STRUCTURAL INVESTIGATION OF THE NATURAL PRODUCTS COMPOSITION OF SELECTED SOUTH AFRICAN SEAWEEDS.
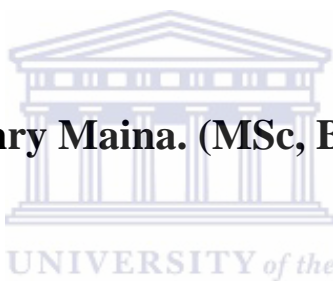
UNIVERSITY of the WESTERN CAPE

By

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A thesis submitted in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in the Faculty of Natural Sciences University of the Western Cape

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ABSTRACT

Recently, a great deal of interest has developed towards the isolation of bioactive compounds from marine sources due to their numerous health benefits. Furthermore, marine algae are valuable sources of structurally diverse metabolites with scientifically proven therapeutic claims. The cell walls are rich in sulfated polysaccharides such as fucoidans in brown algae, carrageenans in red algae and ulvans in green algae. These sulfated polysaccharides exhibit many beneficial biological activities such as anticoagulant, antiviral, antioxidative, anticancer and immunomodulating activities. They have great potential for further development as products in cosmeceutical, pharmaceutical and nutraceutical areas. Although the mechanism of action is still not clear, their biological activities could be mainly attributed to their major secondary metabolites namely; phlorotannins, terpenoids and fucoidans. There was use of comprehensive chromatographic separations and a full analysis of isolates using one or other of the spectroscopic techniques. Antioxidant and cytotoxicity tests were perfomed in details for *Ecklonia maxima*. Furthermore, structural and electronic features of the phlorotannins were compared in an attempt to provide an explanation for the differences in their radical scavenging properties. In this regard, two main radical scavenging mechanisms, hydrogen atom transfer (HAT) and electron transfer (ET), were assessed in order to determine the preferred mode of radical scavenging. Fully relaxed geometry optimizations of the neutral and the radical species were performed utilizing DFT/B3LYP and DFT/UB3LYP methods respectively. In further studies, the structural and functional properties of sulfated polysaccharides from the three brown and one red seaweeds were investigated. This was through detailed analysis of chemical composition of crude and purified polysaccharides using PMP-derivatization of hydrolysed sugars, anion exchange, molecular weight determination, ion chromatography, FT-IR, NMR to

methylation analysis. The work reports isolation and characterization of compounds from four algae: *Ecklonia maxima*, phlorotannin derivatives, namely phloroglucinol (**22**), eckol (**23**), 7-phloroeckol (**24**), 2-phloroeckol (**25**) and a sterol, 24-ethylidine cholesterol (**26**); *Splachinidium rugosum*, 24-ethylidine cholesterol (**26**), 1, 3-Dicapryloyl-2-oleoylglycerol (**27**), *E*-3,7,11,15-tetramethylhexa dec-2-en-1-ol (phytol) (**28**); *Macrocystis angustifolia*, 24-ethylidine cholesterol (**26**); a red seaweed *Aeodes orbitosa*, and *E*-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol (**28**) and 17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7, 8,9,11,12,14,15,16,17-dodecahydro-1 *H*-cyclopenta[*a*]phenanthren-3-ol (*β*-sitosterol) (**29**). Experimental findings and theoretical predictions of phlorotannins indicated that the radical scavenging activities followed the order **22** < **23** < **25** < **24**. Theoretical studies further indicated the ET mechanism is more significant than the HAT mechanism due to the high BDE values. Their polysaccharide structures were tentatively shown to have a backbone of $(1\rightarrow3)$ and $(1\rightarrow4)$ linkages with sulfate groups at *O*-2 and *O*-2, 3 positions. The only red algae studied contained, 2-*O*-methyl-D-galactose with $(1\rightarrow3)$ and $(1\rightarrow4)$-glycosidic linkages possessing sulfate groups at positions 2 and 6.

KEYWORDS: brown algae; phlorotannins; fucoidan, radical scavenging activity; hydrogen atom transfer mechanism.

DECLARATION

I, Henry Maina Mwangi hereby declare that this work is my original dissertation and to my knowledge, it has not been submitted anywhere else for the award of a degree at any other University.

Date …………………………………. Signed …...

ACKNOWLEDGEMENTS

DEDICATION

Special thanks to my loving wife Monica and children Edward and Winnie, who has been an inspiration to my struggle over the years. Thanks to my adoring Mum and Dad for their boundless moral support and love in my endeavors into this long journey of my studies. And may the Lord God bless them in a mighty way in everything they do.

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LIST OF PUBLICATIONS

Parts of the work reported in this thesis have been published, submitted or prepared for submission as manuscripts for publication in some journals.

I.   **Henry, M. Mwangi**, Christie, N, Van Der Westhuizen, J., Jeanine Marnewick, J. and Wilfred, T. Mabusela and Mwadham, M. K (2013). Isolation, identification and radical scavenging activity of phlorotannin derivatives from brown algae, *Eklonia maxima species*: An Experimental and theoretical study. Free Radicals and Antioxidants 3: S1-S3 http://dx.doi.org/10.1016/j.fra .2013.10.006.

II. **Henry, M. Mwangi.**, Martin, O. Onani., Van Der Westhuizen, J., and Wilfred, T. Mabusela. Phloroglucinol and sterol derivatives from the brown alga *Ecklonia maxima* and their anticancer properties. (Manuscript under review)

III. **Henry, M. Mwangi.**, Van Der Westhuizen, J., and Wilfred, T. Mabusela. Sulfated polysaccharide from the brown seaweed *Ecklonia maxima*, *Splachinidium rugosum* and *Macrocystis angustifolia.* (Manuscript)

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Chapter 7.0 Conclusion and future recommendations

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List of abbreviations

AAPH- 2, 2′-azobis (2-amidino-propane) dihydrochloride

ABTS (2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid

AcOH- Acetic acid

ALT- Alanine transaminase

AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AOC- Antioxidant capacity

APT- Attached Proton Test

AUC- Area under curve

B3LYP- Becke's three parameter hybrid functional Lee-Yang-Parr

BDE- Bond dissociation enthalpy

COSY- Correlation Spectroscopy

DEAE- Diethylaminoethyl

DCM- Dichloromethane

DEPT- Distortion Enhanced by Polarization Transfer

DFT- Density Functional Theory

DMSO- Dimethylsulfoxide

DPPH- 2, 2-diphenyl-1-picrylhydrazine

ELISA- Enzyme-linked immunosorbent *assay*

ESI-MS- Electrospray ionization trap mass spectrometry

ES-MS- Electrospray Ionization Mass Spectrometry

EtOAc- Ethyl acetate

ET- Electron transfer

FAB- Fast atom bombardment

FDA- Food and Drug Administration

FID- Flame ionization detector

FRAP- Ferric reducing antioxidant power

GAE- Gallic acid equivalents

GC-Gas Chromatography

GLC- Gas-liquid chromatography

HAT- Hydrogen atom transfer

HMBC- Heteronuclear Multiple Bond Correlation

HMQC- Heteronuclear Multiple Quantum Coherence

HIV- Human Immune Virus

HPLC- high performance liquid chromatography

HSV- Herpes Simplex Virus

HSQC- Hetero-nuclear Single Quantum Coherence

IC- Ion chromatography

IEC- Ion Exchange Chromatography

IHB- Intra-molecular hydrogen bonds

IP- Ionization potential

IR- Infra Red

LAV- Low Activity Virus

LC_{50}- Lethal concentration 50

MDR- Multiple Drug Resistance

MeOH- Methanol

MIC- Minimum Inhibitory Concentration

MS- Mass Spectrometry

MTT- 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

NMR- Nuclear Magnetic Resonance

NOESY- Nuclear Overhauser Enhancement Spectroscopy

ORAC- Oxygen radical absorption capacity

PCM- Polarizable continuum model

PHL- Public Health Laboratory

PMAA- Partially methylated alditol acetates

PMP-1-phenyl-3-methyl-5-pyrazolone

PG-1- Protegrin-1

PG-1- Protegrin-2

PC- Paper Chromatography

PUFA- Polyunsaturated fatty acid

R&D-Research and Development

RFIC- Reagent-Free™ Ion Chromatography

ROS- Reactive oxygen species

RPMI- Roswell Park Memorial Institute

RT- Reverse Transcriptase

TAC- Total antioxidant capacity

TAS- Total antioxidant status

TCM-Traditional Chinese Medicine

TEAC- Trolox equivalent antioxidant capacity

TES- Triethylsilylane

TFA- Trifluoroacetic acid

TLC- Thin Layer Chromatography

TMS- Tetramethylsilane

TMSO- trimethylsilyl methanesulfonate

TE- Trolox® equivalents

TPTZ- Tripyridyltriazine

UV- Ultra Violet

VLC- Vacuum Liquid Chromatography

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CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Seaweed is the collective term for a heterogeneous group of marine plants with a long fossil history. The term encompasses macroscopic, multicellular, benthic marine algae that comprise members of brown, red and green algae (McHugh, 2003). These oceanic algae (seaweeds) are plants without vascular system. However, their cells agglomerate into strings or blades (Sharma, *et al*., 2013). They have a low content in lipids, high concentration in polysaccharides, natural richness in minerals, polyunsaturated fatty acids and vitamins as well as bioactive molecules, which make them to be a good source of nutraceuticals (Rupe´rez and Saura-Calixto, 2001). Two major types of algae that have been identified are the microalgae which are found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton and the macroalgae which occupy the littoral zone. Seaweeds grow in the intertidal as well as in the sub-tidal area up to a certain depth where very little photosynthetic light is available. They are classified into brown algae (phaeophyta), green algae (chlorophyta) and red algae (rhodophyta) on the basis of their morphological appearance and chemical composition. They are an excellent source of vitamins A, B_1, B_{12}, C, D and E, pantothenic acid, niacin, riboflavin, and folic acid as well as minerals such as Ca, P, Na, K (Dhargalkar and Pereira., 2005).

Although seaweeds are exposed to the adverse environmental conditions like ultraviolet light and high oxygen concentrations that lead to the formation of free radicals and other strong oxidizing agents, they do not show serious photodynamic damage *in vivo*. They are able to generate the necessary compounds to protect themselves from external factors such as pollution, stress and UV radiation. This fact suggests that marine algae, like photosynthesizing plants,

possess anti-oxidative mechanisms and/or compounds which act as anti-oxidant agents. At the same time, some species have been found to contain polysaccharides, glycoproteins and other secondary metabolites with antimicrobial (Cox *et al*., 2009; Gupta *et al.*, 2010a), antitumoral (Koyanagi, *et al*., 2003; Zubia *et al*., 2009) or anti-viral activity (Artan *et al*., 2008; Hemmingson *et al*., 2006; Zhu *et al.*, 2003; 2004). Amongst all the three types, the highest phytochemical content has been reported from brown seaweeds (Seafood plus, 2008).

There are important metabolites from the division Phaeophyta which has 13 orders according to the classification of Bold and Wynne (1985) comprises of polyphloroglucinol phenolic compounds, (Ahn *et al*., 2004; Chandini *et al*., 2008), non-polyphenolic secondary metabolites such as terpenes (Siamopoulou *et al*., 2004), carotenoids such as fucoxanthin (**1**), (Borchardt *et al*., 2001), oxylipins (Kupper *et al*., 2006; Rorrer *et al*., 1995) and polysaccharides. However, only three orders namely Laminariales, Fucales and Dictyotales have been extensively researched for their phytochemical composition. The most studied species of these orders are *Laminaria*, *Ecklonia*, *Undaria*, *Himanthalia* and *Dictyota*.

(**1**)
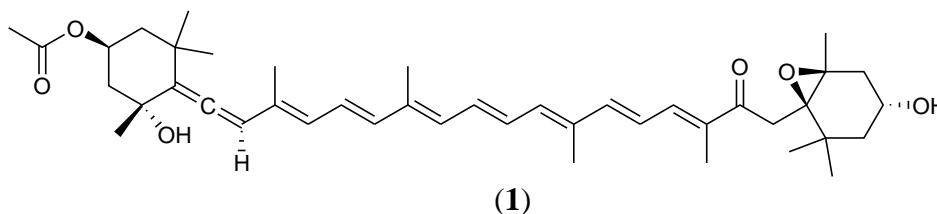
1.1.1 Medicinal uses of seaweeds

Seaweed usage dates back to 4th and 6th century BC in Japan and China, respectively for *Laminaria* species (Madhusudan, *et al*., 2011; Wang, *et al*, 2008). Their oldest medicinal references are rooted in Traditional Chinese Medicine, (TCM) (Hetzel, 1989; Liu, *et al*., 2012). However, the algae origins and use varies according to species and location. In the history of

East Asia, seaweed usage was predominantly nutritional but medicinal uses were also notable. Wakame (*Unnidaria pinnifida*) and Kombu (*Laminaria japonica*) remain as Asian nutritional staples for iodine content and high fibre sources. The Chinese text Materia Medica outlines the ancient use for treating goiter, phlegm accumulation, and "cleansing heat," a key principle to restoring balance in TCM (Hsu *et al*, 1988). Usage in the Western world occurred later, with earliest documentation dating back to the Greek and Roman empires, where mucilage was used to treat rashes and burns (Karleskint, *et al*., 2010). Atlantic wakame (*Alaria esculenta*) is more commonly found around The British Isles (Guiry, 2010). European uses began at sea involving scurvy treatment and parasite elimination (Karleskint, *et al*., 2010). Administration of given seaweed is dependent on species and the type of ailment. For nutritional purposes seaweed is ingested raw or cooked, fresh or dried (McHugh, 2003). Medicinal uses ranges from physical application, to multiple herbal extractions/concoctions, utilized for severe goiter treatment (Bensky and Barolet, 1990; McLaughlin, 1991).

Seaweeds therefore offer a wide range of therapeutic possibilities both internally and externally. However, newer notable therapies include, but are not limited to: treatment against obesity and benefits from antioxidant, antiviral and anti-cancer properties (Smith, 2004). Various studies have described different active components, depending on the nature of ailment being treated. Brown seaweed in particular has a combination of active components. The term is for species in which compound **1** predominates over chlorophyll, giving the plant its brown colour and majorities are found in cold waters (Guiry, 2010; McHugh, 2003). The most widely reported phytochemicals thus far include: phlorotannins (phenols), fucoxanthins, and fucoidan. Botanically, a particular seaweed's placement as brown, red or green is determined first by its photosynthetic pigments, reproductive mode, micro and macro morphologies, and finally by its

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phycopolymers. However, the obvious visual color of a particular seaweed species may not necessarily match its taxonomic color.

Primarily, terrestrial plants have been studied extensively, especially in search for compounds that may treat diseases that face modern mankind. Most medicines are made up of compounds that have been obtained directly from such plants or animals. However, marine plants on the other hand have not played as significant a role as terrestrial plants. The literature on algae and their uses outlines just how much work has been done on seaweeds worldwide. They have been used as traditional medicines by maritime nations as vermifuges (US Army Department Headquarters 2004), ointments and treatment of coughs, wounds, gout, goitre, hypertension, venereal diseases, cancer and a variety of other illnesses. A potent vermifuge like kainic acid (**2**) has been produced by a red alga, e.g. *Digenea simplex* (Moloney, 1998). Consequently, crude extracts of many species have been shown to possess antioxidant and anti-inflammatory activities (Kim, *et al*., 2009; Nakamura, *et al*., 1996), radical scavenging activity (Li, *et al*., 2009), anti-cancer (Hashida, *et al*., 2008), anti-allergic (Li, *et al*., 2008), anti-plasmin (Nakayama, *et al*., 1989), antibacterial (Nagayama, *et al*., 2002), HIV-1 reverse transcriptase and protease inhibitor activity (Queiroz, 2008; Artan, *et al*., 2008), acetyl cholinesterase inhibitory activity (Myung, *et al*., 2005; Yoon, *et al*., 2008), and tyrosinase inhibitory activity (Heo, *et al*., 2009, 2005). From the crude extracts, pure compounds have been isolated and characterized.

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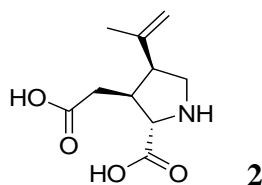
Furthermore, although seaweeds and their constituents have a promising drug research and development period ahead of them, there are noted adverse side-effects. Studies have

associated algae with adverse effects to some extent like: iodine poisoning (Natural Standard, 2009); hypothyroidism (Crawford, *et al.*, 2010); thyrotoxicosis (Bocanegra, *et al*, 2009) and high platelet aggregation via high concentrations of fucoidan (de Azevedo *et al*, 2009). Primarily, the side-effects may be due to excessive dosage.

There are other types of seaweeds used topically in facial treatments, body wraps, and baths, with claims of improvements in blood circulation, detoxification, acne treatment, skin moisturizing, purification, exfoliation, or rejuvenating effects (deRoeck-Holtzhauer, 1991). The use of marine algae in this manner constitutes one aspect of therapy, a discipline based on the belief that "The Sea washes away all of the ills of mankind," (Euripides, 480 BC). Furthermore, many medical experts in the modern world are using topical seaweed body therapies to promote skin beauty and health. Seaweed body treatments are believed to provide surface minerals that can diffuse into the skin, and they provide pleasurable and relaxing experiences when applied in the correct context. Invariably, cosmetic therapists have the belief that marine algae can enhance blood circulation, stimulate local metabolism in the skin, flush out toxins, and assist in improving skin tone and smoothness, perhaps by inducing a mild dermal brasion (deRoeck-Holtzhauer, 1991). Certain seaweed applications act as moisturizers and toners for the skin. Primarily, specific applications on body treatments include attempts to detoxify the body, treat cellulite, enhance lymphatic drainage, clear limb swelling (oedema), and even provide rejuvenating qualities (deRoeck-Holtzhauer, 1991). Seaweeds "scrubs" are also excellent for skin exfoliation. The evidence-base for these beneficial effects of the topical application of the algae remains arguable in the medical literature (deRoeck-Holtzhauer, 1991). In addition to the extensive topical use of seaweed, many types of macroalgae are treasured as dietary inclusions, supplements, or sources of pharmaceuticals (Maeda, *et al*., 2005).

1.1.2 Consumption of seaweeds

Seaweeds have been consumed in Asia since ancient times, but only to a much lesser extent in the rest of the world (Chapman, *et al*., 1980; Indergaard, and Ostgaard 1991). Nutraceutical seaweeds are a rich source of dietary fiber, minerals, and proteins (Lahaye, 1991; Lahaye, and Kaeffer 1997; Mabeau and Fleurence 1993; Fleurence, 1999; Rupe´rez and Saura-Calixto 2001; Jiménez-Escrig and Sánchez-Muniz, 2000). Consumption of unprocessed dried seaweeds can yield many healing benefits. Many physical ailments in both humans and animals may be steadily alleviated with the simple addition of seaweeds to their respective diets. Although therapeutic seaweed constituents can be extracted as crude or pure isolates, chronic conditions usually demand patients eating the whole seaweed material and not just the extracts. Preference is that the seaweed should be eaten raw (Ryan, 2008). Consequently, all essential minerals are provided by dietary seaweeds and no land plant comes close to seaweeds as sources of metabolically-required minerals (Bergner, 1997).

Seaweeds can provide minerals often absent from freshwater and food crops grown on mineral-depleted soils. In addition to their regular consumption, farming on food crops can use copious amounts of seaweeds for mulch and fertilizer. Their extracts may also be sprayed directly onto leaves for foliar feeding through the stomata. Invariably, algae are 20-50 % dry weight mineral (Katsutosi, 2002). This figure is estimated by burning off seaweed's organic material and weighing the remaining ash. The elements abundant in seaweeds include: potassium, sodium, calcium, magnesium, zinc, copper, chlorine, sulfur, phosphorous, vanadium, cobalt, manganese, selenium, bromine, iodine, arsenic, iron, and fluorine. The large brown seaweeds and various kelps tend to contain more minerals per unit weight than red seaweeds. Many human body organs require particular mineral elements as part(s) of their functional

structure and an example is iron for hemoglobin in blood and iodine for thyroxine in the thyroid gland (Katsutosi, 2002).

1.2 Polyphenolics

Polyphenolic secondary metabolites are a large and diverse group of organic chemical compounds which exist both in aquatic macrophytes and in terrestrial plants (Waterman, *et al*., 1994). They comprise tannins, a widespread family of phenolic metabolites present in many plants, which have received much attention from ecologists during recent times (Karban, *et al*., 1997). The word "tannin" comes from the ancient Celtic word for oak, which is a typical source for tannins. These have been traditionally used for many years to tan animal hides to leather (Hagerman, 2002). To date, the term is widely used for large, water-soluble polyphenolic molecules having protein precipitation ability (Strack, 1997). They are divided into three chemically distinct groups based on their structures. There are the hydrolysable ones characterized by a central polyhydroxyl moiety esterified with gallic acid (Gross, 1999). They occur in some green algae and are widely distributed in angiosperms (Waterman, *et al*., 1994). Secondly, the flavonoid-based tannins which are found mainly in woody plants like tea and cocoa (Santos-Buelga and Scalbert 2000). The third group is the phlorotannins, which consist of polymers of phloroglucinol units and is restricted to the brown algae (Ragan, *et al*., 1986).

During the last two decades, the roles and functions of phlorotannins have been the subject of many studies, particularly their possession of many biological activities. Recently, phloroglucinol derivatives from brown algae have received much interest due to their broad therapeutic properties. Furthermore, these phlorotannins have been shown to exhibit activities that include anti-diabetes (Okada, *et al*., 2004), anti-oxidation (Ahn, *et al*., 2007; Shibata, *et al*., 2008), anti-cancer (Kim, *et al*., 2006), anti-inflammation (Kang, *et al*., 2007; Crockett, *et al*.,

2008; Jesse, *et al*., 2007), anti-HIV (Ahn, *et al*., 2004; Schaeffer and Krylov, 2000). Recently, there have been several reports about anti-allergic activity from the phloroglucinol compounds (Sugiura, *et al*., 2006; 2007).

Tannins and related phenolic substances have primary roles by serving as osmoregulatory substances in sea grasses and cell wall components in both marine vascular plants and algae (Arnold, *et al*., 2002). Consequently, the tannins affect palatability due to their astringent taste and may act as potential antioxidant agents. It is also possible that they interact strongly in redox reactions of plants (Da´valos, *et al*., 2003; Luck, *et al.*, 1994; Larson, 1997).

1.2.1 Phlorotannins

In marine brown algae (class Phaeophyceae), the only group of tannins present is the phlorotannins. They accumulate in large quantities and may help protect the algae against predators or epiphytes (Ragan, *et al*., 1978). Most of these materials exist as intermediate molecular weight "oligomers" and high-molecular weight "polymers" based on phloroglucinol (1,3,5-trihydroxybenzene), (Audibert, *et al*, 2010; Apostolidis and Lee, 2010; Fretheim, 1974; Sattler, *et al*., 1974a; 1977b; Ragan, *et al*., 1976a; 1976b). Low-molecular weight congeners such as phloroglucinol itself, "dimers" 2,2',4,4',6,6'-hexahydroxybiphenyl (**3**) and "tetramers", like 2,3',4,5',6-penta- hydroxybiphenyl ether (**4**) are usually present in much smaller amounts. They may constitute up to 15 % of the dry weight of brown algae (Ragan, *et al*., 1986; Targett, *et al*., 1998). The molecular weights of phlorotannins vary from 126 Da to 650 kDa, but are most commonly found in the 10 to 100 kDa range (Boettcher, *et al*., 1993; McClintock, *et al*., 2001).
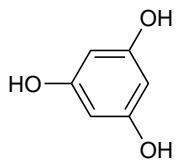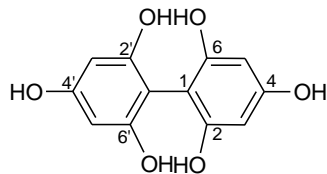
1, 3, 5-trihydroxybenzene   **3**   **4**
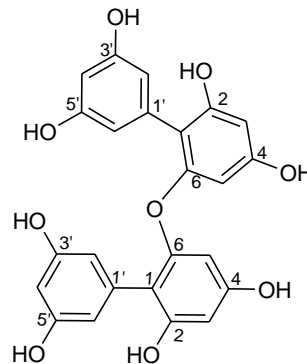
Phlorotannins are one of the most common classes of secondary metabolites in marine angiosperms and macro-algae (Haslam, 1989, Swain, 1979). They are only known from the brown algae and mainly restricted to polymers of phloroglucinol (Ragan, *et al*., 1986). They vary in their linkages between monomeric units, their size and to some extent in the type of substitutions present, that is, hydroxylated groups. However, their abundance and ecological importance in natural systems need to be recognized but the ability to quantitatively measure them in the plant tissues remains a complex issue (Ragan, *et al*., 1986). These compounds are typically large, polar and soluble in water and may be difficult to measure by simple standard techniques as would be the case of analyzing smaller non-polar metabolites such as terpenes and acetogenins. Analysis therefore of such algal polyphenolics is complicated especially with respect to separating the individual compounds from polymeric mixtures (Swain, 1979).

1.2.1.1 Structural diversity

Phlorotannins are dehydro-oligomers or dehydro-polymers of phloroglucinols and intensive investigations have resulted in the structural elucidation of more than 150 compounds (Ragan, *et al*., 1986). Their monomeric units are linked either through aryl-aryl bonds or di-aryl ether bonds forming different sub-groups of phlorotannins (Glombitza and Pauli 2003). When aromatic rings are connected purely by aryl-aryl bonds, a group called fucols is formed, for

example tetrafucol A (**5**). Phlorethols are a group formed solely by aryl-ether bonds, for example, tetraphlorethol B (**6**). Fuhalols are constructed of phloroglucinol units that are connected with para- and ortho- arranged ether bridges with an additional OH- group in every third ring, e.g. tetrafuhalol A (**7**). Consequently, where there exists at least three fused rings, with a centre dioxane, the group is named eckol, e.g. phlorofucofuroeckol (**8**) (Ragan, *et al*., 1986).

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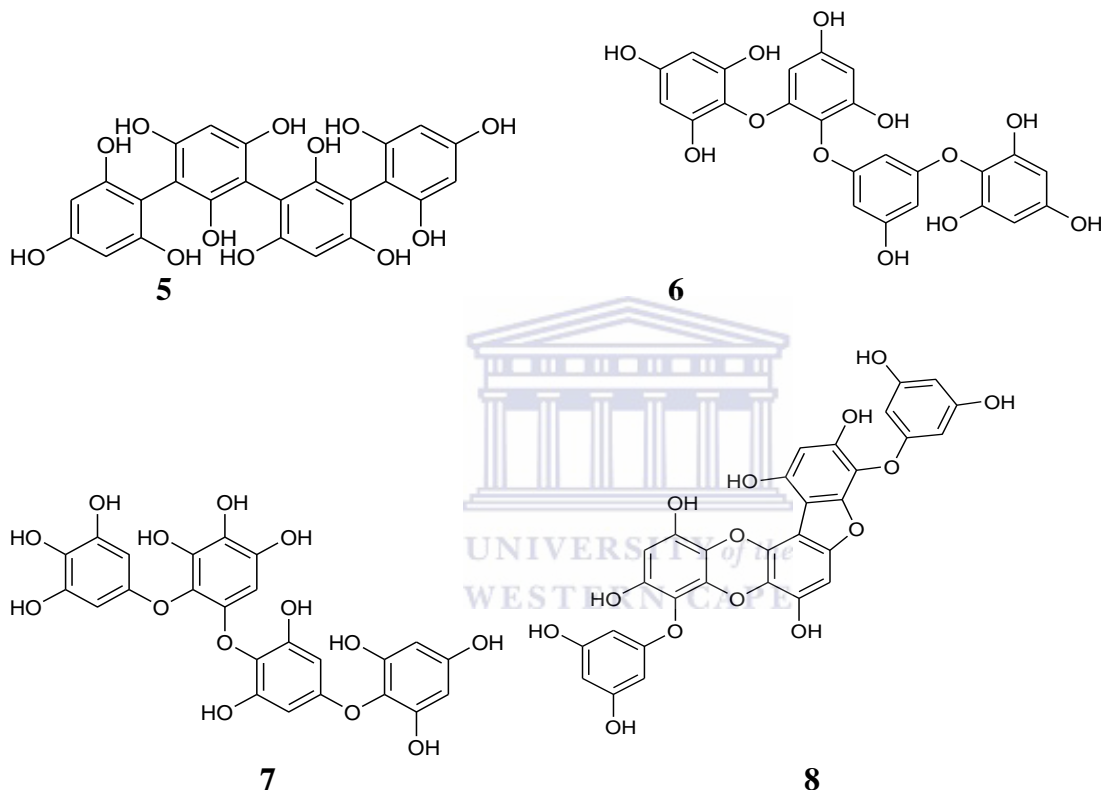
1.2.1.2 Isolation of phlorotannins

There is no single protocol for extracting phlorotannins from all plant material, the procedures used are widely variable (Hagerman, 1988). Use of acetone as the extraction solvent increases the total yield by inhibiting interactions between tannins and proteins (Hagerman, 1988) or even by breaking hydrogen bonds between tannin-protein complexes (Porter, 1989). The well-known feature of free phlorotannins is that they are prone to rapid oxidation (Ragan, *et al*., 1986). In many studies where individual phlorotannins are isolated, they are subjected to

acetylation with acetic anhydride-pyridine to protect them from oxidation (Li, *et al*., 1991b; Glombitza and Pauli, 2003; Sailler and Glombitza 1999). Since free phenols are needed for bioactivity testing, an efficient method for conversion of per-acetylated polyphenolics into free phenolics is by reductive ester cleavage. The cleavage process using lithium aluminium hydride with tetrahydrofuran as solvent is suitable (Keusgen and Glombitza 1997). Subsequently, the addition of ascorbic acid has been effectively used in studies of other tannin groups to prevent oxidation of the extract (Peng and Jay-Allemand 1991; Santos-Buelga, *et al*., 2003). Ascobic acid acts as both an antioxidant and a pro-oxidant (Santos-Buelga, *et al*., 2003). It is believed that the addition of an antioxidant agent increases the stability of tannins.

1.2.1.3 Spectrometric and chromatographic analysis of phlorotannins

In addition to the total content of phlorotannins, knowledge of variation of individual phlorotannin oligomers and polymers would be useful, since their ecological activities and biochemical functions differ from each other. Traditionally, studies of plant phenolics have focused on the total phenolic content of extracts, although it is well known that the group consists of a complex set of different types of individual phenolics. Hydrolysable and condensed tannins are nowadays commonly analysed with modern separation methods (Stern *et al.*, 1996b; Amsler, *et al*., 2006). Due to the high solubility (in water or organic solvents) of phlorotannins, high performance liquid chromatography (HPLC) can offer a suitable tool for qualitative analysis. Modern detection methods, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR), can be combined with HPLC, allowing rapid structural analysis and identification of compounds with minimal manipulation of the sample (Miliauskas, *et al.*, 2006; Schutz, *et al.*, 2006).

Application of liquid chromatography also allows large-scale isolation and purification of

individual compounds. Despite extensive research on the HPLC of plant phenolics, analogous studies for brown algal phlorotannins are rare. The analysis of algal polyphenolics has been suggested to be hampered by the difficulty of separating individual compounds from the polymeric mixture that occur naturally (Stern *et al.*, 1996b). Phlorotannins are difficult to isolate quantitatively due to their large size, structural similarity and reactivity with other compounds. In brown algal studies, HPLC has been applied for isolation of compounds from crude extracts. Per-acetylated alcoholic extracts are eluted through silica columns with various combinations of chloroform, hexane, methanol or ethanol with UV detection varying from 235 nm to 275 nm. Phloroglucinol is reported to have a UV absorbance maximum around 280 nm and the phlorotannin fraction around 260 nm (Swanson and Druehl, 2002; Henry, *et al.*, 2004). The techniques most useful for elucidating structures of phlorotannins include ^{1}H and ^{13}C NMR spectroscopy (Ragan, 1985; Koch *et al.*, 1980; McInnes *et al.*, 1985). The use of mass spectrometry for their analysis has been rare, partly due to low ionisation of acetylated derivatives (Koch *et al.*, 1980; Ragan and Jamieson, 1982; Ragan, 1985; Ragan and Glombitza 1986).

1.2.1.4 Biological roles of phlorotannins

1.2.1.4.1 Primary roles of phlorotannins

Phenolic substances in brown algae are found in physodes, membrane-bound vesicles, which are accumulated at the zygote periphery early in development and are secreted into the primary zygote wall (Schoenwaelder and Clayton, 1998). Physodes, together with other wall components, contribute to the development of the cell wall (Schoenwaelder and Wiencke, 2000). It has been suggested that phlorotannins become components of brown algal cell walls when physodes fuse with the cell membrane and then are secreted from cells, complexing finally with

alginic acid (Lee, *et al*., 2009; Schoenwaelder and Clayon, 1998; Arnold and Targett, 2003). Cell walls of brown algae are mainly composed of polysaccharides: alginic acid, alginates and fucans (sulfated polysaccharides), which comprise up to 40 % of the thallus dry weight (Breton, *et al*., 2011; Mabeau and Kloareg, 1987; Van den Hoeck *et al.*, 1995). Based on microscopic studies, Schoenwaelder and Clayton (1998) suggested that phlorotannins therefore are likely to be integral structural components of brown-algal cell walls. Primarily, phenolic compounds are bound with four major types of bonds; hydrophobic, hydrogen, ionic, and covalent in order of increasing strength (Appel, 1993). Possible linkages between the cell wall and phlorotannins are the ester bonds and the hemiacetal bond, both of which are covalent in nature, thus requiring strong conditions to degrade.

1.2.1.4.2 Secondary roles of phlorotannins

The secondary roles of soluble phlorotannins have been widely studied. They are generally measured as contents of total soluble phlorotannins, which are known to vary due to environmental factors as well as between species and among algal genotypes and populations. Phlorotannin content is found to have elastic responses to environmental factors such as light and nutrient availability, ultraviolet radiation and the intensity of herbivory (Connan, *et al*., 2004; Targett, *et al*., 1998; Amsler, *et al*., 2006; Jormalainen and Honkanen, 2008). Furthermore, they can serve in both constitutive and induced defence against herbivory (Amsler, *et al*., 2006). The inducible defences of plants are phenotypic responses that act to reduce or redistribute the damage inflicted by grazers and therefore decrease the negative fitness consequences of herbivores (Karban, *et al*., 1997).

