ppm and the olefinic protons at δ 5.14 and 4.98 ppm are linearly ^{1}H-^{1}H COSY correlated with the methylene protons at δ 1.68 ppm and the methine proton at δ 3.6 ppm. The coupling constant values have been calculated to be in conformity with the *E*-configuration. This established the structure of 5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one system. The proton at 13-H (δ 5.53 ppm) showed ^{1}H-^{1}H COSY correlation to 14-H (δ 5.81 ppm), which showed linear ^{1}H-^{1}H COSY correlation to 15-H (δ 2.02 ppm) and 16-H (δ 4.33 ppm). The methine proton at δ 4.33 ppm appeared downfield, and has been established due to the presence of a beta—C=O(CH_2)- group. Strong ^{1}H-^{1}H COSY correlation between the proton signals (methylene) at δ 2.07, 1.36, 1.26, 2.75, 2.14, 2.03, 1.90, 1.69, 1.30, 1.45, and 0.97, which in combination of mass spectroscopic analyses established the tetradecanone moiety. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 30 carbon atoms in the molecule (Figure 4.37 & 4.38). In the ^{1}H–^{1}H COSY and HMBC spectra, couplings were apparent as described in the figure support the presence

Figure 4.41. *Acanthophora spicifera* collected from Puthumadom of Gulf of Mannar

of the established skeleton of 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one.

Anjaneyulu et al. (1984) isolated l,7-dlhydroxy-3-methoxy-2-methyl-dibenzo(2,3-6,7) oxepin from the heartwood of *Bauhiniaracemosa*. Greca et al. (1993) isolated a novel dihydrodibenzoxepin from *Juncus effuses. A dihydrodibenzoxepin from Bauhinia variegate* was isolated from the root bark of *Bauhinia variegata* (Reddy et al. 2003), 10,11-dihydro-dibenz[b,f]oxepin-2,4-diol, and 10,11-dihydro-4-methoxy-dibenz[b,f]oxepin-2-ol were isolated from the chloroform soluble fraction of *Dioscorea opposite* (Yang et al. 2009).

4.4. Isolation and Characterization of Antioxidant Secondary Metabolites from *Acanthophora spicifera* (M.Vahl) Børgesen

4.4.1. Bioassay Guided Chromatographic Purification of the Methanol Extract of *A. spicifera*

The MeOH extract of *A. spicifera* (2g) collected from the Gulf of Mannar (Figure 4.41) was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from n-hexane, n-hexane: EtOAc and finally EtOAc: MeOH and the collected fractions were concentrated under vacuum and TLC evaluated and fractions with same TLC profile were pooled together to furnish six fractions (As_{1-6}).The sub fraction AS_1 which showed good antioxidant activity (65.62% DPPH and 27.11% ABTS scavenging ability) and Fe^{2+} ion chelating ability (19.82%) was further purified using preparative thin layer chromatography (20% EtOAc: *n*-hexane) to furnish three sub fractions ($AS_{1,1-3}$). The sub fraction $AS_{1,2}$ exhibited a higher radical scavenging potential and ion chelating ability than other corresponding sub fractions, was further purified using preparative thin layer chromatography (20% EtOAc: *n*-Hexane) to furnish four sub fractions ($AS_{1,2,1-4}$). $AS_{1,2,3}$ ((6*Z*)-methyl 8-(2-(*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-imethylnon-6-enoate) was obtained as a brown oily compound with a significantly higher ($P<0.05$) DPPH (72.62%) and ABTS (30.77%) radical scavenging potential and Fe^{2+} ion chelating ability (21.94%) than the other corresponding sub fractions.

The active crude sub fraction AS_8 (78.46% DPPH and 32.96% ABTS scavenging ability and 22.29% Fe^{2+} ion chelating ability) was purified using column chromatography using silica column (60-120 mesh) with a stepwise gradient of solvents from $CHCl_3$, $CHCl_3$: MeOH and finally with MeOH (Figure 4.42). Collected fractions were concentrated under vacuum and TLC evaluated and fractions with same TLC profile were pooled together to furnish six fractions ($AS_{8,1-6}$). The crude sub fraction $AS_{8,6}$ with high potential (75.53%, 30.95%, 22.41% for DPPH and ABTS radical scavenging ability and Fe^{2+} ion chelating ability respectively) was further purified using preparative thin layer chromatography (5% MeOH: MDC) to furnish $AS_{8,6,6}$ (1-((4*Z*)-2,3,7,8-

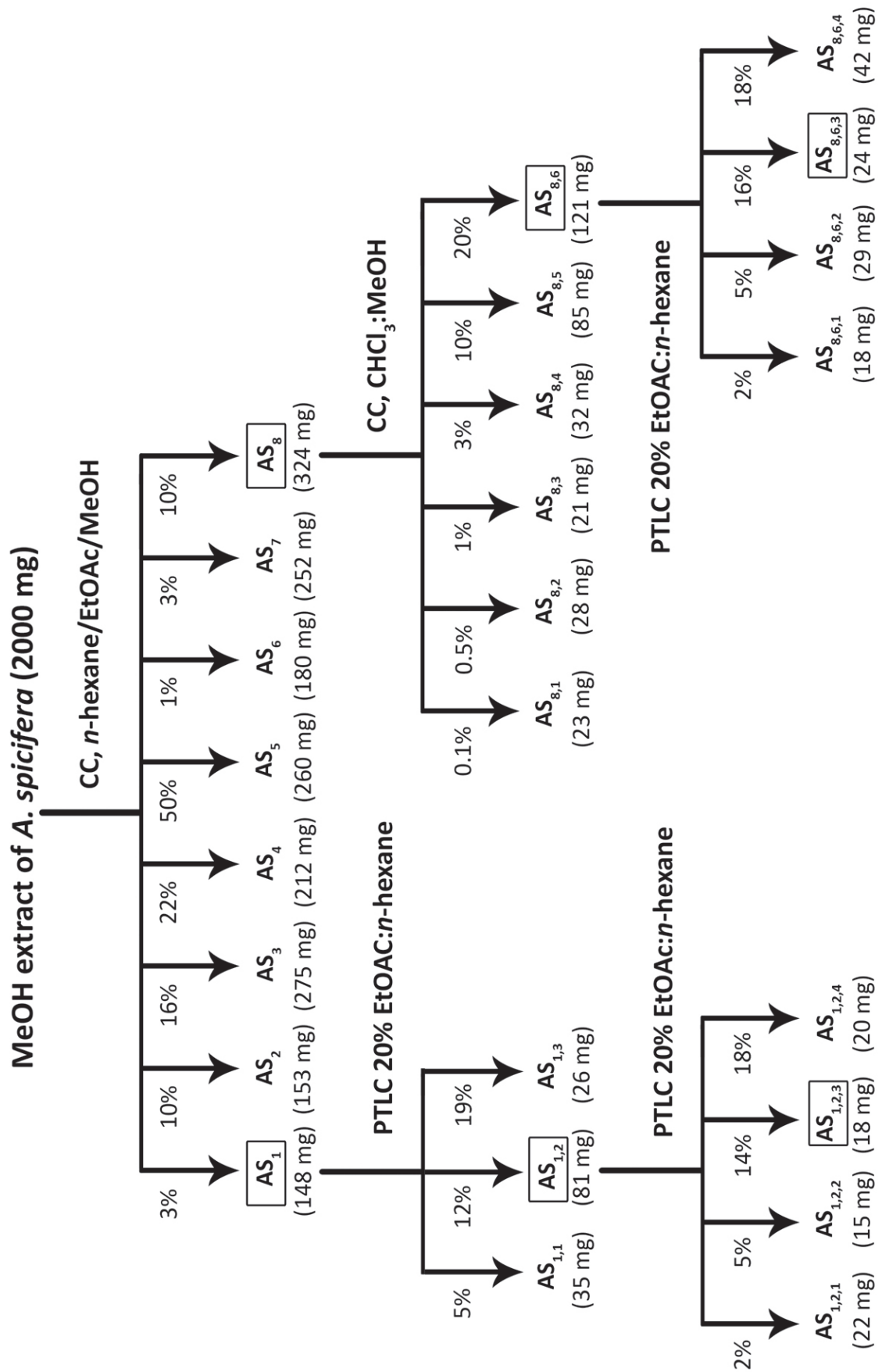


Figure 4.42. Schematic diagram representing the purification of MeOH extract of A. spicifera (AS). CC: column chromatography, PTLC: preparative thin layer chromatography

tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one.) a brown oily compound with a radical scavenging potential of >78% DPPH and > 29% ABTS free radical scavenging potential and > 20% ion chelating potential.

The compounds belonging to 1-3-[3, 5-dimethyl-2-hexenyl] phenyl-1-ethanone have been separated with the hexane/$CHCl_3$ gradient, and the one with propyl acetate group at the fourth position of 1-(3-((*E*)-3, 5-dimethylhex-2-enyl) phenyl) ethanone moiety was demonstrated to possess potent antioxidative activity. The presence of 3, 5-dimethylhex-2-ene, propyl acetate, and 1-(3-(3, 5-dimethylhex-2-enyl) phenyl) ethanone groups have been confirmed by detailed NMR and mass spectroscopic experiments.

The structural characterization were carried out be detailed spectroscopic techniques and are discussed in following sections (4.4.2 and 4.4.3)

4.4.2. Structural Characterization of 3-Hexyl-5, 6-dihydro-6-undecylpyran-2-one

3-Hexyl-5,6-dihydro-6-undecylpyran-2-one: White semisolid; UV (MeOH) λ_{max} (log ε): 226 nm (3.61); TLC (Si gel GF_{254} 15 mm; EtOAc/*n*-hexane20:80, v/v); R_f: 0.75; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) O, 6.20 R_t: 24.22 min.; Elemental analysis found:C,78.51;H,11.97;O,9.52. IR (KBr, cm^{-1}) ν_{max} 723.33 cm^{-1} γr(long chain methyl), 812.06 cm^{-1} δ(=C-H out of plane), 1377.22 cm^{-1} γr(C-H), 1464.02 cm^{-1} δ(C-H alkanes), 1564.32 cm^{-1} ν(C–C stretch), 1653.05, 1683.91, 1742.74 cm^{-1} ν(C=O), 2361.91, 2728.40, 2873.07, 2957.94 cm^{-1} ν(C-H alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.8; EIMS *m/e* (rel. int. %): 337 $[M+1]^+$(13), 98(100), 28(72), 252(53), 337(48), 45(45), 142 (21), 254 (12), 251(08), 253(6), 22(8). HRMS (ESI) *m/e*: calcd. for $C_{22}H_{40}O_2$ 336.5543; found 336.5586 $[M+H]^+$.

The molecular ion peak appeared at *m/e*= 336, which underwent fragmentation to result the fragment peaks at *m/e*=86 (C_6H_{13}), 155 ($C_{11}H_{23}$), 98 ($C_5H_6O_2$) established the structure. The fragment peak at *m/e*= 98 is a base peak with a cyclic structure (5, 6-dihydropyran-2-one) (Figure 4.44).

The IR band at 723.33 cm^{-1} is due to long chain ν(methyl group). The ν(C-H long alkane chain) was observed at 2361.91, 2728.40, 2873.07 and 2957.94 cm^{-1}. The strong band at 1464.02cm^{-1} is also due to δ(C-H alkanes). The bands at 1653.05 1683.91and 1742.74 cm^{-1} are assigned due to ν(C=O vibrations) (Figure 4.46).

The C^{13} NMR spectra displayed a signal at δ 179.20 ppm indicating that the compound has a carbonyl functional group (Figure 4.48 & 4.49). The ^{13}C signals at δ 130.88 and 128.80

Table 4.7. Antioxidant and Fe^{2+} chelating potential (%) of the different fractions obtained by the purification of the MeOH extract of *A. spicifera*

Sl NO	Sample ID	Solvent System	Yield (g)	ABTS Radical Scavenging Activity (%)	DPPH Radical Scavenging Activity (%)	Fe^{2+} ion Chelating Ability (%)
1	AS_1	3	148	27.11±0.36	65.62±0.88	19.82±0.26
2	AS_2	10	153	21.66±0.29	54.38±0.73	16.18±0.21
3	AS_3	16	275	12.82±0.17	30.25±0.41	8.978±0.12
4	AS_4	22	212	24.46±0.33	60.42±0.81	18.20±0.24
5	AS_5	50	260	8.999±0.12	22.40±0.30	6.458±0.08
6	AS_6	1	180	18.06±0.24	43.35±0.58	12.04±0.16
7	AS_7	3	252	25.70±0.35	62.47±0.85	22.29±0.28
8	AS_8	10	324	32.96±0.44	78.46±1.06	22.29±0.30
AS_1						
1	$AS_{1,1}$	5	35	11.39±0.15	29.17±0.39	8.683±0.11
2	$AS_{1,2}$	12	81	25.61±0.34	62.50±0.84	17.60±0.23
3	$AS_{1,3}$	19	26	15.38±0.20	43.38±0.58	12.05±0.16
$AS_{1,2}$						
1	$AS_{1,2,1}$	2	22	25.70±0.34	65.53±0.88	18.61±0.25
2	$AS_{1,2,2}$	5	15	20.08±0.27	48.60±0.65	14.13±0.19
3	$AS_{1,2,3}$	14	18	30.77±0.41	72.62±0.98	21.94±0.29
4	$AS_{1,2,4}$	18	20	15.04±0.20	37.76±0.51	10.88±0.14
AS_8						
1	$AS_{8,1}$	0.1	23	12.41±0.16	37.37±0.50	10.86±0.14
2	$AS_{8,2}$	0.5	29	21.66±0.34	53.41±0.99	17.15±0.28
3	$AS_{8,3}$	1	21	23.08±0.31	54.48±0.73	16.45±0.22
4	$AS_{8,4}$	3	32	14.61±0.46	48.61±1.20	16.37±0.35
5	$AS_{8,5}$	10	121	20.65±0.28	48.73±0.66	14.68±0.19
6	$AS_{8,6}$	20	85	30.95±0.41	75.53±1.02	22.41±0.30
$AS_{8,6}$						
1	$AS_{8,6,1}$	0.2	18	24.11±0.44	53.81±1.09	21.44±0.30
2	$AS_{8,6,2}$	0.5	29	19.69±0.40	43.72±1.13	15.07±0.27
3	$AS_{8,6,3}$	0.6	24	31.91±0.43	78.82±1.06	24.17±0.32
4	$AS_{8,6,4}$	1.8	42	20.50±0.27	52.28±0.70	13.44±0.18

Data are the mean values of the triplicate and expressed as mean ± standard deviation. The concentration of the solvent fractions used were 0.1 mg/mL for DPPH radical scavenging activity, and Fe^{2+} ion chelating activity; and 0.1 µg/mL for ABTS radical scavenging activity.

indicate the olefinic carbons, whilst the signal at δ 130.88 ppm doesn't have any HSQC signal thereby indicating the presence of a quaternary carbon atom. The ^{13}C-NMR signal at δ 128.80 ppm gives a downfield proton signal at δ 7.42 ppm as established by HSQC experiment. These results also indicate that the position of the proton at δ 7.42 ppm is at the trans position with respect to the carbonyl compound. The ^{13}C signal at C-5 (δ 68.17 ppm) is attached to the oxygen of the 5,6-dihydropyran-2-one ring system. The C-5 signal at δ 68.17 ppm shows HSQC signal at H-5 (δ 4.3 ppm). the downfield shift of C-5 appeared to be due to the alpha—OC(=O) group in the 5,6-dihydropyran-2-one system. The C-4 (δ 29.66 ppm) exhibited the HSQC correlation with the proton signal at δ 1.6 ppm (H-4), whilst the proton at δ 4.3 ppm (H-5) displayed ^{1}H-^{1}H COSY correlation with the signal at δ 2.28 ppm (H-6). The proton at δ 4.43 ppm (5-H) showed strong ^{1}H-^{1}H COSY correlation to 6-H (2.3 ppm), which showed linear ^{1}H-^{1}H-COSY correlation to 7-H (δ 1.31 ppm) and 8-H (δ 1.3 ppm) (Figure 4.50). Strong ^{1}H-^{1}H-COSY correlation between the proton signals (methylene) at δ 1.35, 1.20, 1.32, 0.81, and 0.85 (Figure 4.47 & 4.50), which in combination of mass spectroscopic analyses established the dodecane moiety attached with the 5,6-dihydropyran-2-one group. The ^{13}C NMR spectrum in combination with HSQC, HMBC and DEPT experiments indicated the occurrence of 22 carbon atoms in the molecule. In the ^{1}H–^{1}H COSY and HMBC spectra (Figure 4.51), couplings were apparent as described in the figure support the presence of the established skeleton of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one.

There are several reports that compounds with pyran related structures are isolated from various sources such as (-)-5,6-dihydro-6-undecyl-2H-pyran-2-one, and (-)-5,6-dihydro-6-tridecyl-2H-pyran-2-one were isolated from the methanol extract of the stem bark of *Horsfieldia superb* (Al-Mekhlafi et al. 2013), prenylated pyran-2-one have been isolated from the roots of *Thapsia transtagana* (Rubal et al. 2007), 2-methyl-pyran-4-one-3-O-b-D-glucopyranoside isolated from leaves of *Punica granatum* (Balwani et al 2011) etc.

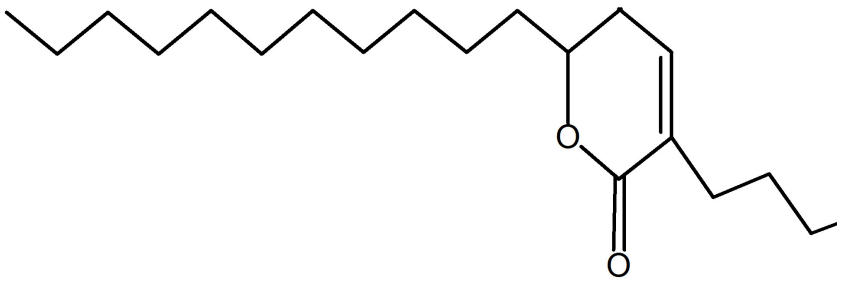


Figure 4.43. 3-Hexyl-5,6-dihydro-6-undecylpyran-2-one

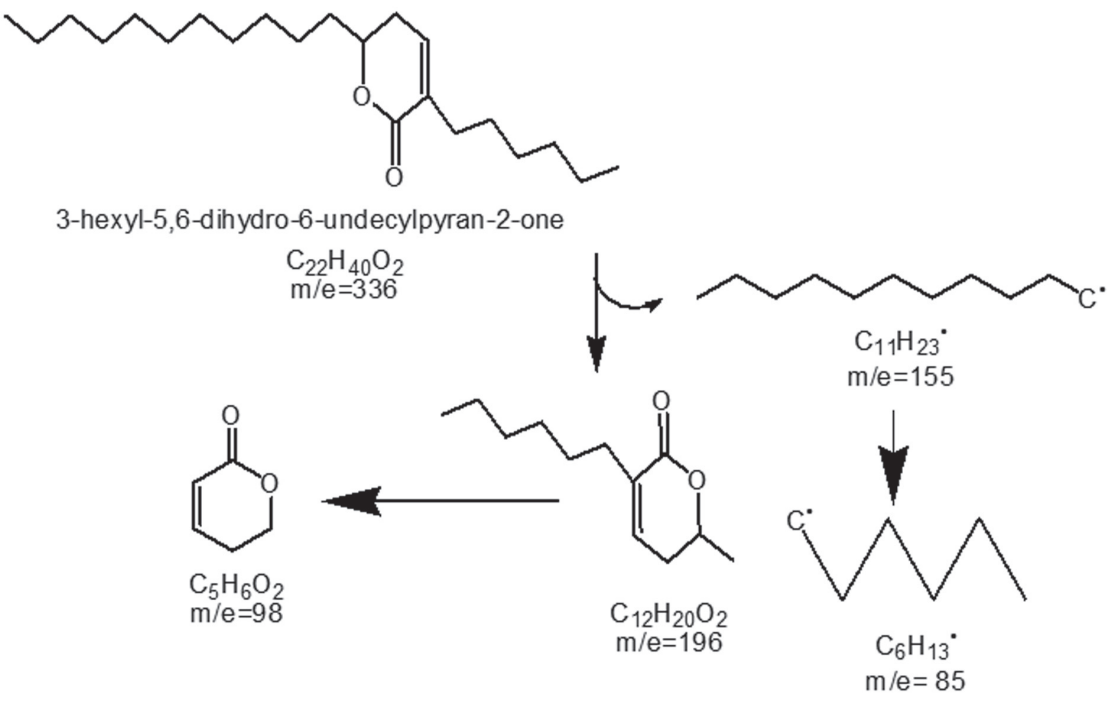


Figure 4.44. MS splitting of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one

Table 4.8. NMR spectroscopic data of 3-hexyl-5, 6-dihydro-6-undecylpyran-2-one in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^1H NMR(int., mult., *J* in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	179.20				
2	130.88				
2a	38.73	2a-H,2a'-H	1.3,1.6	2b-H	C-1,3,4
2b	29.65	2b-H	1.2	2a'-H	
2c	29.59	2c-H	1.2		
2d	30.36	2d-H	1.6	2d-H	C-1,2
2e	29.24	2e-H	1.2	2c-H	
2f	24.70	2f-H	1.2		
3	128.80	3-H	7.4		
4	29.66	4-H,4'-H	1.6		
5	68.17	5-H	4.3		
6	33.94	6-H,6'-H	2.28,2.25	5-H	C-1,5
7	29.68	7-H	1.31		C-5,4
8	29.66	8-H	1.58	7-H	
9	31.92	9-H	1.56	8-H	C-10,5
10	29.59	10-H	1.2	11-H	
11	29.43	11-H	1.2		
12	29.35	12-H	1.32		
13	23.24	13-H	1.2	14-H	C-12
14	22.98	14-H	0.81	13-H	C-13
15	22.68	15-H	0.8	15-H	
16	14.11	16-H	0.85		C-15,14

[a] NMR spectra recorded using spectrometers. Bruker AVANCE III 500 MHz (AV 500)
[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments.