1.2.1.4.3 Medicinal roles of phlorotannins

There are versatile, medicinal benefits of certain types of seaweed and their derivatives that have been highlighted in literature (Van Den Hoek, *et al*., 1995). While the value of seaweed-derived compounds has attracted much attention as thermogenic agents, seaweeds contain several other functional components that benefit an individual with excessive body weight conditions (Holt, 2008). Primarily, the brown algal phlorotannins have been studied and results have shown their potential health benefits with promising effects against radical-mediated oxidative stress, photodamage, cancer, allergy, diabetes, inflammation, viral and antimicrobial infections (Kim and Himaya, 2011; Zhang, *et al*., 2008; Shanab, 2007). Furthermore, having this vast range of biological activities, they are believed to be candidates for development as nutraceuticals and pharmaceuticals (Apstolidis and Lee, 2010; Yangthong, *et al*., 2009). Medicinal values are related to their structure and primarily the degree of polymerization where the oligo-phenols are generally considered more active than highly polymerized compounds (Toth and Pavia, 2001). A summary of some marine algal phlorotannins and their possible pharmacological activities are shown in Table 1.

Table 1: Bioactive algal derived phlorotannins with possible human health effect

	Specific compound	Health effect	References
E. cava	Dioxinodehydroeckol	Anti-cancer	Kong, *et al*., 2009
E.bicyclis and *E. cava*	Dieckol	Anti-diabetic	Okada, *et al*., 2004; Lee, *et al*., 2009
E. bicyclis	Eckol		Okada, *et al*., 2004
E. stolonifera	Phlorofucofuroeckol-A		Jung, *et al*., 2008
I. okamurae	Diphlorethohydroxycarmalol		Heo, *et al*., 2009a
E. stolonifera	Eckol Phlorofucofuroeckol A Dieckol	Anti-hypertension	Jung, *et al*., 2006
E. cava *E. stolonifera*	Dieckol Eckol	Anti-photoaging	Heo, *et al*., 2009b Joe, *et al*., 2006
E. cava *E. bicyclis*	Dieckol Eckol Fucofuroeckol-A	MMP inhibition	Ryu, *et al*., 2009a Lee, 2010
E. bicyclis *E. cava* *E. kurome*	8,8´-Bieckol Phlorofucofuroeckol A Eckol Dieckol	Anti-oxidation	Shibata, *et al*., 2008

E. cava'	6,6´-Bieckol 8,8´-Bieckol 8,8´-Dieckol	Inhibitor of HIV	Artan, *et al*., 2008 Ahn, *et al*., 2004
E. cava *E. arborea* *E. stolonifera*	6,6´-Bieckol Phlorofucofuroeckol B Eckol Dieckol	Anti-allergy	Le et al. 2009 Sugiura, *et al*., 2007 Joe, *et al*., 2006
E. cava *I. okamurae*	Dieckol Diphlorethohydroxycar malol	Whitening effect and Anti-diabetic	Heo, *et al*., 2009, 2010
E. cava	Dioxinodehydroeckol	Anti-cancer	Kong, *et al*., 2009
E. kurome	Phlorofucofuroeckol A	Algicidal	Nagayama, *et al*., 2003
(Kim and Himaya, 2011; Gupta, S. and Abu-Ghannam, N., 2011)			

1.3 Seaweed carotenoids

Carotenoids cannot transmit sunlight into photosynthetic pathways, but they function as accessory pigments. A prominent accessory, or "helper" pigment, is fucoxanthin (**1**), which is responsible for the "brownish" appearance of kelp (Yan, *et al*., 1999). The ability of many types of seaweeds to be adjunctive to weight control has been known for many years (Miyashita, 2007; Maeda, *et al*., 2005, 2006; deRoeck-Holtzhauer, 1991). Recent interests lies with the ability of antioxidant compounds in these seaweeds, specifically compound **1** that promotes weight loss and has some support for its role as an enhancer of body metabolism i.e. thermogenic effect, with direct effect on fat stores (Miyashita, 2007; Maeda, *et al*., 2005, 2006; Jimenez-Escrig, 2001).

Furthermore, there are also those complex sugars described earlier referred to as fucoids (glucans), which may stimulate immune function. Overall, there is a large amount of scientific literature on the use of seaweeds as health food, soil fertilizers, and sources of medicinal compounds.

Carotenoids are strong antioxidants, related to well-known nutraceuticals such as canthaxanthins (**9**), astaxanthins (**10**), lycopene (**11**), violaxanthin (**12**), and neoxanthin (**13**) (Maeda, *et al*., 2005, 2006). The potent and versatile antioxidant actions of them are implicated in cancer protection, immune function, and anti-aging. The anti-aging benefits have been touted for years (Maeda, *et al*., 2005, 2006). Compound **1**, for example, appears to stimulate proteins that cause the oxidation of fats and its conversion to energy or heat (thermogenesis) (Maeda, *et al*., 2005).

9   **10**

11   **12**

13
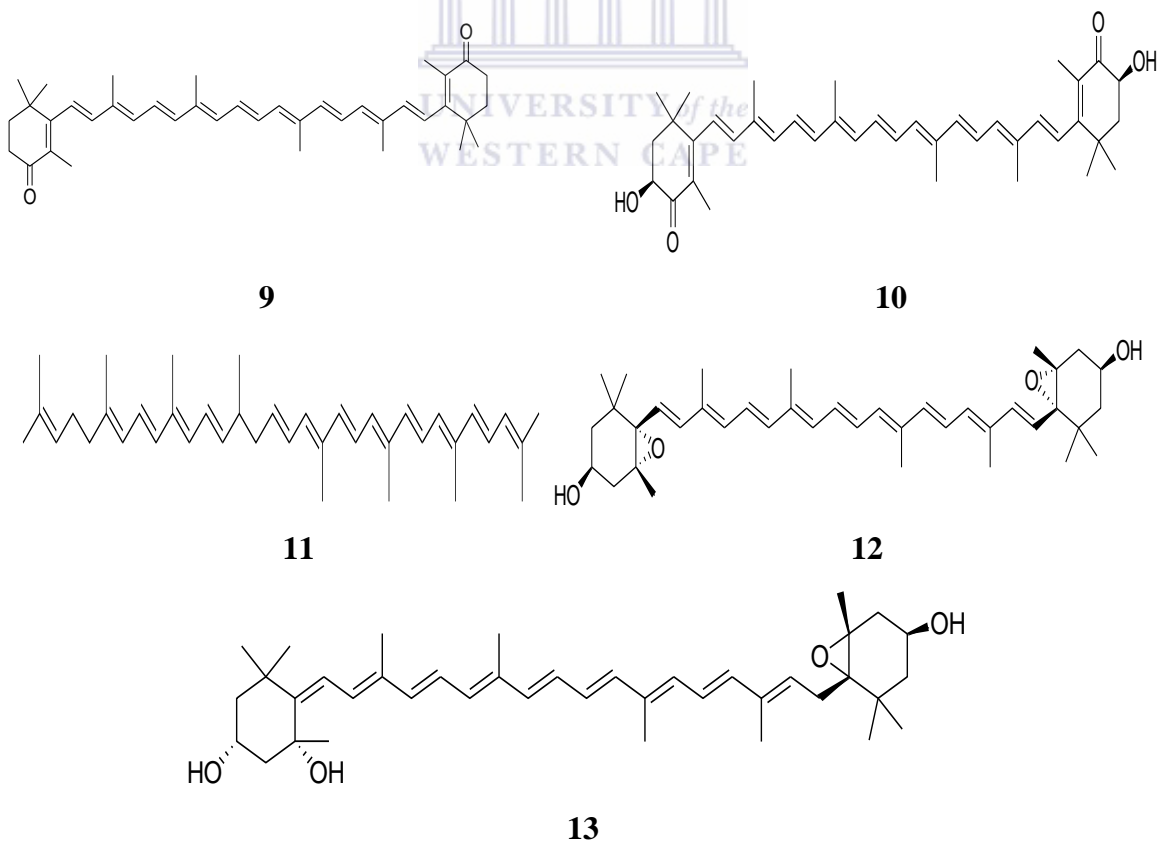
1.4 Seaweeds triterpenoid saponins

Saponins are a vast group of glycosides widely distributed in water plants. Their surface-active properties are what distinguishes them from other glycosides. They dissolve in water to form colloidal solutions that foam upon shaking (Tyler *et al*., 1981). They have been sought after in the pharmaceutical industry since some form the starting point for the semi-synthesis of steroidal drugs. Many have pharmacological properties (Estrada *et al*., 2000) and are used in phytotherapy and in the cosmetic industry. Furthermore, the presence of these saponins justifies the efficacy of algae as skin care products. According to Liu and Henkel (2002), saponins and polyphenols are key ingredients in traditional medicines and they are responsible for most of the observed biological effects. Saponins are classified into two groups based on the nature of their aglycone skeleton. The first group consists of the steroidal saponins, while the second group consists of the triterpenoid saponins, which are the most common and occur mainly in the dicotyledonous angiosperms (Bruneton, 1995). Steroidal saponins consist of a steroidal aglycone (**14**), a C-27 spirostane skeleton, generally comprising of a six-ring structure. Triterpenoid saponins consist of a triterpenoid aglycone (**15**), which consists of a C30 skeleton, comprising of a pentacyclic structure.

14   **15**   R= sugar moiety
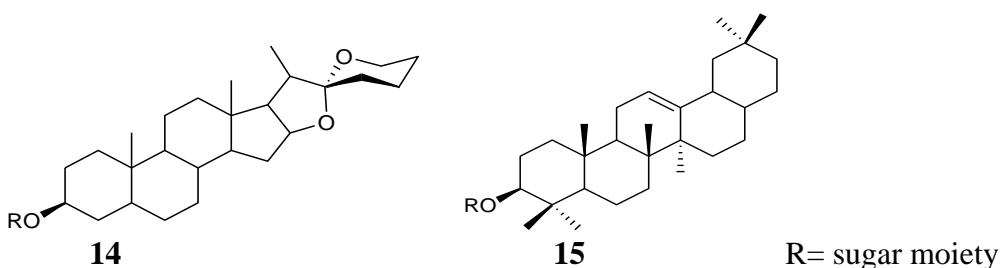
1.5 Seaweed lipids

Seaweeds have been studied for long and valued for their production of industrially important polysaccharides. However, they have not been considered in depth as sources of lipids

since they are found in small quantities (Ramavat, *et al*., 1997). Nonetheless, interest has been on the rise due to recognition of important bioactive molecules like compound **1** as pigments and conjugated fatty acids that possess important physiological effects in the treatment of tumors. Furthermore, there are phospholipids containing polyunsaturated fatty acids having strong anti-cancer effects (Noguchi, *et al*., 2001). Examples of PUFAs have members like arachidonic acid (**16**), linoleic acid (**17**) and eicosapentaenoic acid (**18**) amongst others.
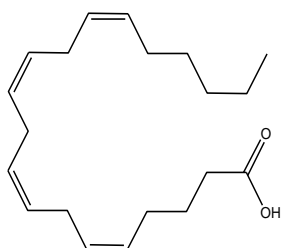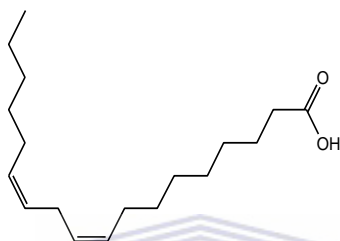
| **16** | **17** | **18** |

1.6 Polysaccharides

Polysaccharides are polymers of various monosaccharides and major components of marine algae representing over 60 % of dry weight (Rioux, *et al*., 2007a; Marinho-Soriano, *et al*., 2006). Each polymer has specific chemical characteristics, particularly in terms of molecular mass, degree of polymerization, degree of branching, types of monosaccharide units and types of linkage. These characteristics vary among them within and between algal species. Some monosaccharide units bear certain substituents such as sulfate which have an important effect on functionality. For example, the presence, position and type of sulfate groups determine the physicochemical properties of polysaccharides such as fucoidan (Rioux, 2007a). Marine algae are known to have large amounts of material which are not digestable in the human digestive system and therefore may serve as new sources of dietary fiber, prebiotics or other functional ingredients (Lahaye, 1991; Mabeau and Fleurence., 1993).

Seaweed fiber is interesting because its consumption has been associated with a signific-

ant reduction of chronic diseases such as diabetes, obesity, heart diseases, cancer and so on. It has been shown that the physiological effects of the fiber are related to their properties hence classified into soluble and insoluble fractions (Roehring, 1988). Soluble ones slow down digestion and absorption of nutrients by increasing viscosity and might thereby decrease blood sugar and cholesterol. In contrast, the insoluble type decreases intestinal retention time and faecal mass (Mabeau and Fleurence., 1993). There are three groups of algal polysaccharides based on their roles: structural, intercellular mucilage and storage polysaccharide. Composition differs widely among green, brown and red algae, and in general offer a wide range of commercially valuable physicochemical properties for the food, pharmaceutical, cosmetics and other industries. Therefore, seaweeds serve as good sources of polysaccharides and their uses in preparation of grafted materials with biomedical applications adds value to them (Sharma, *et al*., 2013).

There are a class of macromolecules which are increasingly gaining attention in the biochemical and medical areas due to their immunomodulatory and anticancer effects (Koyanagi, *et al*., 2003; Zubia *et al*., 2009). These are present primarily in the cell walls and the composition varies according to season, age, species and geographic location. Their ability to form branched structures distinguishes them from other biological macromolecules such as proteins and nucleic acids, which occur only as linear polymers.

Polysaccharides are known to include not only those substances composed of glycosidically linked sugar residues, but also molecules that contain polymeric saccharide structure linked via covalent bonds to amino acids, peptides, proteins, lipids and other structures. These are referred to as glycans. A polysaccharide containing only one kind of monosaccharide molecule is classified as a homopolysaccharide, or homoglycan, whereas those containing more

than one are heteropolysaccharides. The most common constituent in them is D-glucose, but D-fructose, D-galactose, L-galactose, D-mannose, L-arabinose, and D-xylose are also frequent. Some monosaccharide derivatives found in them include the amino sugars (D-glucosamine and D-galactosamine) as well as their derivatives (*N*-acetylneuraminic acid and *N*-acetylmuramic acid) and simple sugar acids (glucuronic and iduronic acid) (Zubia *et al*., 2009).

Chemical structures of polysaccharides have been investigated extensively in the past (Chizhov *et al*., 1998; Duarte *et al*., 2001; Fleury *et al*., 1991; Graham and Wilcox, 2000). Fucoidans and laminarans are the most interesting ones for their potential biological activities whereas alginates are mainly used as food ingredients. Fucoidan is a complex sulfated polysaccharide, usually isolated from brown algae whose main monosaccharide unit is L-fucose (Barashkov, 1972; Usov *et al*., 1988). The exact structural characteristics have not yet been fully elucidated, and only little regularity is known at the present time. The molecular weight of various fucoidan fractions ranges in order of magnitude from 10^3 to 10^5 kDa which influences the solubility of these polysaccharides and determines their extraction properties. Variations are observed between species which have an impact on the determination of the polysaccharide structure. These sulfated fucoidans have been proven to possess a wide range of bioactivities such as antiviral, anti-ulcer and anti-tumoral properties (Shaeffer *et al*., 2000, Nagaoka *et al*., 1999, Grauffel *et al*., 1989, Pereira *et al*., 1999). Structures of polysaccharides varies from one part of the plant to the other (Chevolot *et al*., 1999). The yield and composition depends on environmental factors, type of species from which they are obtained (Lahaye and Robic 2007) and the season (Abdel-Fattah, *et al.,* 1973; Lahaye, *et al*., 1999; Medcalf *et al*., 1975; Robic *et al*., 2009c) as well as the extraction method employed (Percival 1964; Medcalf *et al*., 1975; Ray, and Lahaye 1995; Robic *et al*., 2008).

Brown seaweeds, as algae of interest, primarily are known to produce three different polysaccharides; alginates, fucoidans and laminarans. The last two are the main water-soluble types whereas high-molecular mass alginic acids are alkali-soluble. There are two kinds of acid polysaccharides present in the extracellular matrix; sulfated fucans and alginic acid. Fucans comprises of: fucoidans, xylofucoglycuronans and glycuronogalactofucans (Jimenez-Escrig and Sanches-Muniz., 2000; Bui, *et al*., 2005). Fucoidans (Figure 1) are branched sulfate esters with L-fucose 4-sulfate building blocks as the major component. They are soluble in water and in acid solution (Ruperez *et al*., 2002). Acid hydrolysis of fucoidan yields various amounts of xylose, galactose, and uronic acid, in addition to L-fucose.

These fucans as cell wall polysaccharides are composed of variable amounts of monosaccharides, and their precise chemical structures remains unknown even though some different structural models have been proposed (Percival, *et al*., 1967; Pantakar, *et al*., 1993). Their structures range from high-uronic-acid, low-sulfate, fucose-containing polymers to highly sulfated fucoidan (Maboau, *et al*., 1990). Fucoidans (details in section 1.7) have been extensively studied due to their numerous interesting biological activities. In the past few years, several of their structures have been solved, and many aspects of their biological activity have been elucidated. Among all the diverse sulfated polysaccharides, they appear to be the most prominent from the pharmaceutical view point.
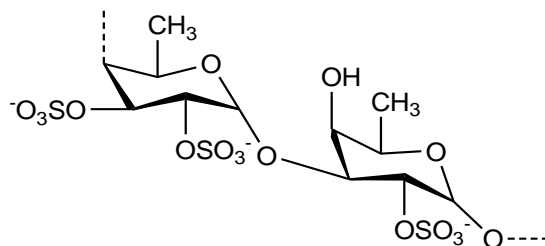
Figure 1: A branched polysaccharide sulfate ester

Cell walls characteristically contain such sulfated polysaccharides which are not found in land plants and have specific functions in therapeutic, medicinal and agricultural applications (Lahayo, *et al.,* 1997; Kloareg and Quatrano 1988; Percival and McDowell 1967). Alginates are mainly cell wall polyuronides which consist of alternating units of mannuronic acid and glucuronic acid, their solubility depending on pH. Their gelling characteristics are influenced by the constituent uronic acid ratios and the presence of divalent cations. Their different thickening and gelling properties find wide applications in the food and pharmaceutical industry (Chapman and Chapman 1980); Indergaard and Minsaas 1991).

1.6.1 Structural analysis of polysaccharides

The process begins with the extraction of a crude polysaccharide that is subjected to some physical methods through hydrolysis. Isolation of more purified samples passes via ion exchange process. Subsequently, derivatization of purified samples in order to determine the monosaccharides composition and their molecular weight.

Therefore, structural analysis requires specialized techniques, which differ significantly from those methods used for small molecules and other biopolymers. For an understanding of the primary structure of a polysaccharide, essential information such as monosaccharide composition, nature and molar ratios of the monosaccharide building blocks is important. Furthermore, linkage patterns and positions between furanosidic and pyranosidic rings should be known. The anomeric configuration of the glycosidic linkages, sequences of monosaccharide residues and repeating units and nature of OH-modifications, such as *O*-phosphorylation, acetylation, *O*-sulfation, etc, must be determined (Cui, *et al*., 2000).

To all natural products, characterization of a polysaccharide requires first that it be taken to the highest possible level of purity, which is reflected by its chemical composition, including

total sugar content, levels of uronic acid, proteins, ash, and moisture. The second step is the determination of monosaccharide composition, both qualitatively and quantitatively. This is readily done using high performance liquid chromatography (HPLC) or gas-liquid chromatography (GLC) after complete acid hydrolysis (Zhang, 2001; Cui, *et al*., 2000).

The ring size and glycosidic linkage positions of the sugar units are then established by methylation analysis and reductive cleavage. Furthermore, the anomeric configuration can be determined with modern techniques, such as fast atom bombardment (FAB) Mass Spectrometry to afford more structural information (Khoo and Dell 1990). Two dimensional (2D) NMR spectroscopy has proved to be the method of choice for the purpose due to the characteristic chemical shifts and coupling constants of the anomeric signals from both ^{1}H and ^{13}C. Invariably, the use of highly specific, well characterized and purified enzymes can also give information leading to linkage position and configuration of the hydrolyzed sugar residues.

1.6.1.1 GC/MS (Gas Chromatography/Mass Spectrometry)

Preliminary analysis involves the generation of hydrolyzates by use of acid like trifluoroacetic acid. Certain conditions of temperatures must be met during the process. The samples are reduced then acetylated in order to generate alditol acetates. Such acetates are soluble in chloroform which is a volatile solvent used during their GC analysis. By gas chromatography, all vaporisable (organic) substances can be separated. Common mobile phases used are helium, argon and nitrogen. Samples are injected as fluids into the gas flow using a syringe. Due to different characteristics in absorption and solubility, the substances migrate with different velocities through the stationary phase (Cui, et al., 2000). At the end of the column, there is a detection system that measures the leaving substances. Polysaccharides are not measured directly and hence must be hydrolysed to produce monosaccharides. The smaller

molecules subsequently get acetylated to form thermal stable ethers that are analysed by GC. The MS system is added to the GC separation that creates charged particles from the eluted molecules. The ions are analysed to provide information about the molecular weight of the compounds present (Cui, et al., 2000).

1.6.1.2 Ion Exchange Chromatography purification

Ion Exchange Chromatography belongs to the liquid separation systems. Separation of the sample molecules results from their charges. In this case, the anionic polysaccharides bind to the positively charged ligands attached to the filling material of the column (Sinner, *et al*., 1975). Using increasing ionic strength NaCl salt gradient, the different parts of the sample can be eluted. Some examples of the matrix used may consist of special cellulose or DEAE sepharose (Cui, *et al*., 2000; Sinner, *et al*., 1975).

1.6.1.3 Derivatization of carbohydrates for HPLC analysis

Derivatization of carbohydrates normally results in improved retention and separation of isomers on C-18 reversed phase columns and traditionally has provided UV absorbance or fluorescence, allowing the use of detectors (Fu and O'Neil 1995; Kang, *et al*., 1990; Meyer, *et al*., 2001). Consequently, many of the derivatives also provide increased MS sensitivities due to the higher ionization and fragmentation efficiencies relative to the underivatized carbohydrates (Saba, *et al*., 2001a). The derivatization of reducing carbohydrates exploits the open ring aldehyde form of carbohydrates. Techniques such as benzoylation or the use of hydrazine derivatives result in anomeric mixtures for reducing carbohydrates (Usui, *et al*., 2007; Lamari, *et al*., 2003; Perez and Colon 1996). Reductive amination, resulting in a single derivative, however, requires a two-step reaction process (Lamari, *et al*., 2003; Dalpathado, *et al*., 2005). A simple and quantitative derivatization process for reducing carbohydrates using 1-phenyl-3-methyl-5-

25

pyrazolone (PMP) (**19**) forming a single bis-PMP carbohydrate (**20**) derivative has been described (Figure.2) (Fu and O'Neil 1995; Honda, *et al*., 1989; Zhang, *et al*., 2003; Saba, *et al*., 1999). The mass spectral properties of these PMP carbohydrate derivatives provide excellent positive ion electrospray ionization and fragmentation efficiency for detection. The derivatives also exhibit enhanced retention and separation properties with compatible mobile phases.
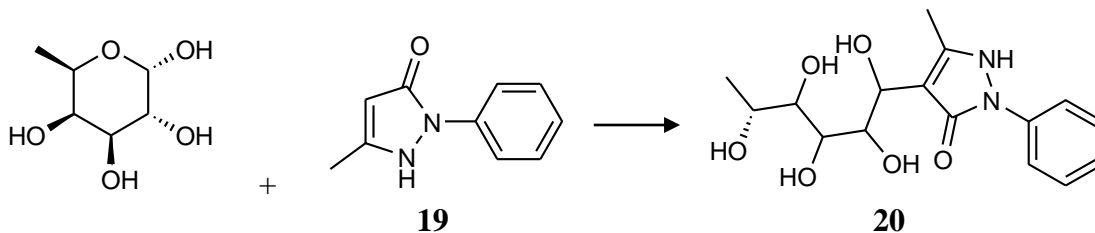
Figure 2: Derivatization of a reducing carbohydrate with PMP

1.6.1.4 Determination of linkage patterns

Methylation analysis followed by reductive cleavage, procedures have been used to determine the structure of carbohydrates for a long time and it is still the most powerful method in carbohydrate structural analysis (Ďurindová, *et al*., 1992; Ciucanu and Kerek 1984; Dell, 1990). Primarily, the analysis process consists of two steps; chemical derivatization and Gas-liquid chromatograph-mass spectroscopy (GC-MS). The derivatized polysaccharides after hydrolysis are reduced and acetylated to give volatile products, i.e., partially methylated alditol acetates (PMAA), which can be identified and quantitatively determined by gas-liquid chromatography equipped with a mass spectroscopic detector (GC-MS). The substitution pattern of the *O*- acetyl groups on the PMAA reflects the linkage patterns and ring sizes of the corresponding sugar in the original polymer (Cui, 2005). However, this method gives no information on sequences or the anomeric configuration of the glycosidic linkages. It also cannot distinguish whether an alditol is derived from an aldopyranose or a corresponding aldofuranose (Cui, 2005). These drawbacks may be over come by reductive cleavage method.

In many instances, sequencing of polysaccharides is challenging to achieve due to the heterogeneous nature of the structure, high molecular weight, and poly-dispersity of the polymer chains (Cui, *et al*., 2000). Naturally occurring polysaccharides usually have structural regularity, and some have repeating units. However, these are frequently interrupted by other sugar units or have substitution groups or branches along the polymer chains. For example, with cellulose, a linkage pattern like (1→3) may be inserted to the main molecular chain to form a (1→3) (1→4)-mix-linked D-glucan (Cui, *et al*., 2000). Furthermore, in nature, many polysaccharides contain multiple monosaccharides and some may contain up to six neutral sugars and uronic acids with variations in linkage patterns. Thus, one should appreciate how difficult it could be to elucidate the complete structure of such complex polysaccharides (Cui, *et al*., 2000).

1.6.1.4.1 Reductive Cleavage method

One major drawback of methylation analysis is that some of the structural information, such as anomeric conformation, gets lost during the conversion of sugars into alditols. Furthermore, the analysis may not distinguish if an alditol is from a 4-*O*- linked aldopyranose or from its corresponding 5-*O*- linked aldofuranose because both sugar units will give the same alditol according to a standard methylation procedure (Figure.3). Reductive cleavage hence can solve such problems (Jun and Gary, 1987).
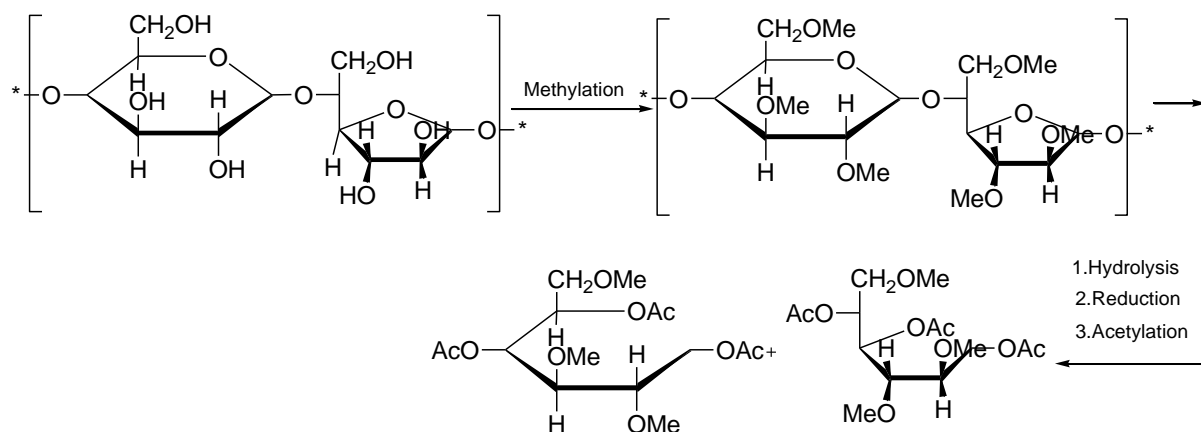
Figure 3: A standard illustration of similar PMAA

However, in the cleavage reduction, the methylated polysaccharide is depolymerized by using triethylsilylane (TES) and trimethylsilyl methanesulfonate (TMSO) to give partially methylated anhydroalditols. Further, these products are acetylated only at the second linkage position of the sugar ring since the anomeric position is deoxygenated. As a result, different reaction products are obtained depending on whether pyranosides or furanosides were cleaved (Figure 4) (Kiwitt-Haschemie, *et al*., 1996).
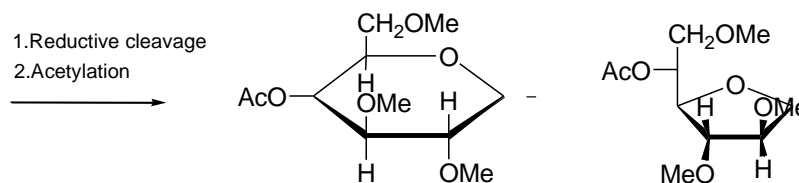
Figure 4: Different products of a 4-*O*-linked aldopyranose and its corresponding 5-*O*-linked aldofuranose

1.6.1.4.2 Peroxidation

Polysaccharides containing free hydroxyl groups have the potential to react with oxidizing agents. The oxidation reaction can be used to elucidate structural information of polysaccharides. For example, vicinyl-glycols react with periodic acid or its salts to form two

aldehydic groups upon the cleavage of the carbon chain as shown in figure 5 (Cui, *et al*., 2000).
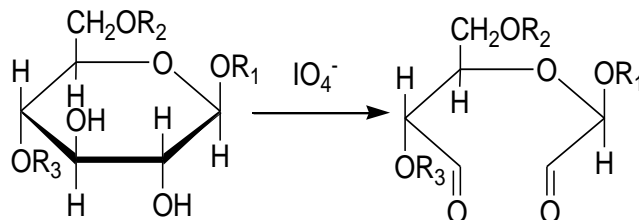
Figure 5: Periodate oxidation of a sugar unit forming aldehydic groups

Furthermore, polysaccharide sugar units with different linkage patterns vary significantly in the way they react with periodates. For example, non-reducing end sugar residue and 1→6-linked non-terminal residues have three adjacent hydroxyl groups; double cleavages will occur and the reaction consumes two molar equivalents of periodate and gives one molecular equivalent of formic acid as shown by Figure 6 (Cui, *et al*., 2000; Angyal and James, 1970).

Primarily, non-terminal units, such as 1, 2-or 1, 4-linked residues consume one equivalent of periodate without formation of formic acid. Sugar units that do not have adjacent hydroxyl groups, such as 1, 3-linked residues or branched at C-2 or C-4 positions will not be affected by this reaction. Nonetheless, a quantitative determination of periodate consumed and the formic acid formed, combined with the information on the sugar units surviving the oxidation reaction, will provide clues to the nature of the glycosidic linkage and other structural features of the polysaccharides (Cui, *et al*., 2000).
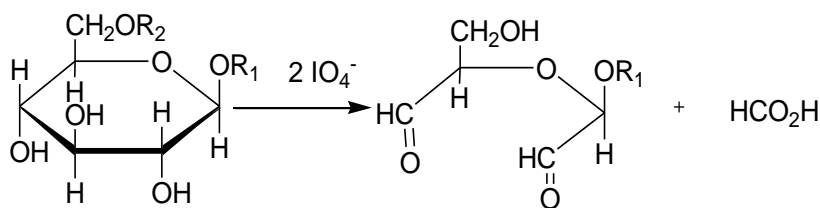
Figure 6: Periodate oxidation of a sugar consuming 2IO$_4^-$ to form two aldehydic groups and formic acid

1.7 Sources and structural characterization of fucoidans

1.7.1 Sources

Fucoidans, sulfated polysaccharides of great interest, are found in various marine sources. This may be from sea cucumber (Ribeiro, *et al*., 1994), sea urchin (Mulloy, *et al*., 1994; Vilela-Silva, *et al*., 2002, 1999) or brown algae (Descamps, *et al*., 2006). In recent years many different algae and invertebrates have been analysed for their content of fucoidans. Recently, interests on them have been focusing primarily on the antitumour, anticoagulant, and antioxidant activities, as well as activities against liver and urinary system failures (Ale, *et al*., 2011). As more scientists continue to explore this unique polysaccharide type, more of its biological health benefits are being discovered (Hayashi, *et al*., 2008; Wijesinghe, and Jeon, 2011). Table 2 shows a summary of the chemical composition of some brown seaweeds of marine origin. All these sources contain different forms of fucoidan. They are extracted in special ways in order to obtain high yields, subsequently analysing the properties of their extracts (Obluchinskaya and Minina, 2004).

Table 2: Brown seaweeds and their chemical composition

Brown seaweed	Chemical composition
Adenocytis utricularis (Ponce, *et al*., 2003)	Fucose, galactose, mannose, sulfate
Ascophyllum nodosum (Percival, *et al*., 1968)	Fucose, xylose, glucuronic acid, sulfate
Bifurcaria bifurcate (Mian and Percival, 1973)	Fucose, xylose, glucuronic acid, sulfate
Dictyota menstrualis (Albuquerque, *et al*., 2004)	Fucose, xylose, uronic acid, galactose, sulfate
Ecklonia kurome (Nishino, *et al*., 1989)	Fucose, galactose, mannose, xylose, glucuronic acid, sulfate
F. distichus (Bilan, *et al*., 2004)	Fucose, sulfate
F. evanescens (Bilan, *et al*., 2002)	Fucose, sulfate
F. serratus L. (Bilan , *et al*., 2006)	Fucose, sulfate
F. vesiculosus (Black, *et al*., 1952; Nishino, *et.*, 1994)	Fucose, sulfate
Hizikia fusiforme (Li, *et al*., 2006)	Fucose, galactose, mannose, xylose, glucuronic acid, sulfate
Laminaria angustata (Kitamura, *et al*., 1991)	Fucose, galactose, sulfate
Lessonia vadosa (Chandia, *et al*., 2003)	Fucose, sulfate
Macrocytis pyrifera (Black, *et al*., 1952)	Fucose, galactose, sulfate
Padina pavonia (Hussein, *et al*., 1980)	Fucose, xylose, mannose, glucose, galactose, sulfate
Pelvetia wrightii (Anno, *et al*., 1996)	Fucose, galactose, sulfate
Sargassum stenophyllum (Duarte, *et al*., 2001)	Fucose, galactose, mannose, sulfate

| *Spatoglossum schroederi* (Rocha, *et al*., 2005) | Fucose, xylose, galactose, sulfate |
| *Undaria pinnatifida* (Lee, *et al*., 2004) | Fucose, galactose, sulfate |

1.7.2 Effect of seasonality and locality on fucoidan levels in brown algae

The fucoidan content differs between the intertidal zone and the zone under the low water line, high and low amounts respectively. Furthermore, there have been discoveries that the sugar content of the algae gradually increases during those periods the algae are exposed to higher amounts of sunlight whose UV light may destroy cell constituents (Honya, *et al*., 1999) as shown in Table 3.

Table 3: Effects of seasonality

	Fucoidan fractions, % of the dried seaweed weight		References
Species	Periods		
	Spring	Autumn	
E. radiata	0.4	1.4	Chapman, 1970
D. aculeata	4.8	2.2	Percival, *et al*., 1974
M. pyrifera	4.1	3.8	Cruz-Suarez, *et al*., 2002

1.7.3 Extraction of fucoidan

Various extraction methods have been studied and employed to produce and preserve high quality fucoidan. The precise structure of fucoidan is still being debated, mainly due to difficulty in extraction and purification procedures (Marais and Jacob, 2001). Most extraction methods tend to extract it as a multicomponent crude form, commonly called 'crude fucoidan' (Eluvakkal, *et al*., 2010). In order to obtain purified fucoidan, ethanol precipitation, ion-exchange

chromatography or gel filtration can be applied to the crude form. Raw seaweeds are usually extracted with acid or base solutions as the solvent (Bilan, *et al*., 2002; Yang, *et al*., 2008) but water is now frequently used to extract so as to maintain the stability of the molecular weight and overall charge of the polysaccharide (Li, *et al*., 2008). Water as a solvent is critical in producing high quality fucoidan, in addition, it ensures that the extracted material retains its natural bioactivity (McNally, 2007).

Extraction with acidic solvents like hydrochloric acid (HCl) gives higher yields of crude fucoidan (Kawamoto, *et al*., 2006) but this has resulted in the extraction of undesirable products such as alginic acid and metals (Hagiwara, 2010) and as such, causing degradation of the fucose chains (Ale, *et al*., 2011). Invariably, the use of solutions of salts like calcium chloride ($CaCl_2$) is effective in removing insoluble components which could affect the purity of the fucoidan (Kawamoto, *et al*., 2006; Umeda, *et al*., 2003) but in turn can lower the yield of crude fucoidan (Hagiwara, 2010). High quality fucoidan should contain less than 0.1 % of contaminating proteins. Therefore, protein content determination must be carried out to estimate the purity of fucoidan and the effectiveness of a given extraction process (Hayakawa, *et al*., 2009). Chemical composition also varies according to the season, geographic location, species, and maturity of the plant (Rioux, *et al*., 2007). The extraction technique therefore used has a large impact on the determination of the final structure (Rioux et al., 2007).

As Ponce *et al.* (2003) reported, fucoidan extracted at room temperature and at 70 °C had completely different chemical compositions. For example, fucoidan of *Adenocytis utricularis* extracted at room temperature was reported to be composed mainly of galactose, fucose and sulfate ester (the "galactofucan") (Ponce, *et al*., 2003). The one extracted at 70 °C contained fucose, accompanied by other monosaccharides mostly mannose, but also glucose, rhamnose,

xylose and galactose, with significant amounts of uronic acids and low proportions of sulfate ester and hence named "uronofucoidan" (Ponce *et al.,* 2003).

1.7.4 Chemical hydrolysis (mild/partial, for structural analysis)

Up to date, chemical hydrolysis seems to be the only possible way to produce fucoidan oligosaccharides as no fucoidanases are available yet (Kim, *et al*., 2008; Chevolot, *et al*., 1999). There are several examples in the literature on how the chemical degradation and modification of polysaccharides may be carried out (Swanson and Cori, 1948). Karlsson and Singh (1999) described the acid hydrolysis of different sulfated polysaccharides (κ-, ι-, λ)-carrageenan, dextran sulfate, heparin and analysed their sensitivity to de-sulfation due to the hydrolysis conditions. One commonly used method of producing bioactive oligosaccharides through chemical hydrolysis has been presented by Yuan and Song (2005) where they obtained oligosaccharides from carrageenan in *Kappaphycus striatum* and compared the anti-tumour activity with other carrageenan polysaccharides. Pomin *et al* analysed selective cleavage and anti-coagulant activity of a sulfated fucan by specifically removing a 2-sulfate ester from a polysaccharide by mild acid hydrolysis and together prepared oligosaccharides with a heparin cofactor II-dependent anti-coagulant (Pomin *et al*., 2005). Nonetheless, enzymatic degradation of polysaccharides may be an alternative tool to chemical hydrolysis. With defined conditions, sulfated oligosaccharides can be produced and subsequently used as standards for analysing enzymatically degraded polysaccharides (Kim, *et al*., 2008).

1.7.5 Structural characterization

The structures of fucoidan from different brown seaweeds have been investigated. Several species of brown seaweeds such as *Fucus vesiculosus* have simple chemical compositions comprising fucose and sulfate ester groups. Most of them are complex in that

besides fucose and sulfate esters, they also contain other monosaccharides such as mannose, galactose, xylose, glucose, and uronic acids as well as acetyl groups and proteins (Yang, *et al*., 2008). These features tend to increase the difficulty in their structural elucidation (Li, *et al*., 2008). However, the structural complexity and chemical composition from algal origin may vary depending on the method of extraction (Grauffel *et al*., 1989). Consequently, each new fucoidan has a unique structural feature which causes it to display unique bioactivity (Li, *et al*., 2008; Hayashi, *et al*., 2008).

Despite all that, some structures or their structural backbones have been elucidated and an extensive review of such has been published (Berteau, *et al*., 2003). Data to date indicate that significant variation exists in the structures particularly with respect to species, local climatic and environmental factors and extraction or purification methods used. However, branched structures have been postulated for several of them (Nagaoka, *et al*., 1999; Chizhov, *et al*., 1999; Marais and Jacob 2001) but the presence of sulfate groups often prevents the unambiguous identification of branching points and determination of their position. In order to identify structural features responsible for biological activities, investigation of sulfated polysaccharides from different species of brown algae needs to be carried out (Painter, 1983; Bilan, *et al*., 2004).

Nonetheless, Chevolot and his team (Chevolot, *et al*., 2001) have succeeded in isolating a disaccharide repeating structure of a low molecular weight fraction of the fucoidan from *Ascophylum nodosum* prepared by acid hydrolysis and partition chromatography, as shown in Figure 7.

$$[\rightarrow 4)\text{-}\alpha\text{-L-Fuc } (2\text{-}SO_3^-)\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fuc } (2, 3\text{-}SO_3^-)\text{-}(1\rightarrow]_n.$$
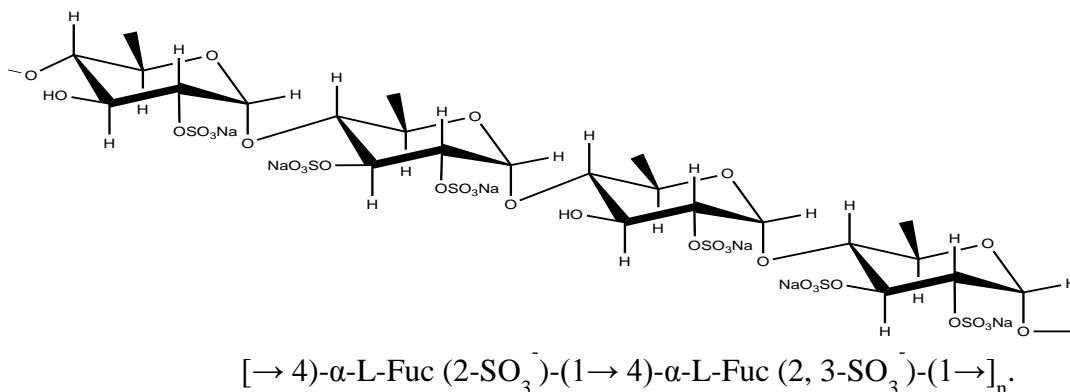
Figure 7: Structure of fucoidan from *Ascophylum nodosum* (Chevolot, *et al*., 2001)

However, there are positions of potential attachment of other carbohydrate units such as L-fucopyranose and D-glucoronic acid and sulfate and acetyl groups (Holtkamp *et al*., 2009) depending on the nature of the polymer. Fucoidan elucidated from *Fucus vesiculosus* is composed of fucose, sulfate and aminoglucose (Black, *et al*., 1952; Nishino, *et al*., 1994; Conchie and Percival 1950; O'Neill, 1954; Newth, 1951). The core regions of fucoidan are primarily a polymer of $(1\rightarrow 3)$ linked fucosyl units with sulfate groups substituted at the C-4 position on some of these sugar residues. Furthermore, similar sugars are attached to the polymer to form branched points, one for every 2 to 3 monosaccharide residues within the chain (Patankar *et al*., 1993). A fucoidan fraction isolated from *Ecklonia kurome* had a highly branched structure, its backbone was made up of $(1\rightarrow 3)$-L-fucosyl units and sulfate groups mainly attached to C-4 (Nishino, *et al*., 1991). Molecular weights reported varied in the range of approximately 100 kDa (Patankar *et al*., 1993) to 1600 kDa (Ruperez *et al*., 2002). Invariably, sulfate groups occupies C-2 and C-3, while 3, 4-diglycosylated and some terminal fucosyl residues are non-sulfated.

1.7.5.1 Position of sulfate groups

The position of sulfate groups is important to the biological activities of sulfated polysaccharides. The methods of determining sulfate presence and position includes; infrared spectroscopy (IR), ion chromatography (IC), desulfation, methylation analysis and

measurement of the stability of the sulfate esters to alkali. The IR spectra of fucoidans have shown that most sulfate groups are in axial positions but some are also in the equatorial positions. This is associated with a strong band at around 842 cm^{-1} and a shoulder at 820 cm^{-1} in the spectra (Zvyagintseva, *et al*., 1999). Infrared spectroscopy is generally the method used for determining the sulfate position. However, erroneous conclusions may be made if the position of sulfate group is determined based only on the infrared spectrum. The C-*O*-S vibration in the 820-850 cm^{-1} region is subject to interference by C-H bending vibrations of reducing sugar end, which will hence affect the judgment on the position of sulfate group. So it is necessary to compare the infrared spectroscopy results with those of stability of the sulfate esters to alkali and methylation analysis (Li, *et al*., 2004).

Mass spectrometry and NMR can also be used in determining the sulfate position. Three isomers 2-*O*-, 3-*O*-, and 4-*O*-sulfated fucose have been analyzed using electrospray ionization trap mass spectrometry (ESI-MS) and capillary electrophoresis. The results showed that it was possible to differentiate between these three positional isomers based on their fragmentation patterns (Tissot *et al*., 2006). It has been shown by NMR that there is some influence from the sulfate groups at C-2 and C-4 on the conformational behavior of fucoidan fragments with a homo-(1→3)-linked backbone (Grachev *et al.,* 2006).

1.7.6 Biological activities of fucoidan

Fucoidans and their oligosaccharides are implicated in various biological activities. Studies have indicated that fucoidan is a non-toxic, non-allergenic, and has no negative effects on the human body after it has been consumed (Shibata, *et al*., 2000). This is further supported by the fact that nutraceutical and food supplements containing them have been marketed for a number of years with no known adverse effects (Choi, *et al*., 2010). No toxicological changes

were observed when rats were orally administered with up to 1000 mg/Kg body weight per day for 28 days, but when the dose was increased to 2000 mg/Kg body weight per day, the plasma alanine transaminase (ALT) level, a biomarker of liver injury, was increased indicating that the consumption of up to 1000 mg/Kg body weight per day was the safest concentration in rodents (Chung, *et al*., 2010). Some of the biological activities are, anti-tumoral (Siddhanta, *et al*., 2001; Li, *et al*., 2008), anti-coagulant (Farias, *et al*., 2000; Silva *et al*., 2005), anti-viral (Lapshina, *et al*., 2006; Lee, *et al*., 2004b; Witvrouw, *et al*., 1997; Alekseyenko, *et al*., 2007), anti-inflammatory (Siddhanta, *et al*., 2001) and lipid reduction activities (Pomin and Mourao, 2008; Wang *et al*, 2010; Li *et al*, 2006; Li *et al*, 2001). Many researchers have targeted the anticoagulant, anticancer, and antioxidant activities as being the most important ones. The effectiveness is related to their chemical composition (Synytsya, *et al*., 2010).

These potential biological applications makes them targets for interesting scientific research. Furthermore, it is postulated that sulfate groups are essentials for antiviral activity and that the higher sulfate content, the more benefits for antiviral (Qiu, *et al*., 2006; Witvrouw, *et al*., 1997) and anti-tumoral (Koyanagi, *et al*., 2003) properties. Such structures therefore play a major role in the biological activity (Boisson-Vidal, *et al*., 2000, 1995). Because of their heterogenous nature and branching, they may contain monosaccharide components with acetyl groups and irregular amounts of sulfation (Bilan *et al*., 2002). The chemistry of algal polysaccharides have been suggested to affect the virus adsorption and penetration (Damonte, *et al*., 2004). Furthermore, anti-tumour property relies on the inhibition of the proliferation of and the induction of apoptosis to certain selected cell lines (Aisa, *et al*., 2005). Wound healing processes are accelerated because of the activity of fucoidan on the collagen gel contraction (Fujimura, *et al*., 2000). Antibodies against them have been described (Nakagawa, *et al*., 2000)

and hence, they can be used as a detection tool in a patient. Due to complexity and the extraction technique used, every type of fucoidan with its unique structural features may possess varied bioactivities and could potentially be a new drug (Eluvakkal, *et al*., 2010).

1.7.7 Applications of Fucoidan and its Oligosaccharides

Fucoidans are known to have many interesting properties hence they possess a wide range of applications. Some of these are shown in Table 4 (Holtkamp, *et al*., 2009; Sezer and Akbuga, 2006).

Table 4: Applications of fucoidan

Fucoidan source	Applications	References
Ascophyllum nodosum	Modulation of connective tissue proteolysis	Senni, *et al*., 2006
Fucus evanescens	Formation of virus in the cells of tobacco leaves	Lapshina, *et al*., 2007 Alekseyenko, *et al*., 2007
Fucus vesiculosus	1. Inhibition of cellular and neurotoxic effects in rat 2. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors 3. Cell apoptosis 4. Delay of thrombus growth 5. Menstrual cycle length	1. Jhamandas *et al*., 2005 2. Suppiramanian *et al*., 2006 3. Aisa *et al*., 2005 4. Thorlacius *et al*., 2000 5. Skibola, 2004
Laminaria japonica	1. Inhibition of the development of proteinuria 2. Development of sea urchin embryos	1. Zhang *et al*., 2005 2. Kiseleva *et al*., 2005
Undaria pinnatifida	Antiviral effects	Lee, *et al*., 2004a

1.8 Antioxidant properties

Reactive oxygen species (ROS) such as hydroxyl radical (OH·), hydrogen peroxide (H_2O_2), superoxide anion (O_2^{-}·) and singlet oxygen (O·) are derived from normal metabolic processes in the human body. They may also originate from external sources such as exposure to radiation, ozone, cigarette smoking, air pollutants or industrial chemicals (Kalyarat, *et al*., 2006; Sathish, *et al*., 2010). Primarily, these ROS and oxidative stress have been associated with the onset of a variety of chronic disease states in humans that disrupt cellular functions (Patra, *et al*., 2008). They act as major causes of human cancer and other diseases. Therefore, ROS are toxic as they can oxidize biomolecules leading to cell death and tissue injury (Morrisey, *et al*., 1998). However, the risk of diseases can be reduced by increased application and consumption of antioxidants which are abundant in certain foods (Kalyarat, *et al*., 2006; Ramapriya and Usha, 2010). They are believed to be protective to the human body against damage by ROS (Halliwell, *et al*. 1995).

Antioxidants from natural sources are preferred by consumers (Kranl, *et al*., 2005) due to concerns on the toxicity, lipid alteration and carcinogenic effects of synthetic types (Ito, *et al*., 1986; Safer, *et al*., 1999; Grillo, *et al*., 1995). Marine algae are known to be exposed to light and oxygen that leads to the formation of free radicals and other strong oxidizing agents (Dykens *et al*., 1992; Namiki 1990). Absence of oxidative damage in the structural components of seaweeds comprising polyunsaturated fatty acids and their stability to oxidation during storage suggests that their cells have protective antioxidative defense mechanism (Matsukawa, *et al*., 1997; Ramarathnam, *et al*., 1995; Jiménez- Escrig, *et al*., 2001). Furthermore, these marine algae contain phenolics which are good antioxidants (Ragan and Glombitza, 1986; Pavia, *et al*., 1996). These phenolics behave as ROS scavengers, enzyme modulators, and metal chelators preventing

lipid peroxidation (Rodrigo and Bosco, 2006). Polyphenols act as reducing agents, and together with other dietary reducing agents like carotenoids, vitamin C and E, act as antioxidants, protecting the body's tissues against oxidative stress and associated pathologies like cancer (Tapiero, *et al*. 2002; Heo and Jeon, 2008).

Since marine algae are a rich source of minerals, proteins and vitamins (Yan, *et al*., 1998), a documented antioxidant activity of these seaweeds would elevate their value in nutraceutical, cosmeceutical and pharmaceutical supplements. There is need to carry out more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action as no single assay accurately reflects all of the radical sources or all antioxidants in a mixed or complex system (Prior, *et al*. 2005, 2009; Frankel, *et al*., 2000). Studies have shown that antioxidant substances which scavenge free radicals play an important role in the prevention of free radical-induced diseases. By donating hydrogen radicals, the primary radicals are reduced to non-radical chemical compounds and are then converted to oxidize antioxidant radicals (Jadhav, *et al*., 1995; Yamaguchi, *et al*., 1998). In the long term, algal species identified as having high levels of antioxidant activity may be viable candidates in the design for further studies. This would contribute to unravel novel treatment strategies for disorders associated with free radicals induced tissue damage (Vadlapudi, *et al.,* 2010).

The skin possesses an elaborate antioxidant defense system to protect it from oxidative stress, but excessive exposure to reactive oxygen species can shift the prooxidant-antioxidant balance towards the more oxidative state. Invariably, the resulting oxidative stress causes many adverse effects and pathological conditions (Ananthaswamy and Kanjilal, 1996; Finkel and Holbrook, 2000). Under these circumstances, regular intake of dietary antioxidants or treatment of the skin with products containing UV protective ingredients may be a useful strategy for

preventing UV-B induced damage. Among them, antioxidant activity related to UV protection has been of intense interest due to the currently growing demand from the cosmeceutical industry where they are interested in anti-aging and whitening natural products. Recent studies have demonstrated that the protective effect against oxidative stress induced by ROS and UV radiation is correlated with the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, and is also affected by other factors, such as other proton-donating groups (-NH, -SH), etc (Rice-Evans *et al*., 1995; Lien *et al*., 1999).

1.9 General information on algae

1.9.1 Class Phaeophyceae

The Phaeophyceae or brown algae, is a large group of mostly marine multicellular algae, including many seaweeds of colder waters. They are unique in being very far removed phylogenetically from all other eukaryotic macrophytes (Van den Hoeck, *et al.*, 1995). The phylogenetic distinctiveness of the Phaeophyceae makes them ideal subjects for testing and extending ideas based on the studies of vascular plants in trophically analogous but phylogenetically distinct organisms. Furthermore, they are also very important members of many marine communities ranging from the tropics to Polar Regions and often dominate these communities in terms of structure and biomass, particularly in temperate and polar waters (Amsler, *et al*., 2006).

They are the most complex forms of algae, commonly adapted in the marine environment. The morphology of members of this group varies dramatically; species range in size and form from microscopic filaments, through to sacs, tubes, and blades, to massive leathery kelps that can be meters in length. Some brown algae are strongly seasonal in their distribution whilst others are perennial. Growth is typically seasonal, with peak production tending to occur

during summer months when temperatures are higher and there are more sunlight hours. They play an important role in marine environments, both as food and for the habitats they form. Brown algae exist in a wide range of sizes and forms; micro-algae and macro-algae as suggested earlier. The smallest members of the microalgae grow as tiny, feathery tufts of threadlike cells no more than a few centimeters long (Connor, *et al*., 1989). The other groups grow into much larger sizes. The rockweeds and leathery kelps are often the most conspicuous algae in their habitats. In form, brown algae primarily range from small crusts or cushions to leafy free-floating mats as with species *Sargassum*. They also consist of delicate felt-like strands of cells, as in *Ectocarpus*, or of foot-long flattened branches resembling a fan, as in *Padina,* two-foot-tall sea palm *Postelsia* (Abbot and Hollenberg, 1976). *Macrocystis*, kelp of the order Laminariales may reach up to 60 m in length, and forms prominent underwater forests. Another example is *Ecklonia*, which creates unique habitats in the cold Benguela waters of the Atlantic Ocean (Abbot and Hollenberg, 1976).

Many brown algae, such as members of the order Fucales, commonly grow along rocky seashores. Some members of the class are used as food for humans. Worldwide, there are about 1500-2000 species of brown algae (Van den Hoek, *et al*., 1995). Some species are of sufficient commercial importance and are interesting in their bioactive compounds, such as *Ascophylum nodosum*, that have become subject of extensive research in their own right (Senn, 1987). These brown algae belong to a very large category, division Heterokontophyta, a eukaryotic group of organisms distinguished most prominently by having chloroplasts surrounded by four membranes, suggesting an origin from a symbiotic relationship between a basal eukaryote and another eukaryotic organism. Most of them contain the pigment fucoxanthin (**1**), beta carotene and chlorophyll which are responsible for the distinctive greenish-brown colour that gives them

their name.

Regardless of size or form, two visible features set the Phaeophyceae apart from all other algae. Firstly, the members of the group possess a characteristic color that ranges from an olive-green to various shades of brown. A particular shade depends upon the amount of fucoxanthin (**1**) present in the alga (Bold, *et al*., 1987). Secondly, all brown algae are multicellular, that is, no known species exist as single cells or as colonies of cells, and they are the only major group of seaweeds that does not include such forms. However, this may be the result of classification rather than a consequence of evolution, as all the groups hypothesized to be the closest relatives of the brown seaweeds include single-celled or colonial forms. Whatever their form, the body of all brown algae is termed a thallus, indicating that it lacks the complex xylem and phloem of vascular plants. Although not all brown algae are structurally complex, those that are, typically possess one or more characteristic parts. They have a holdfast which is a root-like structure present at the base of the alga. This serves to anchor the alga in place on the substrate where it grows, and thus prevents the alga from being carried away by the current. A stipe is a stalk or stem-like structure present in an alga. It may grow as a short structure near the base of the alga as in *Laminaria*, or may develop into a large, complex structure running throughout the algal body as in *Macrocystis*. The stipe may be relatively flexible and elastic in species that grow in strong currents, or may be more rigid in species like *Ecklonia* that are exposed to the atmosphere at low tide.

Many algae have a flattened portion that may resemble a leaf, and this is termed as a blade, lamina, or frond. These blades are attached directly to the stipe, to a holdfast with no stipe present, or there may be an air bladder between the stipe and blade. The surface of the lamina or blade may be smooth or wrinkled, its tissues may be thin and flexible or thick and

leathery. In some species, the surface of the blade is coated with slime to discourage the attachment of epiphytes or to deter herbivores. Blades are also often the parts of the alga that bear the reproductive structures. Gas-filled floats called pneumatocysts provide bouyancy in many kelps and members of the Fucales. These bladder-like structures occur in or near the lamina, so that they are held nearer the water surface and thus receives more light for photosynthesis.

With the exception of the Fucales, all brown algae have a life cycle with an alternation between haploid and diploid forms. Ecologically, brown algae have adapted to a wide variety of marine ecological niches including the tidal splash zone, rock pools, the whole intertidal zone and relatively deep near shore waters in temperate areas throughout the world (Lane *et al*., 2006). They are an important constituent of some brackish water ecosystems, and four species are restricted to life in fresh water (Lee, 2008). A large number of Phaeophyceae are intertidal or upper littoral, and they are predominantly cool and cold water organisms that benefit from nutrients in up welling cold water currents and inflows from land (Lee, 2008). Brown algae growing in brackish waters are almost solely asexual (Lee, 2008). The chemistry of brown algae indicates that they have cellulose walls with alginic acid and also contain the polysaccharide fucoidan in the amorphous sections of their cell walls (Lee, 2008). They produce a specific type of tannin called phlorotannins.

Although there are many brown seaweed species available in the coastal regions of South Africa, particular interest is on the *Ecklonia maxima*, *Splachinidium rugosum, Macrocystis angustifolia*, *,* and *Aeodes orbitosa*, (a red seaweed) kelp that flourishes on rocky reefs off the coast of the western tip where the pristine, icy Benguela current sweeps up from Antarctica. *Ecklonia maxima* are a special kelp which is already used in industry as a raw material towards a

variety of commercial products. The kelp together with *Macrocystis angustifolia* is ecologically and economically important due to their large sizes (up to 30m), high abundance, and high productivity (Lewis, *et al*., 1986). The water currents keep their kelp fronds in constant motion, bathing them in the tumultuous, nutrient-rich ocean, causing them to grow extremely rapidly. Their emersion periods lead to both direct exposure to sunlight and potential photo-oxidative stress. To protect themselves against harmful solar radiations, especially against UV, they produce photo-protective compounds that comprise of several kinds of phenols and pigments. They also contain polysaccharides that act as natural bioactive compounds whose research interests have increased in recent years. Among them are fucoidans (sulfated polymers of fucose) with a wide range of applications from medicinal, nutraceutical, to cosmeceutical.

1.10 Problem statement

All brown seaweeds contain polysaccharides like fucoidans, laminarans, and alginates. Fucoidans and laminarans are complex polysaccharides known for their major biological impact (Thrombosis Research 2000). Brown seaweeds have been shown to contain effective ingredients and are emerging as leading materials in the nutraceutical, cosmeceutical and pharmaceutical fields. Through modern biotechnological extraction processes, concentrated extracts of the brown/red algae may be a very useful source of therapeutic agents. Many of them possess several biological activities, for example, anti-cancer, anti-oxidation, (protection against free radicals) and anti-allergy properties. Lack of scientific backup information despite the enormous benefits, a comprehensive investigation for the presence of chemical constituents implicated on selected brown seaweeds will be carried out.

1.11 Justification

Seaweeds have been consumed in Asia since ancient times, and to a much lesser extent in the rest of the world. Since Africa has a range of macro-algae which have the potential for therapeutic applications in modern society, studies to understand their activity, efficiency and safety need to be pursued more vigorously. The relationships of structure and activity are important for the study. This research should reveal the main constituents which may be used as part of commercial products for the cosmeceutical and pharmaceutical industries.

1.12 Hypothesis

1. Seaweeds are considered a source of bioactive compounds as they produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities.

2. They produce polysaccharides, glycoproteins or other secondary metabolites with antimicrobial, anti-viral, antitumor and anticancer activities.

3. These compounds may remain bioactive following isolation from the algal source.

1.13 Objectives

1.13.1 General objectives

Preparation of crude extracts from brown seaweeds, both aqueous and organic solvent based; isolation and structural elucidation of secondary metabolites.

1.13.2 Specific objectives

1. To perform extractions of crude extracts from brown seaweeds, both aqueous and organic solvent based.

2. Carry out phytochemical screening of the crude extracts.

   a) Screening of crude organic extracts for various classes of compounds present.

b) Hydrolysis and chromatographic analysis of aqueous extracts for monosaccharide composition.

3. To conduct selected bioassays of the crude extracts.

4. To carry out fractionation of the active extracts, towards

   a) Isolation of pure compounds using various chromatographic techniques.

   b) Purification by ethanol/complexation precipitation and gel filtration or IEC of aqueous solvent based polysaccharides.

5. Characterization of the pure isolates.

   a) Pure compounds to be characterized with a combination of various spectroscopic techniques.

   b) Methylation analysis, Partial degradation.

6. To carry out anti-microbial, antioxidant or anticancer bioassay of some isolated pure compounds.

7. To compare experimental and theoretical study of radical scavenging activity of phlorotannin derivatives by computational models
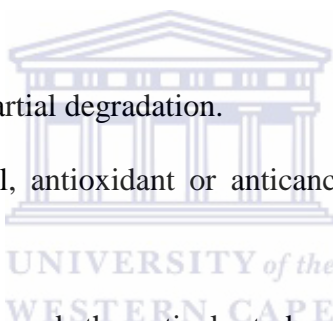
CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental activities

2.1.1 Reagents, equipment and materials

2.1.1.1 Chemicals and solvents

Chemicals used in this study were purchased from Sigma- Aldrich Chemical Company, Kimix or Locab, South Africa and they were of analytical grade.

Standard monosaccharides: glucose, xylose, galactose, mannose, arabinose, rhamnose and fucose and Dextran standards (10,000, 70,000, 110,000, 150,000 and 500,000 Da). DEAE-Sepharose 60, and Sephacryl S-400HR. Phenol-sulfuric acid, acetone, methanol, sulphuric acid, acetic acid, PMP, Dichloromethane (DCM), n-hexane, ethylacetate (EtOAc), chloroform ($CHCl_3$), vanillin, deuteriated methanol. Folin-Ciocalteu reagent, sodium carbonate, gallic acid standard, phosphate buffer, Fluorescein sodium salt, AAPH, and Trolox® (standard and control). Acetate buffer, glacial acetic acid, HCl, TPTZ, Iron (III) chloride hexahydrate, ascorbic acid, Brine shrimp eggs (*Artemia salina*), sea salt.

Tissue culture reagents: Dimethylsulfoxide (DMSO), Tetrazolium salt 3-[4, 5-diphenyl-tetrazolium bromide (MTT), PBS (pH 7.2), Roswell Park Memorial Institute (RPMI-1640) and Trypsin prepared at 0.25%. Cell lines: lung cancer (H157), breast cancer cells (MCF7) and human ovarian cancer (*HeLa*).

2.1.1.2 Equipment

BÜCHI Rotavapor RE 111 with a water bath at 40 ^{0}C and 14 mbar, UV-Vis spectrophotometer, HPLC, GC-2014 gas chromatograph equipped with flame ionization detector (FID) on a DB 225 capillary column (30 m×0.25 mm×0.25 μm). Samples at 10 μl were injected into the GC analyzer and the column temperature was programmed at 225 ^{0}C. Injector and

detector temperatures were 225 ^{0}C and 215 ^{0}C, respectively. Helium gas was used as the carrier gas, FT-IR, IC and a freeze-drier. Dialysis tubes (Spectrapor membrane tubing 6,000-8,000 Da), Dionex ICS-1600 Reagent-Free™ Ion Chromatography (RFIC) System, Varian-600 NMR machine. Centrifuge 5810R, Balance (4 decimal places), 15 ml and 50 ml Conical tubes with screw cap, Eppendorf pipettes and tips, 12 Channel pipette and solution basis, Eppendorf tubes (1.5ml), Media bottle (100ml, 1L), 96 well clear plate (visible range), Multiskan plate reader, Polytron homogenizer, Gilson pipetting aid with 10 ml disposable serological pipettes. A Fluorescence plate reader, Black 96-well plate, pH meter, Media bottle (1 L, 50 ml, 100 ml, 250 ml), Tube rotator, Sample concentrator. Conical flask, aquarium aerator pump, Pasteur pipette, ELISA plate reader and incubator.

2.1.1.3 Chromatography

Aluminium pre-coated plates of silica gel 60 F_{254} with a 0.2 mm layer thickness, paper TLC. Preparative thin layer chromatography (PTLC) silica gel 60 F_{254} pre-coated on glass plates (20 x 20), column chromatography packed with silica gel 60 (0.040-0.063mm) (230-400 Mesh ASTM, Merck)

2.1.1.3.1 Column chromatography (CC)

Glass columns (20-25 mm diameter) wet packed with silica gel 60 (0.040-0.063mm) (230-400 Mesh ASTM, Merck) were used for column chromatography. Size-exclusion chromatography was carried out using Sephadex® LH-20 (Pharmacia), eluting with ethanol. A DEAE-Sepharose-6B (300ml) suspended in excess buffer (500 ml) glass column (18 cm x 2.6 cm) with glass wool rinsed with 1M NaCl as a buffer and swirled slowly.

2.1.1.4 Spectroscopy

2.1.1.4.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were acquired on a Varian-200, 400 and 600 NMR machine based at the Department of Chemistry, University of the Western Cape, University of Cape Town and University of Free State, South Africa respectively. Deuteriated water (D_2O), methanol (CD_3OD) and chloroform ($CDCl_3$) were used as the solvents. Chemical shifts were recorded in δ (ppm).

2.1.2 Seaweed material

2.1.2.1 Collection and preliminary treatments

In the present work, *Ecklonia maxima*, the major alga of interest, was harvested twice, summer collection done in January of 2011 and the winter material in May at Kommetjie, south of Cape Town near the Cape of Good Hope, South Africa. The seaweed was blended into a paste that was freeze-dried and then kept at 4 °C until use. The freeze-dried samples were subjected to both aqueous and organic solvent extraction processes.