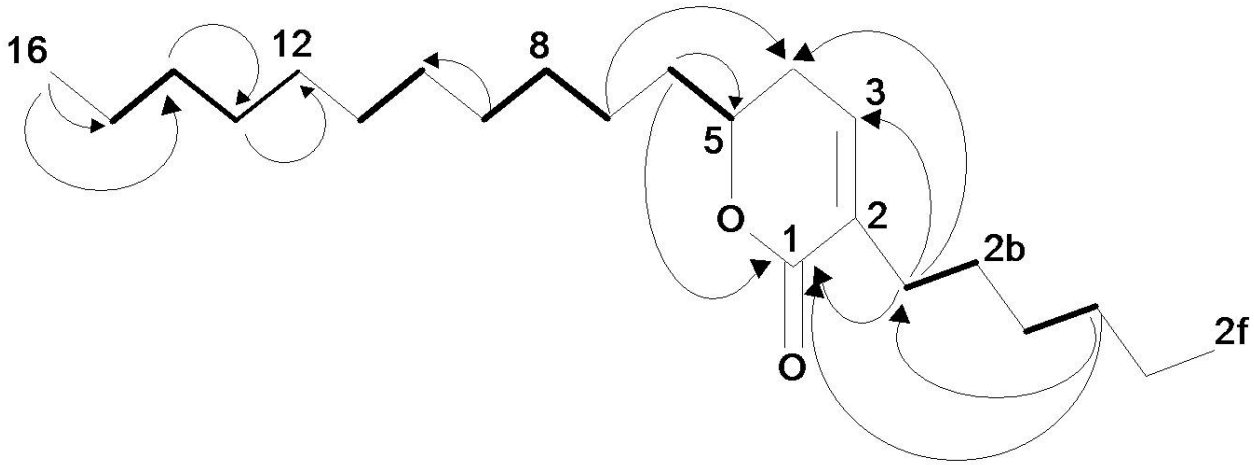


Figure 4.45. 2D NMR correlations as observed in 3-hexyl-5, 6-dihydro-6-undecylpyran-2-one. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

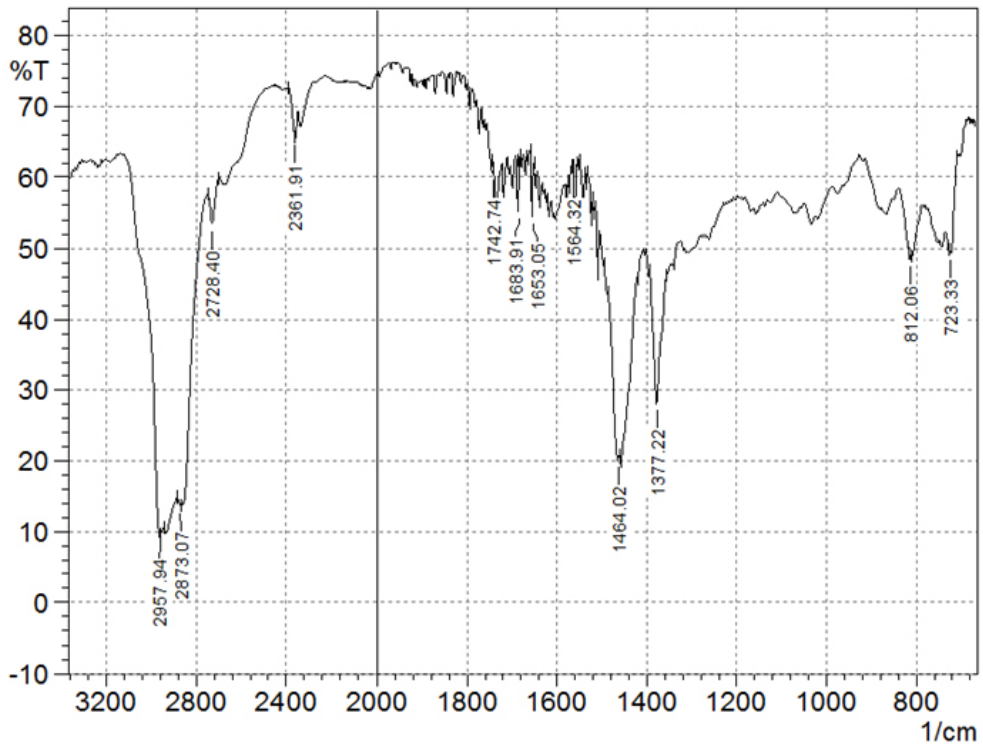
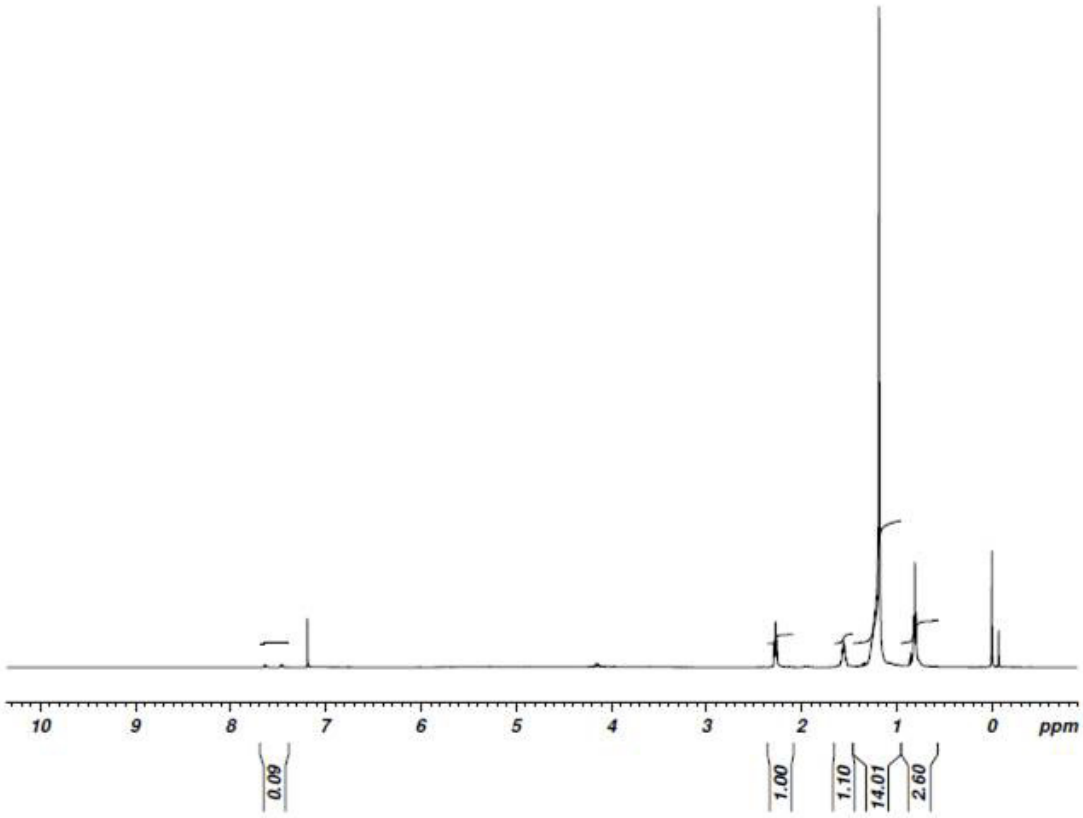


Figure 4.46. IR spectrum of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one

A



B

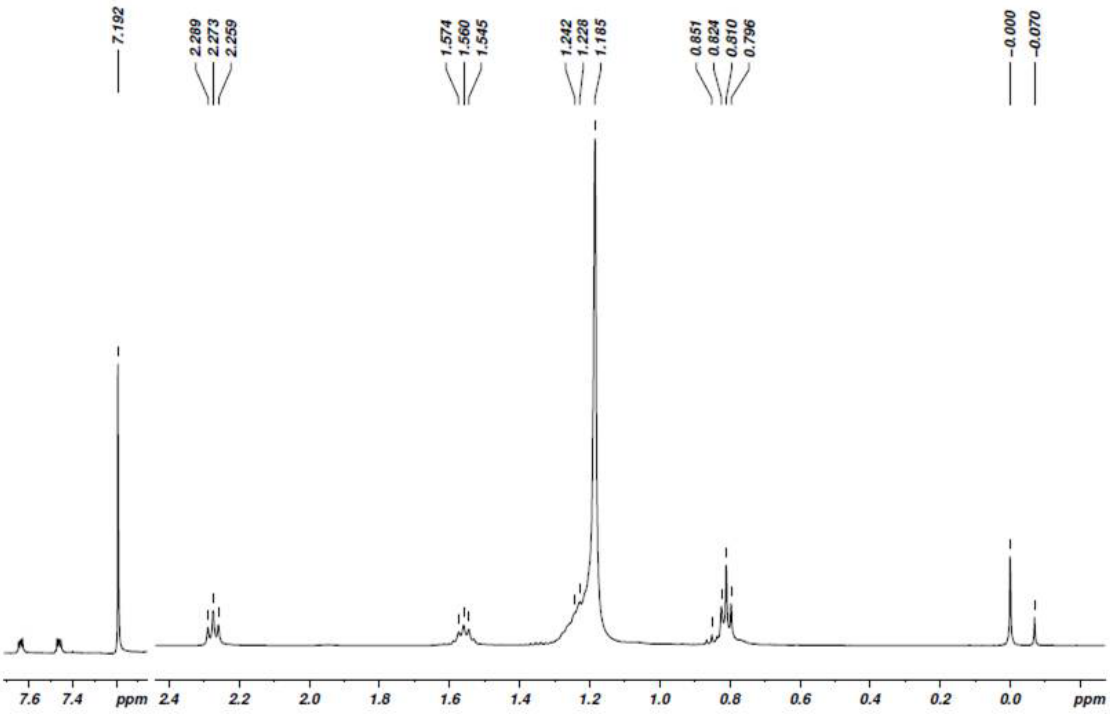


Figure 4.47. Proton NMR spectra of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one (A - full view, B - expanded view)

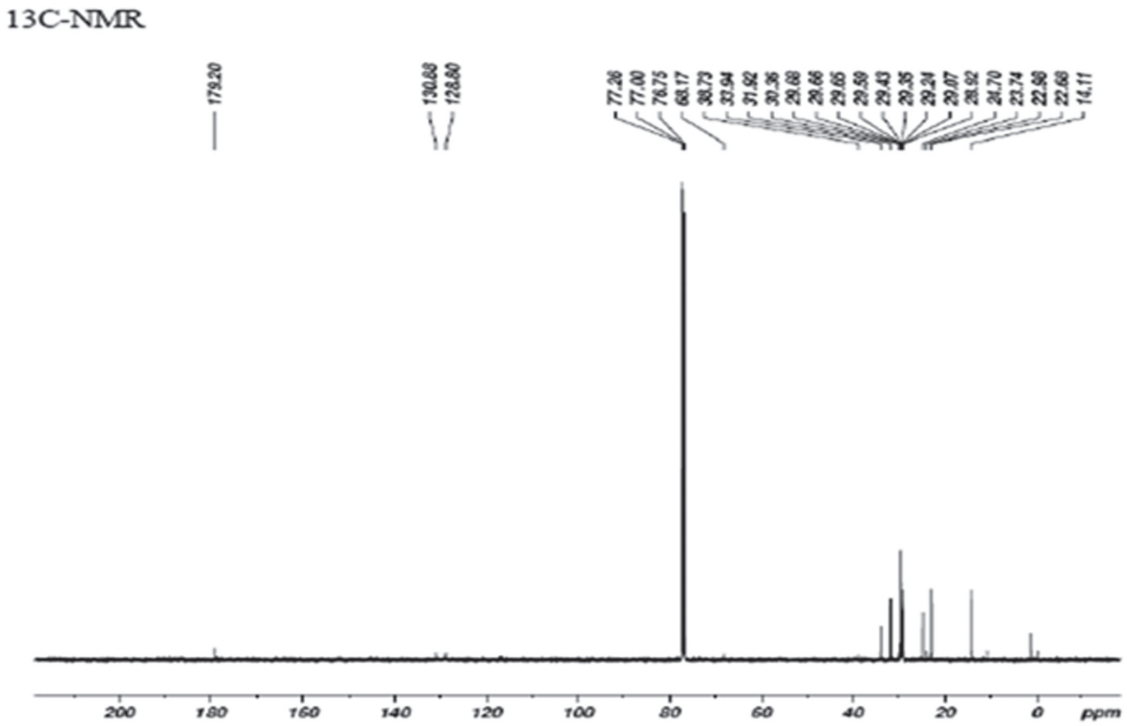


Figure 4.48. ^{13}C NMR spectrum of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one

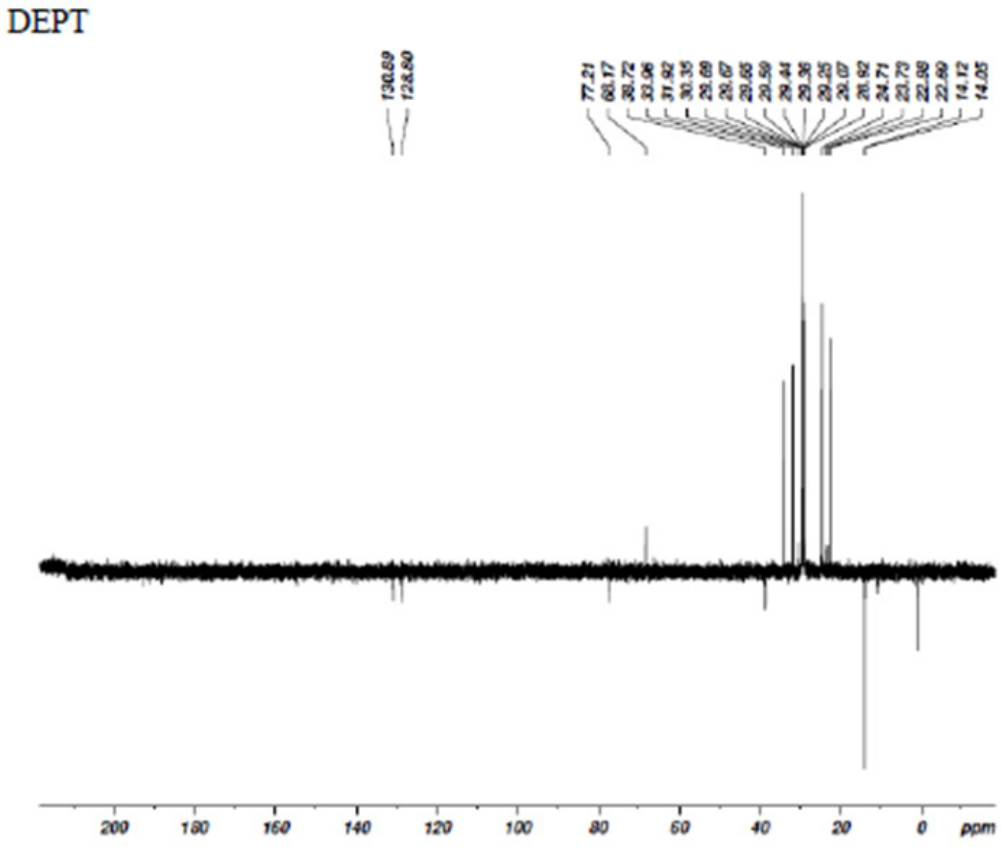
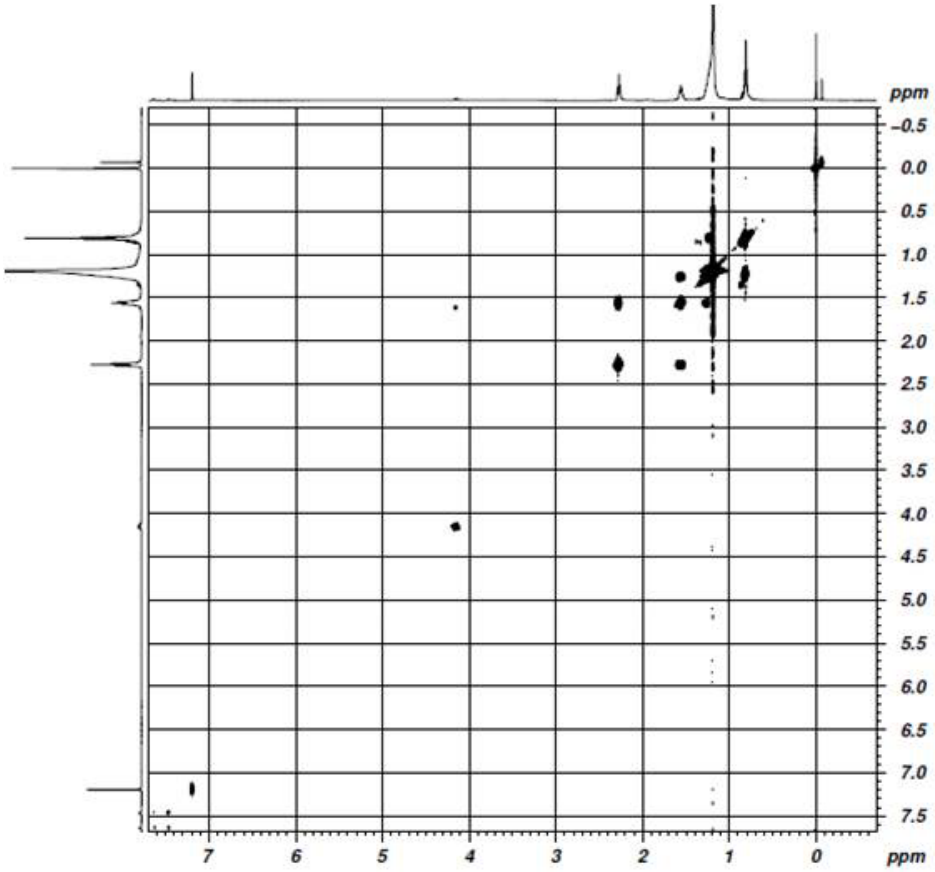


Figure 4.49. DEPT spectrum of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one

A



B

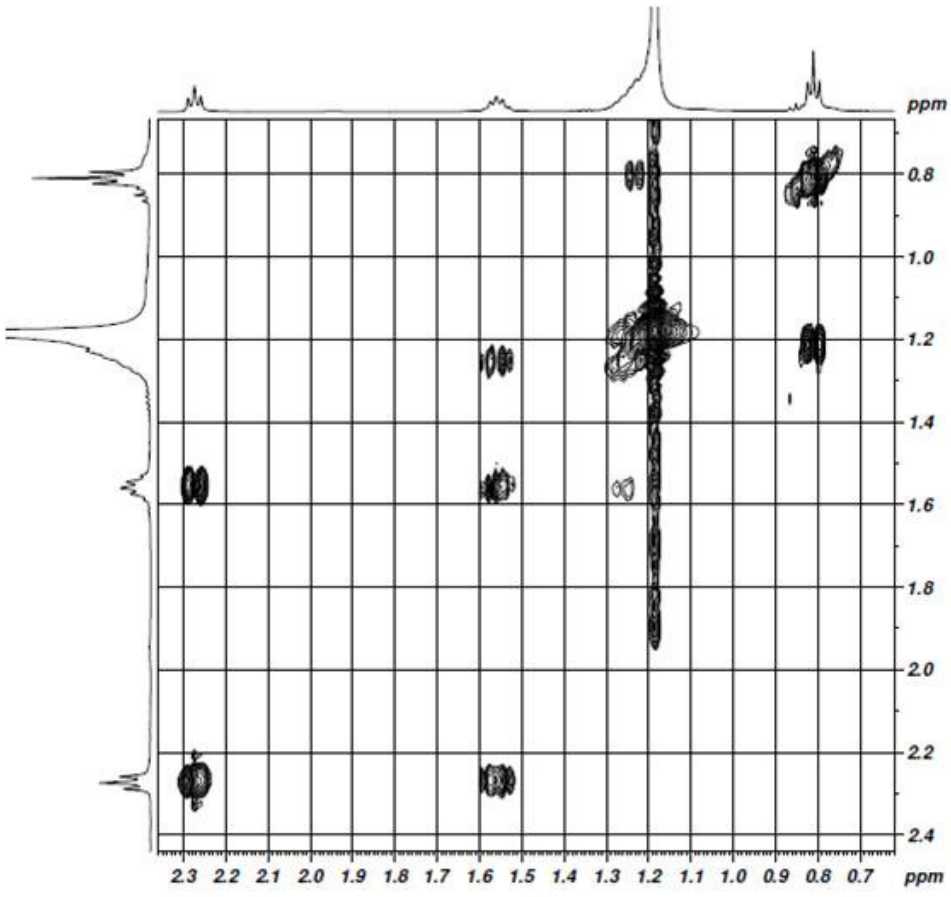
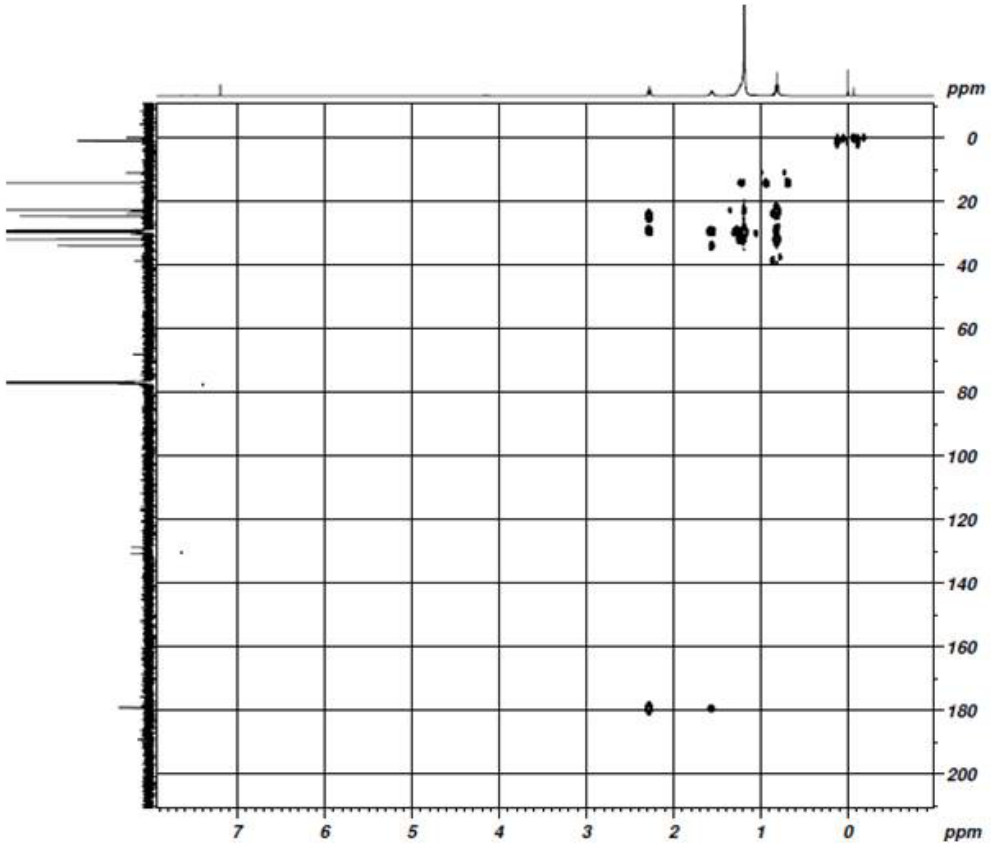


Figure 4.50. ^{1}H-^{1}H-COSY spectra of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one (A - full view, B - expanded view)

A



B

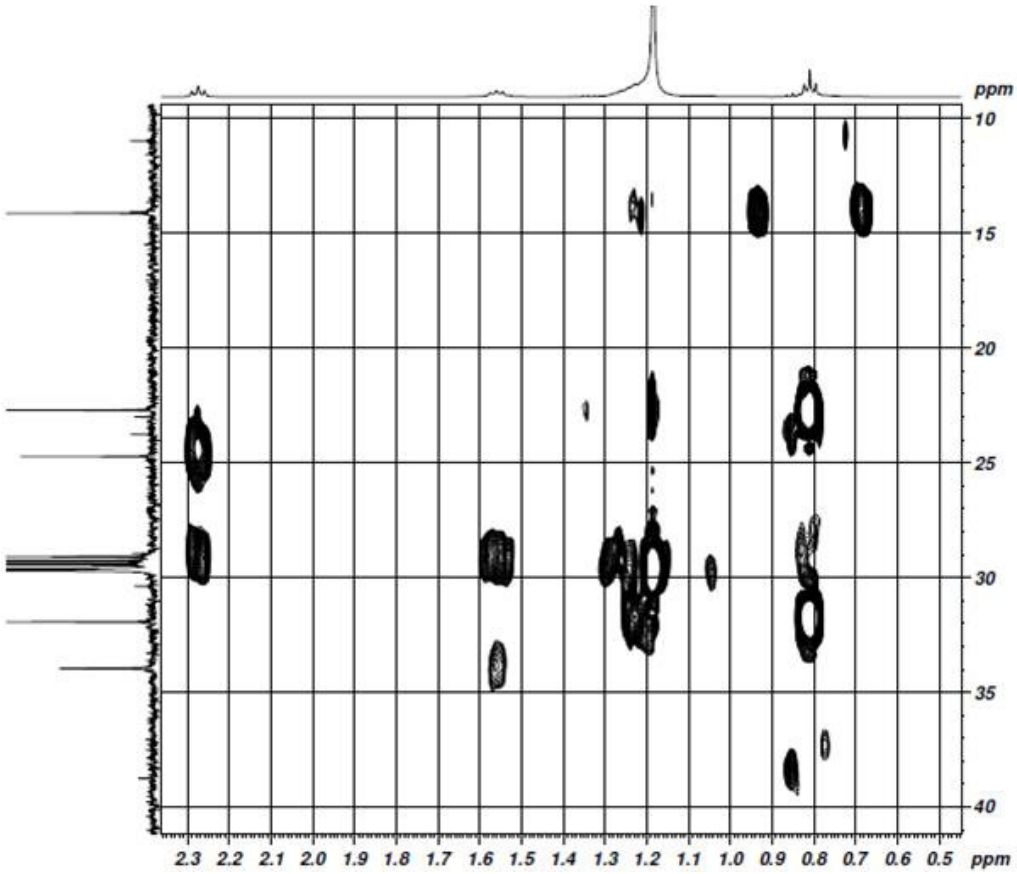


Figure 4.51. HMBC spectra of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one (A - full view, B - expanded view)

4.4.3. Structural Characterization of 3-hexyl-5,6-dihydro-6-undecylpyran-2-one and butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate

Butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate: Light yellow semisolid; UV (MeOH) λ_{max} (log ε): 267 nm (4.61); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:90, v/ v) R_f: 0.45; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) R_t: 9.45 min.; Elemental analysis found:C,76.16;H,8.63;O,15.22; IR (KBr, cm^{-1}) ν_{max} 721.40 cm^{-1} γr(C-H alkanes), 1377.22 cm^{-1} γr(C-H), 1457.27 cm^{-1} δ(C-H alkanes), 1558.54 cm^{-1} 1717.67, 1739.85 cm^{-1} ν(C=O stretchig), 2727.44, 2852.81, 2925.15, 2954.08 cm^{-1} ν(C-H alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.9; EIMS *m/e* (rel. int. %): 316 (8) $[M+1]^+$, 274.36(21), 230.35(17), 120.15(100), 188.35(75), 112.21(46), 103.13(6), 91.13(62), 72.61(62); HRMS (ESI) *m/e*: calcd. for $C_{20}H_{27}O_3$ 315.4424; found 315.4468 $[M+H]^+$.