2.1.2.2 Extraction of organic and sulfated polysaccharide

The extraction and isolation of the crude organic extracts and polysaccharides was performed first by macerating the plant material with 80 % methanol, both summer and winter collections respectively. The process was performed three times, preserving the filtrate for partitioning with organic solvents, the residue subsequently dissolved in water at room temperature. Continuous stirring of the residue was done for 5 hours to obtain crude polysaccharide. The extract was filtered with a muslin cloth and performed twice. It was centrifuged for 20 minutes at 3500 revolutions and the supernatant was concentrated using a rotary evaporator (BÜCHI Rotavapor R 200) to a minimal volume and then freeze-dried. The product obtained was marked as HM1 (summer) and HM2 (winter) respectively. The

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polysaccharide yield of these HM1 and HM2 was calculated as percent dry weight of the seaweed material (Scheme 1).
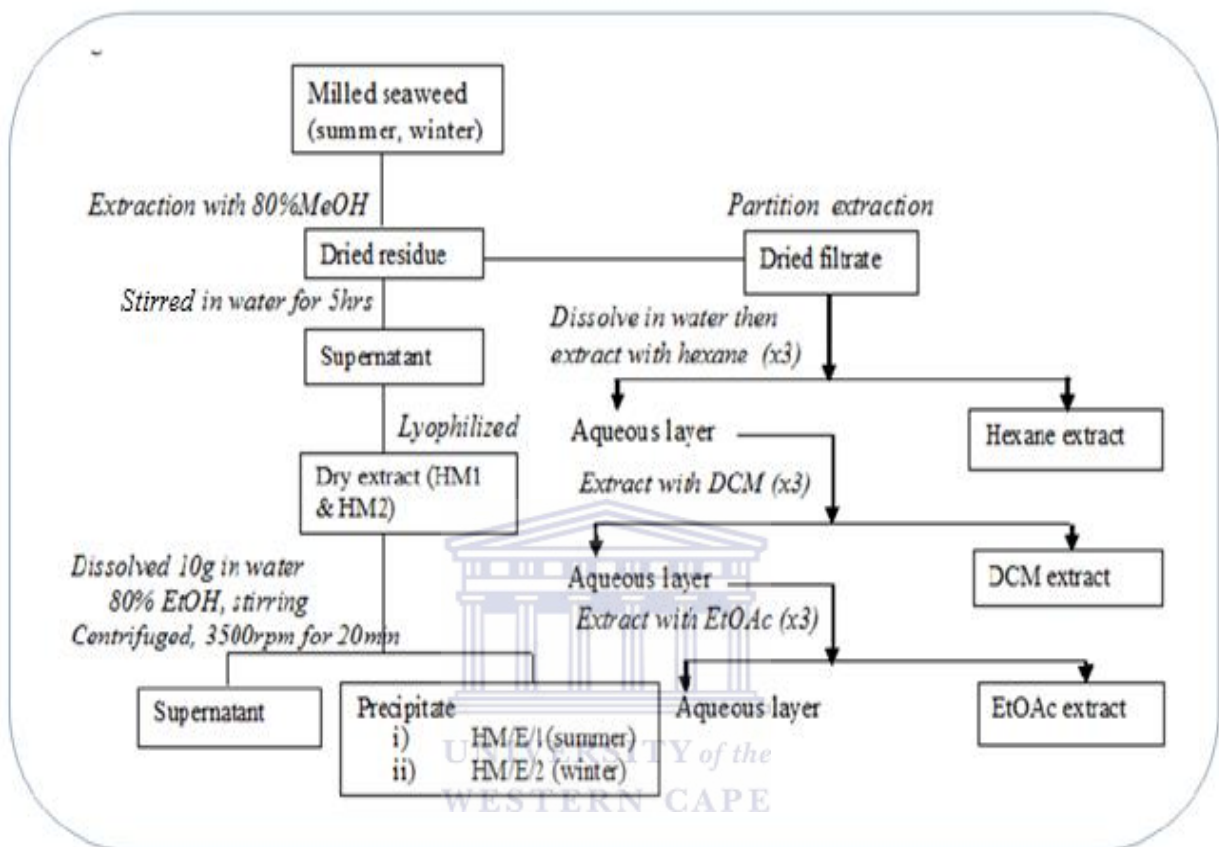
Scheme 1: A flow chart showing fractionation of polysaccharide to obtain aqueous and organic extracts

2.2 Extraction and isolation of organic extracts

2.2.1 Preparation of the crude extracts

Briefly, the preserved methanol filtrate was evaporated at 40 ^{0}C using a rotar vapor to obtain a methanol extract. Subsequently, it was subjected to partition extraction process developed based on solvents of increasing polarity. The extract was first suspended in distilled water and partitioned with hexane, dichloromethane and ethyl acetate repectively (Scheme 1). A profile of compounds present in the respective extracts was developed using TLC technique. The

various extracts were subjected to a technique of lethality assay in order to establish presence of active principles.

2.3 Chemical and biological evaluations

2.3.1 Brine shrimp (*Artemia salina*) lethality assay

The method was done following Solis protocol with some modification, (Solis *et al*., 1993). Brine shrimp eggs (*Artemia salina*) were obtained and hatched by placing 1 g of these in 1L of artificial sea water prepared using 40 gm of sea salt in a large conical flask as an artificially partitioned dam. Incubation of the shrimp eggs was kept for 48 h at 27 ^{0}C under continuous illumination and an aquarium aerator pump for agitation and aeration of the eggs for oxygenation. The larvae (nauplii) were observed to be attracted to one side of the vessel towards the light source where they were easily collected for the assay. Samples were prepared by dissolving them in dimethylsulfoxide (DMSO) and diluted with artificial sea water so that final concentration did not exceed 0.05 %. Different stock solutions of the test samples were prepared at 1000 μl and from this; 100 μl and 10 μl of the solutions were prepared. Each dosage was tested in triplicate. The larvae (nauplii) were collected with a Pasteur pipette after attracting the organisms to one side of the vessel with a light source. Ten nauplii were placed in each of a series of the test tubes containing the test samples at varying concentrations. A control experiment was prepared in the same way except that the test samples were omitted. The suspensions of nauplii were incubated at 25 ^{0}C for 24 h. The number of dead nauplii were counted and recorded as a lethality data. The mortality values were used for calculating the LC_{50} at 95 % confidence limit by the Finney Probit analysis program (Meyer *et al*., 1982). LC_{50} values greater than 1000 ppm or within that range were considered inactive (Mc Laughlin *et al*., 1991).

2.3.2 Isolation of the pure compounds

The EtOAc fraction (5.0 g) that contained yellow spots was subjected to chromatography on a silica gel column, eluting with 500 ml volumes of EtOAc: methanol (10:1-5), yielding 15 sub-fractions (E01-E015). The fractions were subjected to further TLC analysis using DCM-MeOH (9.5:0.5) ratio. Pooling of fractions with similar TLC profiles was done.

Fraction E001 (50 mg) was purified by high performance liquid chromatography (HPLC) system equipped with two pumps, degasser, auto-sampler, and a controller. The temperature of the column was set at 30 °C and 254 nm UV. The column consisted of a C18, (150×4.5 mm, 5 μm) with mobile phase A consisting of water/formic acid (100:0.1) and mobile phase B consisting of acetonitrile/formic acid (100:0.1). The chromatographic flow rate was set to 0.70 ml/min and maintained at 82:18 ratio respectively. Other frations, E004 (55 mg), E006 (40 mg), E009 (32 mg) were also subjected to similar runs using identical solvent conditions. The DCM extract was subjected to a preparative TLC. Finally, the purified compounds were characterized by using their ^{1}H and ^{13}C NMR data and 2D experiments.

2.4 Chemical evaluation

2.4.1 Anti-oxidant assay

With these chemical assays, there is need to carry out more than one type of antioxidant activity measurement taking into account the various mechanisms of antioxidant action (Frankel *et al*., 2000; Prior *et al.,* 1999), as no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system (Prior *et al*. 2005). In the present study, we evaluated the antioxidant activities of the seaweed using:

a) Oxygen radical absorption capacity (ORAC); acts on H^+ ion transfer.

b) Ferric reducing antioxidant power (FRAP); simple electron transfer.

c) Trolox equivalent antioxidant capacity (TEAC)

These analytical methods are routinely used to assess antioxidant activities *in vitro*. Estimation of the total phenolic contents of the seaweed was done using the classical Folin-Ciocalteu reagent. This investigated the relationship between the total antioxidant capacities and phenolic contents in the seaweed samples.

2.4.2 Total phenolic content evaluation

The total phenolic content of the crude extracts and pure isolates was determined according to the Folin-Ciocalteu method with minor modifications using gallic acid as a standard and expressing results as gallic acid equivalents (GAE) (Velioglu, *et al*., 1998; Jiménez-Escrig, *et al*., 2001). The level of total phenol determined according to the Folin-Ciocalteu method are not absolute measurements of the amounts of phenolic materials but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. Data presented was an average of three measurements.

2.4.2.1 Preparation of reagents

Methanol (10 %) was prepared by adding 100 ml of methanol to 900 ml H_2O. Folin-Ciocalteu reagent (in a 15 ml screw cap tube), 1 ml added to 9 ml H_2O and mixed well. For sodium carbonate, 7.5 % was prepared by weighing 7.50 g Na_2CO_3 and adding 100 ml H_2O. Gallic acid standard prepared by dissolving 40 mg (0.040 g) in 50 ml 10% methanol to give a stock standard concentration of 800 mg/L. A control solution was made by dissolving 10 mg (0.010 g) gallic acid in 50 ml 10 % MeOH (200 mg/L).

2.4.2.2 Sample preparation

Approximately 1 mg of each of the samples was weighed into 1.5 ml Eppendorf tubes. Methanol (1 ml) was added with the aid of a Gilson pipette to dissolve the samples. They were

then vortexed to mix thoroughly for 5 minutes. The supernatants were used directly after a suitable dilution for the entire assay. Twenty five micro litre (25 μl) of the diluted samples were added to 125 μl of diluted Folin-Ciocalteu reagent and the contents mixed thoroughly. After 5 minutes, 100 μl of saturated sodium carbonate (75 g/L) was added and the mixture again mixed. The controls containing all the reaction reagents except the sample were also set. After 2 hours of incubation at room temperature, the absorbance was measured at 734 nm. Gallic acid diluted to varying concentrations (0-625 mg/L) was used for the standard calibration curve. The results were expressed as gallic acid equivalent (GAE)/g dry weight of macro algae and comparing to a gallic acid calibration curve.

2.4.2.3 Preparation of standard series

Six Eppendorf tubes were selected and marked A-F. An amount of standard stock solution and diluents to each tube were done as described in Table 5. A clear 96 well plate was used for the experiment.
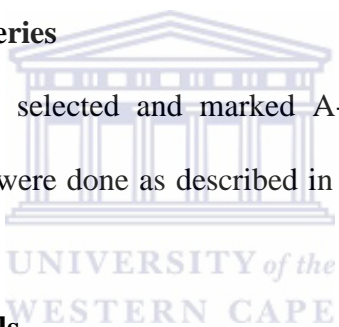
Table 5: Gallic acid standard wells

Tube	Gallic acid stock solution (μl)	MeOH (μl)	Concentration (mg/L)	Well number
A	0	1000	0	A1-A3
B	25	975	20	A4-A6
C	62.5	937.5	50	A7-A9
D	125	875	100	A10-A12
E	312	688	250	B1-B3
F	625	375	500	B4-B6

The 25 μl of the standards from tubes A-E gallic acid were added to the designated wells in a clear well plate. Initial wells, A1-A3 were filled with 25 μl of the blank whereas the control wells setup was from B7-B12. For the sample wells, 25 μl of each of the nine samples was added in triplicate to the wells (C1-E3). This was followed by adding 125 μl Folin-Ciocalteu reagent to each well using a 12 point multichannel pipette. After 5 minutes, 100μl Na_2CO_3 was further added to each well. The plate was left for 2 hours at room temperature before taking the readings on the multiskan spectrum plate reader.

2.4.2.4 Data analysis

All the data was expressed as gallic acid milligrams equivalents per gram of sample (mg GAE/g). Samples with high values of polyphenols were diluted either two-fold or four-fold as the case may be. For four-fold dilutions, the samples were diluted by pipetting 6.25 μl of the sample supernatant into a new Eppendorf and 18.75 μl of methanol added to effect the dilution. The assay was then repeated with the diluted sample and the readings determined.

2.4.3 ORAC assay (OXYGEN RADICAL ABSORBANCE CAPACITY)

This is a fluorescence-based method which was first developed by Glazers laboratory (Glazer *et al.,* 1989) and depends on the unique properties of phycoerythrin, which is used as a target of free radicals. It is based on the discovery that the fluorescence of phycoerythrin changes with respect to time upon damage caused by peroxyl or hydroxyl radical attack. It measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators. Antioxidants are considered to protect the fluorescent molecule from the oxidative degeneration. The ORAC assay as published by Cao *et al.,* 1993 differs from the Glazer method in that the reaction is driven to completion

while the Glazer method looks at what is reported as the flat period. In the ORAC assay, the antioxidant capacity is quantified by calculating the net protection area under the time recorded fluorescence decay curve of the β-PE in the presence of an antioxidant. The method was modified in 1995 by Prior *et al* through automation using the COBAS FARA II centrifugal analyzer and fluorescein salt instead of β-phycoerythrin (Prior, *et al*., 2001).

The β-PE has a few disadvantages in that it is approximately 30 % pure due to its isolation process and inconsistent from the one sample to the next. Its variable reactivity to peroxyl radicals is also inconsistent. Invariably, β-PE can be photo-bleached upon exposure to excitation light for a certain time. In terms of cost, β-PE is much more expensive than fluorescein and is for these reasons that fluorescein has prevailed. A further improved method of ORAC assay has been developed and validated using fluorescein as the fluorescent probe (Ou, *et al*., 2001). This modification provides a direct measure of hydrophilic chain breaking antioxidant capacity against peroxyl radical. The ORAC assay combines both inhibition time and inhibition percentage of free radical action by antioxidants using an area under curve technique for overall quantification. However, it is usually not possible to determine directly the contribution of specific phytonutrients to the total ORAC value (Ou, *et al*., 2001).

This is a simple, sensitive, and reliable method used to measure the peroxyl radical absorbing capacity of antioxidants using 2, 2′-azobis (2-amidino-propane) dihydrochloride (AAPH) as a peroxyl radical generator. The degeneration of fluorescein is measured as the presence of the antioxidant slows the fluorescence decay. Decay curves (fluorescence intensity vs. time) are recorded and the area between the two decay curves (with or without antioxidant) gets calculated. Subsequently, the degree of antioxidant-mediated protection is quantified using the antioxidant Trolox® (a water soluble analogue of vitamin E) as a standard by which all other

antioxidant compounds are compared. Different concentrations of Trolox® are used to make a standard curve and test samples are compared. This assay expresses the results as ORAC units or Trolox® equivalents, which correspond to the amount of Trolox micromoles that have the same antioxidant activity as one liter of the tested solution (Cao, *et al*., 1993, 1995).

The method is used with a fluorometry micro-plate reader using a 96-well plate to perform simultaneous kinetic analysis of many samples as required. Although it was originally developed for plasma samples, it has been successfully applied in other fields of study. The performance involves using a fluorescence spectrophotometer until zero fluorescence occurs. The results are reported as a net protection area under the quenching curve of Fluorescein in the presence of an antioxidant. Calculation of the ORAC value is by dividing the area under the sample curve by the area under the Trolox® curve with both areas being corrected by subtracting the area under the blank curve. The ORAC assay uses a biological relevant source and is unique in its analysis in that it takes into account the inhibition time and degree of inhibition into a single quantity by the area under the curve. The ORAC method is not affected by dilution (Cao, *et al*., 1995).

2.4.3.1 Preparation of reagents

The phosphate buffer, 75 mM, pH 7.4 where (1.035 g of $NaH_2PO_4.H_2O$ in 100 ml H_2O as 1st solution; 1.335 g $Na_2HPO_4.2H_2O$ in 100 ml H_2O as 2nd solution. 18 ml of 1st solution mixed with 82 ml of 2nd solution) was prepared. Fluorescein sodium salt (0.0225 g $C_{20}H_{10}Na_2O_5$ in 50 ml phosphate buffer), Trolox® (standard): 500 µM Stock solution (0.00625 g 6-hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid was added to 50 ml phosphate buffer); Trolox® (control): 250 µM Stock solution (0.00312 g 6-hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid was added to 50 ml phosphate buffer) were prepared.

2.4.3.2 Sample analysis

For the Trolox® standard series, 6 Eppendorf tubes were marked from A-F. The amount of standard stock solution and diluents were added to each tube as described in Table 6. To the experimental analysis, "ORAC" measure of the excitation wavelength was set at 485 nm while the emission wavelength set at 530 nm.

Table 6: Trolox® standard series

Tube	Standard concentration μM	Trolox® stock solution μl	Phosphate buffer μl	Well number
A	0	0	750	A1-A3
B	83	125	625	A4-A6
C	167	250	500	A7-A9
D	250	375	375	A10-A12
E	333	500	250	B1-B3
F	417	625	125	B4-B6

To the Trolox® standard wells, 12 μl of the standard tubes A-F were added per well in the designated 96-microwell black opaque plate (A4-B6). A similar amount of the Trolox® control 12 μl was added into the control wells (B7-B12). For the samples, 12 μl of each of the earlier prepared nine sample supernatant was added in triplicate to wells (C1-E3). This was followed by adding 138 μl of the fluorescein stock prepared by adding 10 μl of the stock solution to 2 ml phosphate buffer in an Eppendorf tube and 240 μl of this solution in 15 ml buffer solution using a multichannel pipette into each well of a 96-microwell black opaque plate. Then a transfer of 50 μl of AAPH solution to each well was prepared by adding 6 ml of the phosphate buffer to the

AAPH weighed earlier and mixing them well until dissolved. The final volume of the assay was 200 μl. Finally, the 96-microwell plate was inserted into the fluorometer and the readings taken.

2.4.3.3 Data analysis

Fluorescein consumption was evaluated from the decrease in the sample fluorescence intensity (excitation 485 nm, emission 530 nm) using a time base scan program. The fluorescence was then monitored kinetically with data taken every minute. The ORAC values were calculated using a regression equation: $Y = a + bX + cX^2$ between Trolox® concentration (Y) (μM) and the net area under the fluorescence decay curve (X). Data was expressed as micromoles of Trolox® equivalents (TE) per milligram of sample (μmole Trolox®/mg of sample). Trolox® in the experiment was used as a control sample.

2.4.4 FRAP assay (FERRIC REDUCING ANTIOXIDANT POWER)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain (Benzie and Strain, 1999). FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. It is a technique that applies an oxidation/reduction reaction to measure the ability of a sample to reduce Fe^{3+} to Fe^{2+}. A given antioxidant donates electrons in the same manner as a reductant in an oxidation/reduction, so it is assumed that the FRAP assay is a method for evaluating antioxidant capacity. However, it does not directly measure the antioxidant capacity of a potential antioxidant. Also, since there are no free radicals introduced into the system, there is no way of comparing the antioxidant capacity towards different kinds of radicals (Benzie and Strain, 1999).

The FRAP assay gives fast, reproducible results with plasma, single antioxidants in a pure solution, or with mixtures of antioxidants in an aqueous solution. The assay is not

concentration-dependent, showing no deviation from the expected linear trend of the results going through the origin. At low pH, reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to the ferrous (Fe^{2+}) occurs giving an intense blue colour and can be monitored by measuring the change in absorption at 593nm. The reaction is non-specific, in that any half-reaction that has a lower redox potential, under reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric (Fe^{3+}) to ferrous (Fe^{2+}) reaction. The change in absorbance, therefore, is directly related to the reducing power of the electron donating antioxidants present in the reaction mixture.

2.4.4.1 Preparation of reagents

An acetate buffer, 300 mM, pH 3.6 (1.627 g sodium acetate added to 16 ml glacial acetic acid) was prepared and making to 1L with distilled water. The 40 mM HCl, (1.46 ml of concentrated HCl (32 % HCl) and making it to 1L), fresh 10 mM 2, 4, 6-tri [2-pyridyl]-s-triazine (0.0093g TPTZ added to 3 ml of 40 mM HCl), Iron (III) chloride hexahydrate, 20 mM (0.054 g $FeCl_3.6H_2O$ dissolved in 10 ml distilled water) and a standard 1.0 mM L-Ascorbic acid solution containing 0.0088 g Ascorbic acid in 50 ml distilled water were also prepared. 125 µl of the stock solution was diluted with 1375 µl water to obtain a concentration of 83 µM. The control was made from L-Ascorbic acid 400 µM (0.00352 g in 50 ml distilled water then diluting 311 µl of this solution with 1189 µl water to obtain a concentration of 83 µM).

2.4.4.2 Sample analysis

FRAP reagent was prepared in a 50 ml conical tube by mixing 30 ml acetate buffer, 3 ml TPTZ solution, 3 ml $FeCl_3$ solution and 6.6 ml distilled water, forming a straw colored solution. The multiskan program from the computer was then set to read data at 593 nm. The preparation of the

standard series involved taking 6 Eppendorf tubes and marking them A-F. Standard stock solution was added and diluents to each tube made as described in the Table 7.

Table 7: Ascorbic acid standard wells

Tube	Ascorbic acid stock solution μl	Distilled water μl	Standard concentration μM	Well number
A	0	1000	0	A1-A3
B	50	950	50	A4-A6
C	100	900	100	A7-A9
D	200	800	200	A10-A12
E	500	500	500	B1-B3
F	1000	0	1000	B4-B6

To the ascorbic acid standard wells, 10 μl of the standard (tubes A-E) was added to each of the designated wells (A4-B6) in the clear 96-well plate. Then 10 μl of control solution was equally added to the control wells (B7-B12) and samples to the wells (C1-E3) in triplicate. Addition of 300 μl FRAP reagent was then done to each well using a multichannel pipette. The final volume of the assay in each well was 310 μl. The plate was incubated for 30 minutes in an incubating oven set at 37 °C after which the 96-well plate was ran in the machine.

2.4.4.3 Data analysis and calculations

The data results were expressed as micromoles of ascorbic acid equivalents per gram of sample (μmole AAE/g of sample).

FRAP of sample = (Change in absorbance of sample) x FRAP value of standard (1000 μM)
                        Change in absorbance of standard

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2.4.5 (TEAC/ABTS) Trolox equivalent antioxidant capacity assay

The TEAC/ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6- sulfonic acid)) assay is one based on the inhibition by antioxidants of the absorbance of the radical cation 2,2′- azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS$^{\bullet+}$), which has a characteristic long-wavelength absorption spectrum showing main absorption maxima at 415 nm, and secondary absorption maxima at 660, 734, and 820 nm. This ABTS radical cation scavenging assay was first developed by Miller *et al*, for the determination of the total antioxidant status (TAS) of body fluids (Miller, *et al*., 1993). Antioxidants added to this system can either scavenge the ABTS$^{\bullet+}$ formed or interfere in the radical generating process. Measurement of the absorbance at a specific time after addition enables the calculation of a percentage scavenging. Determination of the TEAC value is based on the ability of the antioxidant to scavenge the ABTS$^{\bullet+}$ radical cation relative to the ABTS$^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue-Trolox$^{®}$ (Miller, *et al*., 1993).

2.4.5.1 Preparation of reagents

ABTS (2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt, 7 mM was prepared by adding 0.0192 g of ABTS into 5 ml distilled water while 88 μl of the potassium-peroxodisulfate solution was added to 5 ml of the ABTS solution. Standard Trolox$^{®}$ (6-Hydrox-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid):1.0 mM was freshly prepared using 0.0125 g Trolox in 50 ml of ethanol. For the control, 200 μM was prepared by dissolving 0.0025 g Trolox$^{®}$ in 50 ml of ethanol and used when fresh.

2.4.5.2 Sample analysis

The TEAC Readings were taken at 734 nm on the multiskan spectrum plate reader and the TEAC standard series were prepared as shown in Table 8 and set at 25 ^{0}C. Data was

expressed as µmole Trolox® equivalents per gram of sample (µmole Trolox® /g of sample).

Preparation of standard series was carried out by taking 6 Eppendorf tubes and marking them A-
F. The amount of standard stock solution and diluents were added to each tube as described in
Table 8.

Table 8: Trolox® standard wells

Tube	Trolox® Standard µl	Ethanol µl	Trolox® conc. µM	Well number
A	0	1000	0	A1-A3
B	50	950	50	A4-A6
C	100	900	100	A7-A9
D	150	850	150	A10-A12
E	250	750	250	B1-B3
F	500	500	500	B4-B6

To the Trolox® standard wells, 25 µl of the standard (tubes A-E) per well was added to each of
the designated wells (A4-B6) in a clear 96 well plate. Then 25 µl of the control solution was
added to the control wells (B7-B12). Sample wells (C1-E3) were filled with 25 µl of the samples
and in triplicate. 300 µl of diluted ABTS mix was then added to each of the wells using a
multichannel pipette. The 96-well plate was kept for 30 minutes at room temperature before
taking a reading.

2.4.5.3 Data analysis

Inhibition of the sample was calculated by the following equation:

$$\text{Inhibition} = [(A_0 - A_1)/A_0]$$

*A_0 expresses the absorbance of control; A_1 expresses the absorbance of the tested seaweed
extract.*

65

The ABTS radical anion scavenging assay was expressed as Trolox® equivalent antioxidant capacity (TEAC) and defined as μmol of Trolox® equivalents per 1 g of sample.

2.5 Computational theoretical studies on radical scavenging activity

The antioxidant radical scavenging activity is mainly governed by the hydrogen atom transfer (HAT) and electron transfer mechanism (Kozlowski, *et al*., 2007; Trouillas, *et al* 2008; Wright, *et al*., 2001 and Chiodo, *et al*., 2010). In the HAT mechanism, the antiradical properties of phenol derivatives (ArOH) are related to their ability to transfer their phenolic H-atom to a free radical. The H-atom abstraction is described by the reaction:

$$R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet} \tag{1}$$

The termination of further chain reactions depends strongly on the stability of the $ArO^{\bullet}$ radical species formed. This means that factors enhancing the stability of the $ArO^{\bullet}$ radical increases the antiradical activity. The ability of phenolic antioxidants to donate a hydrogen atom is mainly governed by the homolytic O-H bond dissociation enthalpy (BDE):

$$BDE = H_r(ArO^{\bullet}) + H_h(H^{\bullet}) - H_p(ArOH) \tag{2}$$

Where H_r *is the enthalpy of the radical generated by H abstraction, H_h is the enthalpy of the H atom, and H_p is the enthalpy of parent compound.*

A low O-H BDE value, usually associated with greater ability to donate the H atom, corresponds to high radical scavenging ability by the phenolic compound (Leopoldini, *et al*., 2004; Kabanda, 2012). The electron transfer (ET) mechanism on the other hand is governed by the capacity of the studied compound to transfer an electron and is better described in terms of the ionization potential (IP).

$$R^{\bullet} + ArOH \rightarrow ArOH^{\bullet +} + R^{-} \tag{3}$$

The stability of the radical cation is better described by the IP value; the lower the IP, the easier is the electron abstraction. This radical stability is determined by the calculation of stabilization energies ($\Delta E iso$), as shown in equation 4 for the electron transfer, where the phloroglucinol derivatives are represented by ArOH and the phloroglucinol molecule (eckol) is represented by PhOH.

$$\Delta E_{iso} = [ArOH^{\cdot+} + PhOH] - [ArOH + PhOH^{\cdot+}] \qquad (4)$$

2.5.1 Materials and methods

2.5.1.1 Computational details

Fully relaxed geometry optimizations of the neutral and the radical species were performed utilizing DFT/B3LYP and DFT/UB3LYP methods respectively, where B3LYP is the Becke's Three Parameter Hybrid Functional using the Lee-Yang-Parr correlation functional (Becker, *et al*., 1993). Calculations were performed with the 6-31+G* basis sets to take into consideration the effects of hydrogen bonding. Accuracy of the energy evaluation in the case of systems involving open-shell species is sensitive to spin contamination. The Spin contaminations of radicals $\langle S^2 \rangle$ values, calculated for all radical species, had a 0.77- 0.79 range, which was close to the value of 0.75 (corresponding to the pure doublet wave function). Consequently, after the annihilation of the first spin contaminant, they dropped to correct value of 0.75. Therefore, the results of the calculations were less affected by spin contamination and did not bias the found enthalpies. Since the species had more conformers, all of them were investigated. The conformer with the lowest electronic energy was used in this work. Frequency calculations were performed on fully optimized conformers to determine the nature of the stationary points and to obtain thermochemical quantities necessary for the estimation of BDE and IP values (Becker, *et al*., 1993).

The estimation of the O-H BDE values in the presence of a solvent was done by using the total free solvation energies of the neutral and the radical species, as suggested elsewhere (Tomasi, *et al*., 2005). Solvent effects on geometries and relative conformational stabilities were taken into consideration using the polarizable continuum model (PCM) (Cancès, *et al*., 1997) in the integral equation formalism (IEF) framework (Frisch, *et al*., 2003). All calculations were performed with Gaussian 03 (Shao, *et al*., 2006) and Spartan 10.V1.01 program (Mammino and Kabanda, 2008). Gaussian 03 allowed solution-phase geometry optimization where the approach was used for the parent molecules and their respective radicals and anions. All enthalpies were calculated at 298 K. The schematic representations were drawn using the ChemOffice package in the UltraChem 2010 version and conformers were drawn using Spartan 10.V1.01 program (Mammino and Kabanda, 2008).

2.6 Assessment of cell viability

Cell viability was tested using 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide (MTT) to purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells, and hence it provides a means of assessing the viability (cell counting) and the proliferation of cells. It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth. The cytotoxicity was evaluated by MTT reduction assay as described by Mosmann (1983) with some modifications.

2.6.1 Culturing of cells

HeLa, H157 and MCF7 were cultured in RPM1-1640 media in T25 flask, 25 cm^3 tissue culture flasks and were allowed to grow to 90 % confluency in an incubator set at 37 ^{0}C containing 5 % CO_2 atmospheric pressure for 24 hrs. The process was carried out before they were trypsinized.

2.6.2 Cell viability using MTT assay

The isolates were first spiked separately onto one cell line *HeLa* in order to determine which one would have the most significant effect on cell proliferation. This procedure was carried out in order to assess any possible synergic effects of the isolates on the cell line of choice. MTT preliminary tests showed that compounds **23**, **24**, **25** and **26** produced proliferation activity of 43.63%, 91.79%, 99.76% and 68.94% respectively at a concentration of 125 µg/ml following 24 hrs incubation in relation to the control. Isolates **23** and **26** produced activities that were lower than that of the control at all the concentrations and incubation periods tested but only **23** was subjected to further analysis.

The anti-proliferation activities of isolate **23** were pursued further on the three cell lines (*HeLa*, H157 and MCF7) which were seeded in 96-well plates at an initial density of 5×10^3 cells/mL per well. After the cells had been incubated for 24 hrs at 37 °C, various concentrations of the phlorotannin were added in the growth medium with quadruplicate wells for each dilution, and the incubation was continued further for 24 hrs. MTT solution (20 µl) at a concentration of 5 mg/ml was added and the wells were incubated again under 5 % CO_2 at 37 °C. After 4 h of the incubation, the plates were shaken for 5 min and the supernatants were aspirated. The culture and excess MTT was removed from each well, subsequently 150 µl 0.04 M DMSO was added and the plates shaken to solubilize the formazan crystals. The absorbance was measured via ELISA

plate reader at the wavelength of 560 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The data was expressed as mean percentages of the viable cells versus the respective control. The cytotoxicity was expressed as 50 % cytotoxic concentration (IC_{50}), which was the concentration of the test substances that inhibited up to 50 % of the growth of various cell lines. The percentage growth proliferation was calculated using the formula,

$$\% \text{ cell proliferation} = \{At/Ac\} \times 100$$

Where; *At =Absorbance value of test compound*
Ac =Absorbance value of control

2.7 Precipitation and purification crude polysaccharide

2.7.1 Ethanol precipitation

In order to obtain a more purified crude polysaccharide, 10 g of each of the freeze-dried samples was dissolved in 50 ml of water. The mixture was then stirred for 1 hour, precipitated with 400 ml of 80% ethanol overnight. Centrifugation at 3500 rpm for 20 minutes led to the collection of the precipitated polysaccharide material. The precipitation process was repeated 3 times. The final precipitate was re-dissolved in 50ml water, evaporated to a small volume then freeze-dried. The samples obtained, were weighed and stored until further use.

2.7.2 Chemical composition of the crude of polysaccharide

2.7.2.1 Monosaccharide determination

Preliminary analysis of the monosaccharide composition of the precipitated crude polysaccharides was conducted by treating 50 mg of each sample with 0.5M trifluoroacetic acid (TFA) at 100 ^{0}C for 18 h, after which the hydrolysate was co-evaporated with methanol (40 ml) under reduced pressure to remove the acid. Methanol co-evaporation was repeated five times to obtain dry residue. The residue was dissolved in a methanol-water mixture (4:1) and 2 drops of

25% ammonia solution added to neutralize any residual acid. Sodium borohydride (NaBH₄, 100 mg) was added and the contents mixed with gentle swirling then allowed to stand overnight. The reaction mixture was then treated with glacial acetic acid (AcOH) to about pH 6, followed by further co-evaporation (five times) with methanol to remove the formed boric acid as its methyl ester. The resulting dry residue was subsequently treated with acetic anhydride (5 ml), and the mixture heated at 100 ^{0}C in a steam-bath for 2 hr with occasional swirling. A sample mixture containing rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glu) as monosaccharide standards, was also dissolved in 0.5 ml distilled water and treated with sodium borohydride (NaBH₄). These resulting alditols were acetylated as described above according to the method described by Blakeney *et al* (1983) with slight modifications.

Then crushed ice was added followed by vigorous stirring for 1hr in order to destroy excess acetic anhydride. Addition of chloroform (CHCl₃) followed with vigorous shaking and then allowing the two layers to separate. The organic layer was washed 3 times with distilled water before drying with anhydrous Na₂SO₄. Filtration followed by evaporation resulted in a residue composed of alditol acetates that were subsequently analysed by GC. The peak areas of the samples and monosaccharide alditol standards were compared in turn, in order to calculate the content of neutral sugars. Confirmatory tests of the identity of the neutral sugars were done by subjecting the alditol acetates to GC/MS. The retention times of the peaks from the test samples were compared to those of the standard derivatives.

2.7.3 Paper chromatography (PC)

Five standard monosaccharides and the hydrolyzates of treated polysaccharide (2 µl per spot) were applied on a special paper plate which was developed in a PC chamber. The sugar

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composition was determined by this technique in an eluent mixture of chloroform: methanol: water (10:10:3), for 18 hrs. After drying, the plate was sprayed with anisidine hydrochloride dissolved in methanol, ethanol and butanol in a ratio of 1:1.5:6 (v: v: v) respectively and then heated in oven at 105 °C for 1 h.

2.7.4 Fucose content determination

Free fucose was determined by use of cysteine-sulphuric acid method for methyl pentoses (Dische and Shettles, 1948). Five replicate fucose standard samples were prepared in different concentrations with deionized water ranging from 20-200 µg/ml. Commercial L-fucose (Sigma-Aldrich) was used as the standard. All the polysaccharide samples were prepared at a concentration of 200 µg/ml. Each sample solution of exactly 1 ml was placed into separate test tubes and cooled in an ice water bath. Subsequently, 4.5 ml of sulfuric acid reagent prepared by adding six volumes of the concentrated acid with one volume of water was added into each tube and vortexed. The tubes were warmed in a 25 ^{0}C water bath for 5 minutes, and then subjected to boiling water for 10 minutes. They were then cooled under running tap water and 100 µl of 5% cysteine hydrochloride solution in deionized water was added to each tube and vortexed. Absorbance was read at 396 nm and 427 nm, after zeroing the spectrophotometer with a water blank treated in the same manner. Absorbance values were calculated by using the following equation: Absorbance = (A396 nm - A427 nm). This corrects for the presence of hexoses (Dische and Shettles, 1948). The total fucose in milligrams was calculated using the regression curve equation obtained from the standard curve.

2.7.5 Sulfate content

Measurement of sulfate in the fucoidan was based on the barium sulphate ($BaSO_4$) determination using barium chloride ($BaCl_2$) (Dodgson, *et al*., 1962) with slight modifications,

whereby sulfate content was estimated turbidimetrically as $BaSO_4$. The conditioning reagent was prepared by mixing 5 ml glycerol, 3 ml concentrated hydrochloric acid, and 30 ml deionized water, 10 ml isopropyl alcohol and 10 ml 1M sodium chloride into a large beaker with mechanical stirring overnight. Four replicate samples were prepared by weighing 5 mg of dried fucoidan into separate ampoule tubes containing 5 ml 2 M TFA. The samples were subjected to acid hydrolysis for 18 hours at 100 ^{0}C. A solution of potassium sulfate standards with concentrations ranging from 20-100 µg/ml of sulfate was prepared. A 5 ml sample solution was put into a test tube and conditioning reagent (5 ml) was added and stirred mechanically at a constant speed. $BaCl_2$ (0.15 g) was added, stirred for exactly 1 minute and then left standing for 4-6 minutes to allow the $BaSO_4$ precipitate to form. Absorbance was measured at 420 nm using a spectrophotometer after zeroing with water blank that was treated in the same manner. Potassium sulfate data was used to plot a standard graph providing a regression curve.

2.7.6 Uronic acid determination

The contents of uronic acid of purified samples were determined colorimetrically, and the glucuronic acid was used to make a standard curve. The method of Bitter & Muir (1962) was followed to estimate the uronic acid content in fucoidan. This was with some modifications of the original procedure developed by (Dische, 1962; Dische and Shettles, 1948). The modified procedure was reported to have less interference, a more stable color formation, and reacted faster (Bitter and Muir, 1962). Absorbance was measured at 525nm after zeroing the spectrophotometer with a water blank treated in the same way as the samples. Glucuronic acid standard stock solutions were made in ultra-pure water using amounts 0, 5, 10, 20, 40, 50 and 100 µg quantities for the generation of a calibration curve. The curve was used for calculating the uronic acid content of the polysaccharide samples.

2.7.7 Derivatization with PMP reagent

PMP derivatization of the monosaccharides was carried out as described by Daotian and Roger, (1995), Zhang, *et al*. (2003), Andersen, *et al*, (2003), Honda, *et al*, (2003), Lv, *et al*, (2009) with appropriate modification. Ethanol treated samples of the algae powder (0.020 g) was dissolved in 2 M trifluoroacetic acid (5 ml) in an ampoule. The ampoule was sealed and kept under hot water at 100 ^{0}C for 18h. After cooling to room temperature, the reaction mixture was washed with 30 ml MeOH three times to remove the acid. The residue was then dissolved in 1 ml of deionized water. 200 µl of a mixture of standard monosaccharide solution, or the hydrolyzed polysaccharide samples were placed in the 2.0 ml Eppendoff centrifuge tubes, respectively, then 100 µl of 0.5M methanol solution of PMP and 200 µl of ammonia were added to each. The mixture was allowed to react for 30 min in a 70 ^{0}C water bath, then cooled to room temperature and neutralized with 200 µl of formic acid. The resulting solution was separated by liquid-liquid extraction using 900 µl volume of isoamyl acetate two times and then 900 µl chloroform once, respectively. After being shaken vigorously and centrifuged for 20 min at 14,000 rpm, the organic phase was carefully discarded to remove the excess reagents. Then the aqueous layer was filtered through a 0.45 µl membrane and diluted with ultra-pure water before HPLC analysis.

The reversed-phase chromatographic separation of the PMP carbohydrate derivative hydrolyzates were analyzed by a HPLC method (Liu, *et al*., 2007) on an Agilent 1100 series. The process was performed on an instrument equipped with two pumps, degasser, autosampler, and a controller. The temperature of the column was set at 30 °C and 254 nm UV. The column consisted of a C18, (250×4.6 mm, 5 µm) with mobile phase A consisting of water/formic acid (100:0.1) and mobile phase B consisting of acetonitrile/formic acid (100:0.1). The

chromatographic flow rate was set to 1.0 ml/min and maintained at 82:18 ratios respectively. The mobile-phase eluent was directed from the HPLC column through a switch valve and diverted to waste for the first 5 min. An injection volume of 20 µl was used.

2.7.8 Fractionation of the crude polysaccharide
2.7.8.1 Anion exchange

Crude fucoidan can be purified into fractions using ion-exchange chromatography, a technique which separates molecules based on the overall charge of the molecule. As fucoidans generally have an overall negative charge due to their sulfate groups, they can bind with anion exchangers, which contain positively charged functional groups such as diethylaminoethyl (DEAE) (Chotigeat, *et al*., 2004; Huang and Lam, 2011). Each of the ethanol-treated samples, (300 mg) was dissolved in 2 ml distilled water, loaded on the DEAE- Sepharose-6B column and eluted with 60 ml distilled water at a flow rate of 1ml/min. This was followed by elution with sodium chloride (NaCl) at increasing concentrations 0.1M, 0.2M up to 1M NaCl each time to a minimum of 5 ml in each tube. Test tubes containing the eluted samples were transferred (1 ml) into more robust glass test tubes in order to monitor sugar content using the 40% phenol-sulfuric acid test solution, each time up to the absence of a positive reaction of eluate for carbohydrates (Tatsuga, *et al*., 2005). The absorbance was measured at 490 nm for any indication of sugars on the basis of the Phenol-H_2SO_4 method (Dubois *et al*. 1956). Fractions of peaks with positive response according to the elution profile graph prepared were pooled together separately, concentrated, and dialyzed against distilled water at room temperature for 3 days with water change daily in order to remove NaCl from the fractions. Dialysis tubes (Spectrapor membrane tubing) with a molecular weight cut-off membrane of 6,000-8,000 Da were used (Arthur H. Thomas Co. Philadelphia, Pa, USA) and fractions were then lyophilized using a VIRTIS 4K fre-

eze dryer for 48 hours.

2.7.9 Determination of molecular weight

To determine the molecular weight of fucoidan and its fractions, gel permeation chromatography was applied using Sephacryl S-400HR column (16×70 cm). By applying molecules through the gel, small molecules get trapped in the gel beads and those with a larger molecular weight will flow through the gel. Consequently, this technique allows larger molecules to elute first, followed by smaller molecules which are held longer inside the beads (Garrett and Grisham, 1999). Dextran standards with molecular weights ranging from 10,000 to 500,000 Da were used as standard molecular weight markers. Sephacryl S-400HR was suspended in 100 ml 1M NaCl excess buffer and allowed to swell and settle at room temperature for 5 hours. Fresh 50 ml buffer was then added to make slurry that subsequently was poured into the glass column to the intended height and formed the stationary phase. The gel suspension was poured using a glass rod as a guide with its end touching the inner wall until approximately 80% of the column was filled. The outlet tap was opened to allow flow of the buffer by gravity, settling the gel. The column was then washed with 50 ml of buffer in order to further pack the bed and also equilibrate the gel. The setup was placed on top of a fraction collector and the outlet tap closed until required.

The Dextran standards of known average molecular weights and a selected fraction from the samples were dissolved in distilled water at a concentration of 1 mg/ml. All the standards were subjected to gel-filtration chromatography first in order to give data for a calibration curve. Elution process was carried out using 1M NaCl at a flow rate of 0.5 ml/min and collecting 2 ml fractions, then applying the Phenol-H_2SO_4 method (Dubois *et al*., 1956) to monitor the polysaccharide content. The molecular weights of the unknown sample were calculated from a

calibration curve, in which the logarithm of the molecular weight of the Dextran standards was plotted as a function of the absorbance.

2.7.10 Ion chromatography

Ion chromatography is a form of liquid chromatography that uses ion-exchange resins to separate atomic or molecular ions based on their interaction with the resin. Its greatest utility is for analysis of anions for which there are no other rapid analytical methods. The method (IC) provides a convenient and simple method for the direct determination of anions in a single analysis (Miskaki, *et al*., 2007). It is also commonly used for cations and biochemical species such as amino acids and proteins. Most ion-exchange separations are performed with pumps and metal columns. It is the only chromatographic technique that can provide quantitative analysis of anions at the ppm level. Aqueous solutions require filtration, dilution, and cleaning to remove interferences if required for analysis. Stock anion standard solutions (1000 mg/L) for several of the analytes of interest, were obtained from a commercial source (Miskaki, *et al*., 2007).

Composite working standard solution at a lower analyte concentration was prepared from the 1000-mg/L stock standard solution. Seven levels of calibration standards were used in this study to find out if their concentrations could be found in a fucoidan material. Approximately 1 mg of purified polysaccharide fraction was hydrolysed then dissolved into 10 ml of deionised water. The hydrolysed oligosaccharides were extracted with water to remove ions from their surfaces to obtain an aqueous solution for analysis. The samples were placed on a vortex to fully disperse them and then filtered through a 0.45 µm Supor® membrane filter. A Dionex ICS-1600 Reagent-Free™ Ion Chromatography (RFIC) System was used in this work with $NaHCO_3$ as the electrolytic eluent. Deionized water, (type I reagent-grade, 18 MΩ-cm resistivity), sodium and potassium salts were used in preparing anion standards.

2.7.11 Infra-red spectroscopy

About 2 mg of pure dry sample was pressed into KBr disc for infrared spectra (IR) absorption measurement. A whole IR spectrum acquired in the transmission mode within the range of (300-4000 cm^{-1}) was recorded on a Perkin-Elmer 735 IR 560 spectrophotometer.

2.7.12 NMR spectroscopy

A portion of the freeze-dried sample of treated fraction 4 (7 mg) winter collection was deuterium-exchanged by lyophilisation with D_2O for a minimum of three times. Subsequently, the sample was dissolved in 1 ml of D_2O and filtered through 0.45-μm filters to remove any insoluble material. The deuterium-exchanged sample was subjected to a ^{1}HNMR analysis; chemical shifts were expressed in ppm.

2.7.13 Methylation analysis

In polysaccharide structural studies, methylation analysis is a method for determining the mode of linkage of monosaccharide constituents, and may be described as the process of methylating a polysaccharide, followed by hydrolysis of the methylated product, conversion of the resulting partially methylated monosaccharides into alditol acetates, and subsequent analysis by GC and GC-MS (Ciucanu, *et al*., 1984; Dell, 1990). The method is generally carried out as follows: - a polysaccharide material (5-20 mg) was placed in a dry 50ml three necked round bottomed flask. A rubber septum cap was attached at one end followed by evacuation and flushing out air with nitrogen for 20 minutes. Dry DMSO, 5 ml, was added using a dry glass pipette and the reaction mixture stirred for 30 minutes at room temperature. The solubilization process was achieved by constant stirring and at elevated temperatures of up to 40 °C. Dry powdered sodium hydroxide of about 100 mg was quickly added with vigorous stirring for 1 hour still under nitrogen. The mixture was frozen with ice water before introducing 4 ml of

methyl iodide, with stirring, under a slow flow of nitrogen. The reaction was allowed to continue overnight at room temperature. The methylated product was subsequently extracted with 15 ml DCM-10 ml water, shaking and removing the top aqueous layer using a separating funnel. Further 10 ml volumes of deionized water were added three more times to the DCM layer, the mixture vortexed and subsequently discarding the top water layer after each washing. To minimize undermethylation of the polysaccharide, silver oxide-methyl iodide (Purdie, *et al*., 1903) was added to the methylated product and continuous stirring under reflux was continued for two days. The extraction process with the DCM-water mixture was repeated. To remove traces of water, anhydrous magnesium sulfate was added, filtered through glass wool in a Pasteur pipette. The methylated product was isolated by evaporating the DCM on a rotary evaporator under reduced pressure.

The subsequent hydrolysis process was then carried out as described in section 2.7.2.1 in order to obtain partially methylated alditol acetates (PMAA). The products were characterized by GC-MS with mass fragmentation patterns obtained referenced to validated peak reports found in literature. Sugar residues in the sample were identified by comparing their mass fragmentation pattern with previous standards reports.

2.7.14 Desulfation and methylation of fucans

Desulfation of the sulfated L-fucan samples was performed by solvolysis in dimethylsulfoxide as described previously for desulfation of other types of polysaccharides (Falshaw, *et al*., 2005; Mourão, 2004). The polysaccharide was converted into the pyridinium salts. Briefly, a sample (20 mg) was dissolved in a mixture of anhydrous DMSO-MeOH-pyridine (89:10:1, v/v/v) (8 ml) and heated at $100\ ^0C$ for 14 h. After cooling to room temperature, distilled water (5 ml) was added to the resulting mixture; dialyzed overnight against distilled water and

then freeze-dried to afford the partially desulfated fucan. The desulfated polysaccharide (5 mg) was subjected to methylation process as described previously.

CHAPTER 3

3.0 *Ecklonia maxima*

3.1 Introduction

3.1.1 Ecklonia kelp

Ecklonia kelp flourishes in nutrient-rich ocean currents. Ecologically, this kelp forms a canopy that provides an important complex, three-dimensional habitat for thousands of species of fish, invertebrates and other seaweeds. Invariably, the kelp beds are among the most productive ecosystems on earth, supporting high primary production levels. This high productivity forms the base of many coastal food webs in cool water environments worldwide. Hence, both directly and indirectly, they are an important food source for a large variety of invertebrates, fish, mammals and seabirds. Furthermore, kelp is tough and resilient, and stretching into sea, often for many kilometers, they help break the great force of the waves offering protection to the near shore ecosystem.

Ecklonia kelp has developed a strong and rubbery cell structure which enables it to thrive in the violent wave action present on the coastline. The strength of this marine plant is due to the presence of high levels of organic gels within its tissues. In addition to organic gels, the kelp has significant amounts of vitamins, minerals, enzymes, and amino acids. This kelp forms huge forests in the sub-tidal pools (Lane *et al*., 2006). Their holdfasts grip tightly around the rocks where other smaller algae anchor themselves for support in a symbiotic relationship. In South Africa, the seaweed industry is based on *Ecklonia* and *Laminaria* among other brown and red seaweeds. Here, kelp is widely used as a fertilizer and is harvested extensively as feed for commercially well-known abalone. Other applications in huge markets exist internationally from the harvested kelp for alginate production. This is a substance of considerable importance as it is

used as a gelling and emulsifying agent in a number of industries (Lane *et al*., 2006).

In the food industry, it makes water-based products thicker, creamier and more stable over extreme differences in temperature and pH over long periods. In the cosmetic, pharmaceutical, paint, textile and welding industries, alginate aids in the suspension and stabilization of agents over ranges of pH and temperatures. In the paper industry, such are important in enabling the sizing and polishing of finished paper products. They have also been in use in making of fibers for high quality audio speakers. In the medical industry, kelp is equally important. The alginate is used to encapsulate several materials such as tablets in powder form, fracture castings and moulds, and even organs for transplant. The same kelp contains astonishing amounts of vitamins and minerals, iodine being the most important. This is an important mineral in the treatment of goiter and prevention of cretinism. In line with this, kelp is good for weight loss formulas (Lane *et al*., 2006; Anderson, *et al*., 2006).

Recent research has shown a direct relationship between alginate contained in kelp and the prevention of breast cancer. The history of the related kelp *Ecklonia cava* is such that its use as food dates back hundreds of years in Asia (Li, *et al*., 2009). Dried kajime as an example is abundant in the seafood markets. Furtheremore, its powdered form is used as a food additive to give a brown tint to ''mannan'', a traditional food in Japan and Korea. Another one, wakame, is often used to flavor foods, particularly salads and soups. Due to the people in this part of the world appearing to be healthier than those who live in the western half of the globe, much research is being conducted into the potential health benefits of these seaweeds. One of the most promising constituents of the seaweed is fucoxanthin (**1**) (Jeon *et al*, 2010).

3.1.2 Morphology and growth

Ecklonia maxima is a large brown seaweed which is endemic to Southern Africa, and is utilized as a plant growth stimulant, food for the abalone farms and exported in dried form for alginate extraction. It grows in high concentration around the Cape Peninsula although it is also found in other parts of the West coast. It has been found that a cosmetic type cream containing an extract of this alga can help relieve various types of eczema and psoriasis, while consumption of products based on the seaweed have also shown to promote weight loss. On the basis of available literature, compounds of seaweed origin which have been implicated in the aforementioned biological/pharmacological activities include polyphenols fucoxanthins and fucoidans. Data available to date indicate that significant structural variation exists in fucoidans, depending particularly on species of origin, local climatic and environmental factors and extraction/purification methods. Whether such variations result in differences in the bioactivity of fucoidans is an aspect of continuous investigation (Anderson, *et al*., 2006).

The seaweed species is being studied with due consideration to factors like geographical location and harvesting season which may influence the polysaccharides content; this work is designed to study the effects of such differences. On the other hand, the alga is particularly interesting because few studies have been conducted on its polysaccharides and fucoidal properties. Fucoidans are complex carbohydrates with the capacity to retain water. They are natural moisturizers, serving to prevent seaweed from drying out when exposed to the wind and sun at low tide. The purpose of this study is to document the organic and polysaccharides composition characteristics of the alga found in the Western Cape. *Ecklonia maxima,* a sea bamboo, is a species of the kelp forests, native to the southern oceans. It is most typically found along the southern Atlantic coast of Africa from the very south of South Africa up to the north of

Namibia. In these areas the species dominates the shallow, (up to 8 m) temperate-water kelp forests offshore (Anderson *et al*., 1997). The algae remain largely unexploited in the Western province of South Africa.

The South African distribution of the algae extends from Port Nolloth on the west coast to Cape Agulhas on the south coast, where it was found growing in abundance in 1937 (George, 1942). This distribution is not continuous; however, the species is represented by occasional individuals, along the northern and the greater part of the western shores of False Bay. The absence or scarcity in this region is owing to the high sea temperatures which prevail there during the summer months. The high temperature conditions eliminate these algae from the flora of the northern and western parts of False Bay. The species reappears at certain stations along this coast, starting at the mouth of the Steenbras River, and extends eastward beyond False Bay as far as Kommetjie. It dominates the inshore areas of both west coast and transition zone kelp beds. Furthermore, the species has been harvested commercially at Soetwater for many years, for the production of an agricultural growth stimulant (Anderson *et al*., 1997). Since the early 1990's, increasing amounts of algae have been harvested as feed for abalone cultured in land-based farms (Anderson *et al*., 2003). More than 7000 tonnes of fresh fronds are harvested in a year from the beds, and demand is increasing as abalone farms expand (Anderson *et al*., 2006). In the harvested areas, divers cut out all plants with stipes longer than 0.5 metres. A dense bottom cover of small sporophytes appears within a year, and a surface canopy is re-established within 2 to 3 years (Anderson *et al*., 1989).

Amongst other seaweeds, the alga is present in sufficient amounts for commercial exploitation. In order to promote the emergence of this industry, it is important to establish the structural characteristics of polysaccharides with commercial interest. Furthermore, the structural

differences between seaweed species need to be determined. This species is of economic importance, harvested for both an agricultural supplement and as food for abalone bred on farms (Robertson-Anderson *et al*., 2006; Anderson, *et al*., 2006). It is also known to be used as a nutritional supplement for farm animals. The alga has been harvested for the production of a very successful plant growth stimulant and has shown to be a great source of micronutrients.

Plate 1: A photograph of *E. maxima* (Source: www.algaebase.org)

The seaweed possesses a massive holdfast that extends into a long, hollow, gas-filled stipe of up to 15m to 20m in length that extends in a bulb at its tip. The bulb further extends into a flat, solid primary blade from which the secondary blades emerge. These secondary blades can quite easily reach 3m in length. Because of its hollow stipe and bulb, this species is buoyed up in

the water column, its blades frequently breaking the surface of the ocean. The alga constitutes the canopy of the kelp forest at the ocean's surface and it is here that certain types of red seaweed species attach epiphytically (Anderson, *et al*., 2006).

Ecklonia maxima consists of a simple thallus in its juvenile state which is somewhat elongated, with a blade borne on a stipe. In slightly older stages pinnae develop along both margins of the blade. In older plants, the stipe becomes hollow and its terminal portion becomes inflated, forming a large float. Mature plants may attain a length of seven meters or more from the base of the stipe to tips of longest pinnae. Most of the algae kelps have growth points in the middle of the plant, usually where the blades join the stipe. This is an advantage because the blade can continue to grow at its base while the tip is worn and grazed away. These kelps have the most complex tissues of all algae where the stipe has strengthening tissues and tubular cells to transport the food through the plant. The blades are many cells thick with a protective outer layer, pigmented photosynthetic cells and storage tissues containing starch, and tannin, to deter herbivores. Spores are produced in specialised sporangia situated in raised patches (sori) on the blades. During its life cycle, the kelp alternates between the huge spore producing plant (sporophyte) and microscopic male and female plants (gametophytes) that produce the gametes. From the holdfast attached to a rock or to a large holdfast of kelp, a single long stipe rises to the surface waters, where a single large pneumatocyst holds a tangle of blades at the surface (Mann and Kenneth, 2000).

3.2 Experimental results and discussion

3.2.1 Chemical and biological evaluation of organic extract

3.2.1.1 Crude algal extract and phytochemical screening

3.2.1.1.1 Crude extract

Freeze-dried pulverized material of the seaweed (300 g) after treatment with 80% MeOH and then filtered was partitioned by extracting the filtrate with hexane, DCM, and ethyl acetate. The hexane (25.4 g), DCM (22.6 g) and ethyl acetate (15.0 g) extracts were subjected to phytochemical screening for detection of secondary metabolites. The results shown in Table 9 demonstrated presence of phenolics, saponins, flavonoids but no alkaloids.

3.2.1.1.2 Phytochemical screening

Preliminary phytochemical screening of the seaweed samples from the partition extraction process was carried out to determine the secondary metabolites profile of each extract.

Table 9: Phytochemical screening results of the algae extracts

Test material	Phenolics	Saponins	Flavonoids	Alkaloids
Hexane	-	-	-	-
DCM	+	-	+	-
EtOAc	+	-	+	-
MeOH	+	+	+	-

The screening process targeted the detection of natural product classes present in the seaweed extracts. The collection of *Ecklonia maxima* alga material was considered from two separate times of the year. Consequently, it was observed that the material collected in summer was not phytochemically different from that collected during winter other than decreased yields; similar pigment elution profiles were registered. In this regard, winter collection displayed a significant

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concentration of spots compared to the summer as is true for its high biomass stored during spring. Winter harvesting would be highly recommended due to the rich concentrates in the alga.

3.2.2 Brine shrimp lethality bioassay

Brine shrimp larvae (nauplii), a zoological organism, have been a convenient monitor for the screening of bioactive natural products lethality *in vivo* (Meyer, *et al*., 1982). In any of a biological test carried out, a high dosage of any bioactive compound can be toxic and hence pharmacology of such a compound requires preliminary testing from the toxicological results. As a general principle, three concentrations of a natural product extract; 10, 100 and 1000 µg/ml are prepared in test tubes, each with ten nauplii, and in triplicate for each concentration. Subsequently, the number of surviving nauplii counted after 24 hours are used to estimate the LC_{50} with a 95% confidence using the SPSS 16 Finney probit analysis computer programme. This technique was applied as a bioassay for this research work on four samples; Hexane, DCM, Ethyl Acetate and Methanol crude extracts.

Table 10: Number of dead *nauplii* after 24hours

Test materials	Vials at 1000 µg/ml			Vials at 100 µg/ml			Vials at 10 µg/ml			Vials for control expt.
	1	2	3	1	2	3	1	2	3	
Hexane	4	5	4	3	3	2	1	3	2	10
DCM	5	5	4	2	3	3	3	3	3	10
EtOAc	9	7	8	6	4	5	3	4	2	10
MeOH	8	7	7	4	5	4	3	2	1	10

The results in table 10 demonstrated that hexane and DCM extracts showed reduced activity. The highest bioactivity was evident with ethyl acetate and methanolic extracts. From the results, it should be noted that all the extracts may be considered as good candidates for antimicrobial and antiviral agents. Moreover, the lethality of a test substance to brine shrimp nauplii has a possible ability of having antitumor activity or posseses an anti-inflammatory property (Mc Laughlin *et al*., 1991). Subsequently, the results were classified based on the analysis programme as active or inactive at the three test concentrations with all determinations done in triplicate.

Table 11: LC$_{50}$ of the dead nauplii after 24 hours

Test material	1000 μg/ml	100 μg/ml	10 μg/ml	LC$_{50}$	Remarks
Hexane	5	3	2	6921.708	Inactive
DCM	5	3	3	2015.950	Inactive
EtOAc	8	4	3	98.046	Active
MeOH	7	4	2	191.639	Active

The results in Table 11 showed that ethyl acetate and methanol extracts were the most active (LC$_{50}$ < 1000 μg/ml). This could be attributed to the presence of active principles in the extracts. For them being the most polar extracts, they consequently got attributed to their richness in polyphenols that have a lot of biological activity. Furthermore, lethality of any test substance to brine shrimp nauplii has been linked to the possibility of killing cancer cells, bacteria or pests (Meyer *et al*., 1982). In this study, the ethyl acetate fraction in particular has the greatest potential as a candidate for the discovery of cytotoxic compounds. It was therefore deduced that these active samples could be subjected to further pharmacological tests.

3.2.3 Isolation of pure compounds
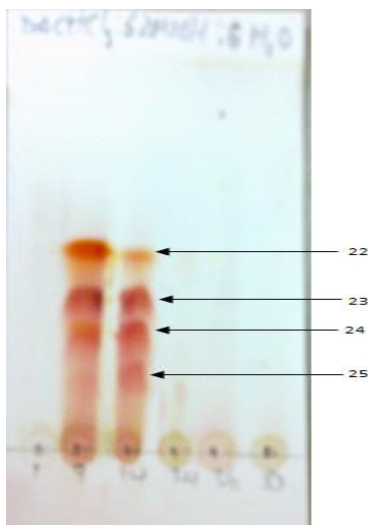
Plate 2: A TLC profile of some characteristic spots of phenolic compounds (yellow) from the EtOAc extract

The fractions E09 to E014 obtained from silica gel column as indicated in section 2.3.2 were combined and ran and chromatographed on a Sephadex column in order to obtain pure isolates. Subsequently, the fractions collected were subjected to HPLC for further purification. After several runs, E001 led to the isolation of compound **22** (25 mg). Further HPLC runs of each of the fractions; E004 (55 mg), E006 (40 mg), E009 (32 mg) using identical solvent conditions, led to the isolation of compound (**23**) (20 mg), compound (**24**) (20 mg) and compound (**25**) (15 mg) respectively. Compound **26** was isolated from a DCM extract using preparative TLC.

3.2.3.1 Physico-chemical data of isolated compounds
3.2.3.1.1 Benzene-1, 3, 5-triol (22)

Off-white powder; ES-MS, m/z 125.05 [M+H]$^+$, ^{1}H NMR (200 MHz, CD$_3$OD) δ 5.66 (3H, *s*, H-2, 4, 5); ^{13}C NMR (CD$_3$OD, 50 MHz) δ 95.5 (C-2, 4, 6); δ 160.1 (C-1, 3, 5).

3.2.3.1.2: 1-(3′,5′-dihydroxyphenoxy) dibenzo[b,e][1,4]dioxine-2,4,7,9-tetraol (23)

Light brown powder; ES-MS, m/z 371.0397 [M + H] $^+$, (calcd, for $C_{18}H_{12}O_9$, 371.0403). ^{1}H NMR (600 MHz, CD$_3$OD) δ 6.17 (1H, s, H-3), δ 5.97 (1H, d, J = 2.6 Hz, H-6), δ 5.96 (1H, d, J = 2.6 Hz, H-8), δ 5.95 (1H, t, J = 2.0 Hz, H-4′), δ 5.96 (2H, d, J =2.0 Hz, H-2′, 6′); ^{13}C NMR(150 MHz, CD$_3$OD); δ 124.2 C-1; δ 145.8 C-2; δ 98.0 (s, C-3); δ 141.9 C-4; δ 123.1 C-4a; δ 142.8 C-5a; δ 94.4 (d, C-6); δ 153.1 C-7; δ 98.4 (d, C-8); δ 145.7 C-9; δ 123.4 C-9a; δ 137.1 C-10a; δ 160.4 C-1′; δ 94.0 (d, C-2′); δ 158.8 C-3′; δ 96.3 (d C-4′); δ 158.7 C-5′; δ 94.0 (d C-6′).

3.2.3.1.3: 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxy

dibenzo-1, 4-dioxine-2,4,9-triol (24)

Light brown powder; ES-MS m/z 495.0570 [M - H]$^+$ (calcd for $C_{24}H_{16}O_{12}$, 495.0564). ^{1}H NMR (600 MHz, CD$_3$OD) δ 5.95 (1H, s, H-3), δ 5.97 (1H, d, J = 1.8 Hz, H-6), δ 5.99 (1H, d, J = 1.8 Hz, H-8), δ 5.93 (2H, s, H-3″, 5″), δ 6.09 (2H, J = 2.1 Hz, H-2′, 6′), δ 5.98 (1H, t, J = 1.8 Hz, H-4′); ^{13}C NMR (150 MHz, CD$_3$OD); δ 123.9 C-1; δ 145.7 C-2; δ 96.6 (s, C-3); δ 141.7 C-4; δ 123.4 C-4a; δ 142.7 C-5a; δ 94.1 (d, C-6); δ 155.0 C-7; δ 98.6 (d, C-8); δ 147.3 C-9; δ 124.9 C-9a; δ 137.3 C-10a; δ 160.6 C-1′; δ 94.4 (d, C-2′); δ 159.0 C-3′; δ 96.5 (d, C-4′); δ 159.0 C-5′; δ 94.4 (d C-6′); δ 123.0 C-1″; δ 150.8 C-2″; δ 94.8 (d, C-3″); δ 153.2 C-4″; δ 94.8 (d, C-5″); δ 150.8 C-6″.

3.2.3.1.4: 1-(3′,5′-dihydroxyphenoxy)-2-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydi

benzo-1,4-dioxine-4,7,9-triol, **(25)**

Light brown powder; ES-MS m/z 495.0561 [M - H]$^+$ (calcd for $C_{24}H_{16}O_{12}$, 495.0564). ^{1}H NMR (600 MHz, CD$_3$OD) δ 5.97 (1H, s, H-3), δ 6.00 (1H, d, J = 2.6 Hz, H-6), δ 6.02 (1H, d, J = 2.6 Hz, H-8), δ 5.95 (2H, s, H-3″, 5″), δ 6.11 (2H, J = 1.8 Hz, H-2′, 6′), δ 6.01 (1H, t, J = 1.8 Hz, H-4′); ^{13}C NMR (150 MHz, CD$_3$OD); ; δ 123.8 C-1; δ 147.3 C-2; δ 96.5 (s, C-3); δ 141.6 C-4; δ

123.1 C-4a; δ 142.6 C-5a; δ 94.6 (*d*, C-6); δ 153.0 C-7; δ 98.7 (*d*, C-8); δ 145.6 C-9; δ 123.4 C-9a; δ 137.2 C-10a; δ 160.5 C-1'; δ 94.3 (*d*, C-2'); δ 158.8 C-3'; δ 96.7 (*d* C-4'); δ 158.8 C-5'; δ 94.3 (*d*, C-6'); δ 125.4; δ 150.7 C-2''; δ 95.0 (*d*, C-3''); δ 154.8 C-4''; δ 95.0 (*d*, C-5''); δ 150.7 C-6''.

3.2.3.1.5: 24-ethylidine cholesterol, (26).

White needles; ES-MS data; *m/z* 411.3584 [M - H]$^+$ (calcd for $C_{29}H_{48}O_1$, 411.3216); ^{1}H NMR (600 MHz, CDCl$_3$) δ 0.87 (1H, *m*, H-1*ax*); δ 1.12 (1H, *m*, H-1*eq*); δ 1.88 (1H, *m*, H-2*ax*); δ 2.03 (1H, *m*, H-2*eq*); δ 3.55 (1H, *m*, H-3); δ 2.20 (1H, *m*, H-4*ax*); δ 2.31 (1H, *m*, H-4*eq*); δ 5.37 (1H, *m*, H-6); δ 1.23 (1H, *m*, H-7*ax*); δ 1.54 (1H, *m*, H-7*eq*); δ 1.55 (1H, *m*, H-8); δ 1.47 (1H, *m*, H-9); δ 0.95 (1H, *m*, H-11*ax*); δ 1.53 (1H, *m*, H-11*eq*); δ 1.10 (1H, *m*, H-12*ax*); δ 1.20 (1H, *m*, H-12*eq*); δ 1.15 (1H, *m*, H-14); δ 1.43 (1H, *m*, H-15*ax*); δ 1.54 (1H, *m*, H-15*eq*); δ 1.00 (1H, *m*, H-16*ax*); δ 1.55 (1H, *m*, H-16*eq*); δ 1.02 (1H, *m*, H-17); δ 0.71 (3H, *s*,H-18); δ 0.99 (3H, *s*, H-19); δ 0.95 (1H, *m*, H-20); δ 1.01 (3H, *d*, *J*=6.6, H-21); δ 1.13 (2H, *m*, H-22); δ 2.08 (2H, *m*, H-23); δ 2.17 (1H, *m*, H-25); δ 1.00 (3H,*d*, *J*=6.9, H-26); δ 0.99 (3H,*d*, *J*=6.9, H-27); δ 5.20 (1H, *q*, H-28); δ 1.59 (3H, *d*, *J*=6.6, H-29). ^{13}C NMR (150 MHz, CDCl$_3$); δ 36.5 (C-1); δ 31.7 (C-2); δ 71.8 (C-3); δ 42.2 (C-4); δ 140.9 (C-5); δ 121.7 (C-6); δ 28.2 (C-7); δ 31.9 (C-8); δ 50.2 (C-9); δ 36.4 (C-10); δ 21.1 (C-11); δ 37.2 (C-12); δ 42.4 (C-13); δ 55.6 (C-14); δ 24.3 (C-15); δ 24.2 (C-16); δ 56.7 (C-17); δ 11.9 (C-18); δ 19.6 (C-19); δ 34.8 (C-20); δ 18.7 (C-21); δ 35.2 (C-22); δ 25.7 (C-23); δ 146.9 (C-24); δ 34.7 (C-25); δ 22.2 (C-26); δ 22.0 (C-27); δ 115.4 (C-28); δ 13.2 (C-29).