The molecular ion peak appeared at m/e= 316, which underwent fragmentation to result the fragment peaks at m/e=230 ($C_{16}H_{22}O$) with the elimination of propyl hydrogen carbonate ($C_4H_8O_3$, m/e=104). The peak at m/e=230 further fragmented to yield m/e=118.13 ($C_5H_{10}O_3$) assigned to be as (E)-3, 5-dimethylhex-2-ene and the base peak (C8H8O, m/e 120), assigned to be acetophenone (Figure 4.53).

The IR band at 721.40 cm^{-1} is due to long chain ν(methyl group). The ν(C-H long alkane chain) was observed at 2727.44, 2852.81, 2925.15 and 2954.08 cm^{-1}. The strong band at 1457.27cm^{-1} is also due to δ(C-H of alkanes). The bands at 1558.54, 1717.67 and 1739.85 cm^{-1} are assigned due to ν(C=O vibrations) (Figure 4.55).

The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 21 carbon atoms in the molecule (Figure 4.57 & 4.58). The C^{13} NMR spectra displayed a signal at δ 179.20 ppm indicating that the compound has a carbonyl functional group. The ^{13}C signals at δ 130.88 and 128.80 indicate the olefinic carbons, whilst the signal at δ 130.87 ppm doesn't have any HSQC signal thereby indicating the presence of a quaternary carbon atom. The ^{13}C -NMR signal at δ 140.69 ppm gives no HSQC signal to conclude the carbon as quaternary. The presence of acetyl group attached to the aryl ring system is established by the typical proton shift of the CH_3 group at δ 2.3 ppm and the corresponding HSQC correlation of the carbon (at 40.6 ppm) and proton (at δ 2,3 ppm). The carbonyl proton of the acetyl group appeared at δ 179.8 ppm, and no HSQC signal confirmed this assignment. The ^{13}C signal at δ 179.8 ppm also established the presence of another carbonyl group and its attachment with the aryl ring system has been assigned by the strong HMBC correlation with the aryl proton at δ 7.76 ppm (Figure 4.60).

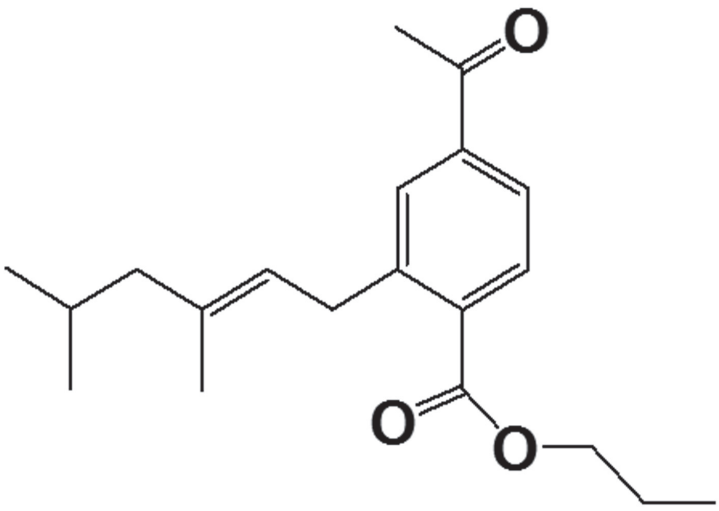


Figure 4.52. Butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate

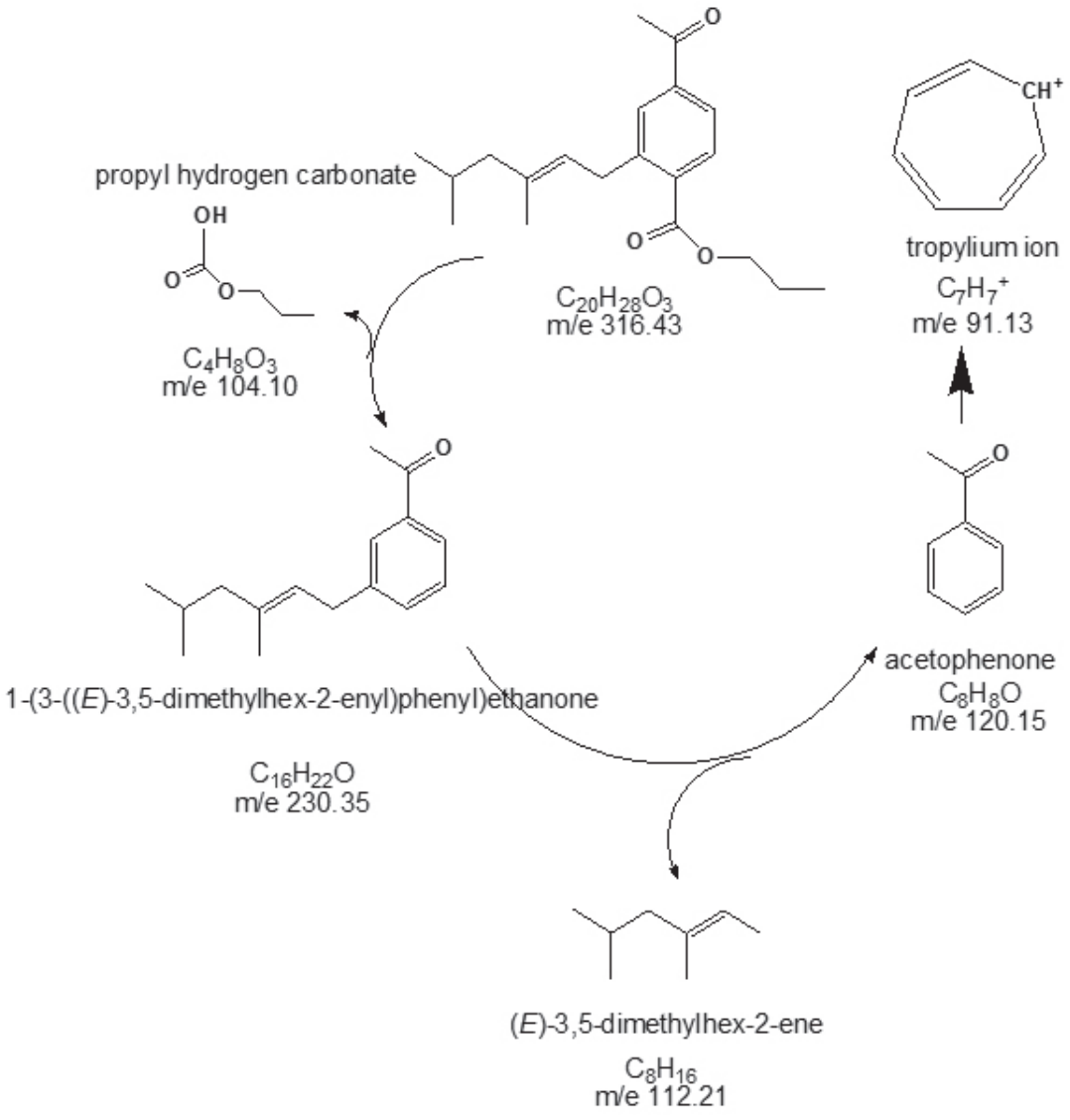


Figure 4.53. GC-MS Splitting patteren of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate

Table 4.9. NMR spectroscopic data of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^1H NMR(int., mult., J in Hz)[b]	^1H-^1H COSY	HMBC(^1H-^{13}C)
1	130.87				
1a	179.8				
1b	65.57	1b-H	4.18,2.4(dt)	1c-H	C-1c,1d
1c	42.3	1c-H	1.7(m)	1b-H	C-1d
1d	23.7	1d-H	0.91(m)	1c-H	
2	140.69				
3	126.73	3-H	7.46(dd)		C-4a
4	130.0				
4a	179.81				
4b	40.58	4b-H	2.3(s)		C-4a
5	128.8	5-H	7.74(m)	6-H	C-4a,4b
6	129.7	6-H	7.76(m)		C-1a,1b
1’	68.16	1’-H	4.3(d)	2’-H	C-2,2’,3”
2’	130.20	2’-H	5.3(t)		
3’	121.73				
3”	35.8	3”-H	2.1(s)		C-5”
4’	57.49	4’-H	2.6 1.3(dd)	5’-H	C-6’,5”,2’
5’	37.46	5’-H	1.2(m)	4’-H	
5”	32.04	5”-H	1.5(d)	5’-H	
6’	33.78	6’-H	1.31(d)	5’-H	

[a] NMR spectra recorded using spectrometers. Bruker AVANCE III 500 MHz (AV 500)

[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^1H-^1H COSY, HMQC, HMBC and NOESY experiments.

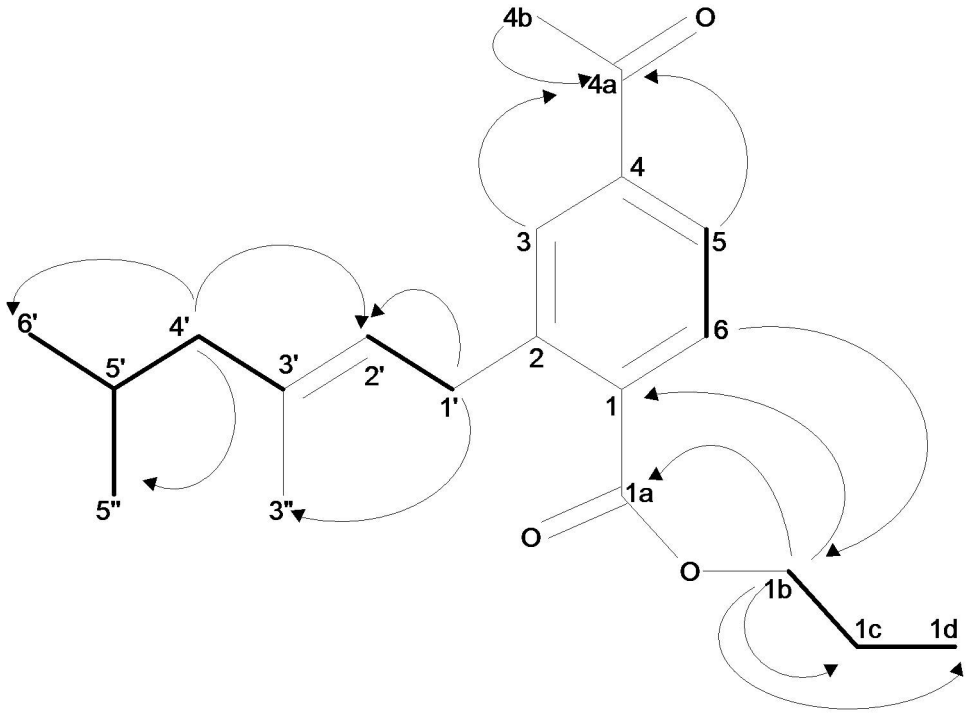


Figure 4.54. 2D NMR correlations as observed in butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

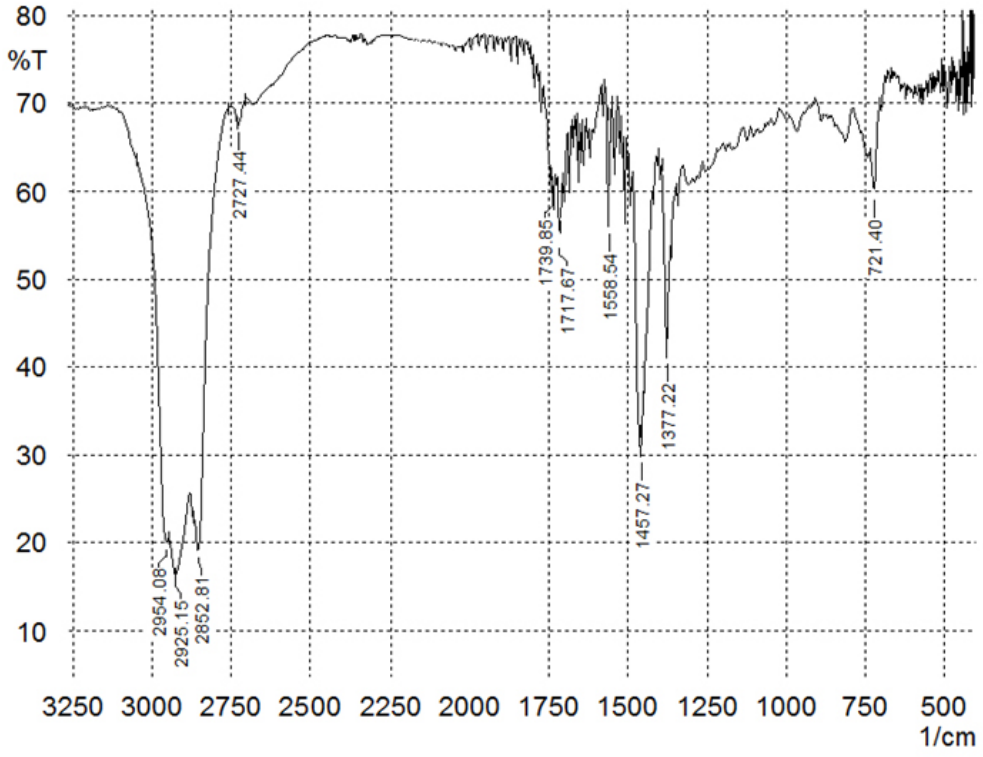


Figure 4.55. IR spectral details of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate

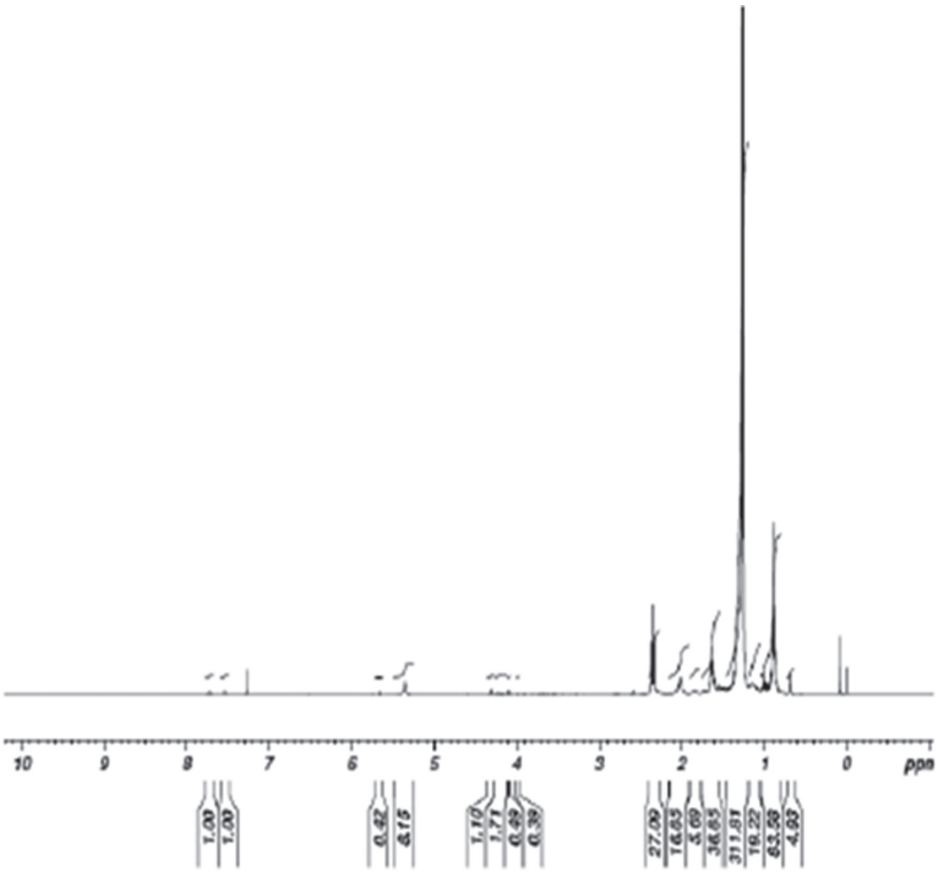


Figure 4.56. Proton NMR spectrum of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate

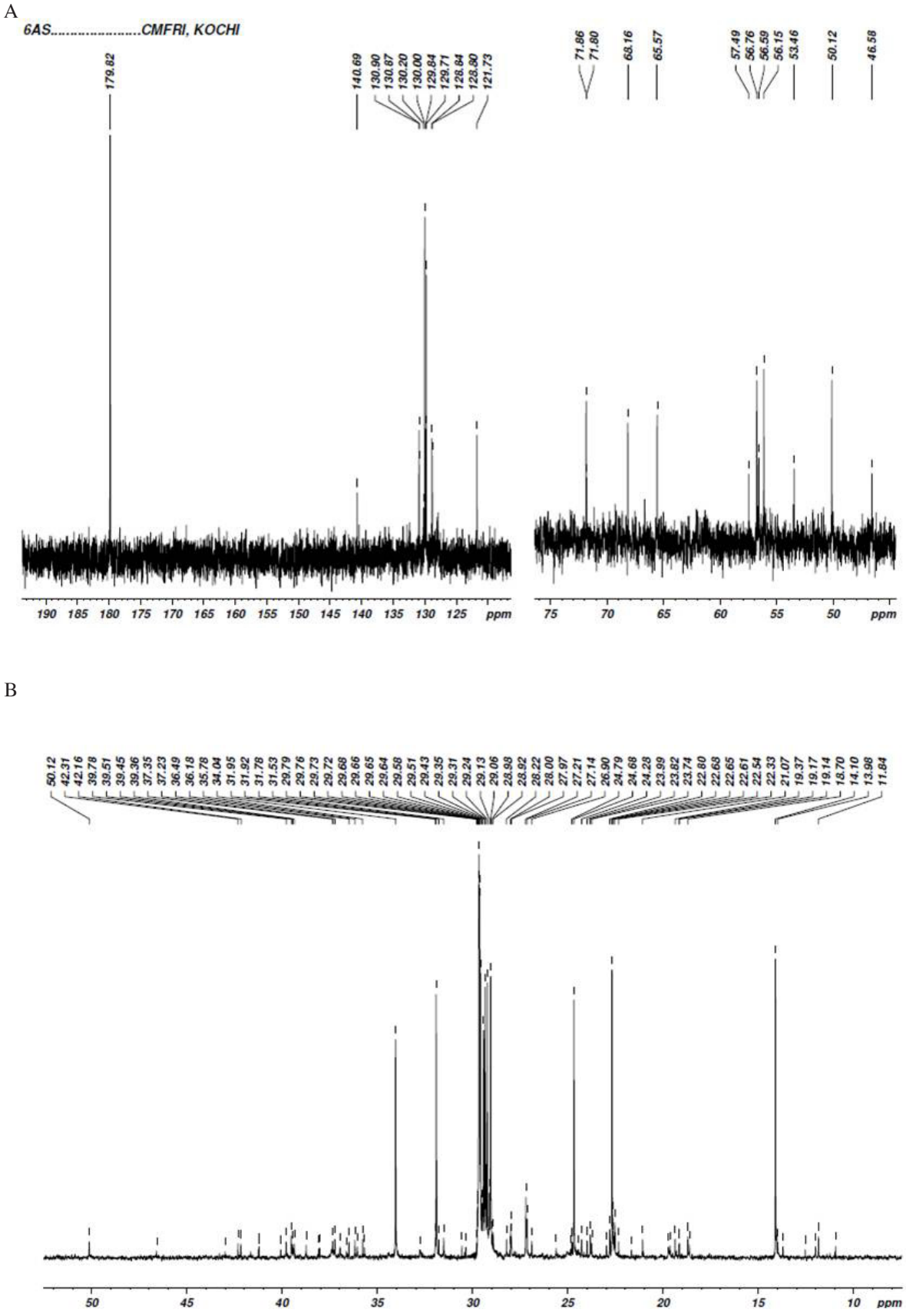
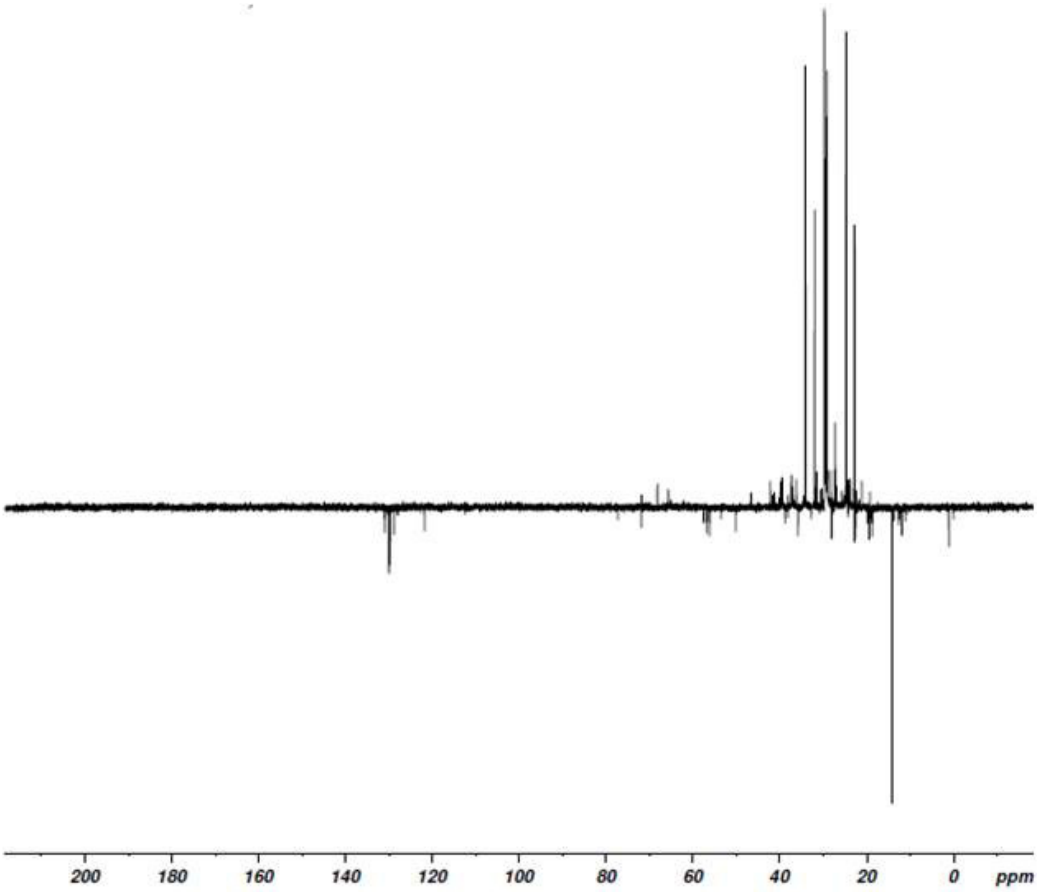