3.2.4 Structural elucidation of compounds

3.2.4.1 Compound 22 (Benzene-1, 3, 5-triol)

Running of fraction E001 through Sephadex using ethanol eluent, an off-white powder that showed a positive colour test of phenolics upon spraying with vanillin reagent was obtained. From the ^{1}H NMR (200 MHz, CD$_3$OD) δ 5.66 (3H, *s*, H-2, 4, 5); ^{13}C NMR (CD$_3$OD, 50 MHz) spectrum displayed a peak at δ 95.5ppm assignable to the protonated carbon atoms (C-2, 4, 6) while the signal at δ 160.1 was due to the hydroxylated carbons (C-1, 3, 5). In conjunction with ES-MS m/z 125.05 [M+H] [+], this was a symmetrical structure with a single signal reflecting that all the protons were in the same chemical environment. A similar compound has been reported by Rengasamy *et al* from the same alga (Rengasamy, *et al*., 2013). It was conclusively identified as phloroglucinol, **Benzene-1, 3, 5-triol (22)**.

22
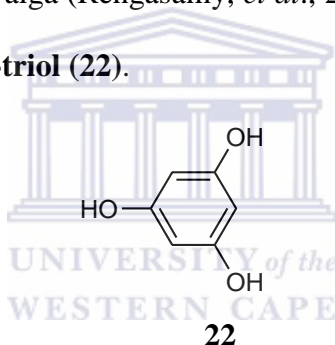
3.2.4.2: Compound 23 (1-(3′, 5′-dihydroxyphenoxy)dibenzo[b,e][1,4]dioxine-2,4,7,9-tetraol)

Preparative TLC of fraction E004 after running it through Sephadex (MeOH-DCM 1:1) gave a light brown powder that showed positive colour test for phenolics with vanillin. The final purification of the individual compound was accomplished by HPLC.

23
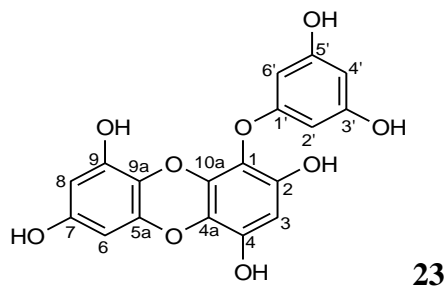
The detailed structure was determined by using 1D (^{1}H, ^{13}C, APT and DEPT 45) and 2D-NMR

experiments HSQC, HMBC and COSY. ^{1}H-^{13}C NMR experiments confirmed the phlorotannin nature of the structure (Table 12), as they displayed signals in the range of δ H 5.8-6.3 and δ C 94-163 ppm, respectively. The quaternary carbons showed characteristic peaks which could be related to the type of bonding they were involved in, i.e. either carbon to carbon, or carbon to oxygen, where the latter may in turn either be in ether-linkage or hydroxylated. Consequently, the signals appearing from δ C 94.0-98.4 ppm could be associated with the protonated carbons, verified by the one-bond HMQC spectrum (one of the peaks accounting for more than one carbon, thus totaling six), whereas the group of signals from δ C 123.1-160.4 ppm was due to non-protonated carbons, which were oxygenated, totaling twelve.

Compound **23** had a molecular formula $C_{18}H_{12}O_9$ as determined from ES-MS, *m/z* 371.0397 [M + H][+], (calcd, 371.0403). ^{13}C NMR displayed carbon resonances for an ether bond (C-1, δ 124.2). In the ^{1}H NMR spectrum, an AB_2 system at δ 5.96 (2H, *J* =2.0 Hz), 5.95 (1H, *J* =2.0 Hz), an AB system at δ 5.97 (1H, *J*= 2.6 Hz), δ 5.96 (1H, *J*= 2.6 Hz) and a singlet at δ 6.17 (1H) were observed. The structure contained CH protons, which were in a very similar chemical environment in the polyphenol. All ^{1}H and ^{13}C NMR signals were assigned with the aid of HMQC and HMBC experiments. Based on the data in Table 12 and values in the literature, (Rengasamy, *et al*., 2013, Fukuyama, *et al*., 1985), compound **23** was identified as an eckol, characterized by the presence of a dibenzodioxin moiety. A similar compound has been reported by Rengasamy *et al* from the same alga (Rengasamy, *et al*., 2013) and previously by others (Fukuyama *et al*., 1985; Craigie *et al*., 1997; Glombitza *et al*., 1977, 1979; Geiselman *et al*., 1981) in extracts from other brown seaweeds. Consequently, it is hereby reported as **1-(3′, 5′-dihydroxyphenoxy) dibenzo [b, e] [1, 4] dioxine-2, 4, 7, 9-tetraol,** an eckol.

Table 12: 1D and 2D ^{1}H (600MHz) and ^{13}C NMR (150 MHz), MeOD data for compound 23

Position	δ ppm	HMQC	HMBC	δ lit
1	124.2			122.9
2	145.8			145.6
3	98.0 (6.17, *s*)	6.17	C-1 C-2, C-4a	97.9 (6.13, *s*)
4	141.9			141.5
4a	123.1			121.9
5a	142.8			142.2
6	94.4 (5.97, *d*, *J*= 2.6)	5.97	C-9a	93.6 (5.78, *J*=2.6)
7	153.1			152.6
8	98.4 (5.96, *d*, *J*= 2.6)	5.96	C-6, C-9a	98.3 (5.95, *J*=2.6)
9	145.7			145.7
9a	123.4			122.3
10a	137.1			136.8
1′	160.4			160.0
2′	94.0 (5.96, *d*, *J*= 2.0)	5.96		93.5 (5.71, *J*=2.0)
3′	158.8			158.4
4′	96.3 (5.95, *d*, *J*= 2.0)	5.95		96.0 (5.79, *J*=2.0)
5′	158.7			158.4
6′	94.0 (5.96, *d*, *J*= 2.0)	5.96		93.5 (5.71, *J*=2.0)

3.2.4.3: Compound 24 (1-(3′, 5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxine-2,4,9-triol)

Fraction E006 from Sephadex (MeOH-DCM 1:1) of the EtOAc extract gave another light brown powder that showed positive colour test for phenolics with vanillin-sulfuric acid.
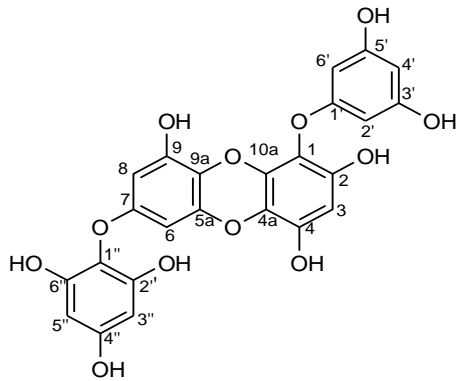
24

Compound **24** had the molecular formula $C_{24}H_{16}O_{12}$ as determined from ES-MS data; m/z 495.0570 $[M - H]^+$ (calcd for $C_{24}H_{16}O_{12}$, 495.0564). The ^1H NMR spectrum showed an AB_2 system at δ 6.09 (2H, $J = 2.1$), 5.98 (1H, t), an AB system at δ 5.99 (1H, $J = 1.8$ Hz), 5.97 (1H, $J = 1.8$ Hz), and two singlets at δ 5.95 (1H) and 5.93 (2H). The signal at δ 5.93 (2H) showed twice the intensity when compared to the other singlet signal at δ 5.95 (1H) and twice the intensity of the doublet resonances at δ 5.97 and δ 5.99. The latter weakly coupled with each other suggesting that they are located in a meta-position relative to each other. Couplings between some of the aromatic protons were lacking indicating a certain degree of symmetrical arrangement in the molecule.

The ^{13}C NMR spectrum indicated the presence of eight non-substituted and 16 O-bearing aromatic carbons between δ 94.4 and δ 160.6 (Table 13). The APT experiment indicated the signals at δ 94.4, and δ 98.6 ppm were due to singly protonated C-atoms, with some peaks accounting for two carbon atoms each. The spectral data is very similar to that of eckol (**23**), except for four additional signals, suggesting that compound **24** was composed of four phloroglucinol units. The position of the extra phloroglucinol moiety was determined to be at C-7 based on the observation that the ^{13}C NMR spectrum for compound **24** were nearly similar with that of compound **23**, except for the downfield shift of the signals due to C-7 and C-9a

which displayed a. A similar phenomenon was reported by Okada *et al* (2004) on elucidation of another phloroglucinol derivative, 7-phloroeckol (Yashihito, *et al*., 2004).

Table 13: 1D and 2D ¹H (600MHz) and ¹³C NMR (150 MHz), MeOD data for compound 24

Position	δ ppm		HMQC	HMBC	δ lit
1	123.9				122.2
2	145.7				145.9
3	96.6	5.95 (*s*)	5.95	C-1, C-2, C-4	98.3
4	141.7				141-9
4a	123.4				123.2
5a	142.7				142.4
6	94.1	5.97(*d, J*=1.8Hz)	5.97	C-7, C-8	93.5
7	155.0				154.5
8	98.6	5.99 (*d, J*=1.8 Hz)	5.99	C-6, C-9, C-9a	98.2
9	147.3				146.1
9a	123.9				124.0
10a	137.3				137.1
1′	160.6				160.3
2′	94.4	6.09 (*d, J*=2.1Hz)	6.09	C-1′, C-3′, C-5′	93.7
3′	159.0				158.8
4′	96.5	5.98 (*t*)	5.98	C-2′, C-3′, C-5′	96.2
5′	159.0				158.8
6′	94.4	6.09 (*d, J*=2.1Hz)	6.09	C-1′, C-3′, C-5′	93.7
1″	123.0				122.6
2″	150.8				151.2
3″	94.8	5.93 (*s*)	5.93	C-4″, C-5″	94.9
4″	153.2				154.8
5″	94.8	5.93 (*s*)	5.93	C-2″, C-4″	94.9
6″	150.8				151.2
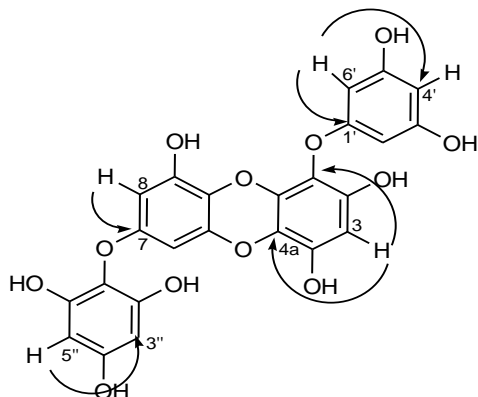
Figure 8: Important HMBC for compound 24

The chemical structure we propose has a similar skeleton to one that was characterized by Okada in extracts from *Ecklonia cava* (Okada, *et al*., 2004). However, it has not previously been elucidated from *Ecklonia maxima* and consequently, it was concluded that compound **24** is **1-(3′, 5′-dihydroxyphenoxy)-7-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxine -2,4,9-triol,** namely 7-phloroeckol.

3.2.4.4: Compound 25 (1-(3′, 5′-dihydroxyphenoxy)-2-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxine-4,7,9-triol)

Fraction E009 obtained from Sephadex (MeOH-DCM 1:1) of the EtOAc extract was subjected to preparative TLC. It gave a light brown powder that showed positive colour test for phenolics with vanillin-sulfuric acid. The final purification of the individual compound was also accomplished by HPLC.

25
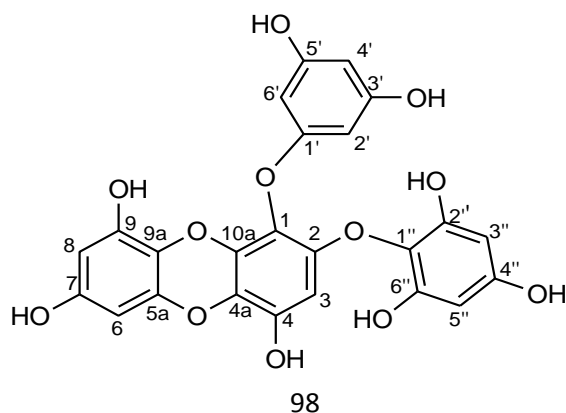
Compound **25** had the molecular formula $C_{24}H_{16}O_{12}$ as determined from its ES-MS data; m/z 495.0561 $[M - H]^+$ (calcd for $C_{24}H_{16}O_{12}$, 495.0564). The ^{1}H NMR spectrum indicated an AB_2 system with signals at δ 6.11 (2H, d, $J = 1.8$ Hz) and 6.01 (t). Proton "A" coupled equally to each "B" protons due to their chemical and magnetical equivalence. An AB system [δ 6.02 (1H, d, $J = 2.6$ Hz) and 6.00 (1H, d, $J = 2.6$ Hz)], which displayed reflection symmetry, two singlets at δ 5.97 (1H, s), 5.95 (2H, s) and eight phenolic hydroxyl proton signals were observed. In the ^{13}C NMR spectrum, eight non-substituted and 16 O-bearing aromatic carbon signals were observed (Table 14). The ^{13}C NMR spectrum is very similar to that of eckol (**23**), except for the four extra signals, indicating that compound **25** was composed of four phloroglucinol units. The linkage position of the additional phloroglucinol moiety was determined to be at C-2 based on the fact that the ^{13}C NMR signals for the basic skeleton in **25** were nearly the same with those of **23** except for C-1, C-2 and C-3. The signals of C-1 and C-2 were observed at downfield shift compared to those of **23**. A similar structure was reported by Fukuyama *et al* on elucidation of another phloroglucinol derivative, 2-phloroeckol (Okada, *et al*., 2004; Suzuki, *et al*., 2003 and Fukuyama, *et al*., 1985). Consequently, this compound has not previously been elucidated from *Ecklonia maxima* and do hereby report and assign it as **1-(3′, 5′-dihydroxyphenoxy)-2-(2″,4″,6″-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxine-4,7,9-triol**, namely 2-phlo -eckol.

Table 14: 1D and 2D ¹H (600 MHz) and¹³C NMR (150 MHz), MeOD data for compound 25

Position	δ ppm	HMQC	HMBC	δ lit
1	123.8			122.9
2	147.3			147.6
3	96.5	5.97 (*s*)	C-1 C-2, C-4, C-4a	96.2
4	141.6			141.5
4a	123.1			123.2
5a	142.6			142.0
6	94.6	6.00 (*d, J*= 2.6 Hz)	C-9a	93.9
7	153.0			153.0
8	98.7	6.02 (*d, J*= 2.6 Hz)	C-6, C-9a	98.7
9	145.6			146.0
9a	123.4			122.7
10a	137.2			137.3
1′	160.5			160.4
2′	94.3	6.11 (*d, J*= 1.8 Hz)	C-4′	94.2
3′	158.8			158.6
4′	96.7	6.01 (*t*)	C-2′, C-6′	96.4
5′	158.8			158.6
6′	94.3	6.11 (*d, J*= 1.8 Hz)		94.2
1″	125.4			122.6
2″	150.7			151.0
3″	95.0	5.95 (*s*)	C-4″, C-6″	94.9
4″	154.8			154.7
5″	95.0	5.95 (*s*)		94.9
6″	150.7			151.0
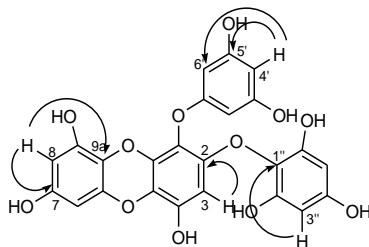
Figure 9: Important HMBC for compound 25

3.2.4.5: Compound 26 (24-ethylidine cholesterol)

Repeated column chromatography of fraction 10 of the initial DCM extract followed by a preparative TLC yielded **26** as white needles (10 mg) that gave a positive colour test for steroids with vanillin-sulfuric acid.

26
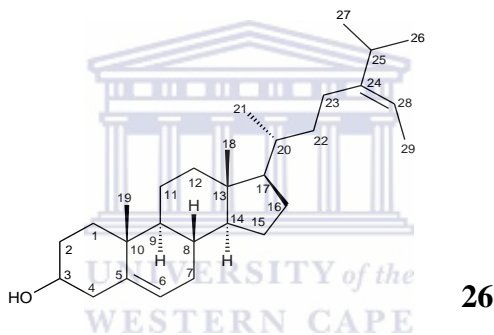
Compound **26** had a molecular formula $C_{29}H_{48}O_1$ as determined from its ES-MS data; *m/z* 411.3584 $[M - H]^+$ (calcd for $C_{29}H_{48}O_1$, 411.3216). The steroidal nature of **26** was deduced from a combination of ^{13}C, HMQC, DEPT-145 and APT NMR data, which revealed the presence of 29 carbons (6 x CH_3, 10 x CH_2, 9 x CH and 4 quaternary centers), four of which were olefinic (C-5, C-6, C-24, C-28). The downfield signal due to δ 71.8 ppm was suggestive of a C-atom that is linked to an oxygen atom and hence could be attributed to C-3. ^{1}H- NMR data revealed the presence of two methyl groups attached to tertiary centers: δ 0.71 (3H, *s*) and δ 0.99 (3H, *s*); four methyl groups attached to secondary centers: δ 0.99 (6H, *d*, *J*= 6.9Hz), δ 1.01 (3H, *d*, *J*= 6.6Hz) and δ 1.59 (3H, *d*, *J*= 6.6Hz), in agreement with the presence of a steroidal skeleton. The signal at δ 5.37 (1H, *m*) is characteristic of H-6 of sterols while the peak at δ 5.20 (1H, *q*) is typical of

the H-28 in the side chain of these structural types. The compound was thus identified as **24-ethylidine cholesterol**, a fucosterol.

^{1}H NMR spectral data (Table 15) showed an olefinic proton of a sharp triplet at δ 5.37 (H-6) and another olefinic proton as a quartet at δ 5.20 (*J*= 6.6 Hz, H-28). Three doublets at δ 1.01 (3H, *J*, 6.6 Hz, H-21), 0.99 (6H, *J*, 6.9 Hz, H-26, H-27), and 1.59 (3H, *J*, 6.6 Hz, H-29). There were two sharp singlets at δ 0.71 (3H, *s*, H-18) and δ 0.99 (3H, *s*, H-19). The signal due to H-3*ax* proton resonated at the expected chemical shift δ 3.55 (1H, *m*,) for such sterols confirming C-5/C6 unsaturated sterol (Forgo *et al*, 2004). The downfield chemical shift of the C-3 methine proton at δ 3.55 was in agreement with the de-shielding of the axial proton by the C-5/C-6 double bond. H-3 (δ 3.55) revealed HMBCs with C-4 (δ 42.2) and C-2 (δ 31.7). Axial-equatorial coupling was observed between H-3*ax* and H-2*eq* (δ 2.03, 1H, *m*) while the axial-axial coupling was observed for H-2*ax* (1.88, 1H, *m*). Similarly COSY interaction between H-3*ax* and H-4*ax* (δ 2.20, 1H, *m*) as well as H-4*eq* (δ 2.31, 1H, *m*) was noted. COSY interactions were observed between H-2*ax* (1.88) and H-1*eq* (δ 1.12, 1H, *m*). H-6 showed interactions with H-7*eq* (δ 1.54, 1H, *m*) and H-7*ax* (δ 1.23, 1H, *m*) in the COSY spectrum. H-28 (δ 5.20, 1H, *q*) showed a strong COSY interaction to H-29 (δ 1.59, 3H, *d*, J= 6.6Hz). The two olefinic carbon signals at C-5 (δ 140.9) and C-6 (δ 121.7) were indicative of the double bond at C5/C6 of the steroidal skeleton (Forgo *et al*, 2004). The signal at δ 5.37 was assigned to H-6 due to the HMBC to C-4 (δ 42.2), C-8 (δ 31.9) and C-10 (δ 36.4). All-important HMBCs are represented as in Figure 10.

The structure of this steroid **26** was further established by comparison of its physical and spectroscopic data with that previously reported (Shen, *et al*., 1997). The position of the double bond at C-24 was determined by the observation of a signal for H-25 at about δ 2.2 ppm. This proton is known to resonate at about 2.2 ppm in fucosterol and at 2.8 ppm in isofucosterol (Forst,

et al., 1968). The signal of the C-21 methyl proton appeared at about δ 0.99, rather than at 0.95

ppm as in isofucosterol, providing further confirmation for the presence of a trans-double bond at

C-24/28 of **26** (Forst, *et al*., 1968; Rubinstein, *et al*., 1976).

Table 15: 1D and 2D ¹H (600MHz) and ¹³C NMR (150 MHz), CDCl₃ data for compound 26

Position	δ ¹³C	δ ¹H	COSY	HSQC	HMBC	Fucosterol
1	37.2	*ax*, 0.87, *eq*, 1.12 (2H, *m*)	H-2*eq*	0.87, 1.12	C-2	37.2
2	31.7	*ax*,1.88, *eq*, 2.03 (2H, *m*)	H-1*eq* H-3*ax*	1.88, 2.03	C-1, C-3	31.6
3	71.8	3.55 (1H, *m*)	H-2*ax* H-2*eq* H-4*ax* H-4*eq*	3.55	C-2, C-4	71.8
4	42.2	*ax*, 2.20, *eq*, 2.31 (2H, *m*)	H-3*ax*	2.20. 2.31	C-6, C-5, C-2, C-10	42.2
5	140.9	-		-		140.7
6	121.7	5.37 (1H, *m*)	H-7*eq*	5.37	C-4, C-8, C-10	121.7
7	31.9	*ax*, 1.23, *eq*, 1.54 (2H, *m*)	H-6	1.23, 1.54	C-6	31.9
8	31.9	1.55 (1H, *m*)		1.54		31.9
9	50.2	1.47 (1H, *m*)		1.47	C-8	50.1
10	36.4	-		-		36.5
11	21.1	*ax*, 0.95, *eq*, 1.52 (2H, *m*)		0.95, 1.52		21.0
12	39.2	*ax*, 1.10, *eq*, 1.20 (2H, *m*)		1.10, 1.20	C-13	39.7
13	42.4	-		-		42.3
14	55.6	1.15 (1H, *m*)		1.15		56.7
15	24.3	*ax*, 1.43, *eq*, 1.54 (2H, *m*)		1.43, 1.55		24.3
16	28.2	*ax*, 1.00, *eq*, 1.55 (2H, *m*)		1.00, 1.55		28.2
17	56.7	1.02 (1H, *m*)		1.02		55.7
18	11.9	0.71 (3H, *s*)		0.71		11.8
19	19.6	0.99 (3H, *s*)		0.99	C-5, C-9	19.4
20	36.5	1.74 (1H, *m*)		0.95	C-21	36.4
21	18.7	1.01 (3H, *d*, *J*=6.6)		1.01		18.7
22	35.2	1.13 (2H, *m*)		1.13		35.2
23	25.7	2.08 (2H, *m*)		2.08	C-24, C-28	25.6
24	146.9	-		-		146.9
25	34.7	2.17 (1H, *m*)		2.17		34.7
26	22.2	1.00 (3H, *d*, *J*=6.9)		0.99	C-24	22.2
27	22.0	0.99 (3H, *d*, *J*=6.9)		0.99	C-24	22.1
28	115.4	5.20 (1H, *q*)	H-29	5.20	C-23, C-25, C-29	115.5
29	13.2	1.59 (3H, *d*, *J*=6.6)	H-28	1.59		13.1
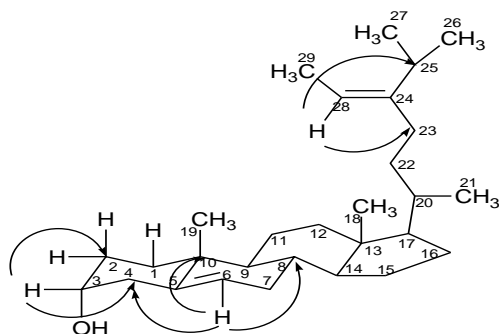
Figure 10: Important HMBC for compound 26

3.2.5 Chemical evaluation.

3.2.5.1 Total phenolic content evaluation.

There was a wide range of phenol concentration outcomes from the extracts and isolates. The total phenolic content of the different samples of the 4 crude and 5 pure isolates was evaluated, using the Folin-Ciocalteu method. The variation of phenolic content was quite significant (Table 16). Consequently, the phenolic content of crude extracts varied from 0.081 of the methanol extract to 0.329 mg GAE/g ethyl acetate. The estimation of phenolic compounds was carried out in triplicate, and the results were averaged. A calibration curve of gallic acid was prepared (in methanol), and the results, determined by the regression equation of $y = 0.007x + 0.056$; $R^2 = 0.998$ mg GAE/g dry alga extracts as shown in Figure 11.
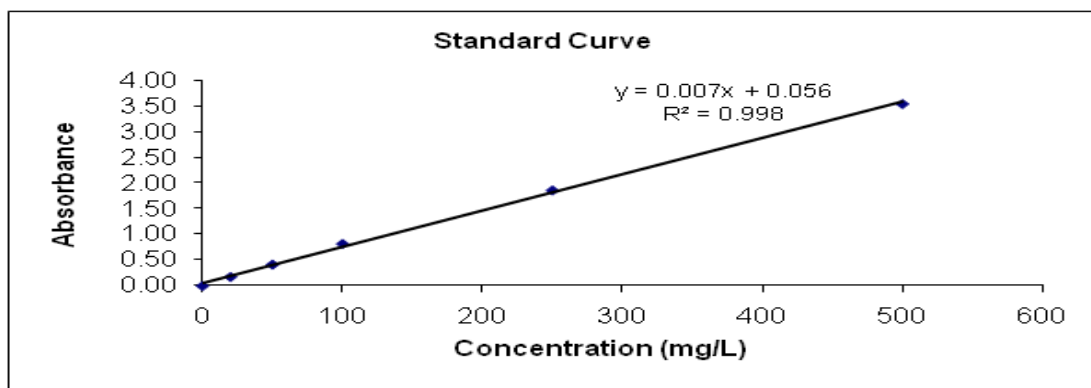
Figure 11: A standard calibration curve of absorbance of gallic acid equivalent mg (GAE)/L of macro algae concentration.

Table 16: Concentration in mg GAE/g of extracts and isolates

Extract/ isolate	Hexane	DCM	EtOAc	MeOH	Comp'd 22	Comp'd 23	Comp'd 24	HM/E/5 (N/D)	Comp'd 25
mg GAE/g	0.124	0.135	0.329	0.081	0.394	4.553	26.570	1.743	5.987
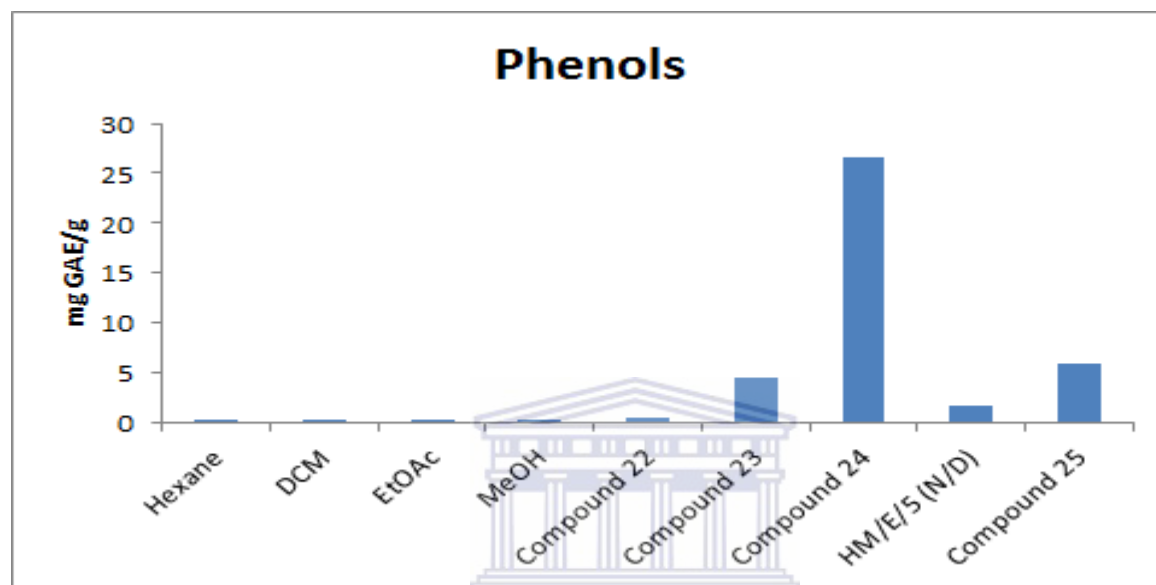
Figure 12: Bar Chart of the phenol content in mg GAE/g of extracts and isolates in *E. maxima*.

Figure 12 is a bar chart showing the phenol content of the extracts and isolates tested. The ethyl acetate fraction of the crude extracts had the highest phenolic content (0.329 mg GAE/g) with methanol sample having the lowest (0.081 mg GAE/g). However, the phenolic content of the pure isolates varied from 0.394 to 26.57 mg GAE/g; Compound **24** had the highest content (26.57 mg GAE/g). The phloroglucinol monomer Compound **22** had the lowest amount (0.394mg GAE/g). According to the tests, various phenolic compounds had different responses in the assay. The polyphenolic isolates were determined to be major contributors to the antioxidant activity from this seaweed. Invariably, the molar responses of the method are proportional to the number of phenolic hydroxyl groups in a given substrate, but the reducing

capacity is enhanced when the phloroglucinol monomers are oriented in a particular arrangement. This property has been elaborated at length under molecular modeling. Primarily, since the structural features of polyphenolic compounds are responsible for antioxidant activity, measurements of total phenols in them is related to their antioxidant activities. In this study, a relatively large number of the isolates were systematically evaluated for the first time, and they established a general relationship between their antioxidant activities with the proposed applications of the seaweed in cosmeceutical industries.

3.2.5.2 ORAC assay

ORAC method is the only one so far that combines the total inhibition time and the percentage of the free-radical damage by the antioxidant into a single quantity, ensuring that, by the end of the process, all the antioxidants present in the sample have reacted with the radicals generated (Zulueta, *et al*., 2009). In this experimental work, the antioxidant capacity of the polyphenols based on hydrogen atom transfer (HAT) reaction was assayed using 2, 2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) as peroxyl radical's source and fluorescein as a molecular probe. Subsequently, the results were reported as the ORAC value, which is the net protection area under the quenching curve of fluorescein in the presence of an antioxidant. The calculation was done by dividing the area under the sample curve by the area under the Trolox® curve with both areas being corrected by subtracting the area under the blank curve. Invariably, one ORAC unit is assigned as being the net protection area provided by 1 µM Trolox® in final concentration. Comparing the area under the curve for the sample to the area under the curve for Trolox®, the results were reported in Trolox® equivalents (TE). The data in Table 17 indicated that antioxidant readings were attributed to micromoles that have the same antioxidant activity as Trolox® equivalent per gram of the tested sample solution. Figure 13 represents data in a bar

graph of µmol TE/g. Compound **24**, a pure isolate recorded the highest value (255.27µmol TE/g) of inhibition followed by compound **25** (71.66µmol TE/g) then compounds **23** (59.21µmol TE/g) and **22**, a phloroglucinol monomer (5.36µmol TE/g), respectively showing the lowest antioxidant property. Invariably, the results implied that structural arrangement of the phloroglucinol units influenced attack of free radical scavengers.

Table 17: Concentration in µmol TE/g of the extracts and isolates

Extract/ isolate	Hexane	DCM	EtOAc	MeOH	Comp'd 22	Comp'd 23	Comp'd 24	HM/E/5 (N/D)	Comp'd 25
µmol TE/g	1.44	1.58	4.01	0.70	5.36	59.21	255.27	1.743	71.66
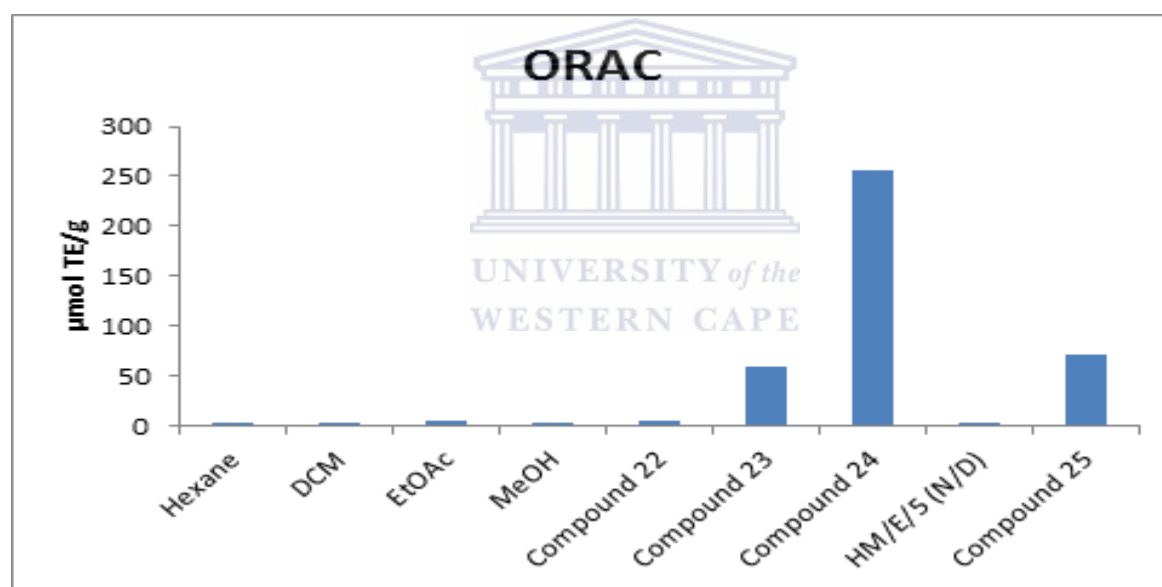
Figure 13: Bar Chart of ORAC values of crude extracts and pure isolates in *E. maxima*.

Furthermore, the net area under curve (AUC) summed over time of different Trolox® standards were plotted as a function of concentration. The subsequent calibration curve was then used to interpolate the antioxidant capacity of unknown samples. Principally, the standard curve is used to quantitate unknown samples. The resultant determinations are expressed as Trolox® equivalents. Table 17 represents summary data of extracts and compounds from the seaweed

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under study claimed to have antioxidant properties and assayed using ORAC. From the experimental data, a standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. The ORAC values of samples were then calculated automatically using computer software to interpolate the sample's Net AUC values against the Trolox® standard curve. Invariably, the loss in fluorescence is a measure of the area under the curve (AUC) of the kinetic plot for each given concentration. The greater the extent of florescent decay, the smaller the expected AUC value would be. The highest concentration of the Trolox® standard tested provided the highest protection, while the lowest concentration tested offered only slight protection above the buffer control. Trolox® equivalents of the samples were calculated using the quadratic relationships ($Y = a + bX + cX^2$ between Trolox® concentration (Y) (μM) and the net area under the Fluorescein decay curve (X) (AUC_{sample} - AUC_{blank}). Data may be expressed as micromoles of Trolox® equivalents (TE) per liter or per gram of sample (μmol of TE/L or μmol of TE/g). In the presence of increasing concentrations of Trolox®, the decay of the curve was delayed.
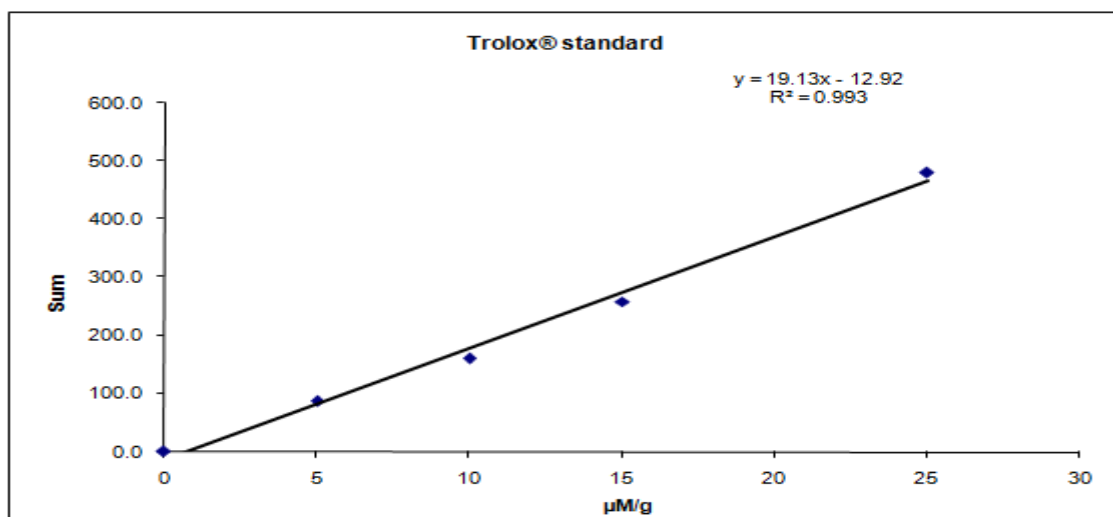
Figure 14: Blank-corrected linear regression curve of Trolox®. The data points were summed over time and were plotted on the y-axis vs. concentration.

Figure 14 shows the consumption of Fluorescein elicited by its incubation in the presence of AAPH, with increasing concentrations of Trolox®. Again in Table 17, the results consequently shows data of the nine samples where all compounds considered in the study demonstrated a good relation between the net area under the curve (AUC) and consumption of Fluorescein to Trolox® equivalence (TE). The fluorescence of each well was measured from the bottom after every one minute at a sensitivity setting of 60 cycles. ORAC values were calculated as described by Cao and Prior (1998, 1999). Furthermore, the AUC and the Net AUC of the standards and samples were determined using a data reduction software applying equations 1 and 2 respectively. The linearity of the plots implies that the ORAC values could be defined by equations (1 and 2):

$$AUC = (0.5 + f_2/f_1 + f_3/f_1 + f_4f_1 + \ldots + f_n/f_1) \text{ x } CT \qquad (1)$$

Where f_1 = initial fluorescence reading at cycle 1, f_n = fluorescence reading at cycle n, and CT = cycle time in minutes.

$$Net \ AUC = AUC_{sample} - AUC_{blank} \qquad (2)$$

Integration of that area under the curve (AUC) is performed up to a time such that (f_n/f_1) reaches a value close to zero. Principally, antioxidant curves (fluorescence versus time) are first normalized to the curve of the blank corresponding to the same assay by multiplying original data by a factor f $_{blank}$, $t = 0$/f $_{sample}$, $t=0$. Subsequently, from the normalized curves, the area under the fluorescence decay curve (AUC) can now be calculated. ORAC-Fluorescein values are then expressed as Trolox® equivalents by using those standard curves calculated for each assay. Further, after subtracting the AUC for the blank, the resultant difference is the protection conferred by the antioxidant compound. Accordingly, the assay expresses results as ORAC units or Trolox® equivalents, which correspond to the amount of Trolox® micromoles that have the

same antioxidant activity as one liter or per gram of the tested solution (Cao, *et al.*, 1993, 1995).
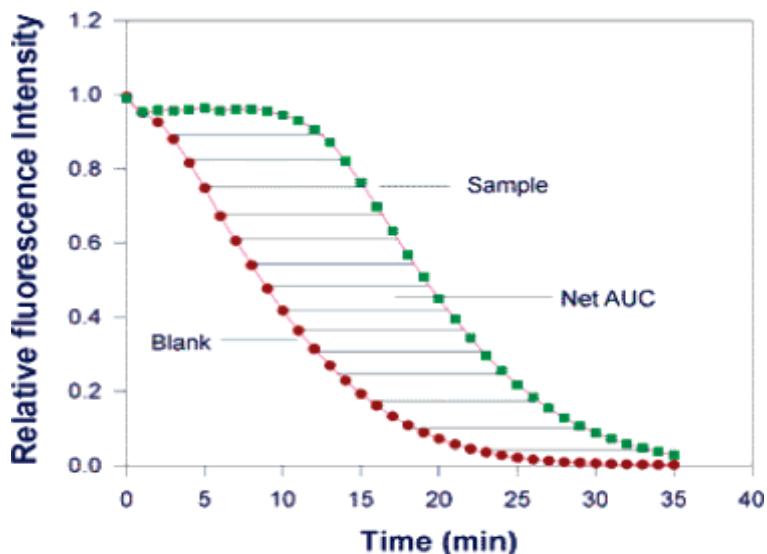
Figure15: ORAC antioxidant activity determination illustration of a tested sample expressed as the net area under the curve (AUC) (Prior, *et al.*, 2005).

In principle, the AUC for standards, samples and blank are calculated and the blank value subtracted. Subsequently, the resultant difference is the protection by the antioxidant sample. The fluorescent intensity decreases as the oxidative degeneration proceeds, and this intensity is typically recorded within half an hour after the addition of a free radical generator (Figure 15).

Interestingly, the assay principle is that over time, reactive oxygen species (ROS) generated from the thermal decomposition of AAPH quenches the signal from the fluorescent probe Fluorescein (Scheme 2). A subsequent addition of an antioxidant produces a more stable fluorescence signal, with signal stability depending on the antioxidant's capacity.

ROS (Reactive Oxygen Species)

Fluorescent Probe + Buffer	Fluorescent Probe + Trolox®	Fluorescent Probe + Sample
↓	↓	↓
Loss of fluorescence	Loss of fluorescence	Loss of fluorescence
↓	↓	↓
Sum Blank	Sum Standard	Sum Sample
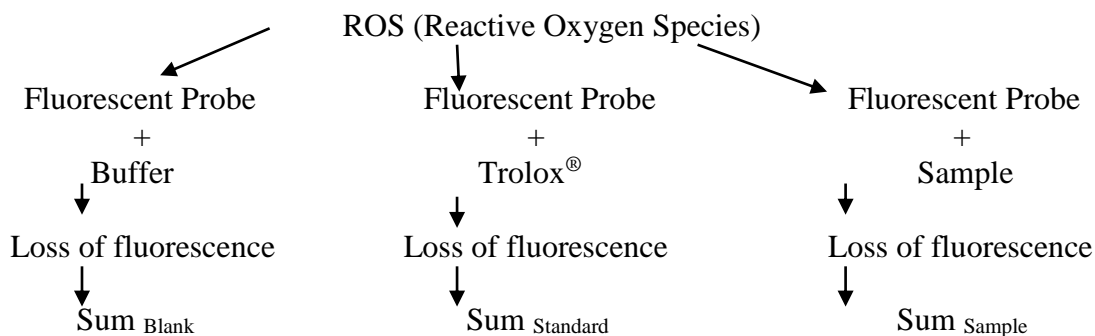
Antioxidant Capacity relating to Trolox® = (Sum Sample - Sum Blank) / (Sum Standard - Sum Blank).

Scheme 2: The ORAC assay principle

The antioxidant capacity of a sample is that net difference between the area under the curve (AUC) of the sample and that of the blank.

The variations between the data in this study are attributed to the protection of polyphenols towards Fluorescein consumption. This could be due to several factors among them the different chemical reactivity involved in the hydrogen abstraction by the peroxyl radical. Nonetheless, it is well known that the first step of Fluorescein oxidation involves hydrogen of the phenolic group being abstracted by the peroxyl radical, forming a stable Fluorescein-phenoxyl radical (Dávalos, *et al*., 2004). Invariably, in air-saturated solution, the generated AAPH radical reacts with O_2 rapidly to give a more stable peroxyl radical ROO˙. However, the presence of antioxidants in the different extracts and isolates protects the extent of Fluoroscein-phenoxyl radical from bleaching by neutralizing the peroxyl-free radical and other free radicals formed in the system. Therefore, one benefit of using the ORAC method to evaluate substances' antioxidant capacity is that it takes into account samples with and without lag phases of their antioxidant capacities. Moreover, ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H-atom transfer (Ou, *et al*., 2001). The loss of fluorescence of Fluorescein is an indication of the

extent of damage from its reaction with the peroxyl radical. In the presence of antioxidant, $ROO^{\bullet}$ abstracts a hydrogen atom from the antioxidant to form hydroperoxide (ROOH) and a stable antioxidant radical (Phenoxyl-$O^{\bullet}$); as a result, the damage to Fluorescein induced by peroxyl radical is inhibited. Invariably, Ou *et al* have shown that under the ORAC experimental conditions, fluorescence decrease is independent of concentrations of Fluorescein but first order with AAPH concentration. The ORAC assay directly measures the antioxidant activities of chain-breaking antioxidants against peroxyl radicals. Therefore, we suggest that ORAC values be used as a guideline for "peroxyl radical absorption capacity" from *Ecklonia maxima*.

Biologically, organisms are able to repair oxidative damage in their bodies and any negative effects that free radicals have on them are well recognized (Varahalaro and Chandrashekar, 2010). Antioxidants can delay or prevent such oxidative stresses applied on our organs. Furthermore, phlorotannins have, in recent years demonstrated total antioxidant capacity/antioxidant capacity (TAC/AOC) (Wang *et al*., 2007). Unlike synthetic antioxidants, phlorotannins are a natural antioxidant and have a large potential for avoiding or delaying free radical-mediated illnesses (Li and Zhao, 2008). Primarily, reactive oxygen species (ROS) are generated continuously in our biological systems through metabolism and environmental sources. Even though our body has its own natural defensive mechanisms against ROS, it cannot prevent the damage entirely. Antioxidants are therefore substances that can prevent these radical reactions by forming stable free radicals out of ROS (Wang, *et al*., 2009). As a result, the food and pharmaceutical industries are considering the use of natural antioxidants which have no side effects and are non-toxic to humans (Wang *et al*., 2007).

3.2.5.3 FRAP assay

Fe^{3+}/Fe^{2+} transformation was investigated in the presence of samples for the measurements of the reductive ability. The μmole AAE/g for the extracts and isolates was presented in Table 18. In the present study, effectiveness of the radical scavenging activity appeared more pronounced with the isolates as compared to the crude extracts. Consequently, the results of the investigations shown in Figure 16 portrays that the extracts of hexane, DCM and methanol recorded a relatively low activity. The FRAP assay at a glance reconfirmed the consistency in the antioxidant capacity of the extracts and isolates as observed with ORAC in which the highest values were recorded in the pure isolates, compound **24** registering the highest. All the pure isolates were obtained from the ethyl acetate extract with biological greater activity.

Table 18: Concentration in μmol AAE/g of the extracts and isolates in FRAP assay

Extract/ isolate	Hexane	DCM	EtOAc	MeOH	Comp'd 22	Comp'd 23	Comp'd 24	HM/E/5 (N/D)	Comp'd 25
μmol AAE/g	0.248	0.256	0.995	0.055	0.411	10.849	93.249	3.841	14.130
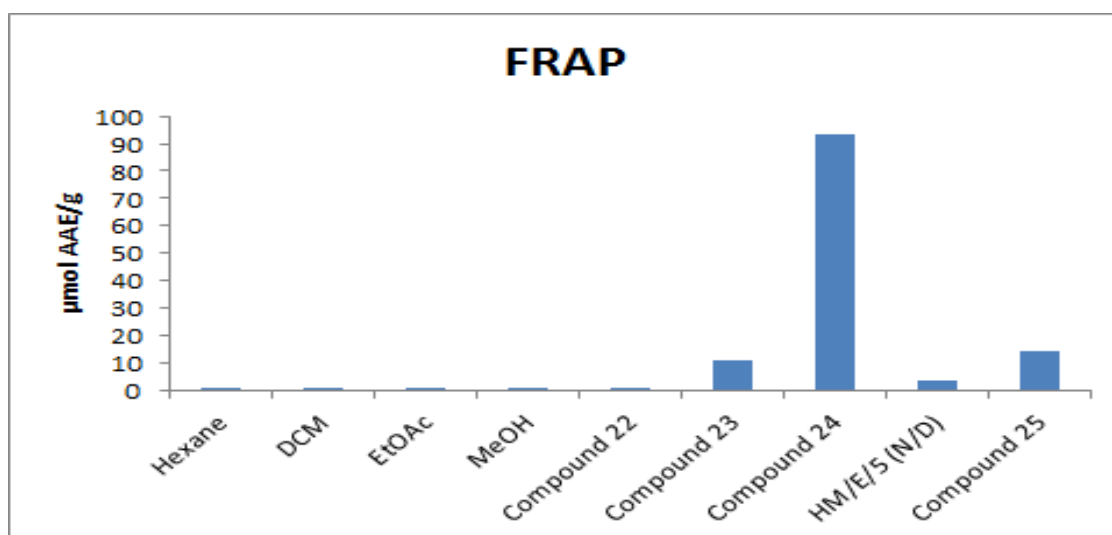
Figure 16: Bar Chart of FRAP values from the extracts and isolates present in *Ecklonia maxima*.

The FRAP principle reflects that relevant chemical reaction of the method involves a single electron reaction between $Fe^{3+}(TPTZ)_2$ and a single electron donor. Benzie and Strain (1999) considered an antioxidant as any species that reduces the oxidizing species that would otherwise damage substrates. Subsequently, the antioxidant activity is then interpreted as the reducing capability. Polyphenols have "total antioxidant power" and hence the "total reducing power". Furthermore, at low pH, reduction of $Fe^{3+}(TPTZ)_2$ complex to ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorption at 593 nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under the reaction conditions, than that of Fe^{3+}/Fe^{2+} half reaction, will drive into the ferrous ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

$$Fe^{3+}(TPTZ)_2 + ROH \longrightarrow Fe^{2+}(TPTZ)_2 + ROH^{+\bullet}$$

A limitation of the FRAP assay is the possible interference due to the UV-Vis absorption at 593 nm by compounds other than $Fe^{2+}(TPTZ)_2$. Therefore, the FRAP assay cannot be used in biological samples alone rather other assays must be incorporated. Primarily, this assay depends on the reduction of a ferric tripyridyltriazine $Fe^{3+}(TPTZ)_2$ complex to the ferrous tripyridyltriazine $Fe^{2+}(TPTZ)_2$ by an antioxidant at a low pH of 3.6. However, a very low pH can significantly inhibit an electron transfer (ET) from the antioxidant to the ferric ion. FRAP results reflect only the antioxidant reducing potential based on ferric ion instead of the antioxidant preventative effect. Clearly, the FRAP assay measures the reducing capability based upon Fe^{3+} which is not relevant to antioxidant activity mechanistically and physiologically, let alone the total antioxidant capacity. Nevertheless, on the basis of these facts, it is not appropriate to use the FRAP value alone as an indicator for "total antioxidant power".

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3.2.5.4 ABT/TEAC assay

The antioxidant capacities of the extracts and isolates were evaluated using an improved ABTS$^{\bullet+}$ radical decolorisation assay, one of the most commonly employed methods for measuring antioxidant capacity, which determines the ability of a compound to scavenge ABTS$^{\bullet+}$ radical. Furthermore, it is recommended for use in extracts because of the long wavelength absorption maximum at 734 nm that eliminates colour interference (Awika, *et al*., 2003). Invariably, this assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavengers of lipid peroxyl radicals).

Table 19: Concentration in µmol TE/g TEAC standard series of extracts and isolates

Extract/ isolate	Hexane	DCM	EtOAc	MeOH	Comp'd 22	Comp'd 23	Comp'd 24	HM/E/5 (N/D)	Comp'd 25
µmol TE/g	0.750	0.880	1.412	0.342	1.693	19.745	83.153	3.875	22.196
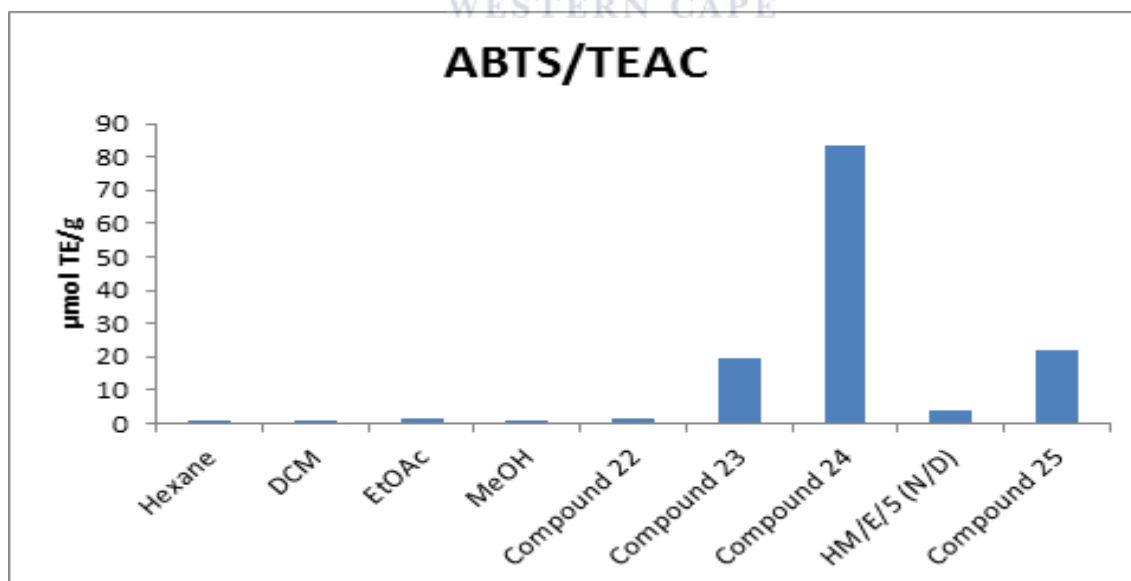
Figure 17: Bar Chart showing the ABTS (TEAC) values

Table 19 shows the antioxidant capacities of the tested extracts and isolates of the seaweed. The alga had very diverse radical scavenging capacity ranging from 0.342-83.153 µmol Trolox$^{®}$E/g. This is so since the samples were from different extracts and also the isolates had differing phloroglucinol monomers in composition. However, isolates with similar number of monomers and equal molecular masses exhibited very different radical scavenging ability. For example, compound **25** (22.196 µmol Trolox$^{®}$E/g) possessed a much lower total antioxidant capacity than compound **24** (88.153 µmol Trolox$^{®}$E/g) yet they are isomers. Pure isolates displayed a much higher total antioxidant capacity (Figure 17) than the crude extracts probably due to interferences from other substances in the extracts. Primarily, the high and varying antioxidant activities of the isolates may be due to their differences in their phenolic hydroxyl patterns. Many hydrophilic polyphenolic compounds in seaweed have strong antioxidant components. This justifies the importance of testing the pure isolates as they are good candidates for future studies.

The antioxidant characteristics from this macro-alga have different polarities, and as a result, compounds were extracted from different fractions. From the less polar solvents, hexane and DCM extracts had antioxidant capacities of 0.750 and 0.880 µmol Trolox® E/g respectively. For the ethyl acetate extract, the antioxidant capacity was at 1.412 µmol Trolox$^{®}$E/g registering the highest antioxidant activity compared to the other crude extracts. This sample was the source of all the pure isolates. To our knowledge, there has been no prior report as to the antioxidant activities of the extracts from these macro-alga *Ecklonia maxima*. Therefore, this study has provided valuable preliminary data, through a demonstration of the high alga antioxidant property from its polyphenolic isolates. The macro-alga therefore serves as a potentially rich source of natural antioxidants. However, from the results of the ABTS radical cation decolorisation assay, the extracts and isolates of the seaweed can eliminate free radicals by

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acting as free radical scavengers or by donating a hydrogen atom to a molecule hence making them behave as primary and secondary antioxidants (Re, *et al.*, 1999; Zhu, *et al*., 2002).

Polyphenols in general contain reducing properties as hydrogen or electron-donating agents, thus are seen as potential free radical scavengers (antioxidants) (Rice-Evans, *et al.*, 1997). Furthermore, polyphenolic compounds are natural antioxidants which are found mostly in plants and seaweeds (Moon, *et al*., 2008; Lim, *et al*., 2002). They are consistent with broad antioxidant activities via both single electron transfer and hydrogen atom transfer system (Prior *et al.*, 2005). Nonetheless, the antioxidant potency of naturally occurring phenolic compounds is known to be strongly related to the structure, in particular to electron delocalization in the aromatic system (Cuvelier, *et al*., 1992; Rice-Evans, *et al.*, 1996).

3.2.6 Computational theoretical studies
3.2.6.1 Conformation and radical stability

Theoretical studies on phloroglucinol have established that an enol form is by large preferred to the keto form, also in relation to the stability of the aromatic ring (Mandix, *et al*., 1993). Furthermore, a study of conformational preferences has identified two possible conformers, differing by the mutual orientation of the OH groups; a conformer with all the OH groups oriented uniformly, and one in which the orientation is not uniform and two of the OH groups are oriented towards the same linear direction (Spoliti, *et al*., 1997). Principally, energy of the former is 1 kcal/mol lower than the energy of the latter and the rest of the molecular geometry is not affected by the difference in the orientation, as long as planarity is maintained (Spoliti, *et al*., 1997).

Consequently, from the B3LYP/6-31+G (d) optimized geometries in this study, corresponding units to the lowest-energy conformer of each of the isolated phloroglucinol

derivatives are shown in Figure 18. Studies on the isolated phloroglucinol moiety have established that there are two conformations, one in which all the phenolic OH groups are oriented in the same way (uniform orientation, PG-1) and the other in which two of the phenolic OH groups are oriented towards each other (non-uniform orientation, PG-2). The conformation with uniform orientation of the phenolic OH has lower energy than the conformation with non-uniform orientation of the OH groups (Kabanda and Mammino, 2010). The lowest-energy conformers of structures **23**, **24** and **25** correspond to geometries in which the number of intramolecular hydrogen bonds (IHB) is maximized.

22 **23** **24** **25**
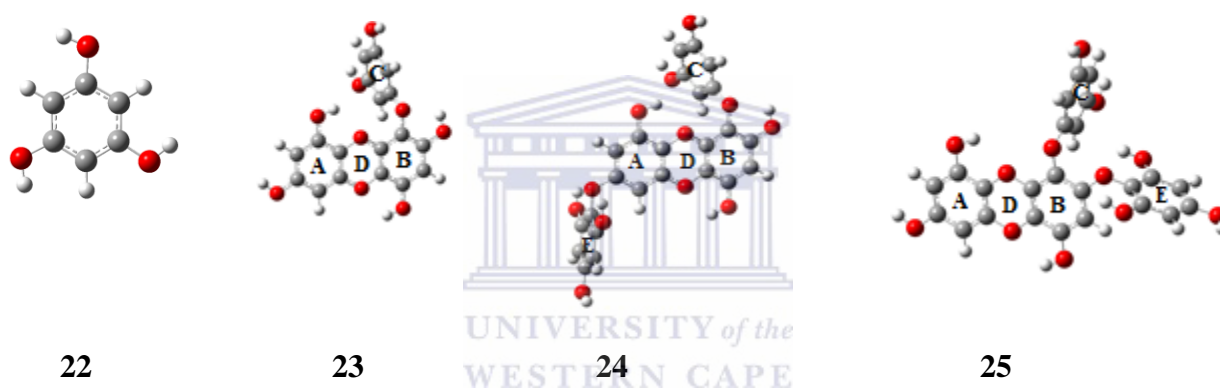
Figure 18: Lowest-energy conformers of the optimized structures for the isolated phloroglucinol derivatives, B3LYP/6-31+G(d) results *in vacuo*.

Structure **23** can form a maximum of three O-H···O intramolecular hydrogen bonds (namely $H_9···O_{10a}$, $H_2···O_1$ and $H_4···O_{4a}$). The H···O bond length is 2.20 Å for $H_9···O_{10a}$ and $H_4···O_{4a}$ and 2.21 Å for $H_2···O_1$, the O···O distance is in the range of 2.72-2.75Å and the OĤO bond angle is $\approx 112°$. These geometric parameters are indicative of weak IHB. In the lowest energy conformer, the phloroglucinol moiety linking to C_1 is oriented in such a way that the C_{10a}-C_1-O_1-$C_{1'}$ torsion angle is -73.8° (i.e., ring C is slightly inclined towards ring D). This orientation affords the formation of a weak O_9-$H_9···\pi$ bond between the phenolic OH at C_9 and the π system of benzene ring C. H_9 is therefore engaged in bifurcated IHB, $H_9···O_{10a}$ and O_9-$H_9···\pi$.

The lowest energy conformer of structure **24** can form a maximum of five O-H···O IHB (H_9···O_{10a}, H_2···O_1, H_4···O_{4a}, $H_{2''}$···$O_{1''}$ and $H_{6''}$···$O_{1''}$) and three unconventional IHB namely, O_9-H_9···π_C, $C_{2'}$-$H_{2'}$···π_B and C_6-H_6···π_E (where π_B, π_C and π_E refers to the π systems of the aromatic ring B, C and E respectively). The C_{10a}-C_1-O_1-$C_{1'}$ torsion angle is -71.6° from the plane of ring B and the C_6-C_7-O_7-$C_{1''}$ torsion angle is 2.6° from the plane of ring A.

The lowest-energy conformer of structure **25** can form a maximum of four O-H···O IHB (H_9···O_{10a}, H_4···O_{4a}, $H_{2''}$···O_2 and $H_{6''}$···O_2) and three unconventional IHB namely C_3-H_3···π_E, $C_{2'}$-$H_{2'}$···π_B and $O_{6''}$-$H_{6''}$···π_C (where π_B, π_C and π_E refers to the π systems of the aromatic ring B, C and E respectively) . The C_{10a}-C_1-O_1-$C_{1'}$ torsion angle is -84.8° from the plane of ring B and the C_3-C_2-O_2-$C_{1''}$ torsion angle is -107.6° from the plane of ring B, suggesting that ring C is inclined towards ring E. The geometric constrictions imposed by the different IHB accounts for the stability of **24** and **25**. For instance, it is noted that the unconventional intramolecular hydrogen bonds involving two aromatic rings (e.g., C_3-H_3···π_E and $C_{2'}$-$H_{2'}$···π_B) impose a perpendicular arrangement of the benzene rings (e.g., the C_7-O_7-$C_{1''}$-$C_{2''}$ torsion angle in **24** is 92.2°). The perpendicularity of benzene rings has been reported to stabilize molecular conformations (Mammino and Kabanda, 2009).

Structures **24** and **25** are structural isomers and a comparison of the lowest-energy conformer of the two indicates that the former is 2.365kcal/mol lower than the latter. The preference of structure **24** (% population of 98.2) to structure **25** (% population of 1.8%) may be related to the fact that the former has an additional O-H···O IHB with respect to the latter, resulting in a five-member ring. IHB are known to determine conformational preferences and also to influence biological activities (Verevkin and Schick, 2004). Furthermore, both

experimental and theoretical studies have established that the strength (kcal/mol) of the O-H···O IHB (resulting in a five member ring) in hydroxybenzenes is in the range of 0.71-2.22 (Mammino and Kabanda, 2011; Bentes, *et al*., 2011). Therefore, the energy difference between structure **24** and **25** is in the upper range of the O-H···O IHB in hydroxybenzenes.

The optimized neutral radical species, necessary to study reaction enthalpies related to the HAT mechanism, were generated from the lowest-energy conformer of each of the studied compounds and are shown in figure 19. The total spin density distribution for each neutral radical species is shown in figure 20 and the relative energies of the neutral radical species are reported in Table 20.

a) Neutral radical species compound **23**
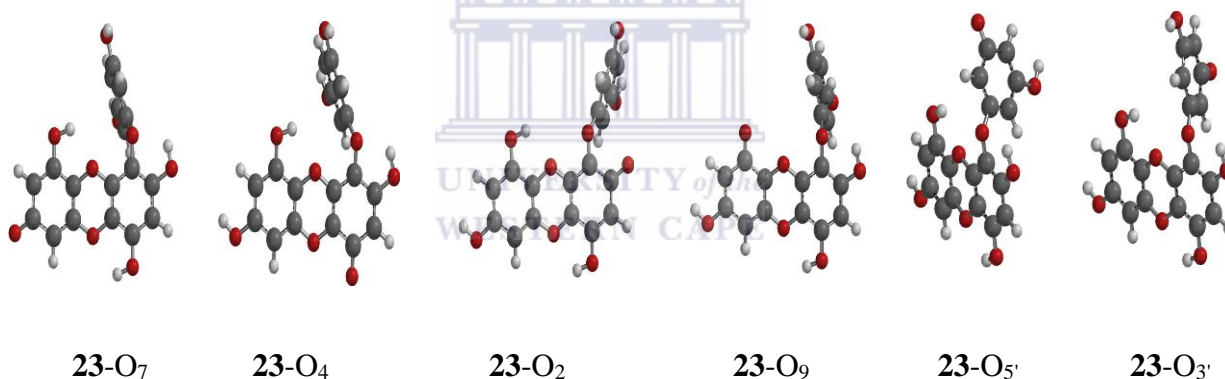
| **23**-O_7 | **23**-O_4 | **23**-O_2 | **23**-O_9 | **23**-$O_{5'}$ | **23**-$O_{3'}$ |

b) Neutral radical species of compound **24**

| **24**-O_4 | **24**-$O_{2''}$ | **24**O_2 | **24**-$O_{6''}$ |

24-O$_{4''}$ 24-O$_{5'}$ 24-O$_9$ 24-O$_{3'}$

c) Neutral radical species of compound **25**

25-O$_7$ **25**-O$_4$ **25**-O$_{6''}$ **25**-O$_9$
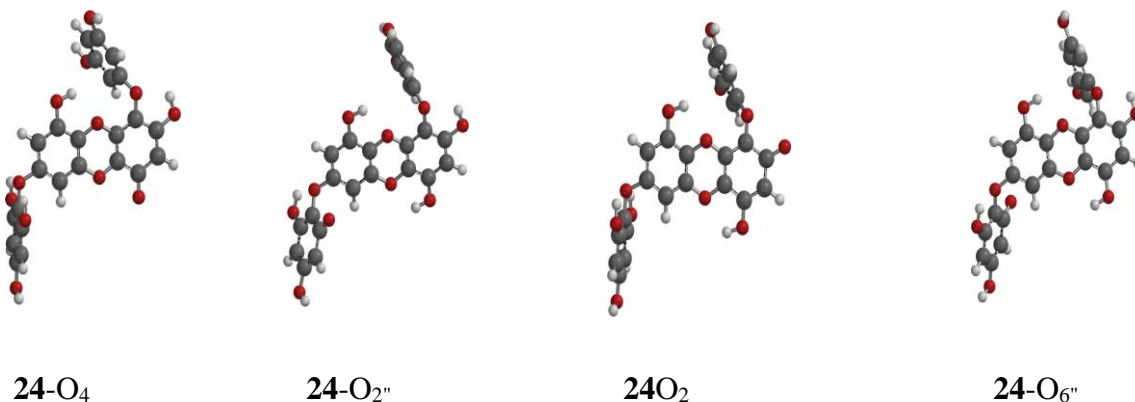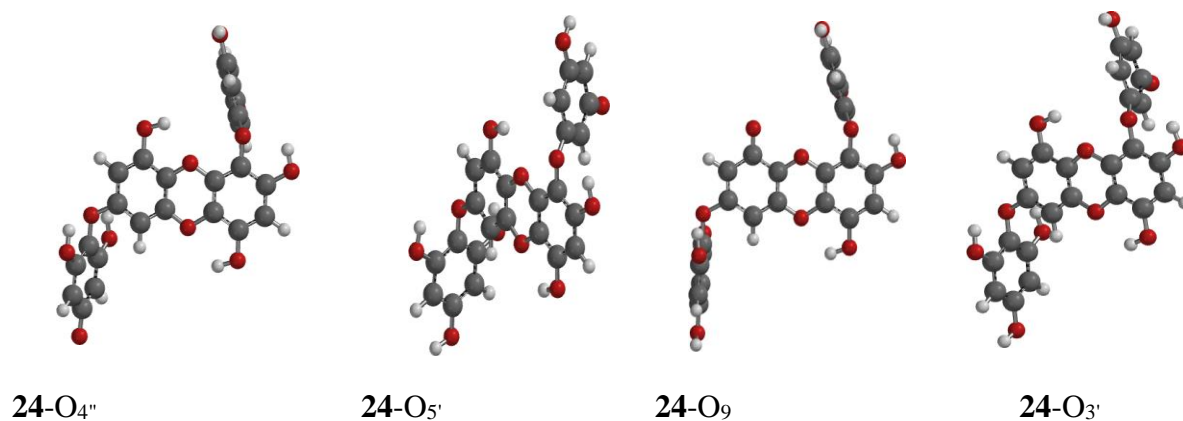
25-O$_{4''}$ **25**-O$_{2''}$ **25**-O$_{3'}$ **25**-O$_{5'}$
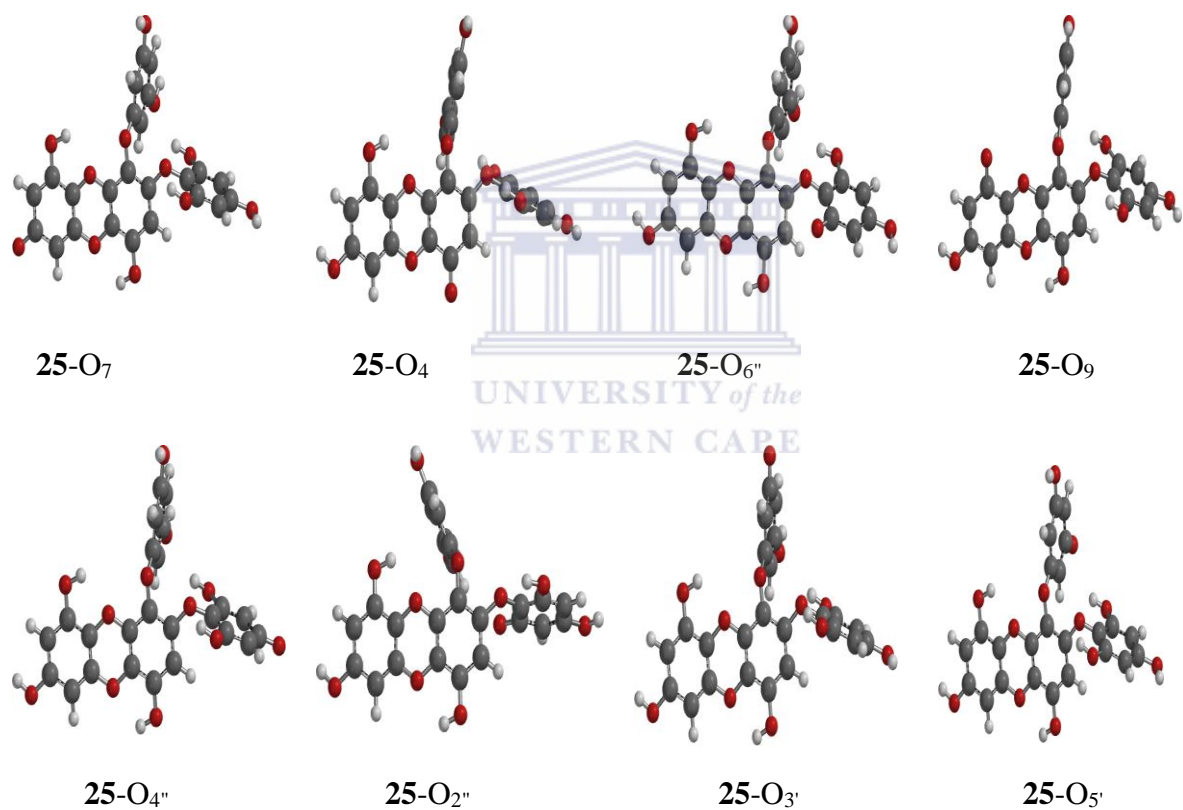
Figure 19. *In vacuo* **B3LYP/6-31+G(d) optimized geometries for the neutral radical species of the isolated phloroglucinol derivatives. The confomers are arranged in order of increasing energy reported in Table 20.**

Table 20: Relative energies (kcal/mol) and BDE values (kcal/mol) for the calculated neutral radical species of the studied compounds, B3LYP/6-31+G(d) results in different media.