Figure 4.57. ^{13}C NMR spectra of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate (A - full view, B - expanded view)

A



B

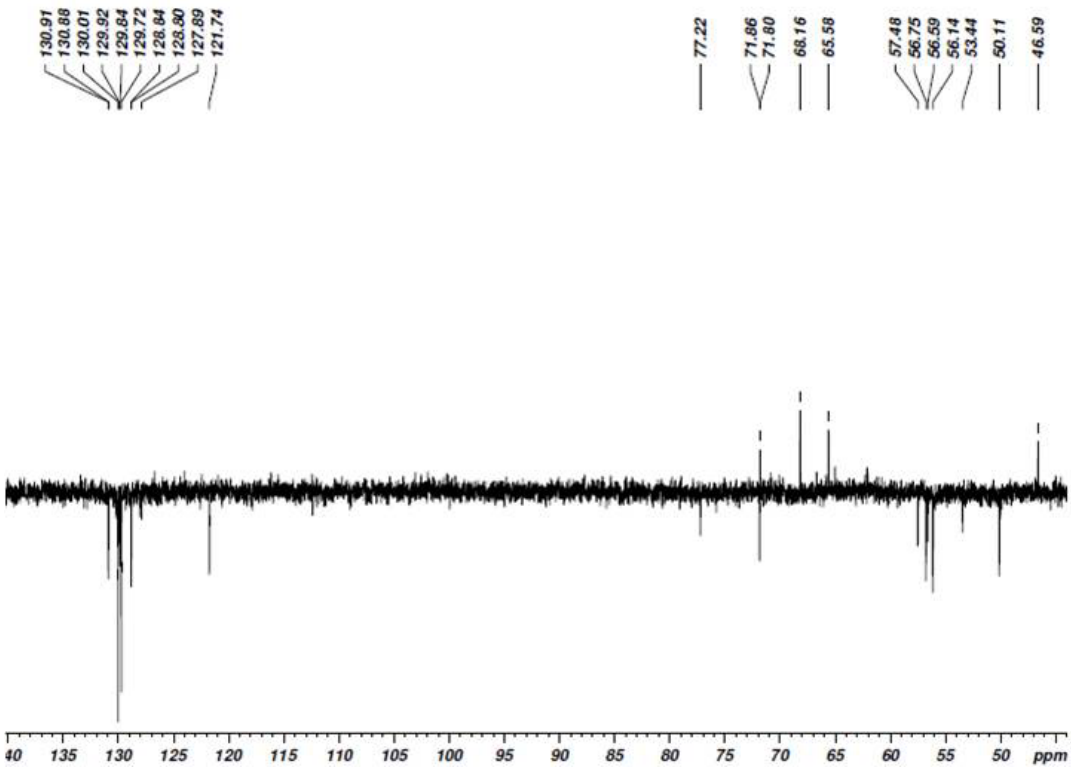
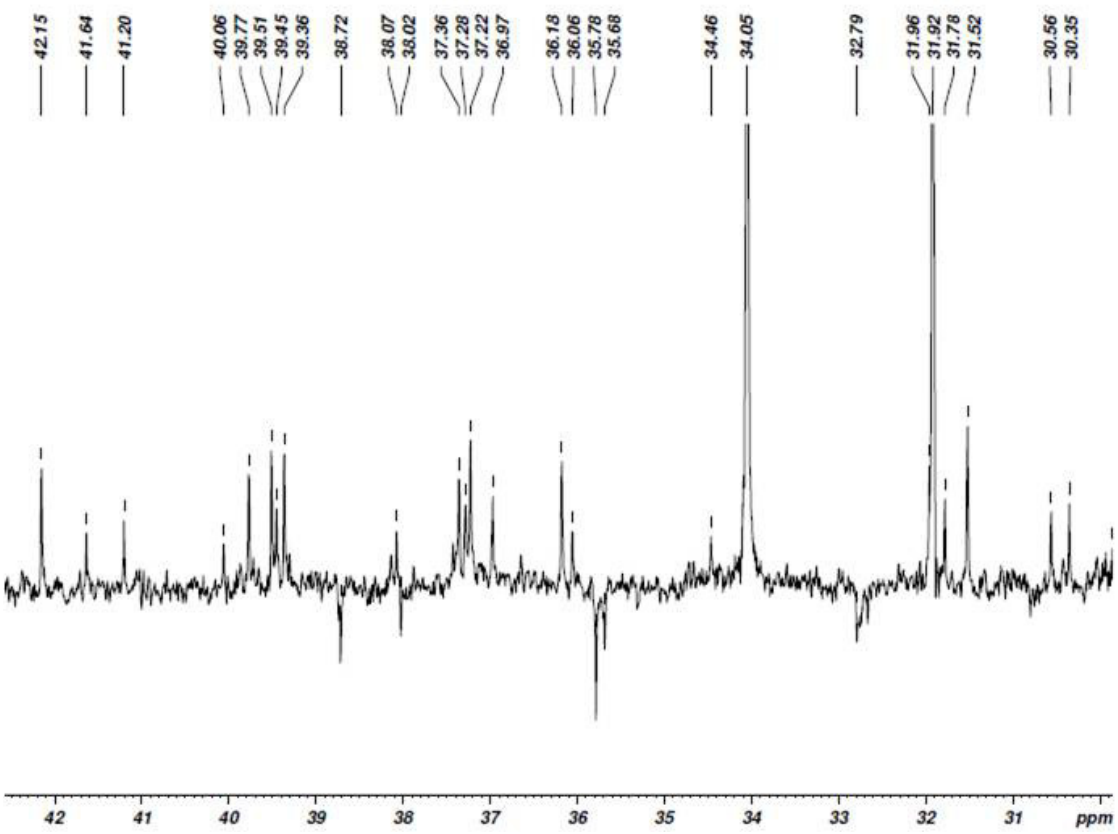


Figure 4.58. DEPT spectra of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate (A - full view, B, C & D - expanded view)

C



D

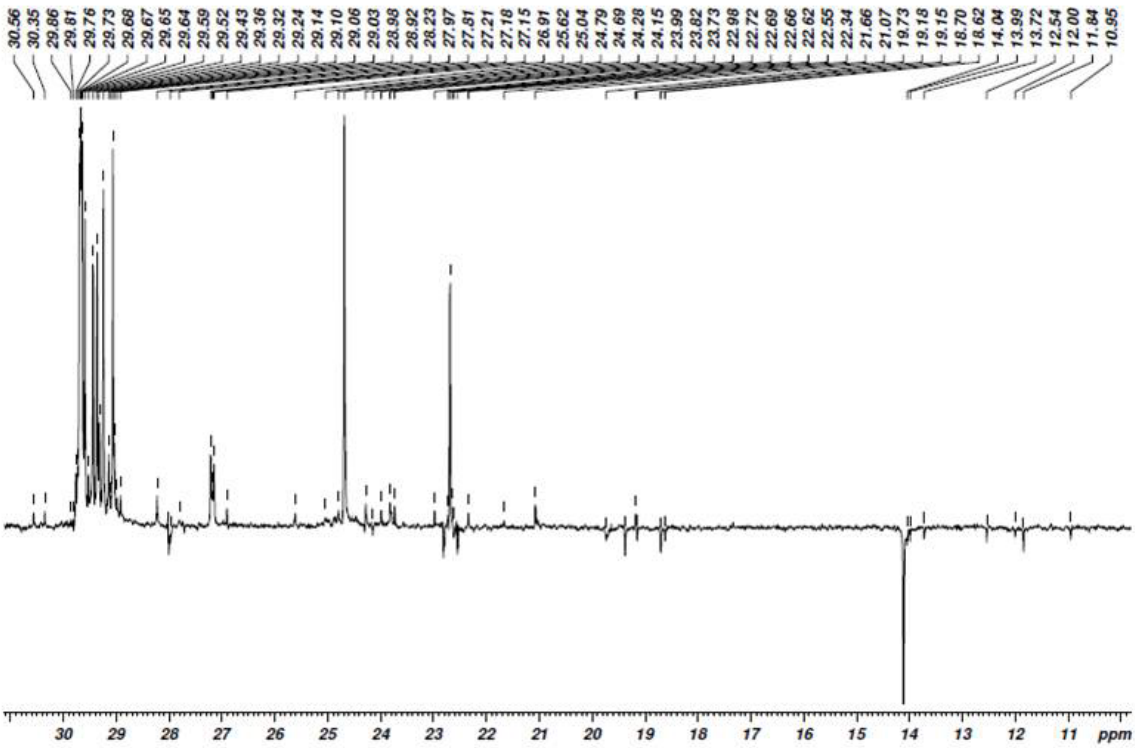
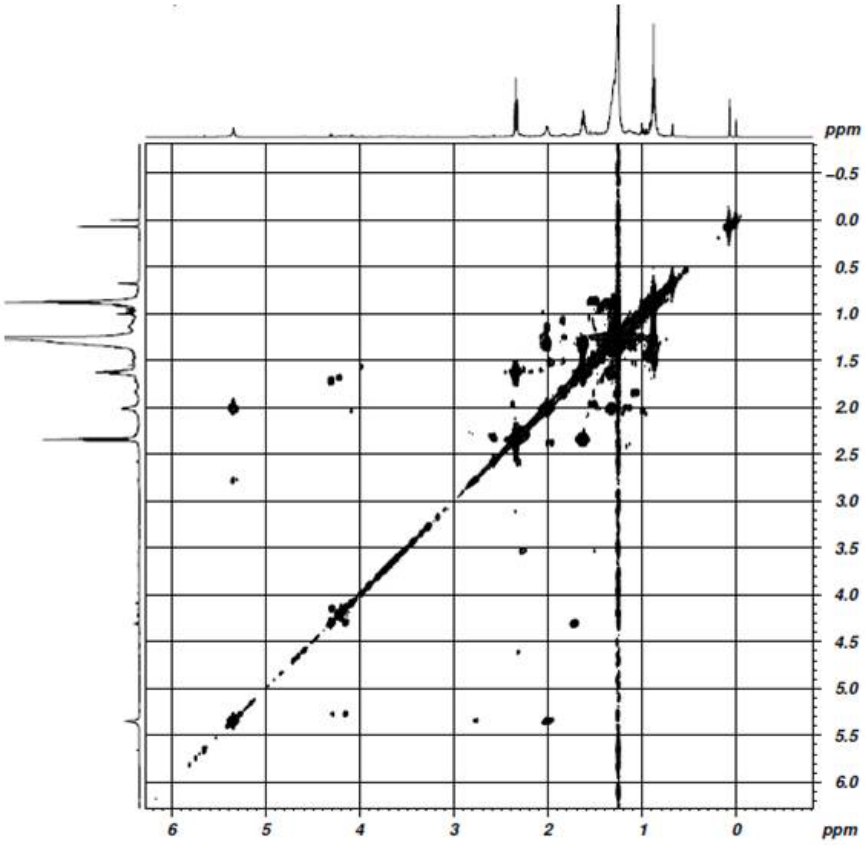


Figure 4.58. DEPT spectra of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate (A - full view, B, C & D - expanded view)

A



B

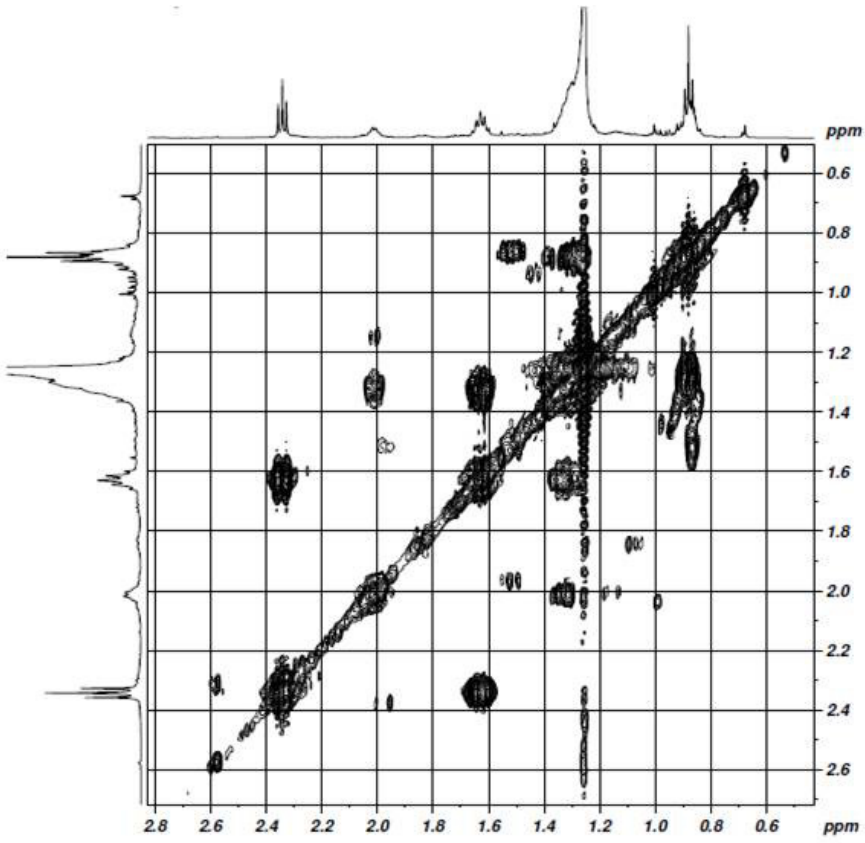
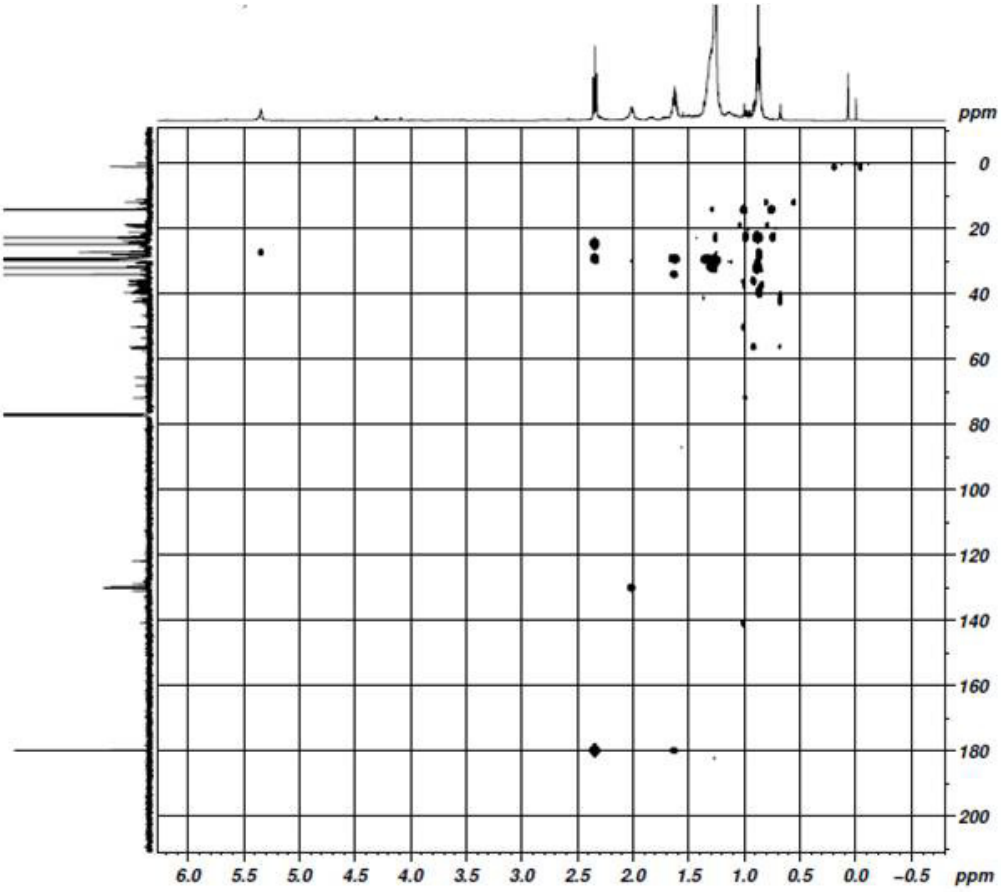


Figure 4.59. ^{1}H-^{1}H-COSY spectra of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate (A - full view, B - expanded view)

A



B

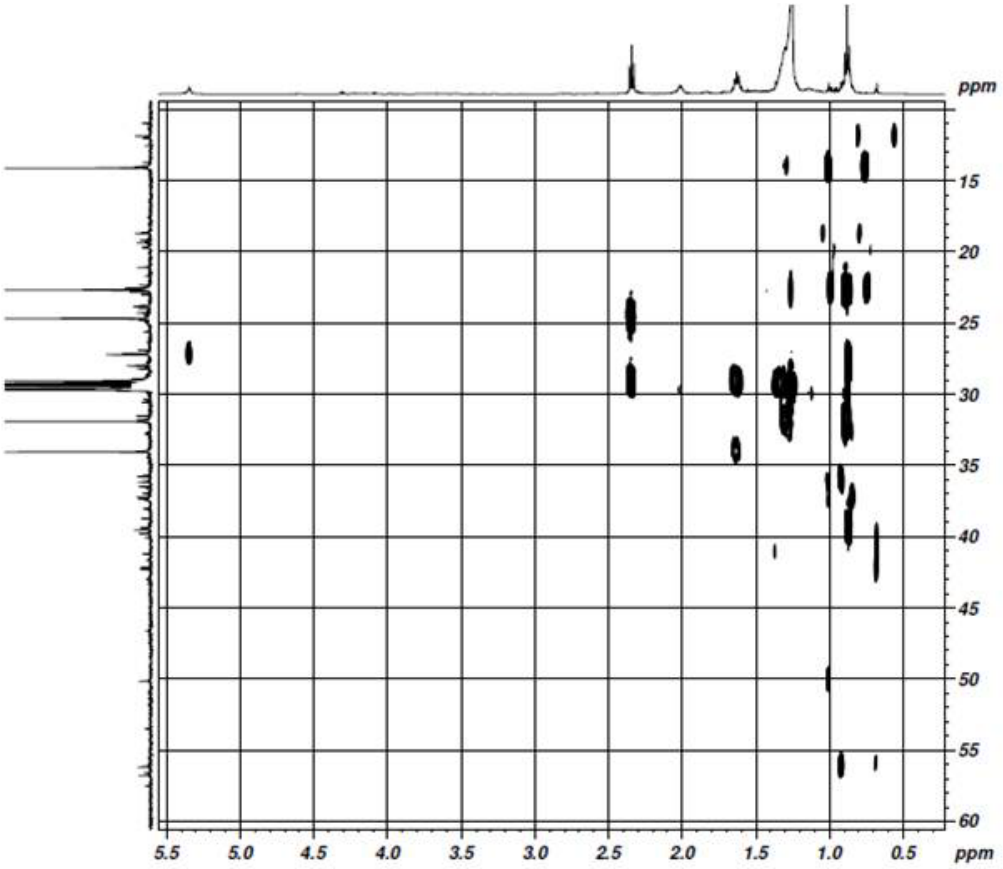


Figure 4.60. HMBC spectra of butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate (A - full view, B - expanded view)

The downfield shift of the methylene proton at δ 4.18 ppm (Figure 4.56) led us to conclude the presence of a adjacent electronegative group (oxygen) as a part of a strong electron withdrawing –C=O group. The ^{13}C-NMR spectrum also confirmed the presence of the –C=O group at δ 179.8 ppm. Strong ^{1}H-^{1}H-COSY correlation between the proton signals (methylene) at δ 4.18, 1.70, and 0.91 ppm, which in combination of mass spectroscopic analyses established the butyl acetate moiety attached with the acetophenone ring system. The methylene signal at δ 4.3 ppm (d) showed ^{1}H-^{1}H-COSY correlation with the olefinic proton at δ 5.3 ppm. The HMBC correlation of the proton at δ 4.3 ppm exhibited strong correlation with the ^{13}C signal at δ 126.7 ppm (aromatic) and the querternary carbon at δ 130.87 ppm, to indicate that this proton at δ 4.3 ppm is attached with the aryl ring system. The olefinic carbon at δ 121.7 ppm did not show any HSQC correlation thereby indicating that this carbon is of quaternary type. The dimethylheptene structure has been confirmed by the ^{13}C NMR spectrum in combination with HSQC, HMBC and DEPT experiments. The (*E*)-configuration of the olefinic bond has been confirmed by the coupling constant calculation.

Several benzoate derivatives are reported to be isolated from the seeds of *Cucurbita pepo* (Tanaka et al. 2013), roots of *Zeyhera digitalis* (Ferreira aet al. 1995) etc. There are another report which showed that the the chromatographic separation of the hexane soluble fraction of the methanol extract of the aerial parts of *Solidago virga-aurea* var. gigantea Mie. (Compositae) led to the isolation of a new benzylbenzoate together with four known benzylbenzoates and their structures were determined as 2-methoxybenzyl-2-hydroxybenzoate, benzyl-2-hydroxy-6-methoxy- benzoate, 2-methoxybenzyl-2,6-dimethoxybenzoate, 2-methoxybenzyl-2-methoxy-6- hydroxybenzoate, and benzyl-2,6-dimethoxybenzoate (Choi et al. 2005).

4.5. Isolation and Characterization of Antioxidant Secondary Metabolites from *Laurencia papillosa* (C. Agardh) Greville

4.5.1. Bioassay Guided Chromatographic Purification of the Methanol Extract of *L. papillosa*

The MeOH extract (2.9g) of *L. papillosa* collected from Gulf of Mannar (Figure 4.61) was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*- hexane, *n*- hexane: EtOAc and finally EtOAc:MeOH. Collected fractions were concentrated under vacuum and TLC evaluated and fractions with same TLC profile were pooled together to get six fractions (LP_{1-6}). The sub fraction LP_3 which exhibited good DPPH (56.52%), ABTS (23.26%) radical scavenging potential and Fe^{2+} ion chelating ability (16.77%) was purified further over silica column (60-120 mesh) with a stepwise gradient of solvents from *n*- hexane, *n*- hexane: EtOAc and finally EtOAc:MeOH, to get six more sub fractions ($LP_{3,1-4}$) (Figure 4.62). The active sub fraction

Figure 4.61. *Laurencia papillosa* collected from Mandapam of Gulf of Mannar

$LP_{3,4}$ thus obtained was repurified to furnish another six sub fractions of which $LP_{3,4,2}$ (12-tridecenyl 2-methylacrylate) was found to be pure and active (64.94% DPPH, 26.29% ABTS radical scavenging ability and 18.45% ion chelating potential).

Another sub fraction $LP_{3,4,4}$ thus obtained also exhibited high radical scavenging potential and thus chromatographically purified further to obtain five sub fractions of which 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal (57.02% and 20.22% for DPPH and ABTS radical scavenging ability respectively and 15.84% ion chelating potential) was found to be pure and endowed with antioxidant potential.

The structural characterization were carried out be detailed spectroscopic techniques and are discussed in following sections (4.5.2 and 4.5.3)

4.5.2. Structural characterization of 2-Tridecenyl 2-methylacrylate

2-Tridecenyl 2-methylacrylate: Amorphous yellow semisolid; UV (MeOH) λ_{max} (log ε): 247 nm (3.91); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 10:90, v/ v) R_f: 0.55; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) O, 7.20 R_t: 24.22 min.; Elemental analysis found:C,72.42;H,7.13;N,3.67;O,16.78 ($C_{31}H_{44}O_7$ requires C, 70.427; H, 8.388; O, 21.19); IR (KBr, cm^{-1}) ν_{max} 720.44 cm^{-1} γr(C-H alkanes), 1152.51 cm^{-1} ν (C-O), 1306.82 1376.26 cm^{-1} γr(C-H), 1462.09 cm^{-1} δ(C-H alkanes), 1600.97, 1663.66 1740.81 cm^{-1} ν(C=O), 2727.44 cm^{-1} ν(C-H alkanes), 2853.78 cm^{-1} ν (C-H alkanes), 2926.11 cm^{-1} ν(C-H alkanes), 2954.08 cm^{-1} ν(C-H alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.11; EIMS *m/e* (rel. int. %): 267 (15) $[M+1]^+$ 184.22(13), 182.16(62), 156.52 (44), 111.42(53), 98.46 (100), 85.28(48), 70.14(65), 56.32(72), 42.18(89). HRMS (ESI) *m/e*: calcd. for $C_{17}H_{30}O_2$ 266.4318; found 266.4354

The molecular ion peak appeared at *m/e* 267, ($C_{17}H_{30}O_2$), which has been fragmented to *m/e* 85 ($C_4H_5O_2$). The fragment peaks at *m/e* 184 ($C_{11}H_{20}O_2$), *m/e* 182 ($C_{13}H_{26}$), *m/e* 112 (C_8H_{16}) and *m/e* 111 (C_7H_{16}) established the structure. The other mass spectroscopic signals have been demonstrated to be present at *m/e* 156, 237, 124, 94, 71, 57, and 28 support the structure (Figure 4.64).

The absorbance at 720.44 cm^{-1} is due to long chain ν(methyl group). The ν (C-H long alkane chain) was observed at 2727.44, 2853.78, 2926.11 and 2954.08cm^{-1}. The strong band at 1462.09cm^{-1} is also due to δ(C-H alkanes). The bands at 1600.97, 1663.66 and 1740.81cm^{-1} are assigned due to ν(C=O vibrations). Bands at 720.44, 1306.82 and 1376.26cm^{-1} are assigned due to γr(C-H alkanes). A ν(C-O band) around 1152.51cm^{-1} was also observed.

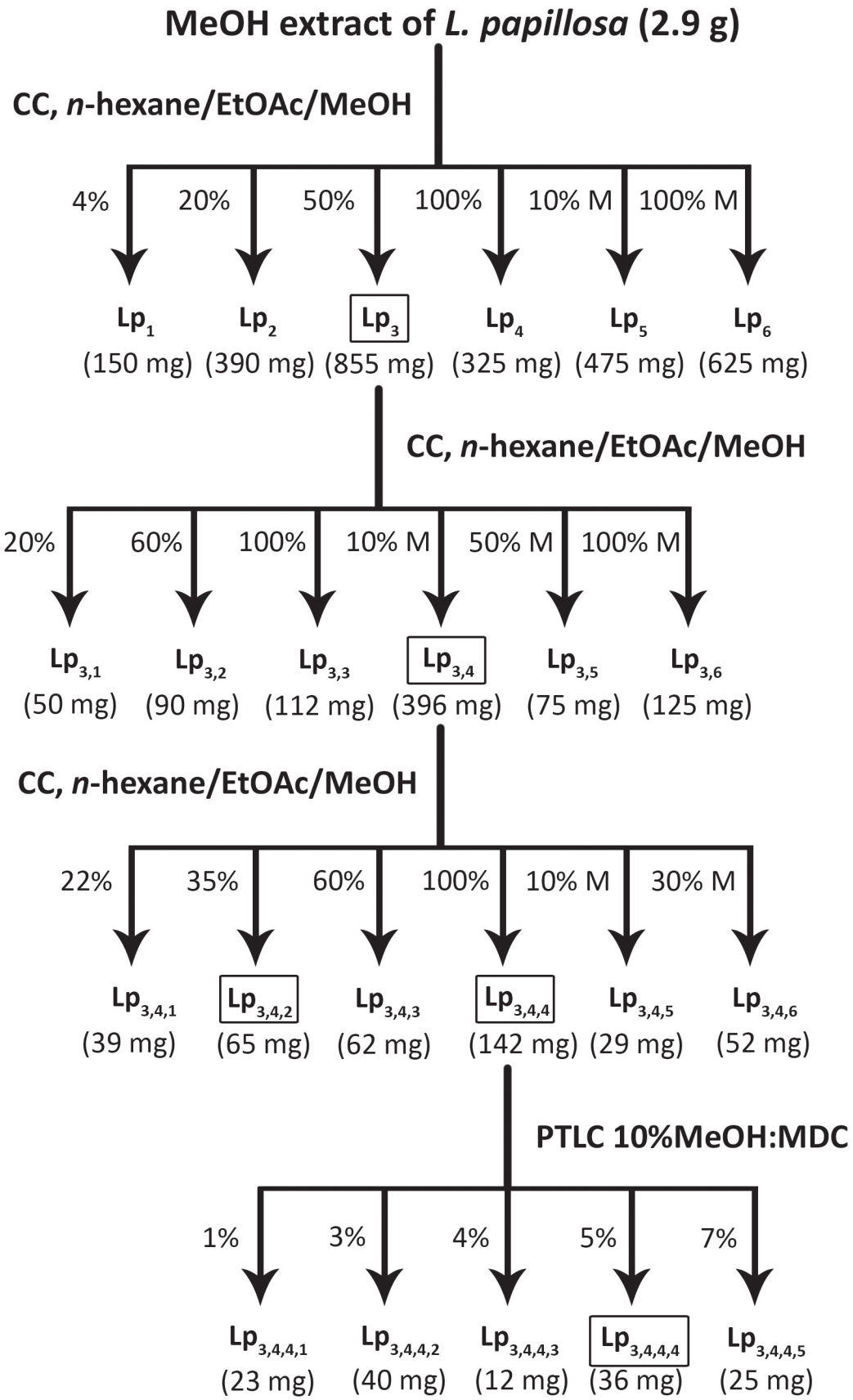


Figure 4.62. Schematic diagram representing the purification of MeOH extract of *L. Papillosa* (LP). CC: column chromatography, PTLC: preparative thin layer chromatography

Table 4. 10. Antioxidant and Fe^{2+} ion chelating potential (%) of the different fractions obtained by the chromatographic purification of the MeOH extract of *L. Papillosa* (LP)

Sl NO	Sample ID	Yield (mg)	Solvent System	ABTS Radical Scavenging Activity (%)	DPPH Radical Scavenging Activity (%)	Fe^{2+} ion Chelating Ability (%)
LP/MeOH						
1	LP_1	150mg	4% E	17.79±0.24	44.29±0.60	13.38±0.18
2	LP_2	390mg	20 E%	17.83±0.24	42.79±0.58	12.73±0.17
3	LP_3	855mg	50%E	23.26±0.31	56.52±0.76	16.77±0.22
4	LP_4	325mg	100% E	19.03±0.25	45.30±0.61	13.64±0.18
5	LP_5	475mg	10% M	19.02±0.25	44.90±0.60	12.93±0.17
6	LP_6	625mg	100% M	15.07±0.20	38.58±0.52	10.71±0.14
LP3						
1	$LP_{3,1}$	50mg	20% E	9.324±0.12	22.75±0.30	6.594±0.08
2	$LP_{3,2}$	90mg	60% E	7.676±0.10	21.64±0.29	6.150±0.08
3	$LP_{3,3}$	112mg	100% E	17.36±0.23	42.89±0.58	11.91±0.16
4	$LP_{3,4}$	396mg	10% M	23.11±0.31	58.93±0.79	17.53±0.23
5	$LP_{3,5}$	75mg	50% M	10.68±0.14	25.85±0.35	7.283±0.09
6	$LP_{3,6}$	125mg	100% M	12.89±0.17	32.37±0.43	8.992±0.12
LP3,4						
1	$LP_{3,4,1}$	39mg	22% E	23.29±0.32	47.32±0.77	12.76±0.22
2	$LP_{3,4,2}$	65mg	35% E	29.69±0.40	72.16±0.97	21.80±0.29
3	$LP_{3,4,3}$	62mg	60% E	20.32±0.27	50.61±0.68	14.71±0.19
4	$LP_{3,4,4}$	142mg	100% E	24.55±0.33	58.93±0.79	16.98±0.23
5	$LP_{3,4,5}$	29mg	10%M	19.69±0.40	52.16±0.97	20.80±0.29
6	$LP_{3,4,6}$	52mg	30% M	21.22±0.28	50.51±0.68	15.03±0.20
LP3,4,4						
1	$LP_{3,4,4,1}$	20mg	10% E	18.04±0.24	42.59±0.57	12.63±0.17
2	$LP_{3,4,4,2}$	40mg	26%E	8.378±0.11	21.44±0.29	6.460±0.08
3	$LP_{3,4,4,3}$	12mg	40% E	8.543±0.11	20.84±0.28	6.007±0.08
4	$LP_{3,4,4,4}$	36mg	80% E	20.22±0.27	57.02±0.77	15.84±0.21
5	$LP_{3,4,4,5}$	25mg	10 % M	19.63±0.26	48.50±0.65	14.06±0.19

Data are the mean values of the triplicate and expressed as mean ± standard deviation. The concentration of the solvent fractions used were 0.1 mg/mL for DPPH radical scavenging activity, and Fe^{2+} ion chelating activity; and 0.1 µg/mL for ABTS radical scavenging activity.

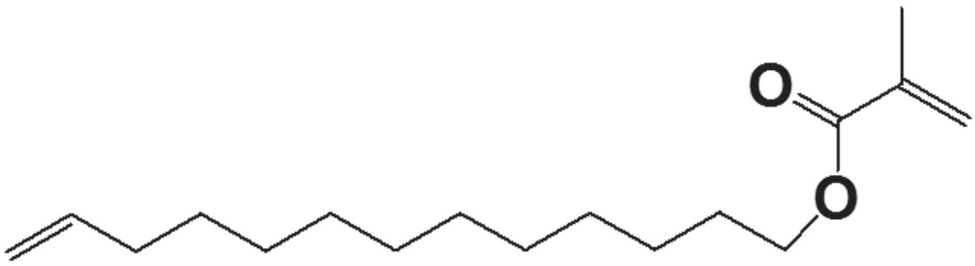


Figure 4.63. 2-Tridecenyl 2-methylacrylate

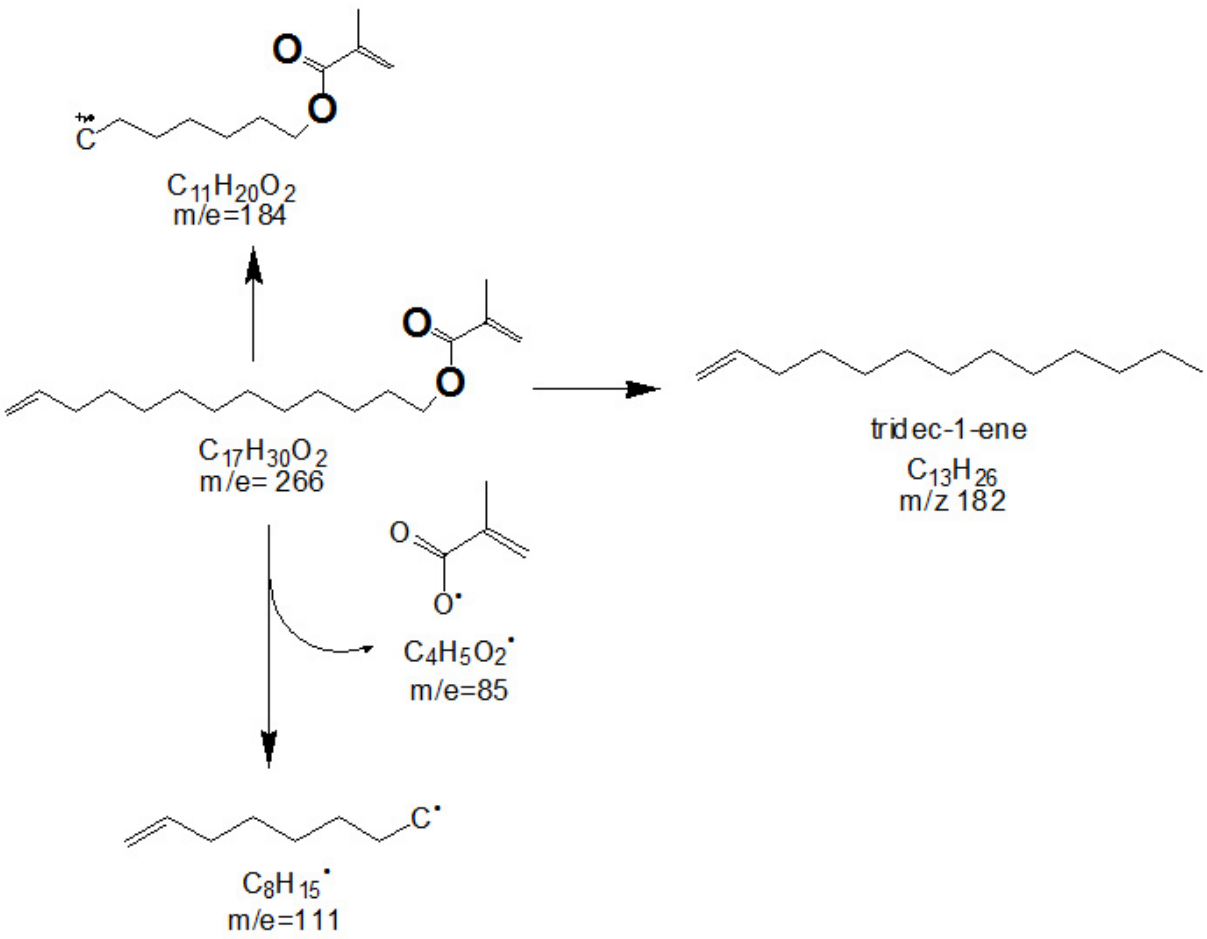


Figure 4.64. GC-MS Spliting pattern of 12-tridecenyl 2-methylacrylate.

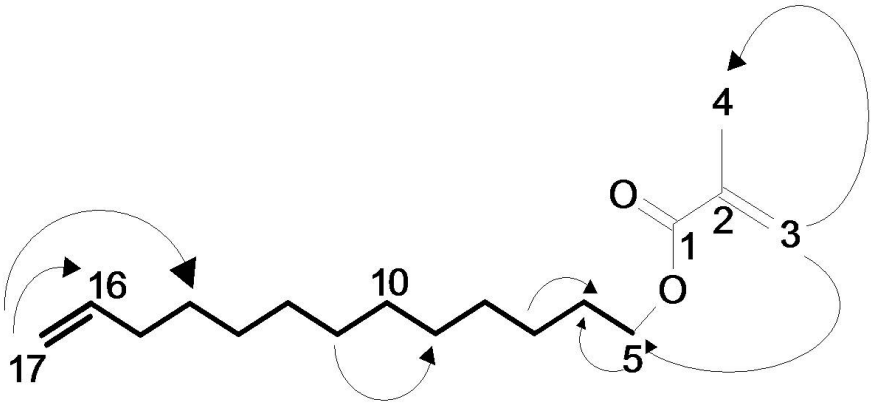


Figure 4.65. 2D NMR correlations as observed in 12-tridecenyl 2-methylacrylate. (A) The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

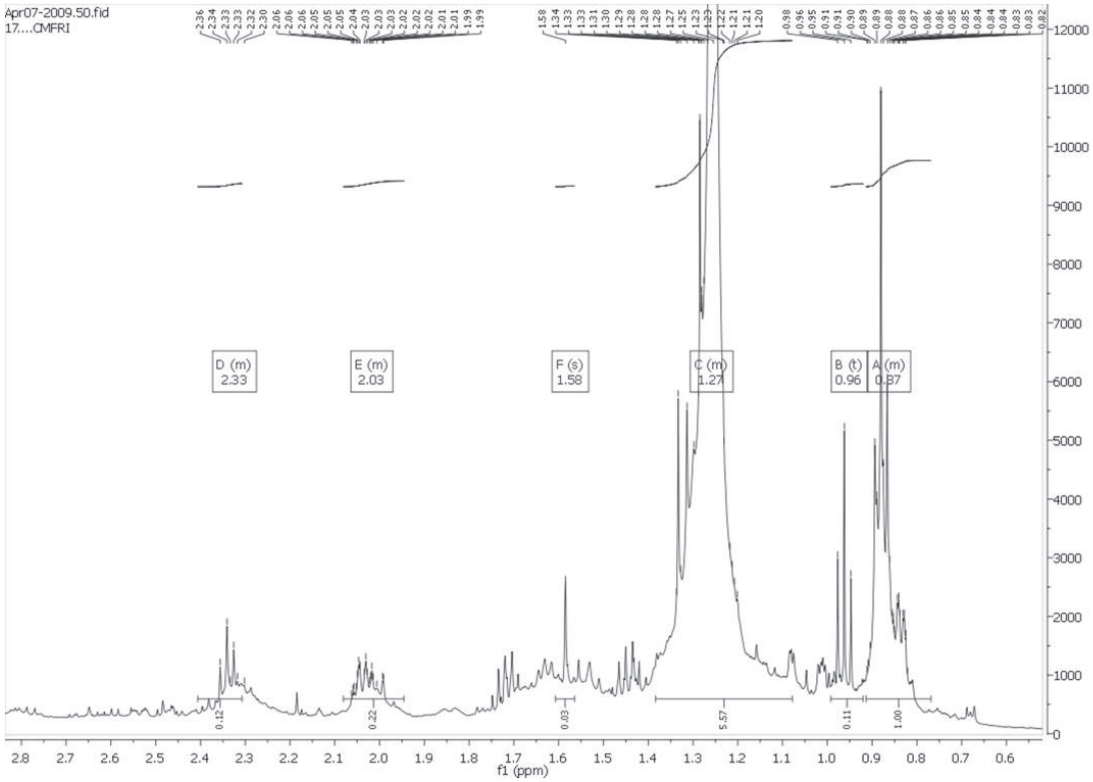
Table 4.11. NMR spectroscopic data of 12-tridecenyl 2-methylacrylate in $CDCl_3$

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR(int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	177.41	2-H			
2	139.28				
3	128.83	3-H	5.72(s)	6-H	C-6,5
4	33.89	4-H	2.39		
5	65.58	5-Ha/5-Hb	4.31/4.1	6b-H	C-3,5,6
6	30.57	6-H	1.7	7-H	
7	33.69	7-H	3.2	6-H	C-6
8	28.52	8-H	2.3	7-H	
9	33.81	9-H	1.5	8-H	
10	28.94	10-H	0.91	11-H, 9-H	
11	29.68	11-H	1.23		C-9,10
12	29.08	12-H	1.10	10-H	
13	30.02	13-H	1.9	10-H	
14	29.35	14-H	1.6		
15	29.24	15-Ha/15-Hb	2.1/2.03		
16	130.91		5.82	15-H	
17	114.05		4.98		C-14,16

[a] NMR spectra recorded using spectrometers. Bruker AVANCE III 500 MHz (AV 500)

[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

A



B

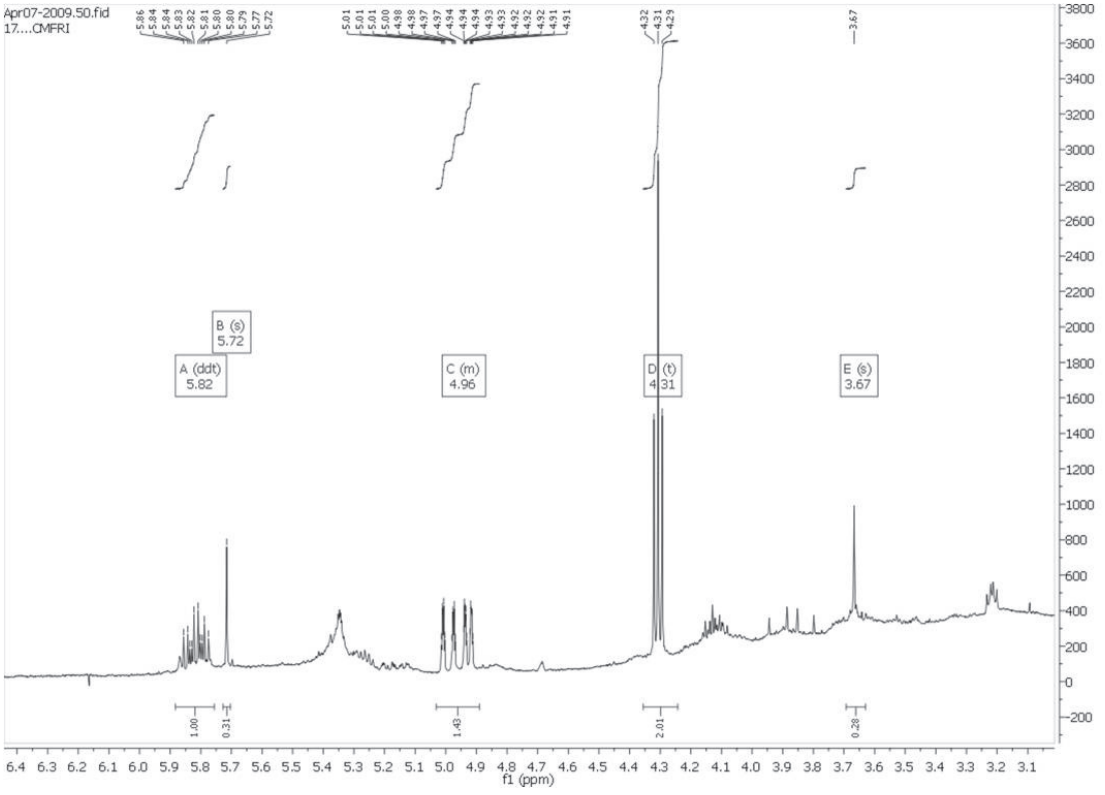


Figure 4.66. Proton NMR spectra of 12-tridecenyl 2-methylacrylate (A - full view, B - expanded view)

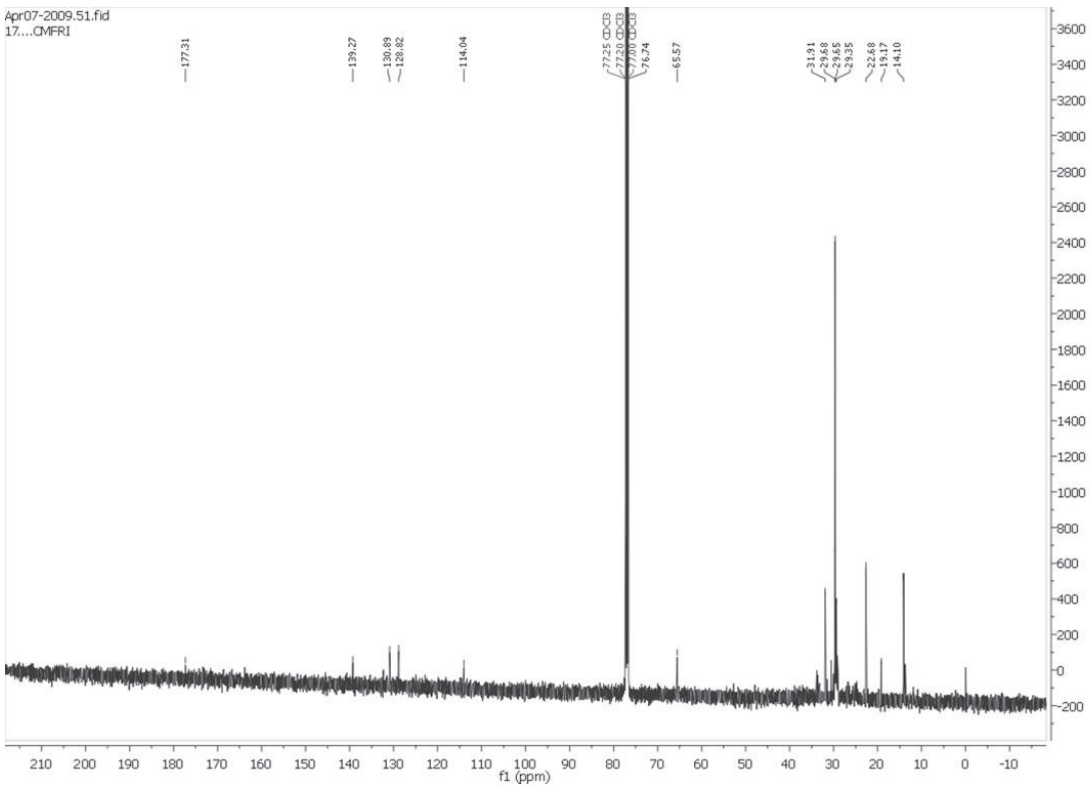


Figure 4.67. ^{13}C NMR spectrum of 12-tridecenyl 2-methylacrylate

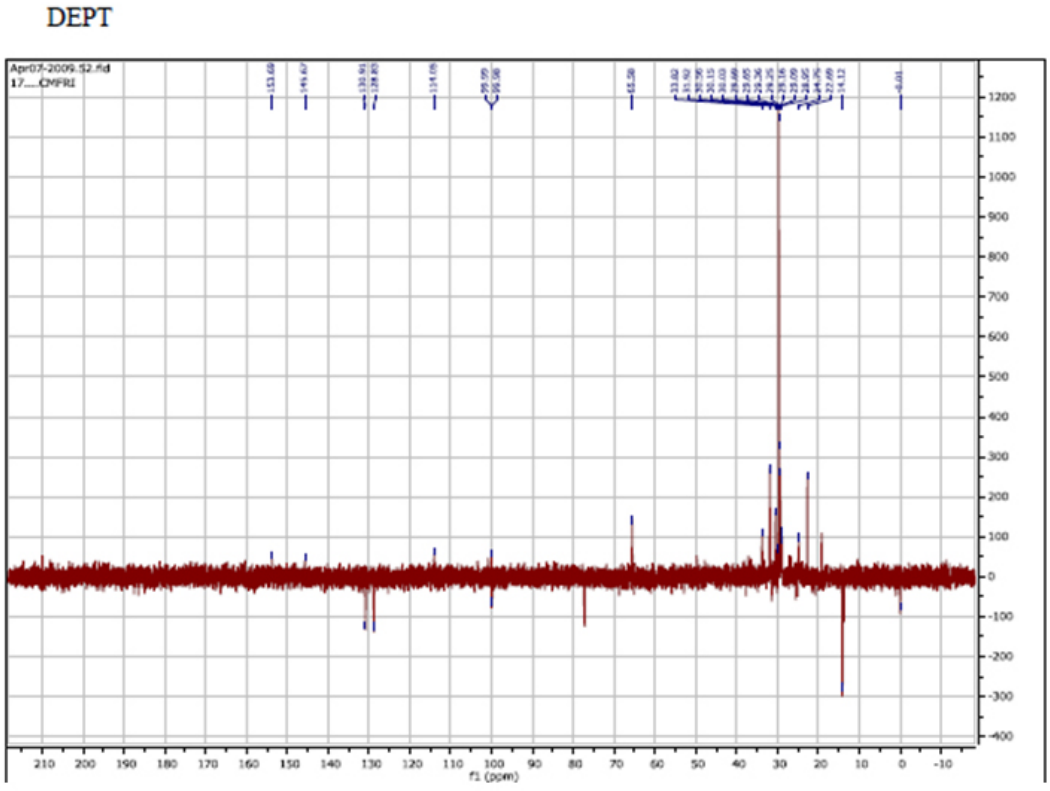


Figure 4.68. DEPT spectrum of 12-tridecenyl 2-methylacrylate

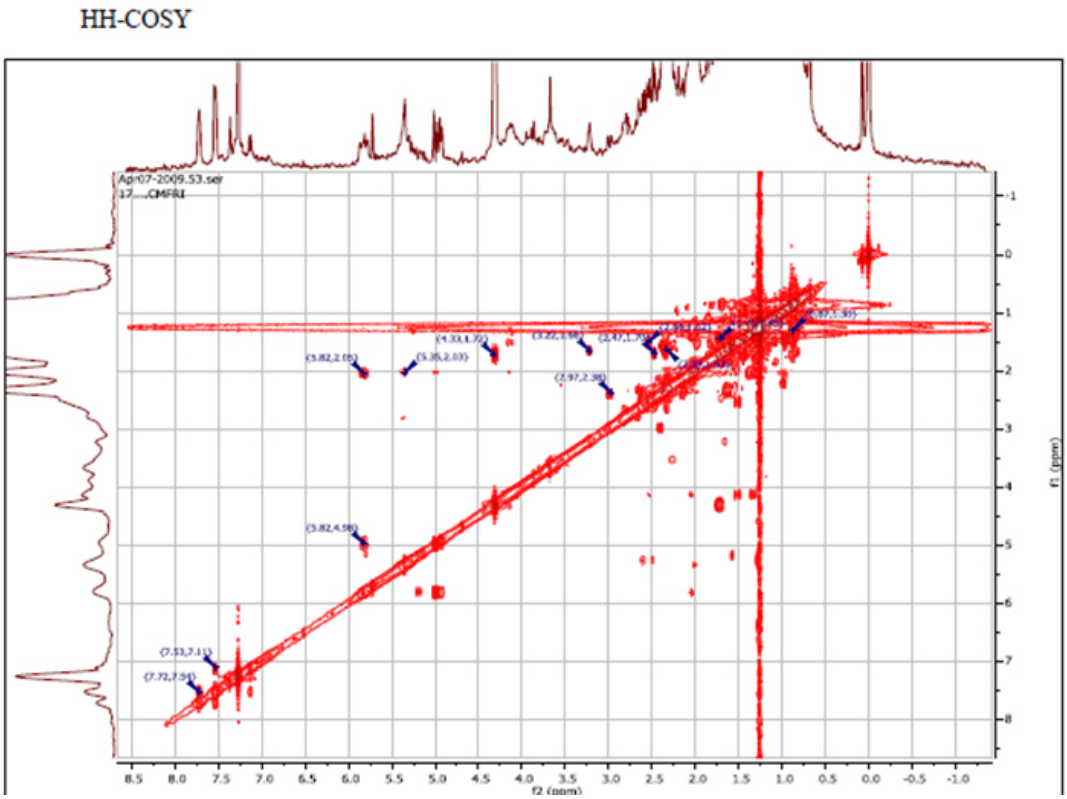


Figure 4.69. ^{1}H-^{1}H-COSY spectrum of 12-tridecenyl 2-methylacrylate

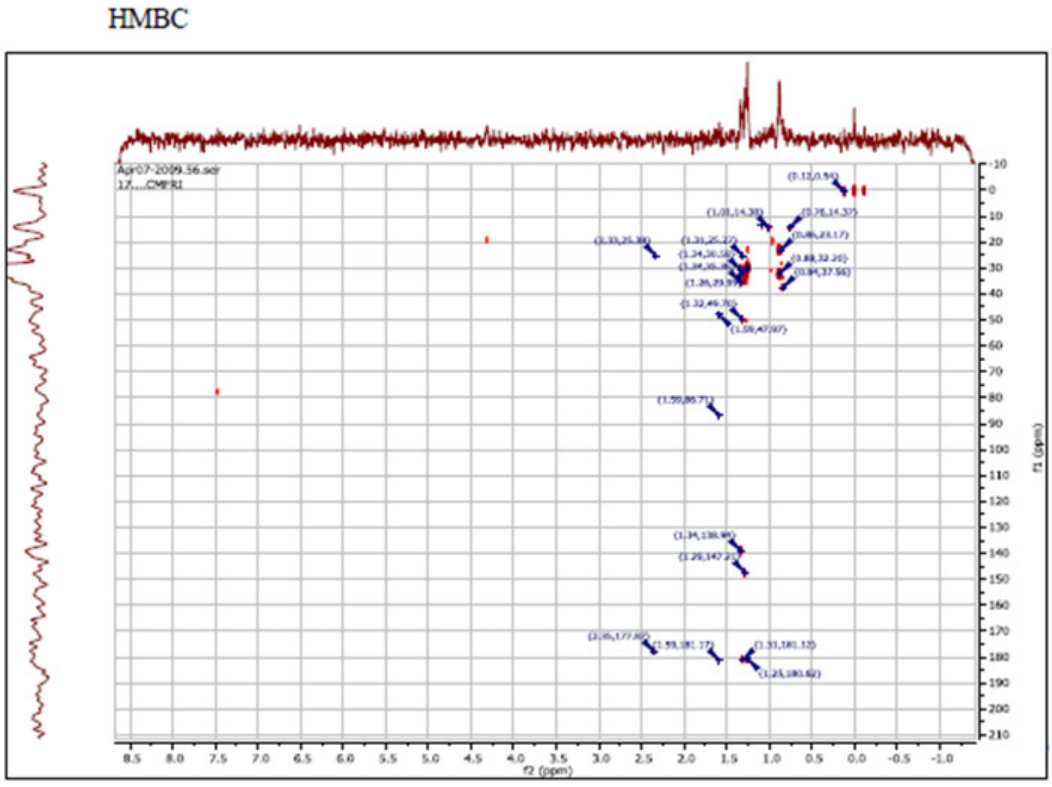


Figure 4.70 HMBC spectrum of 12-tridecenyl 2-methylacrylate

The tridecenyl system has been established by the linear ^{1}H-^{1}H-COSY correlation of the–CH_2 protons, to infer that these methylene groups are adjacent to each other (Figure 4.69). The methylene protons appeared at 2.1 ppm (Figure 4.66) are due to the deshielding effect of the olefinic –CH=CH- group. The methylene protons at δ 1.7 and 4.30 have been found to be further deshielded apparently due to the methyacrylate moiety adjacent to these –CH_2 groups. The olefinic group as in methyacrylate have been established by the proton chemical shift at δ 7.71 ppm; whereas those at δ 5.82, and 7.52 support the presence of the –CH=CH_2 group at the terminal position of the compound (Figure 4.67 & 4.68). The olefinic proton at δ 7.71 ppm established that the -C(=O)O-R moiety of the methacrylate is responsible for the downfield shift. The presence of the olefinic protons has been supported by the ^{13}C-chemical shift at δ 139.28 and 128.83 ppm, which have been assigned to be as –C(Me)=CH2– and -C=C- moieties. The olefinic carbon at 139.28 ppm has been assigned as quaternary because no HSQC correlation is apparent. The presence of carbonyl group has been confirmed by the presence of the quaternary carbon atom at δ 177.41 ppm with no HSQC correlation.

lactucin-8-O-methylacrylate was reported to be isolated from the chloroform extract of the dried fruits of Parmentiera edulis (Perez et al. 2000). Another compound 4,15-isoatriplicolide methylacrylate was also isolated from the chloroform-soluble subfraction of a methanol extract of the whole plant of *Helianthus tuberosus* (Pan et al. 2009).

4.5.3. Structural characterization of 3-(1-Butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

3-(1-Butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal: Amorphous yellow semisolid; UV (MeOH) λ_{max} (log e): 247 nm (3.91); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 10:90, v/ v) R_f: 0.55; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 mL injection volume/$CHCl_3$) R_t: 7.20 min.; Elemental analysis found:C,72.42;H,7.13;N,3.67;O,16.78 ($C_{31}H_{44}O_7$ requires C, 70.427; H, 8.388; O, 21.19); IR (KBr, cm^{-1}) ν_{max} 723.33 cm^{-1} γr(C-H alkanes), 1377.22 cm^{-1} γr(C-H), 1464.02 cm^{-1} δ(C-H alkanes), 1711.88 cm^{-1} ν(C=O), 2852.81, 2924.18, 2953.12 cm^{-1} (C-H ν of alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, see Table 4.12; EIMS *m/e* (rel. int. %): 580 (12) $[M+1]^+$,547(6), 385(18), 356(72), 244(100), 196(26), 144(56), 128 (8), 110(69), 98(52), 71(42); HRMS (ESI) *m/e*: calcd. for $C_{28}H_{42}O_5$ 578.7544; found 578.7572.

The presence of tetraphenone, nonadienone, 3-(tetrahydro-1-oxonaphthalen-7-yl)-3-oxopropanal, and 3-(dihydrooxotetraphenyl)-3-oxopropanal groups have been confirmed by detailed NMR and mass spectroscopic experiments. The compound 3-(1-butyl-7, 12-dihydro-2-methoxy-7-

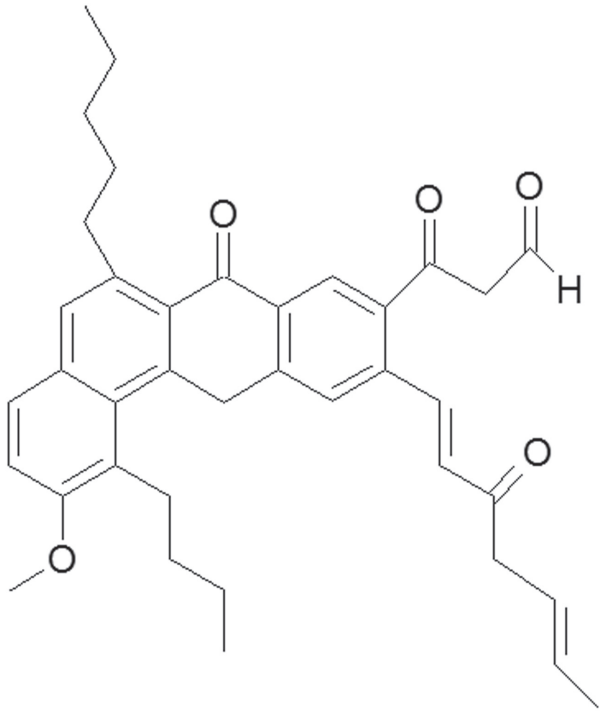


Figure 4.71. 3-(1-Butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

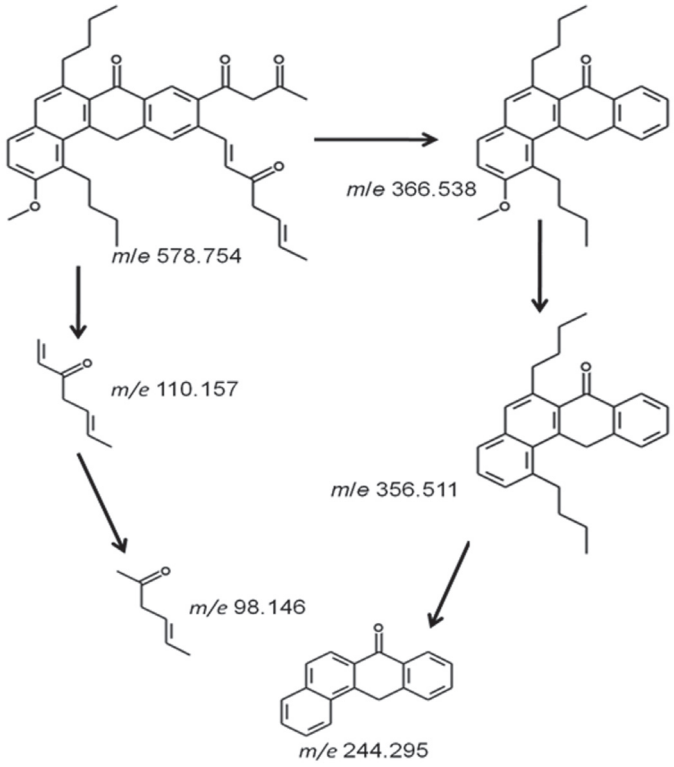


Figure 4.72. GC-MS Splitting patteren of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

Table 4.12 NMR spectroscopic data of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR(int., mult., *J* in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	179.63				
1a	147				
1b	132.32				
2	51.0	2-H	5.91(s)		C-1,1a,1b
2a	110				
2b	120				
3	128	3-H	8.08(m)		C-4,4a,4b,4c
4	135				
4a	197				
4b	59	4b-H	6.51(s)		C-4a,4c
4c	179	4c-H	10.1(s)		
5	127				
5a	142	5a-H	6.60(d)		C-4,5
5b	138	5b-H	6.91(d)		
5c	174				
5d	41	5d-Ha,5d-Hb	5d-Ha at3.7(d) 5d-Hbat 2.9(d)	5c-H	C-5c,5e,5f,5b
5e	129	5e-H	5.6(q)		
5f	114				
5g	21				
6	137				
6a	35	6a-H	2.28(t)	6b-H	C-6,6b,1,7
6b	31	6b-H	1.92(m)		
6c	29	6c-H	1.52(m)	6b-H	C-6a,6b,6d
6d	19	6d-H	0.91(t)		
7	128.46		7.52(m)		C-6,6a,1a
8	129.38				
9	130.15				
10	119.07				
10a	39	10a-H	1.36(t)		C-9,10,10b
10b	34	10b-H	1.26 (m)	10a-H	
10c	32	10c-H	1.22(m)	10b-H	
10d	-R'				
11	126.96				
12	128.4	12-H	7.10(m)	13-H	C-13,8,9,
13	126.3	13-H	7.16(m)	12-H	C-8,10,14
14	51.6	14-H	3.6(s)		

[a] NMR spectra recorded using spectrometers. Bruker AVANCE III 500 MHz (AV 500)

[b]Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments.

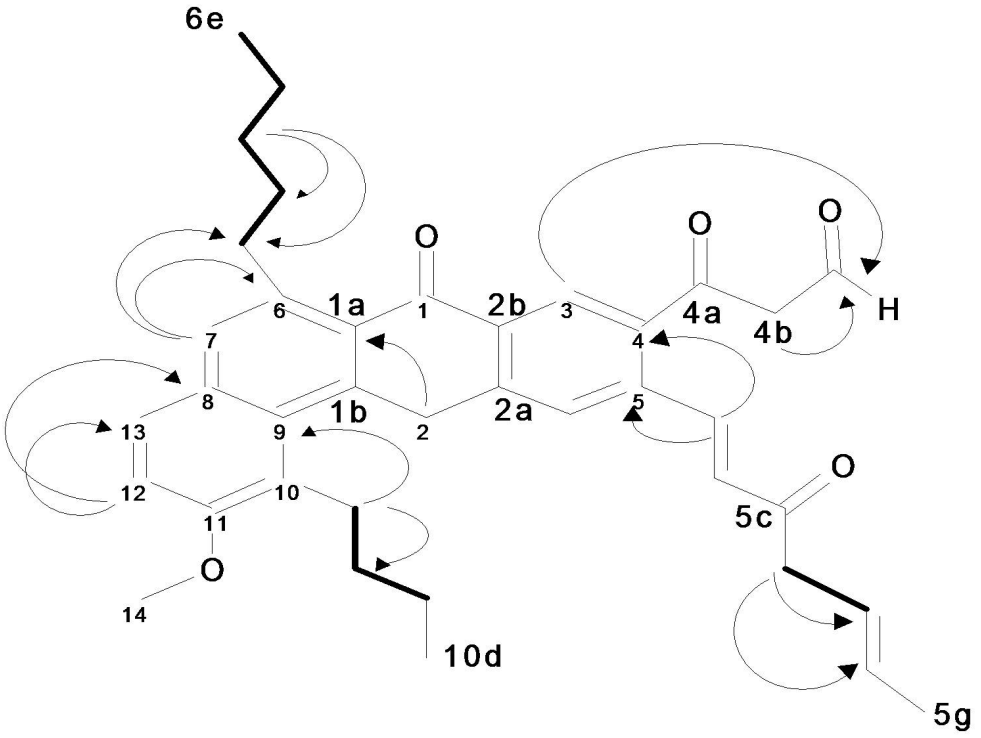


Figure 4.73. 2D NMR correlations as observed in 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal (A) The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

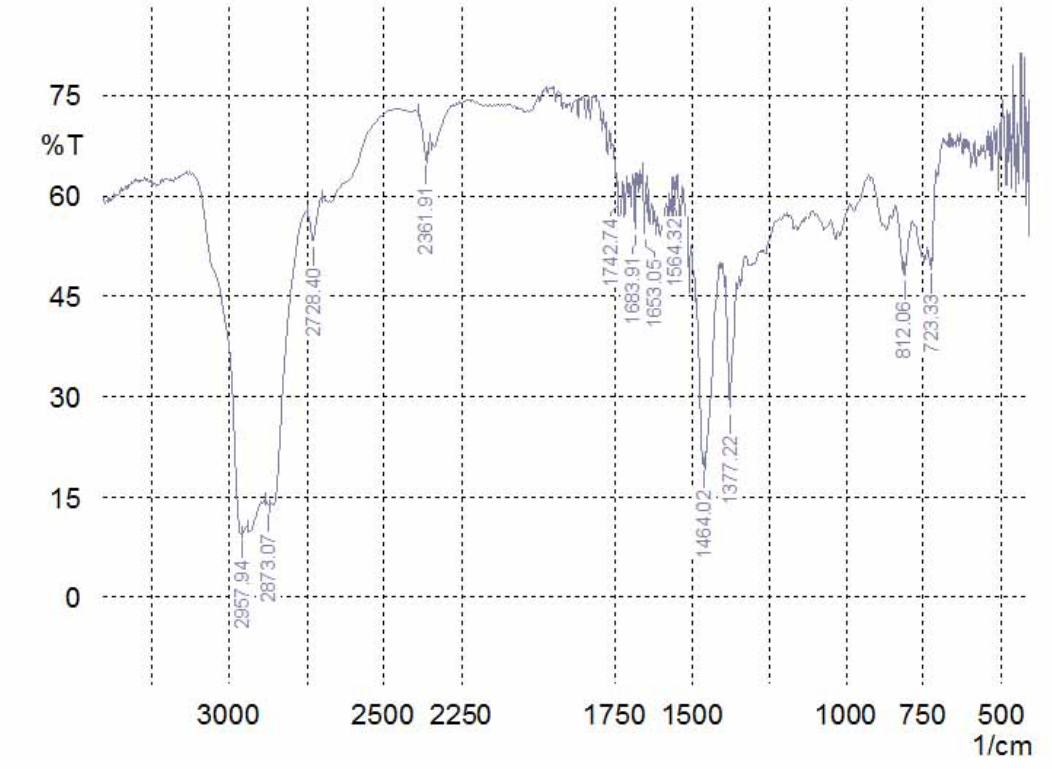
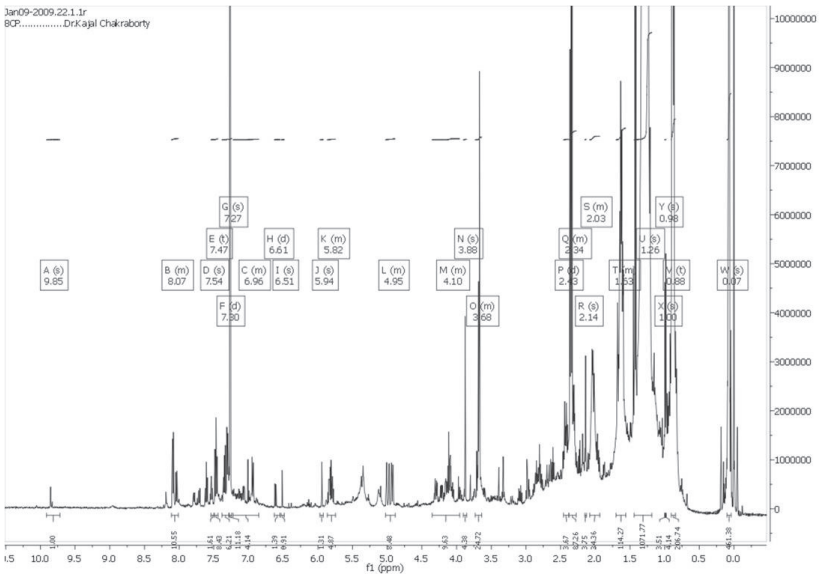


Figure 4.74. IR spectra of of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

A



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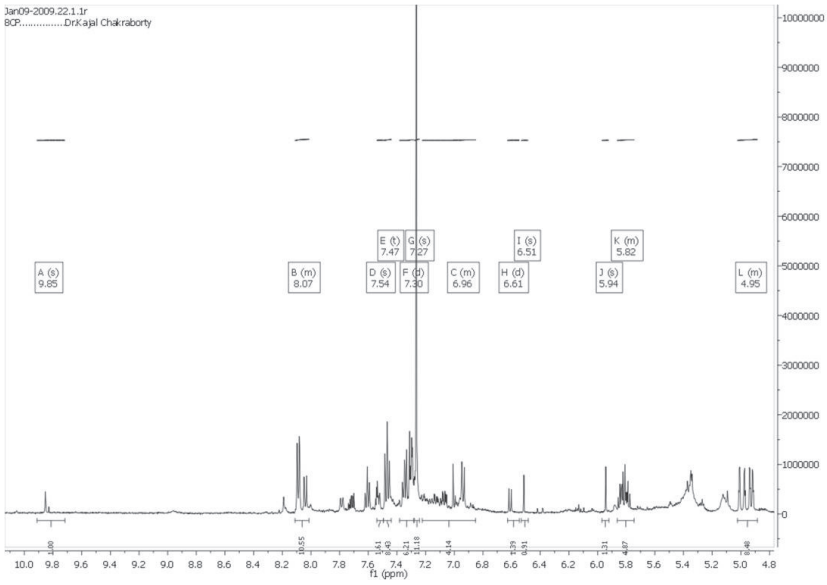

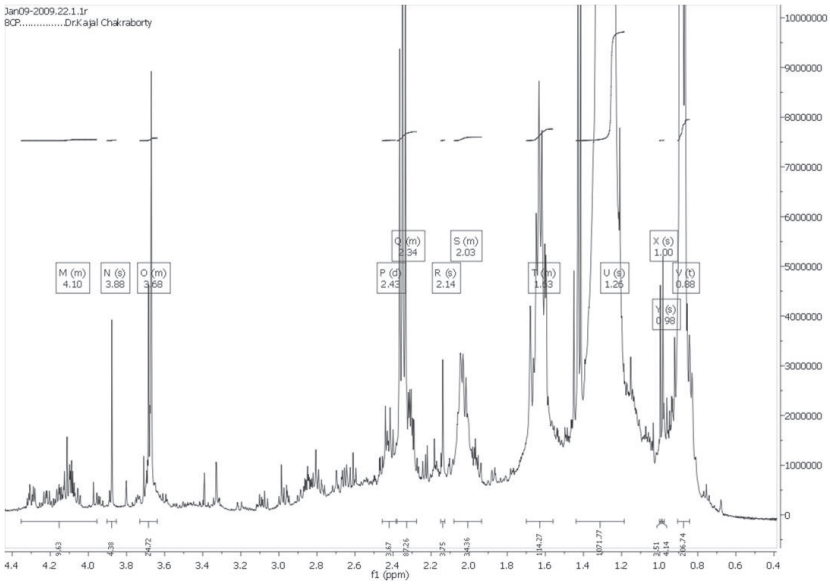

Figure 4.75. Proton NMR spectra of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal (A - full view, B - expanded view)

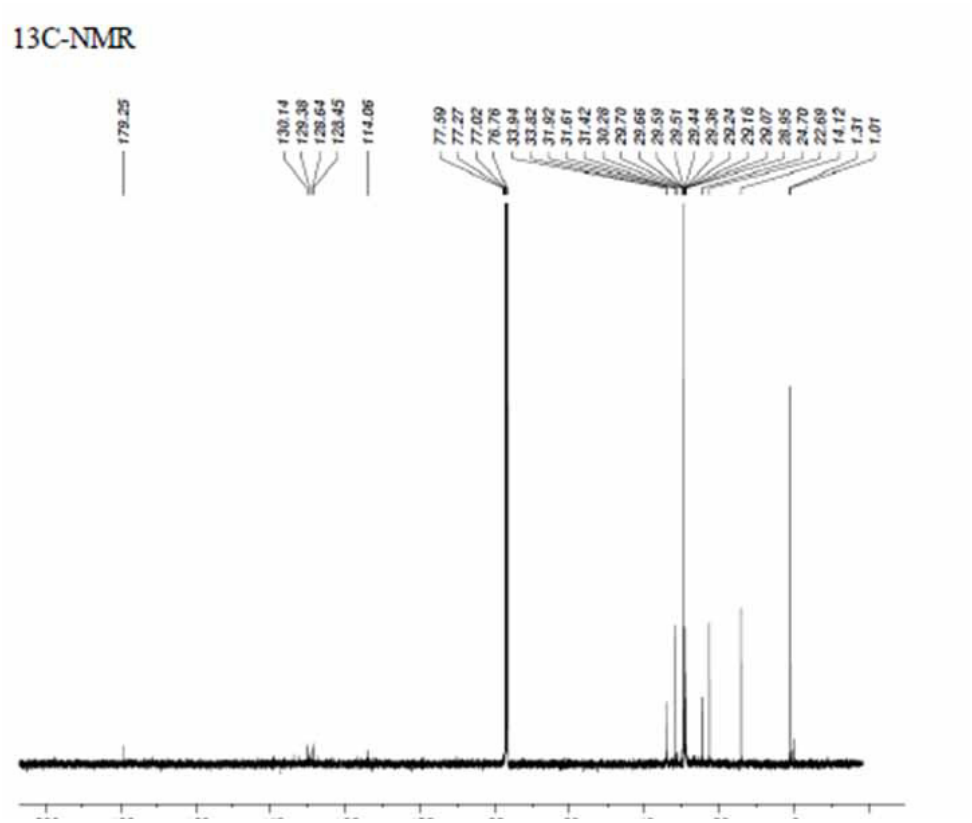

Figure 4.76. ^{13}C NMR spectrum of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

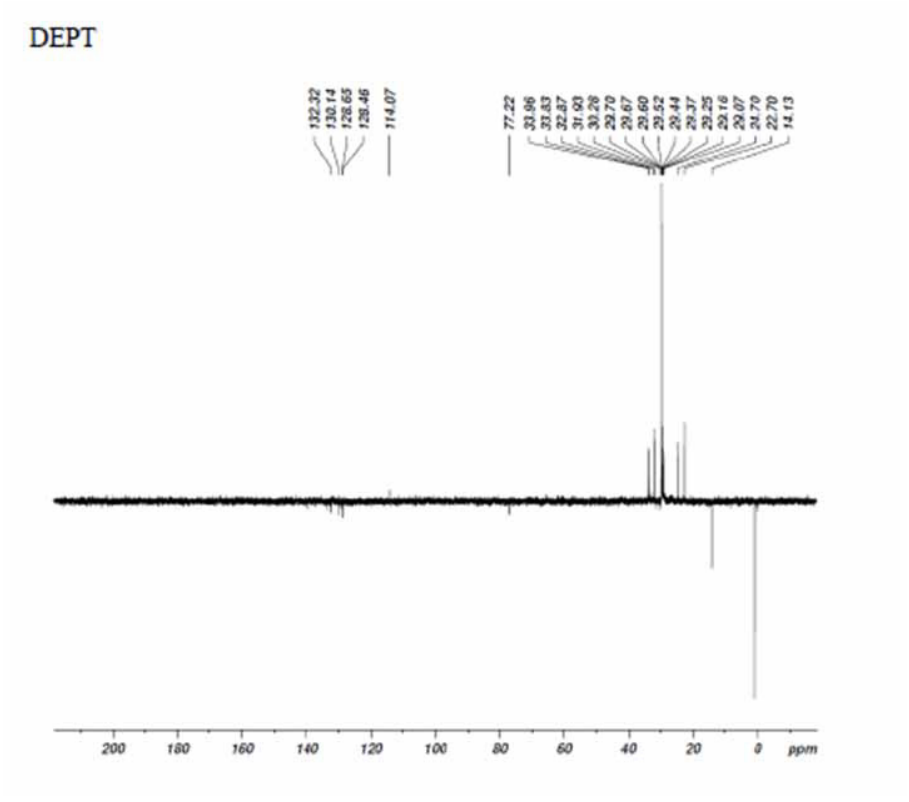

Figure 4.77. DEPT spectrum of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

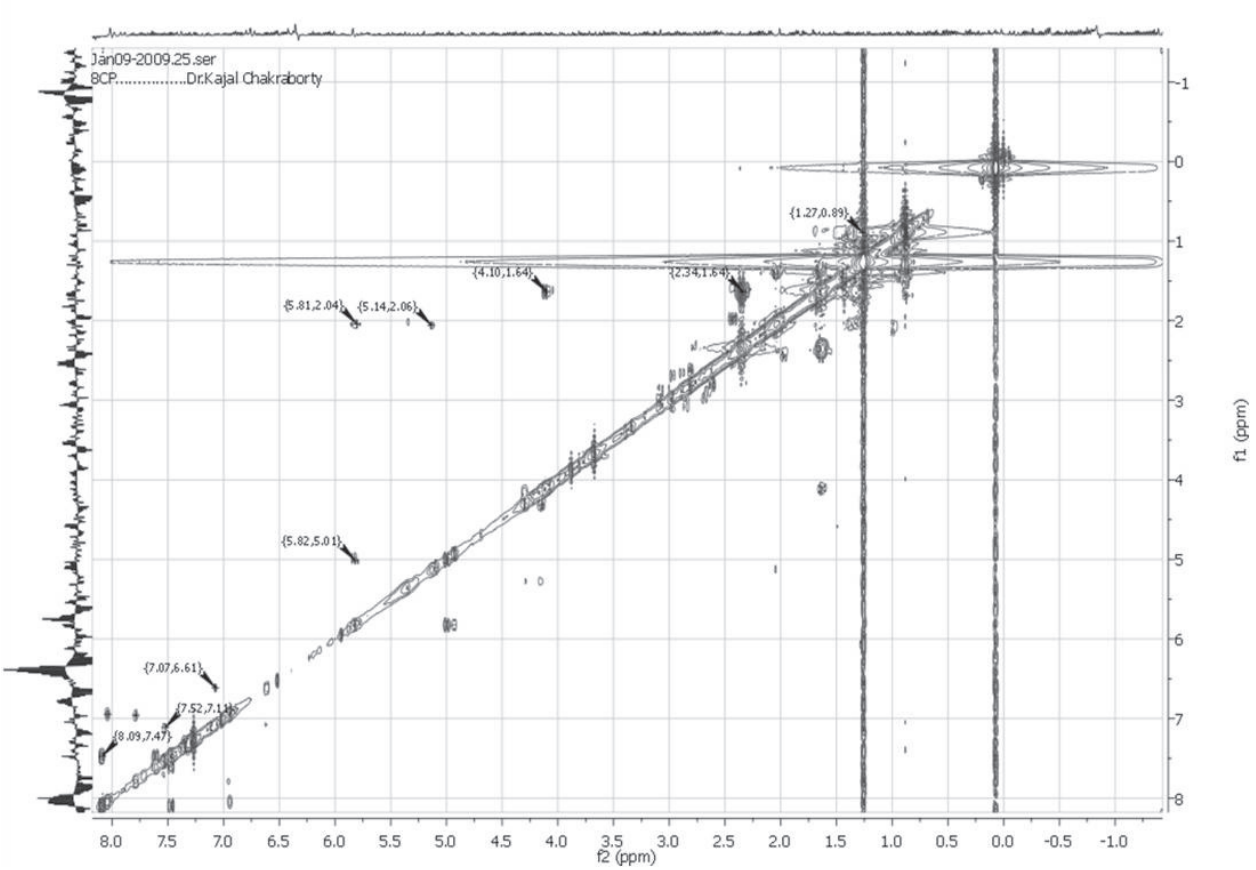

Figure 4.78. ^{1}H-^{1}H-COSY spectrum of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

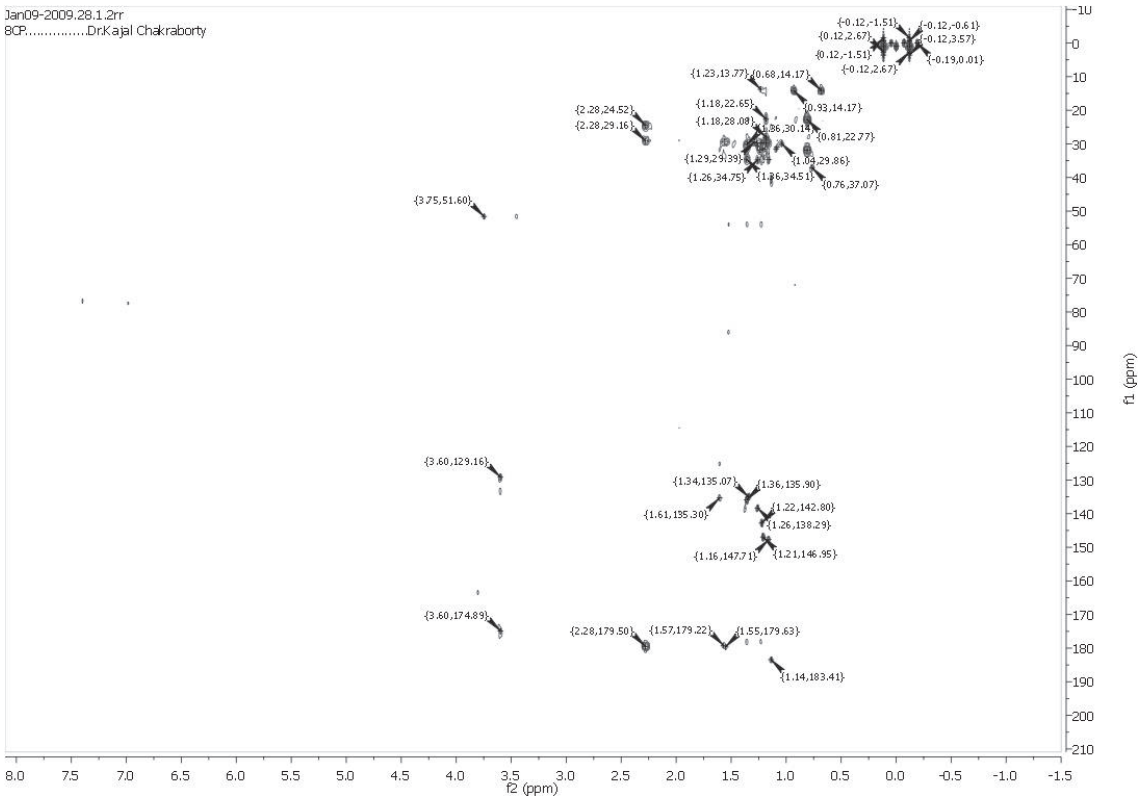

Figure 4.79. HMBC spectrum of 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1E,5E)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal

oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal assigned to possess tetraphen-7(12H)-one group.

The mass spectral pattern showed the molecular ion peak at 580. The base peak at *m/e*=244 due to $C_{18}H_{12}O$ and peaks at *m/e*=366, 356, 110 and 98 supports the molecular structure (Figure 4.72). The aromatic protons appeared at δ 6.8-7.4 ppm, expect one signal appeared downfield at δ 8.1 ppm, which led us to conclude that the aromatic proton is adjacent to a conjugated system (Figure 4.75). The conjugated system assigned to be due to (*Z*)-4-hydroxybut-3-en-2-one or its keto form as 3-oxobutanal. The *Z*-configuration has been assigned by calculating the coupling constant of the olefinic protons at δ 6.60 and 6.91 ppm. The other side chain attached to the tetraphen system is (*E*)-hepta-1,5-dien-3-one as deduced by detailed 2D experiments. The presence of the pentane and butane side chain alongwith the tetraphen moiety has been confirmed by ^{1}H-^{1}H COSY experiments. ^{1}H-^{1}H COSY correlations are apparent between the olefinic protons at δ 6.60 ppm and 6.91 ppm, which in turn exhibited ^{1}H-^{1}H COSY correlation with the protons at δ 3.72 (DEPT methylene signal at δ 41 ppm) and the olefinic protons at δ 5.6 and 5.2 ppm (Figure 4.76, 4.77 & 4.78). This results confirmed the linear structure of the (2*E*, 6*E*)-octa-2, 6-dien-4-one side chain attached to the tetraphen moiety. The E-form of the olefinic protons has been assigned by calculating their coupling constants, which support the assigned configuration. One methylene proton appeared downfield at δ 6.51 ppm (^{13}C δ 59 ppm) to indicate the presence of electronegative centers or conjugated system at close proximity. This methylene group appeared as singlet at δ 6.51 ppm and based on the detailed HMBC and ^{13}C –NMR data (Table 4.12) the structure of 3-oxobutanal linked to the tetraphen has been assigned. The structure of the tetraphen ring system has also been supported by the earlier literature. One singlet methyl group appeared downfield at δ 3.7 ppm has been assigned due to the $–OCH_3$ group, and the HMBC correlation with the aromatic carbon at 126.96 ppm established that the methoxy protons are linked to the tetraphen ring system (Figure 4.79). The ^{13}C-and 2-D NMR assignments have been enlisted in the Table 2. The molecular ion peak appeared at m/e 578, and the carbonyl attached to C7 branch form cyclic structures C_7H_8O (m/e 108). The peaks were observed at *m/e* 71 (due to C_5H_{11}), which also corroborate the structure. The peaks observed at 523,344,110,71,28,97,401,512 also support the structure.

The IR band at 723.33 and 1377.22cm^{-1} are due to long chain ν(methyl group) . The ν(C-H of long alkane chain) was observed at 2852.81, 2924.18 and 2953.12cm^{-1}. The strong band at 1464.02cm^{-1} is also due to δ(C-H alkanes). The band at 1711.88 cm^{-1} is assigned due to ν(C=O vibrations) (Figure 4.74).

4.6. Conclusions

The compounds (9H-fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate and 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid were isolated from the methanol extract of *A. longifolius*. (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon-6-enoate and 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one were isolated from the methanol extract of *P. gymnospora*. Methanol extract of *A. spicifera* upon chromatographic purification yielded propyl 4-acetyl-2-[(*E*)-3, 5-dimethyl-2-hexenyl] benzoate and 3-hexyl-5,6-dihydro-6-undecylpyran-2-one. 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal and 12-tridecenyl 2-methylacrylate were obtained by the bioassay guided purification of the methanol extract of *L. papillosa*. All these compounds exhibited potential redical scavenging and Fe^{2+} ion chelating activities and therefore hence it can be concluded that these compounds may serve as the promising synthetic leads for the development of new generation drug candidates.

CHAPTER 5

Summary

Reactive oxygen species (ROS) are considered to be the major unstable molecules generated due to excessive oxidation. These ROS cause denaturation of cellular machinery and results in ailments such as cancer, atherosclerosis, cardiovascular disorders, inflammation, diabetes mellitus etc, and can also cause ageing. The free radicals can also have harmful effects on foods as production of rancid flavours and odours, reducing the shelf-life, nutritional quality, and safety of food products. The harmful effects of radical associated oxidative stress can be overcome by using antioxidants. Keeping in mind the multiple adverse effects of synthetic antioxidants, there is a need to follow our attention towards natural antioxidants.

Seaweeds are commercially available species, and constitute a major share of marine flora. These species grow under the stressed oceanic conditions, and therefore, are gifted with valuable bioactive molecules with respect to antioxidant properties. It is therefore rational to explore the seaweeds as natural resources to isolate the antioxidant principles. Different seaweeds from the Gulf of Mannar region of Mandapam have been screened for potential antioxidant properties by different model systems. Bioassay guided sequential chromatographic purification yielded an array of pure compounds, which have been assayed for their antioxidant activities. Based upon the results, the compounds exhibiting higher antioxidant potential have been shortlisted for detailed structural characterization.

In this study the seaweeds have been studied based on their abundance in the Gulf of Mannar area. The different brown seaweeds, *Turbinaria conoides, Turbinaria ornata, Anthophycus longifolius, Sargassum plagiophyllum, Sargassum myriocystum, Padina tetrastomatica, Padina gymnospora* and *Stoechospermum marginatum* have been taken into account to evaluate their antioxidant properties. The red seaweeds shortlisted in this study were *Laurencia papillosa, Gelidiella acerosa* and *Acanthophora spicifera*. The antioxidant activities of the methanol extract and solvent fractions (*n*-hexane, dichloromethane and ethyl acetate) of these seaweeds have been evaluated using different *in vitro* systems, *viz* 1,1-dipheny1–2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (H_2O_2)/hydroxyl radical (HO.) scavenging, ferrous ion (Fe^{2+}) chelating ability, thiobarbituric acid reactive species formation inhibition assay and reducing potential. A reversed-phase high-performance liquid chromatography method hyphenated to diode-array detection was also utilized to characterize the solvent extract fingerprints of phenolic acids in the seaweed species.

The ethyl acetate fraction of *Turbinaria conoides* exhibited significantly higher total reduction capability (A_{700nm}1.07, 1 mg/mL), total phenolic content (106 GE/g), scavenging of H_2O_2 (>18%, 1 mg/mL) and hydroxyl radical scavenging activities (64%, 0.6 mg/mL) as compared with *Turbinaria ornata.* The ethyl acetate fraction of *Turbinaria ornata* exhibited higher Fe^{2+} ion chelating (>68%, 0.6 mg/mL), lipid peroxidation inhibitory (7 MDAEC/kg, 2 mg/mL), ABTS radical scavenging (14%, 0.6 µg/mL) and DPPH· radical scavenging activities (64%, 1 mg/mL). The HPLC analysis indicated that gallic acid (21 mg/g) and syringic acid (73 mg/g) were the predominant phenolic acids in the ethyl acetate fraction of *Turbinaria conoides*, whilst epicatechin gallate (205 mg/g) was the major phenolic acid identified in the methanol fraction. Epicatechin gallate (24 mg/g) and syringic acid (15 mg/g) were the major constituents in the methanol fraction of *Turbinaria ornata*, whereas the principle components in ethyl acetate fraction were gallic acid (64 mg/g) and chlorogenic acid (43 mg/g).

The ethyl acetate fraction of *Anthophycus longifolius* exhibited higher total phenolic content (236 GE/g), ABTS radical scavenging (>18%, 0.6 µg/mL), DPPH· radical scavenging (>88%, 1 mg/mL), hydroxyl radical scavenging (>83%, 0.6 mg/mL), reduction capability (Ab_{700nm}1.4, 1 mg/mL) and Fe^{2+} ion chelating activities (>88%, 0.6 mg/mL). The ethyl acetate fraction of *Sargassum plagiophyllum* exhibited higher H_2O_2 scavenging capacity (16 %, 1 mg/mL) and that of *Sargassum myriocystum* exhibited higher lipid peroxidation inhibition ability (4 MDAEC/kg, 2 mg/mL). The HPLC analysis indicated that gallic acid (2 mg/g) and syringic acid (23 mg/g) were the predominant phenolics in the ethyl acetate fraction of *Anthophycus longifolius.* Caffeic acid (<1.3 mg/g), 2,5 dihydroxy benzoic acid (<4.4 mg/g), coumaric acid, ferulic acid, chroman flavanols (epicatechin) and syringic acid (<52 mg/g) were found to be ubiquitous in either of ethyl acetate and methanol fractions of *Sargassum plagiophyllum.* Both the ethyl acetate and methanol fractions of *Sargassum myriocystum* were found to contain coumaric acid, ferulic acid (<16 mg/g), flavanols (epigallocatechin gallate, epicatechin) and syringic acid (<82 mg/g).

In *Padina tetrastomatica,* the ethyl acetate fraction exhibited higher total phenolic content (165 GE/g), hydroxyl radical scavenging (87%, 0.6 mg/mL) and TBARS formation inhibition activities (4 MDAEC /kg, 2 mg/mL). Higher ABTS radical scavenging (29%, 0.6 µg/mL), DPPH· radical scavenging (>77%, 1 mg/mL), reduction capability (A_{700nm}1.48, 1 mg/mL) and Fe^{2+} ion chelating activities (82%, 0.6 mg/mL) were observed with the ethyl acetate fraction of *Stoechospermum marginatum*. Dichloromethane fraction of *Padina gymnospora* exhibited higher hydroxyl radical scavenging activity (>85%, 0.6 mg/mL) and its ethyl acetate fraction exhibited higher H_2O_2 scavenging potential (18%, 1 mg/mL). It was found that these seaweeds contain caffeic acid, 2, 5 dihydroxy benzoic acid, coumaric acid, ferulic acid, and syringic acid as phenolic acids in the

methanol and ethyl acetate fractions. Epicatechin (112 mg/g) was the major phenolic acid identified in the ethyl acetate fraction of *Padina tetrastomatica*. The ethyl acetate fraction of *Padina gymnospora* registered higher amount of 2,5 dihydroxy benzoic acid (163 mg/g), whereas the methanol fraction of this species recorded higher amount of syringic acid (132 mg/g). The dominant phenolic acid present in the ethyl acetate fraction of *Stoechospermum marginatum* was coumaric acid (43 mg/g) where as higher amount of syringic acid (36 mg/g) was found in the methanol fraction.

The ethyl acetate fraction of *Laurencia papillosa* exhibited higher total phenolic content (283 GE/g), ABTS (97%, 0.6 µg/mL) as well as DPPH· radical scavenging activities (97%, 1 mg/mL). Higher hydroxyl radical scavenging (78%, 0.6 mg/mL) and TBARS inhibition abilities (3 MDAEC /kg, 2 mg/mL) were observed with the ethyl acetate fraction of *Gelidiella acerosa,* whereas higher ferrous metal ion chelating activity (63%, 0.6 mg/mL) was observed with its dichloromethane fraction. Dichloromethane fraction of *Acanthophora spicifera* exhibited higher H_2O_2 scavenging ability (18%, 1 mg/mL), whereas a higher reducing capability (Ab_{700nm}1.46, 1 mg/mL) was observed with the ethyl acetate fraction. A higher amount of syringic acid (23 mg/g) was observed with the methanol extract of *Laurencia papillosa* where as its ethyl acetate fraction registered higher amount of epicatechin gallate (33 mg/g) than other phenolic acids. The HPLC analysis indicated that caffeic acid (11 mg/g) was the predominant phenolics in the ethyl acetate fraction of *Gelidiella acerosa*, whilst more polar chlorogenic acid (27 mg/g) was the major phenolics in its methanol fraction. In *Acanthophora spicifera* both methanol and ethyl acetate extracts contain phenolic acids such as epicatechin gallic acid, caffeic acid and 5-caffeoylquinic acid with cyclohexane carboxylic acid derivative (chlorogenic acid).

Based on the abundance and bioassay results obtained for the column fractions, two brown seaweeds (*Anthophycus longifolius* and *Padina gymnospora*) and two red seaweeds (*Acanthophora spicifera* and *Laurencia papillosa*) were shortlisted for further isolation, purification and characterization of antioxidant secondary metabolites.

Bioassay guided chromatographic purification of the methanol extract of *Anthophycus longifolius* yielded (9H-fluoren-9-yl)methyl 1-(propoxycarbonyl) butylcarbamate and 3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid as major antioxidant secondary metabolites with higher DPPH (>76%, 0.1 mg/mL) and ABTS (>30%, 0.1 µg/mL) radical scavenging abilities, and ion chelating potential (>26%, 0.1 mg/mL).

(9H-Fluoren-9-yl) methyl 1-(propoxycarbonyl) butylcarbamate ($C_{23}H_{27}NO_4$ *m/e*= 381.4814), a new derivative of the fluorens was isolated as yellowish amorphous solid with 85% purity. The

molecular ion peak at *m/e* 382 appeared to undergo elimination of C_3H_9+ (45) to yield *m/e* 336 ($C_6H_{12}O_2$), which undergo fragmentation to afford the fragments with *m/e* 264, 201 etc. The IR spectrum recorded absorbance around 1700, 1034 and 1600cm^{-1} due to ν(C=O), ν(C-N) and δ(N-H) vibrations. The ^{1}H NMR in conjugation with ^{13}C-NMR recorded the presence of the methine groups CH δ 7.3-7.7 ppm, which are assigned to be due to fluoren and the downfield shift (about δ 0.6 ppm) of the –CH group is due to the presence of β -O-C=O from the methine group. The methylene groups at δ 3.3 and 4.1 ppm appeared significantly downfield due to the presence of alpha -OC(=O)N and alpha -OC(=O)-C moieties in the methyl 1-(propoxycarbonyl)butylcarbamate side chain. The protons at H-4, and H-8 showed HMBC correlation with C-1, which indicate that C-1 is situated between two aromatic rings. The carbon at C-1 gives HSQC correlation with H-1 (δ 1.60 ppm) to demonstrate a triplet. The H-1 showed ^{1}H-^{1}H COSY correlation with H-11 (δ 2.04 ppm), which showed HMBC correlation with C-12(δ 163.83 ppm). The carbon at C-15(δ 65.7 ppm) exhibited DEPT signal for methylene group, and its downfield shift demonstrates the presence of highly electronegative group at its close proximity.

3-((2*E*,8*E*)-1,4,6,10,12-pentahydroxy-16-oxo-15,21,24-trioxa-bicyclo[17.3.2]tetracosa-2,8-dien-9-ylamino)-22,23-(4-hydroxyphenyl)-2-methoxypropanoic acid, a llight yellow semisolid with molecular formula $C_{28}H_{39}NO_{13}$ (*m/e*= 597.6214) was isolated with 82% purity. The signature mass peaks appeared at *m/e*= 119 ($C_4H_9NO_3$), *m/e*=184 ($C_8H_8O_5$), *m/e*=94, 90, and 89, which supported the molecular structure. The IR spectrum recorded ν_{as} (C=O) vibrations (1740cm^{-1}) of -COOH group, ν(C=O carboxylic acids), ν(C-N) band and γ_w (N-H) vibrations. The –NH and hydroxyl groups appeared as broad singlets at about 4-5 ppm in proton NMR spectra. The phenolic proton appeared at δ 5 ppm due to the deshielding effect of the aryl ring system. The carboxylic –OH appeared downfield at δ 10.2 ppm. Three olefinic protons appeared at δ 4.99-5.82 ppm as established by integrating the number of protons. Two magnetically equivalent –CH_2 groups appeared at δ 4.2, 4.3 ppm, and demonstrated to be a part of (*Z*)-6, 7-dihydro-5H-1, 4-dioxepine ring system of the 3, 4-dihydro-2H-benzo[b] [1, 4] dioxepine-3, 7-diol moiety of the compound. The ^{13}C spectra displayed signal at δ 167.7 ppm and δ 178.6 ppm showed two carbonyl compounds to confirm the structure. The position of the hydroxyl groups was further confirmed from the ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY spectra. In the ^{1}H–^{1}H COSY spectrum, couplings were apparent as described in the figure support the presence of the established skeleton.

The MeOH extract of *Padina gymnospora* upon sequential bioassay guided chromatographic purification yielded two antioxidant secondary metabolites 1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one and (6*Z*)-methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon -6-enoate with >70% DPPH and >23% ABTS radical scavenging ability, and >20% ion chelating potential.

(6*Z*)-Methyl 8-(2-((*E*)-4-ethyl-3-isopropyl-5-methylhept-1-enyl)-tetrahydro-6-oxo-2H-pyran-3-yl)-3,3-dimethylnon -6-enoate was isolated as a yellow amorphous solid with molecular formula $C_{30}H_{52}O_4$ (*m/e*= 476.7545). The molecular ion peak was observed at *m/e*=477. The mass fragments at *m/e*=158, 74, and 60 are the characteristic signature peaks of the proposed structure. The IR spectrum exhibited characteristic bands for ν(C-H alkane chain), δ(C-H alkane chain) and ν(C=O) vibrations to support the structure. The signature peaks of ^{13}C NMR at δ 179.41 and 174.37 ppm indicate the presence of two carbonyl compounds. The signals in olefinic protons appeared at δ 130.20, 130.01, 129.71 and 128.23 ppm, which give HSQC correlation with the protons at 5.26, 5.16, 5.29 and 5.08 ppm, respectively. The linear ^{1}H-^{1}H COSY correlations were apparent between the protons at δ 1.6 ppm (C-4), 2.28 ppm (C-3), 2.24 ppm (C-2) and 4.5 ppm (C-1) that support the valerolactone moiety. These protons showed the HMBC correlation with the carbonyl carbon at C-5 (δ 179.41 ppm), which also support the cyclic valerolactone structure. The proton at C-1 (δ 4.5 ppm) shows ^{1}H-^{1}H COSY correlation with the olefinic proton at δ 5.26 ppm (C-1'), which, in turn realized the ^{1}H-^{1}H COSY correlation with the olefinic proton at δ 5.29 ppm (C-2'). This established the tetrahydro-6-(prop-1-enyl) pyran-2-one moiety of the compound.

1-((4*Z*)-2,3,7,8-tetrahydrobenzo[b]oxepin-2-yl)tetradecan-2-oxo-5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one was isolated as a white semisolid with molecular formula $C_{30}H_{42}O_4$ (*m/e*=466.6422). The IR spectrum exhibited ν(C-H) vibrations, ν(C=C) and ν(C=O 5-membered ring) which supported the proposed structure. The molecular ion peak was observed at *m/e*=467 and the peak at *m/e*=146 is due to the fragment $C_{11}H_{15}$. The peaks at *m/e* 212 and *m/e* 72 were due to the fragments $C_{14}H_{26}O$ (*m/e*=212) and the alkane fragment C_5H_{12} (*m/e*=72). The methine protons appeared at 3.6 ppm, which is connected to the tertiary carbon (δ 51.38 ppm), was established to the part of the substituted dihydrofuran-2(3H)-one ring system. The downfield shift of the proton is due to the electronegative -O-C=O group as established by their HMBC connectivities. The presence of a beta -C=C to the carbon at δ 3.6 ppm is established by the linear ^{1}H-^{1}H COSY connectivities. The structure of the 3-methylisobenzofuran-1(3H)-one system has been established by the HMBC correlation analyses. The structure of (Z)-2,3-dihydrobenzo[b]oxepine has been established by the ^{1}H-^{1}H COSY and HMBC analyses and calculation of the coupling constants (*J*) of the protons at δ 5.53 and 5.81 ppm, which confirm the Z-configuration of the olefinic system. The coupling constant values have been calculated to be in conformity with the *E*-configuration. This established the structure of 5-((*E*)-but-2-enyl)-dihydrofuran-2(3H)-one system.

Two antioxidant secondary metabolites 3-hexyl-5,6-dihydro-6-undecylpyran-2-one and butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate were yielded by repeated bioassay guided chromatographic purification of the methanol extract of the red seaweed *Acanthophora spicifera.*

3-Hexyl-5, 6-dihydro-6-undecylpyran-2-one was isolated as a white semisolid with molecular formula $C_{22}H_{40}O_2$ (*m/e*=336.5543). The molecular ion peak appeared at *m/e*= 336 and the fragment peak at *m/e*= 98 as a base peak with a cyclic structure (5, 6-dihydropyran-2-one) supported the structure. The IR spectrum registered bands at 723, 1464, 1742 and around 2800 cm^{-1}, which are assigned due to ν(methyl group), ν(C-H long alkane chain), ν(C=O vibrations) and δ(C-H alkanes), respectively. The C^{13} NMR spectra displayed a signal at δ 179.20 ppm indicating that the compound has a carbonyl functional group. The ^{13}C signals at δ 130.88 and 128.80 indicate the olefinic carbons, whilst the signal at δ 130.88 ppm doesn't have any HSQC signal thereby indicating the presence of a quaternary carbon atom. The ^{13}C signal at C-5 (δ 68.17 ppm) is attached to the oxygen of the 5,6-dihydropyran-2-one ring system. The downfield shift of C-5 appeared to be due to the α—OC(=O) group in the 5,6-dihydropyran-2-one system. Strong ^{1}H-^{1}H-COSY correlation between the proton signals (methylene) at δ 1.35, 1.20, 1.32, 0.81, and 0.85 ppm, which in combination of mass spectroscopic analyses established the dodecane moiety attached with the 5,6-dihydropyran-2-one group.

Butyl 4-acetyl-2-((*E*)-3,5-dimethylhex-2-enyl)benzoate was isolated as a light yellow semisolid with molecular formula $C_{20}H_{27}O_3$ (*m/e*= 315.4424). The molecular ion peak appeared at *m/e*= 316 and the peak at *m/e* 104.10 (C_4H_8) was assigned to be as (*E*)-3, 5-dimethylhex-2-ene. The base peak at *m/e*=120 (C_8H_8O) was assigned to be due to acetophenone. The IR bands at 721, 1457 and 1739 are assigned due to ν(methyl group), δ(C-H of alkanes) and ν(C=O vibrations). The C^{13} NMR spectra displayed a signal at δ 179.20 ppm indicating that the compound has a carbonyl functional group. The presence of acetyl group attached to the aryl ring system is established by the typical proton shift of the CH_3 group at δ 2.3 ppm and the corresponding HSQC correlation of the carbon (at δ 40.6 ppm) and proton (at δ 2,3 ppm). The ^{13}C signal at δ 179.8 ppm also established the presence of another carbonyl group and its attachment with the aryl ring system has been assigned by the strong HMBC correlation with the aryl proton at δ 7.76 ppm. The ^{13}C-NMR spectrum also confirmed the presence of the –C=O group at δ 179.8 ppm. The olefinic carbon at δ 121.7 ppm did not show any HSQC correlation thereby indicating that this carbon is of quaternary type. The (*E*)-configuration of the olefinic bond has been confirmed by the coupling constant calculation.

Bioassay guided chromatographic purification of the methanol extract of *Laurencia papillosa* yielded two antioxidant secondary metabolites 12-tridecenyl 2-methylacrylate and 3-(1-butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal with >72% DPPH and >29% ABTS radical scavenging ability and >21% ion chelating potential.

2-Tridecenyl 2-methylacrylate was isolated as an amorphous yellow semisolid with molecular formula $C_{17}H_{30}O_2$ (*m/e*= 266.4318). The molecular ion peak appeared at *m/e* 267, ($C_{17}H_{30}O_2$) and the fragment peaks at *m/e*=184 ($C_{11}H_{20}O_2$), *m/e*=182 ($C_{13}H_{26}$), *m/e*=112 (C_8H_{16}) *m/e*=111 (C_7H_{16}) and *m/e*=85 ($C_4H_5O_2$) established the structure. The IR spectra registered bands due to ν(C-H long alkane chain), δ(C-H alkanes), ν(C=O vibrations), γ*r*(C-H alkanes) and ν(C-O band) support the structure. The methylene protons appeared at δ 2.1 ppm are due to the deshielding effect of the olefinic –CH=CH- group. The olefinic group as in methyacrylate have been established by the proton chemical shift at δ 7.71 ppm; whereas those at δ 5.82, and 7.52 ppm support the presence of the $–CH=CH_2$ group at the terminal position of the compound. The olefinic proton at δ 7.71 ppm established that the -C(=O)O-R moiety of the methacrylate is responsible for the downfield shift. The presence of carbonyl group has been confirmed by the presence of the quaternary carbon atom at δ 177.41 ppm with no HSQC correlation.

3-(1-Butyl-7,12-dihydro-2-methoxy-7-oxo-10-((1*E*,5*E*)-3-oxohepta-1,5-dienyl)-6-pentyltetraphen-9-yl)-3-oxopropanal was isolated as an amorphous yellow semisolid with molecular formula $C_{28}H_{42}O_5$ (*m/e* =578.7544). The mass spectral pattern showed the molecular ion peak at 580 and the base peak at *m/e*=244 (due to $C_{18}H_{12}O$). The peaks at *m/e*=366, 356, 110 and 98 support the molecular structure. The IR bands registered the presence of ν(methyl group), ν(C-H of long alkane chain), δ(C-H alkanes) and ν(C=O vibrations). The aromatic protons appeared at δ 6.8-7.4 ppm, expect one signal appeared downfield at δ 8.1 ppm, which led us to conclude that the aromatic proton is adjacent to a conjugated system. The *Z*-configuration has been assigned by calculating the coupling constant of the olefinic protons at δ 6.60 & 6.91 ppm. The other side chain attached to the tetraphen system is (*E*)-hepta-1,5-dien-3-one as deduced by detailed 2D experiments. ^1H-^1H COSY correlations are apparent between the olefinic protons at δ 6.60 ppm and 6.91 ppm, which in turn exhibited ^1H-^1H COSY correlation with the protons at δ 3.72 (DEPT methylene signal at δ 41 ppm), and the olefinic protons at δ 5.6 and 5.2 ppm. This results confirmed the linear structure of the (2*E*, 6*E*)-octa-2, 6-dien-4-one side chain attached to the tetraphen moiety. One singlet methyl group appeared downfield at δ 3.7 ppm has been assigned due to the $–OCH_3$ group, and the HMBC correlation with the aromatic carbon at δ 126.96 ppm established that the methoxy protons are linked to the tetraphen ring system.

Seaweeds as a renewable natural resource of antioxidative compounds stand as potential new generation alternatives to the synthetic antioxidants used in food, pharmaceutical and cosmetic industries. The present study revealed candidates seaweed sp with potential lead molecules for medicinal use. Gulf of Mannar area of Mandapam is considered to be the habitat of diverse seaweeds and identified as a hot spot for searching ocean drugs. Hence the technical programme aimed to

identify prospective species of seaweeds and to characterize the natural lead molecules with antioxidant properties were worked out. The study succeeded in identifying seaweed species with naturally occurring antioxidant compounds and also in characterizing the purified fractions with the modern spectroscopic techniques. The study stands as the first of its kind to establish the bioactive data of the vast majority of eleven seaweed sp abundantly available in this very important region with respect to their antioxidative potential. Bioactivity guided characterization of antioxidative molecules from the selected seaweed sp will serve as the potential synthetic leads for further exploration in healthcare industries and for newgeneration food additives to increase their shelf life.

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ACHIEVEMENTS

Publications:

1. Kajal Chakraborty, Nammunayathuputhenkotta Krishnankartha Praveen, Kodayan Kizekadath Vijayan, Gonugontla Syda Rao (2013) "Evaluation of phenolic contents and antioxidant activities of brown seaweeds belonging to *Turbinaria* spp (Division: Phaeophyta, Family: Sargassaceae) collected from Gulf of Mannar". Asian Pacific Journal of Tropical Biomedicine, 3(1)8-16.
2. Nammunayathu puthenkotta Krishnankartha Praveen, Kajal Chakraborty (2013) Antioxidant and anti-inflammatory potential of the aqueous extract and polysaccharide fraction from brown marine macroalgae *Padina* sp. From Gulf of Mannar of Peninsular India. Journal of Coastal Life Medicine, 1(1) 19-29.
3. Kajal Chakraborty, Deepu Joseph, Nammunayathuputhenkotta Krishnankartha Praveen (2013). Antioxidant activities and phenolic contents of three red seaweeds (Division: Rhodophyta) harvested from the Gulf of Mannar of Peninsular India. Journal of Food Science and Technology, Doi 10.1007/s13197-013-1189-2.

Papers presented at international conference:

1. N. K Praveen, Kajal Chakraborty, K.K Vijayan and G. Syda Rao. Isolation and characterization of antioxidant compounds from *Acanthophora spicifera* and *Padina gymnospora* collected from Gulf of Mannar (9th Indian fisheries forum, 19-23 December 2011, held at Chennai).
2. N. K Praveen, KajalChakraborty and K. K Vijayan. Antioxidant potential of brown seaweeds belonging to *Sargassum* species collected from Gulf of Mannar (Asian Pacific Aquaculture 2011, 18- 20 January 2011, Le Meridian convention centre, Kochi, India).