Structure	Relative energy (kcal/mol)[a]			Bond dissociation enthalpy, BDE (kcal/mol)		
	in vacuo	in CHCl₃	in water	*in vacuo*	in CHCl₃	in water
Phenol[b]	–	–	–	87.675	88.216	90.222
22-O₂	0.000	0.000	0.000	88.977	89.101	90.236
22-O₄	2.094	1.413	0.808	90.300	89.974	90.975
23-O₇	0.000	0.000	1.166	85.149	84.932	86.135
23-O₄	1.893	0.393	0.108	87.068	85.295	85.150
23-O₂	2.156	0.730	0.018	87.434	85.534	84.894
23-O₉	2.948	1.920	0.000	88.144	86.627	84.741
23-O₅'	4.308	4.762	6.042	89.487	89.642	90.918
23-O₃'	5.414	5.456	6.200	90.656	90.341	91.104
24-O₄	0.000	0.000	0.000	87.093	85.515	85.014
24-O₂"	0.247	1.321	2.566	87.325	86.387	86.983
24-O₂	0.271	0.451	0.036	87.434	85.954	85.002
24-O₆"	1.665	2.858	3.448	89.053	88.103	88.040
24-O₄"	1.903	3.333	5.774	88.813	88.970	90.809
24-O₅'	1.884	3.703	5.092	88.988	89.097	89.808
24-O₉	2.733	3.564	1.975	89.867	88.925	86.725
24-O₃'	3.351	4.936	5.811	90.516	90.350	90.550
25-O₇	0.000	0.000	0.691	85.164	86.573	85.347
25-O₄	1.770	0.762	0.000	86.985	85.737	85.929
25-O₆"	2.682	2.821	3.285	87.990	87.786	88.567
25-O₉	3.019	1.583	0.006	88.196	85.750	86.763
25-O₄"	3.188	3.554	4.947	88.372	90.865	88.936
25-O₂"	4.272	4.041	4.086	89.618	89.877	89.253
25-O₃'	4.677	5.522	6.817	89.972	92.572	90.861
25-O₅'	5.118	5.591	6.802	90.470	92.524	90.893

[a] *Relative energy values are taken with respect to the lowest energy conformer in a given media*
[b] *All BDE values are shown relative to phenol calculated at the same level of theory.*

23-O$_7$ **23**-O$_4$ **23**-O$_2$ **23**-O$_9$ **23**-O$_{5'}$ **23**-O$_{3'}$

24-O$_4$ **24**-O$_{2''}$ **24**-O$_2$ **24**-O$_{6''}$ **24**-O$_{4''}$ **24**-O$_{5'}$ **24**-O$_9$ **24**-O$_{3'}$

25-O$_7$ **25**-O$_4$ **25**-O$_{6''}$ **25**-O$_9$

25-O$_{4''}$ **25**-O$_{2''}$ **25**-O$_{3'}$ **25**-O$_{5'}$
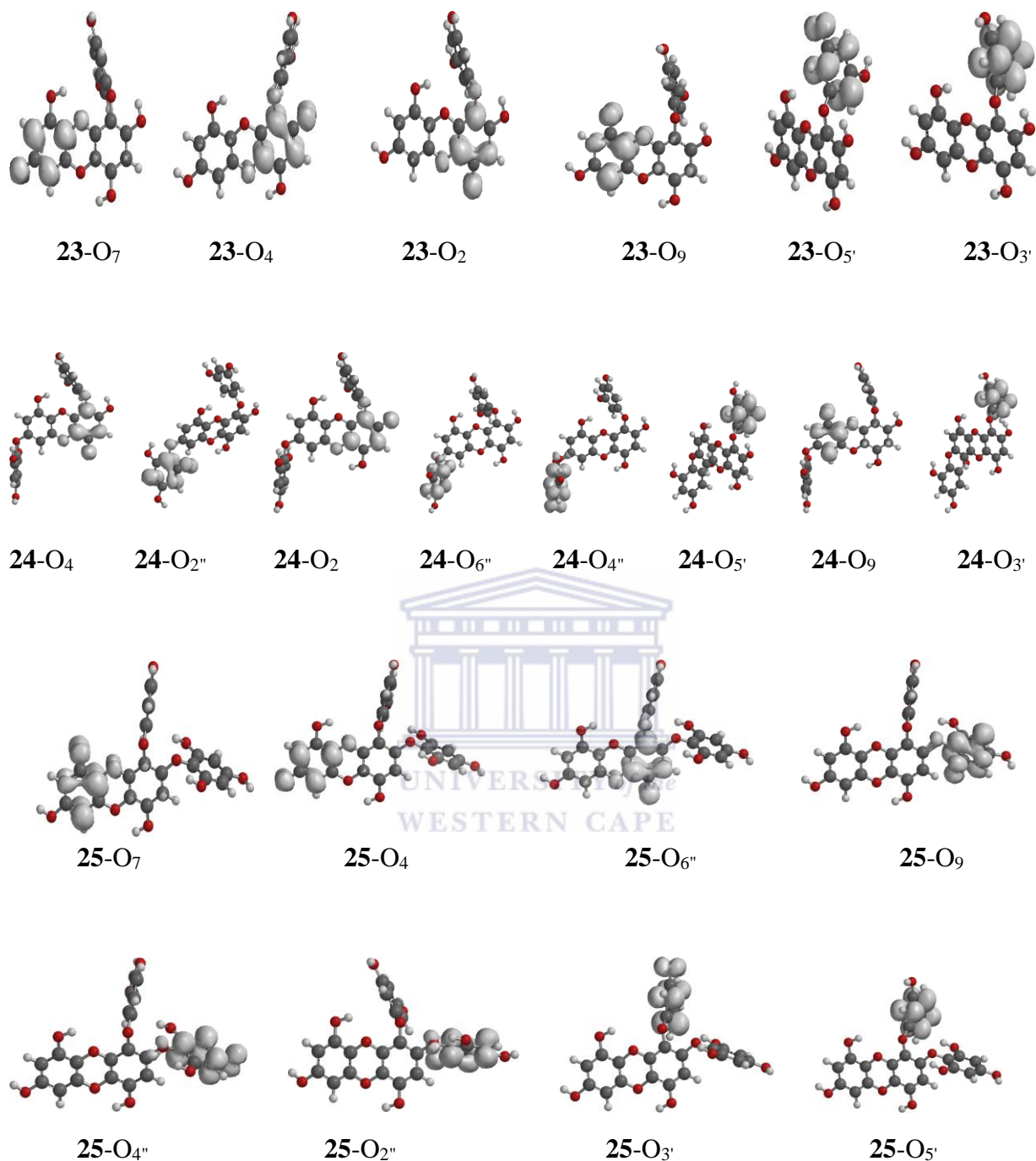
Figure 20: Spin density distributions of investigated phlorotannin neutral radical species, B3LYP/6-31+G(d) *in vacuo*.

The C$_{3h}$ symmetry nature of the lowest-energy conformer of phloroglucinol suggests that only one radical species is generated by the H atom abstraction from each OH group. The H atom abstraction from each OH group present in **23** gives rise to six different radical species. The

23-O_2, **23**-O_4 and **23**-O_9 radical species are formed by the removal of the IHB and are consequently less stable with respect to **23**-O_7 radical species, which is formed from free OH group (i.e., an OH group not engaged in IHB). However, **23**-O_2, **23**-O_4 and **23**-O_9 radical species are more stable than the **23**-$O_{3'}$ and **23**-$O_{5'}$ radical species mainly because the unpaired electron in **23**-O_2, **23**-O_4 and **23**-O_9 radical species are delocalized beyond the ring on which the H atom is removed while in **23**-$O_{3'}$ and **23**-$O_{5'}$ radical species, the unpaired electron are distributed only on the ring with radicalized O atom. This means that the radical species of **23** are stabilized by the presence of IHB and the extent of delocalization of the unpaired electron; depending on the nature of the radical species formed, one of these factors might outweigh the others in contributing to the stabilization of the radical species.

The abstraction of the H atom from each phenolic OH in **24** gives eight different radical species that are stabilized by a number of geometric and electronic factors including IHB, delocalization of the unpaired electron and steric effects. Radical species **24**-O_2, **24**-O_4, **24**-O_9, **24**-$O_{2''}$ and **24**-$O_{6''}$ are formed by the removal of the IHB while radical species **24**-$O_{3'}$, **24**-$O_{5'}$ and **24**-$O_{4''}$ are generated from a free phenolic OH. For the results *in vacuo*, radical species **24**-O_4 has the lowest energy because, despite having fewer IHB than **24**-$O_{3'}$, **24**-$O_{5'}$ and **24**-$O_{4''}$, it distributes the unpaired electron across three rings (A, B and D), which does not happen in latter radical species. Radical species **24**-$O_{2''}$ and **24**-O_2 are 0.331 kcal/mol and 0.343 kcal/mol respectively higher than **24**-O_4 as they combine different stabilization factors; **24**-$O_{2''}$ is stabilized by the OH⋯O IHB and the unconventional $O^{.}$⋯CH bond as a result of the proximity of the radicalized O atom to the CH group of ring C; **24**-O_2 radical species is stabilized more by the delocalization of the unpaired electron further than the ring on which the H atom is abstracted. **24**-$O_{6''}$, **24**-$O_{4''}$ and **24**-$O_{5'}$ radical species are nearly 2 kcal/mol higher than **24**-O_4 radical species;

24-$O_{3'}$ is much higher in energy because of the inability to distribute the unpaired electron beyond the ring with radicalized O atom; **24**-O_9 radical species is the least stabilized probably because it is destabilized by the $O_9 \leftrightarrow O_{9a}$ and $O_9 \leftrightarrow \pi$ repulsions (with the aromatic ring D) as a result of the removal of the IHB.

The abstraction of the H atom from each phenolic OH in **25** also gives eight different radical species. The **25**-O_4, **25**-O_9, **25**-$O_{2''}$ and **25**-$O_{6''}$ radical species are formed by the removal of an IHB and the **25**-O_7, **25**-$O_{3'}$, **25**-$O_{5'}$ and **25**-$O_{4''}$ radical species are formed from a free phenolic OH. For the results *in vacuo*, the **25**-O_7 radical species has the lowest energy because it combines the highest number of IHB and a high tendency to distribute the spin density of the unpaired electron across three rings, A, B and D. The relative energy trend varies in different media, which suggests that different radical species, for a given structure, are stabilized differently by the solvent; for structure **23**, the most stable radical species *in vacuo* and in chloroform is the **23**-O_7 and in water solution the most stable radical species is **23**-O_9; the lowest energy radical species for structure **24** is **24**-O_4 in all the media; the lowest energy radical species for structure **25** is **25**-O_7 *in vacuo* and in chloroform and **25**-O_4 in water solution.

3.2.6.2 The ΔE_{iso} values

The stabilization energy (ΔE_{iso}) is one of the parameters used to predict the ability of antioxidants to scavenge free radical species of phenolic derivatives and a high ΔE_{iso} is indicative of high radical scavenging activity (Borges, *et al*., 2012; Denisov, *et al*., 1987). A meaningful comparison, however, involves structures with similar central moiety which in this work corresponds to structure **23**, **24** and **25**. Table 21 reports the *in vacuo* ΔE_{iso} values among the studied eckol derivatives.

Table 21: Total energy (Hartree) of the lowest-energy conformer of the neutral and radical species and stabilization energy (ΔE_{iso}, kcal/mol)[a].

Compound	$E_{radical}$ (kcal/mol)	$E_{neutral}$ (kcal/mol)	ΔE_{iso}
23	-1369.5122969	-1370.1456359	0.000
24	-1826.2265574	-1826.8634857	2.252
25	-1826.2257923	-1826.8597171	0.368

[a]*The stabilization energy is estimated as the energy difference between the lowest-energy conformer of the neutral species and neutral radical species for a given phlorotannin derivative and expressed as relative energy with respect to compound **23**.*

The results show that **24** has the highest stability with **25** slightly better stabilized than **23**. Invariably, these results indicate that the addition of a phloroglucinol unit (ring E) on the eckol moiety (structure **23**) is preferable on ring A than on ring B. The positive ΔE_{iso} for **24** suggests increased scavenging activity with respect to **23**. Therefore, both experimental findings and theoretical predictions confirm that structure **24** has the highest radical scavenging activities and that **25** is a slightly better radical scavenger than **23**, which is in agreement with the general trend that the stability of radical species, and hence the radical scavenging activity, depends mainly on the number of hydroxyl phenolic groups in the molecule (Leopoldini, *et al*., 2004 a, b, c; Belcastro, *et al*., 2006).

3.2.6.3 The HAT mechanism and the BDE values

The role of the HAT mechanism in determining the free radical scavenging activity of the studied phloroglucinol derivatives is better understood by considering the O-H BDE values. The BDE values, calculated for each phenolic OH of each of the four compounds, are also reported in Table 20. A lower BDE indicates a greater tendency to donate H atom and therefore it corresponds to higher reactivity (Kozlowski, *et al*., 2007). The results indicate that the BDE

value of phloroglucinol (89.0 kcal/mol) is slightly higher than that of a phenol calculated in this work (87.7 kcal/mol) and reported in experimental findings (87.3±1 and 88.2±0.5) (Wayner, *et al*., 1995; Mahoney, *et al*., 1973). Primarily, since phenol is considered a reference compound for understanding the radical scavenging activity of polyphenolic derivatives (ArOH), the results suggest that the scavenging activity of phloroglucinol through the HAT mechanism is minimal.

The BDE values for **23** and **25** indicate that the 7-OH, with the lowest BDE value, is the most reactive site (i.e., the site with the greatest ability to donate H atom) and 3′-OH, with the highest BDE value, has the least ability to donate H atom. The BDE values for **24** indicate the importance of the 4-OH (in the lowest energy radical species) as H atom donor. A comparison of the BDE values across structures suggests that phlorotannins derivatives have better radical scavenging activity than phenol (i.e., they have lower BDE value than phenol) and that the ability to transfer the H atom from the phenolic OH increases in the order **23** > **25** > **24**. The results suggest that structures **23** and **25** preferably scavenge radical species through the HAT mechanism. Both **23** and **25** have lower BDE values than **24** because they have a free phenolic OH attached to ring A (i.e., O_7H_7), which on abstracting an H atom from it gives radical species whose unpaired electron is distributed throughout rings A, B and D (Figure 33), indicating greater delocalization of the unpaired electron. The delocalization of the unpaired electron accounts for the stabilization of the radical species. Structure **24**, which has a phloroglucinol moiety at C_7, does not have a free phenolic OH group from which an abstraction of H atom results in extended delocalization (i.e., beyond the ring on which the H atom is abstracted), and as a result it has higher BDE values than both **23** and **25**. The BDE values of all the studied phloroglucinol derivatives are also lower than the BDE value of phenol (calculated in this work), indicating that in all the media, phlorotannins have the ability to scavenge radical species. The

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trend in the BDE values across structures is different in different media which emphasizes the role of the solvent in stabilizing the radical species; the smallest BDE value, for each structure, often corresponds to the lowest-energy radical species.

The BDE values of each OH group of the four phlorotannins were compared with the BDE value of DPPH-**H** to provide an indication of the reactivity with stable DPPH radical species. The BDE value of DPPH-**H** species is calculated in this work to be 82.324 kcal/mol (experimental value = 80 kcal/mol) (Mahoney, *et al*., 1973). The results therefore suggest that all phlorotannins have higher BDE values than DPPH-**H**, suggesting that reaction equation 1 is not expected to be thermodynamically favorable.

3.3.6.4 The ET mechanism and the IP values

The adiabatic IP values provide information for understanding possibility of the compounds to scavenge free radicals through the electron transfer mechanism. Consequently, the IP values for the studied phlorotannins are reported in Table 22.

Table 22: Calculated ionisation potential for the studied phenolic derivatives, B3LYP/6-31+G (d) results in different media.

Structure	IP (kcal/mol)		
	in vacuo	in CHCl$_3$	in water
Phenol[a]	190.591	158.856	144.453
22	183.188	155.066	140.443
23	162.503	135.623	123.889
24	162.323	138.380	125.561
25	161.711	138.973	127.147

[a] *All IP values are shown relative to phenol calculated at the same level of theory.*

The results indicate that all phloroglucinol derivatives have lower IP values than phenol suggesting that they have the ability to scavenge radical species through the ET mechanism. The scavenging of DPPH radical species by phlorotannins through the ET mechanism was also investigated by determining the IP value for the DPPH-**H** and comparing it with IP values of the studied phlorotannins (Mahoney, *et al*., 1973). The *in vacuo* IP value for DPPH-**H** was calculated to be 168.465 kcal/mol, indicating that the IP values of phlorotannins **23**, **24** and **25** are smaller than that of DPPH-**H**; as a result, reaction equation 3 is expected to be thermodynamically favorable. This means that phlorotannins have high reactivity (i.e., through the ET mechanism) towards scavenging the stable DPPH radical species.

3.2.6.5 Assessment on cell viability

The effects of the phlorotannins were evaluated for their potential to exert cytotoxicity on highly invasive cancer cell lines (*HeLa*, H157 and MCF7) by using MTT assay. The results were expressed as concentration of the phlorotannin required to inhibit tumor cell growth by 50% (IC_{50}). Cytotoxicity of cisplatin, a standard, was evaluated under the same experimental conditions for comparison. The untreated cells without phlorotannins showed 100% cell proliferation, while the viabilities of the cells pre-treated with phlorotannins were decreased with the increased concentrations of the relevant eckols; survival depends on the concentration thereof and an increase leads to decrease in survival.

Table 23: Determination of cytotoxicity by MTT assay

Conc .μg/ml	HeLa			H157			MCF7		
	Absorbance	% Proliferation	IC_{50}	Absorbance	% Proliferation	IC_{50}	Absorbance	% Proliferation	IC_{50}
6.25	0.272357	39.70	< 50	0.449774	97.34	122	0.337917	48.31	< 50
125	0.233748	34.08		0.208261	45.07		0.085190	12.15	
250	0.176023	25.66		0.089592	19.39		0.053105	8.01	
500	0.083662	12.20		0.029397	6.36		0.048455	6.04	
Cispl	0.510261	70		0.503197	70		0.52338	70	
Cont	0.685966	100.00		0.462056	100.00		0.701071	100.00	

Compound **23** proved to possess remarkable antiproliferative activities against the three cancer cell lines compared to the reference drug cisplatin. Furthermore, this compound showed superior cytotoxic activity compared to the other phlorotannins. The cytotoxic effectiveness of this compound to that of cisplatin is several times greater against the cell lines, and this could be attributed to the higher aqueous solubility of the compound, which allows it to dissociate readily in solution, thereby making it bio-available. It is quite potent compared to the commercially available standard. The oligomer contains free phenolic hydroxyl groups which have lower BDE values as outlined earlier. Moreover, on abstracting a H atom from it gives a species whose unpaired electron is distributed throughout the rings making it more sterically demanding compared to the others, thereby permitting high selectivity to protein DNA binding.

The phlorotannin as a natural product, and when considering the proposed mechanism of action of synthetic cisplatin, it is reasonable to suggest that cytotoxicity of phlorotannins is derived from DNA binding. Furthermore, literature has it that those complexes with aromatic

ligands bind to proteins by embolism (Katerina, *et al*., 2012; Erkkila, *et al*., 1999), implying that such natural phlorotannins have a potential as embolic agents. Our initial study therefore, has shown that phlorotannins are promising antitumor agents. However, further biological investigations are needed, especially on those other compounds that showed remarkable cytotoxicity profiles in order to qualify them as potential drugs.

The results of average absorbance and percentage proliferation were tabulated in Table 23 and graphically represented in Figure 21. It was found that the percentage cell proliferation decreased with increasing concentration up to 500µg/ml on *HeLa*, H157 and MCF7 cell lines and the IC_{50} values less than 50µM were considered active.
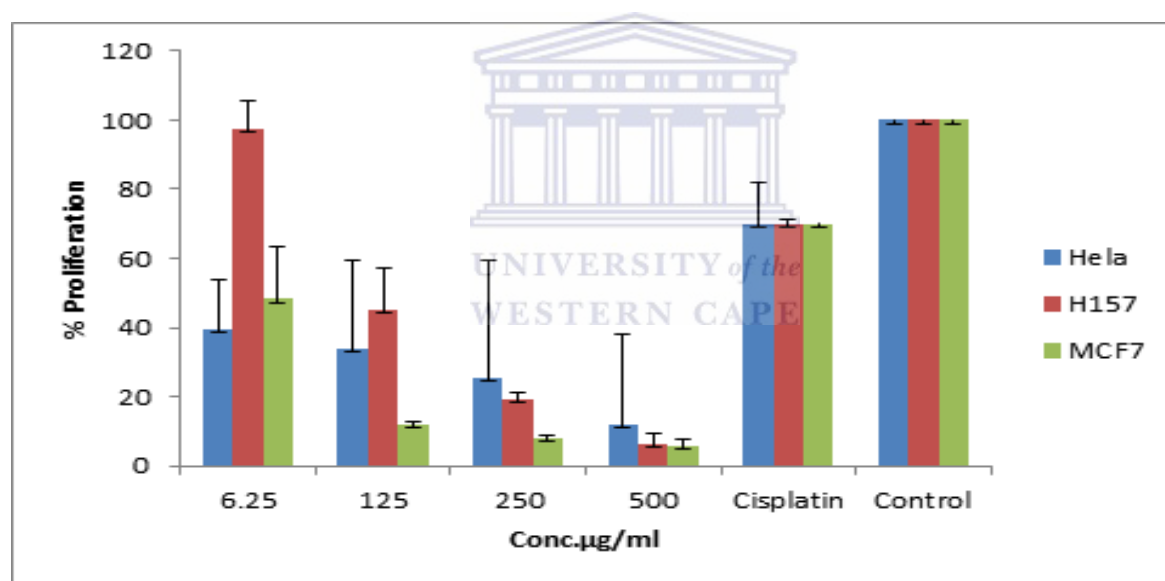
Figure 21: Activity of compound 23 on *HeLa*, H157 and MCF7 cell lines.

The purpose of the study was to evaluate the effectiveness of polyphenols extracted from brown algae in inhibiting carcinogenesis. Our experimental data demonstrated that topical treatment with this brown alga polyphenols decreased *HeLa*, H157 and MCF7 cancer cell lines development. However, 6.25 µM on H157 did not inhibit the cellular growth while 125 µM gave at least 50 % of inhibition. With *Hela,* all the concentrations (6.25, 125, 250, 500 µM) used

inhibited the cellular growth with less than 50 % of inhibition. For MCF7, only 6.25 µM concentration gave at least 50 % of inhibition while the other concentrations killed the cells. The H157 and MCF7 results suggested that the susceptibility of these cell lines on natural phlorotannins may be subjected to future use in cancer application. Compound **23** exhibited different activity on the selected cell lines. The selectivity was due to the sensitivity of a given cell line to the sameor to tissue specific response. Based on this study, it is strongly believed that the cross killing may have occurred due to cytotoxic activity of the samples against the cell lines. Such phlorotannins are phytochemical constituents acting as major components from some brown seaweed which are responsible for the potential cytotoxic activity (Prema, *et al*., 2012).

Marine algae have been well-known as an important source of natural bioactive secondary metabolites including phenols and polyphenols with unique linkages (Torres, *et al*., 2008). *Ecklonia maxima*, which is abundantly distributed along the South African coast, has revealed that it contains a variety of phlorotannins and sterol derivative which have potential defensive or protective functions against cancer cells proliferation. Furthermore, some reports suggest that such phlorotannins from brown algae exhibit antioxidant effects on free radicals hence offering photo-protective effect on cell damage. The viability effect against the selected cell lines may be correlated with the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules. Consequently, our results indicated that compound **23** has functional hydroxyl groups that are well positioned in the dibenzodioxin moiety than other tested phlorotannins; thus, they are effectively exposed to the cells for the relevant action. Cosmeceutical and pharmaceutical industries may target this compound as a suitable natural product lead candidate from marine biomass for cancer management.

3.3 Isolation of water soluble polysaccharide

The milled seaweed was extracted as outlined in Scheme 1. Yields and general analyses of the extracts were determined, with the monosaccharide composition as shown in Table 24. Treatment of the seaweed with 80% aqueous methanol at room temperature removed substantial amounts of materials. Crude polysaccharides were isolated from the alga with water as the extracting solvent. Two collection times of *Ecklonia maxima*, summer and winter, were compared in terms of yield and results obtained gave 56.4% and 59.2% respectively of the freeze-dried material.

3.3.1 Chemical composition of the crude of polysaccharide

The sugar compositional analysis revealing varying proportions of monosaccharides present in the crude polysaccharide of the brown alga is shown in Table 24. Fucose, a predominant neutral sugar constituent of fucoidan was determined to be present together with respective amounts of other units.

Table 24: % composition of the crude polysaccharide

	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
E.maxima-summer	1.9	28.3	-	10.6	42.5	9.6	7.0
-winter	1.7	22.0	-	9.7	48.3	9.2	8.9

Fucoidan content, chemical composition and its structural characteristics vary in relation to the seaweed species, season of harvest, and maturity of the plant (Skriptsova, *et al*., 2009). Furthermore, maximum amounts of fucoidan are found in the sporophylls, but their chemical compositions are quite similar within a given species (Usov, *et al*., 2005). This study established

a correlation between seasonality and fucoidan content, and reported that fucoidan content was highest during winter. During this period, the alga generates its biomass reserve after a rapid growth phase in spring in order to survive the winter where hardly any photosynthesis occurs (Painter, 1983). As a result, a larger amount of fucoidan is found during the winter season. Consequently, the physiological and biochemical processes of the alga varies, resulting in the structure and chemical composition of fucoidan certainly convoy with such changes (Usov, *et al*., 2005). Invariably, environmental conditions have an effect on the chemical composition and structural characteristics of seaweed. Therefore, these changes are likely to affect the chemical composition of fucoidan in a similar manner (Mamatha, *et al*., 2006).

3.3.2 Paper chromatography (PC) analysis

Paper chromatographic (PC) analysis of the monosaccharides present in the hydrolyzate was developed in chloroform: methanol: water (10:10:3), for 18 hrs. The results indicated the presence of the neutral sugars with R_f values similar to those of the standards. There was a major product (oblong spot) relative to fucose and at least minor products (circular spots) contained within the profiles from the origin to the furthest spot. Primarily, the hydrolyzates of the samples and eight standard monosaccharides shared the same fucose, xylose and mannose spots, the others were too faint. GLC analysis of the HM/E/1, HM/E/2 derivatives of the methylated alditol acetates glycosides confirmed these results.

3.3.3 Fucoidan, sulfate and uronic acid composition

Extracted fucoidan from the brown seaweed *Ecklonia maxima* was analyzed for fucose, sulfate, and uronic acid content. Standard samples of commercial L-fucose were prepared in different concentrations with deionized water ranging from 20-200 µg/ml.

3.3.3.1 Fucose content determination

Free fucose was determined by use of cysteine-sulfuric acid method for methyl pentoses. A standard absorbance difference calibration curve for colorimetric determination of fucose content was read between 396 nm and 427 nm (Figure 22). Fucose mg/g dry weight from the chemical analysis of ethanol treated fucoidan derived from the brown seaweed was recorded in Table 25.
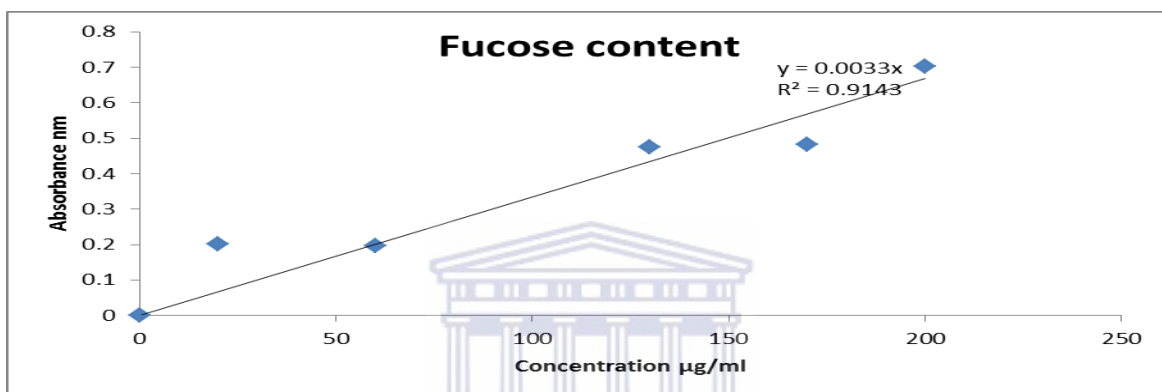
Figure 22: Determination of standard fucose content

Table25: Fucose mg/g dry weight of selected seaweed

Seaweed	Yield (g)	Absorbance	Total fucose (mg/g)
E. maxima (summer)	5.44	0.0664	100.6
(winter)	4.80	0.0817	123.8

E. maxima 0.0664/0.0033 = 20.12µg

(20.12/200) x100 = 10.06%,

10.06 x 5.44g = 0.5473g

In mg/g (0.5473x1000)/5.44 = 100.6mg/g

Fucose, the major sugar constituent of fucoidan, was found to be highest in the winter collection. Primarily, it is worth noting that sugar rhamnose is also a methyl pentose (or a deoxy-hexose)

and may have also contributed to the total amount of fucose estimated in the fucoidan even if present as a minor component of fucoidan.

3.3.3.2 Sulfate and uronic acid content determination
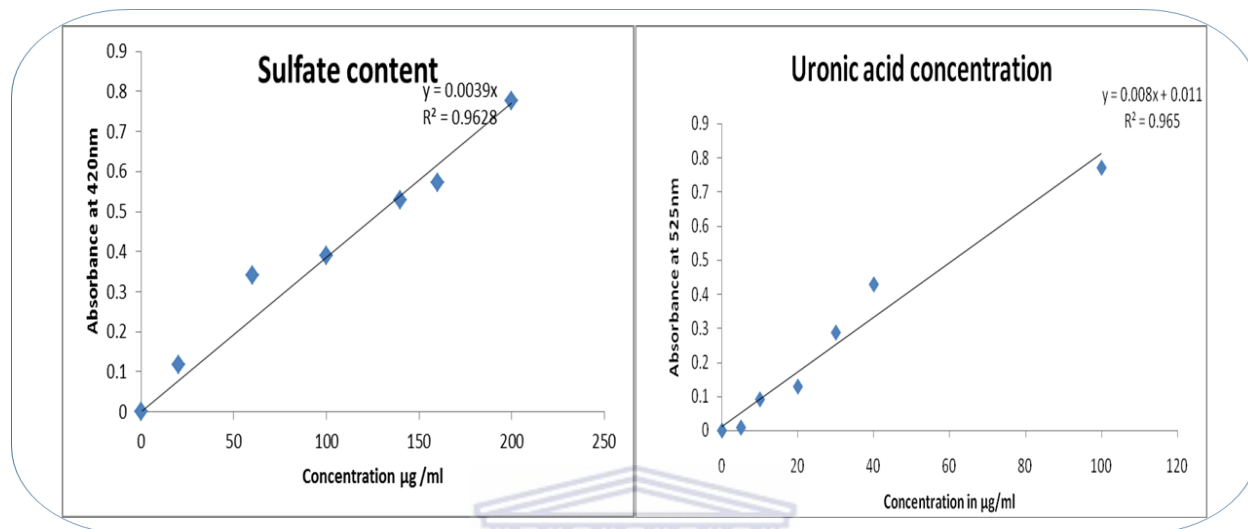
Figure 23: A standard sulfate content and uronic acid determination curves

A standard absorbance calibration curve for colorimetric determination of sulfate content using potassium sulfate was obtained as shown in Figure 23. Table 26 represents a summary of the percentage contents of the sulfate and uronic acid present in the seaweed.

$$0.1425/0.0039 = 36.54\mu g$$

$$(36.54/200) \text{ x}100 = 18.27\%$$

Table 26: % sulfate and uronic acid contents

Seaweed	Yield (g)	Absorbance	% sulfate	Absorbance	% uronic acid
E. maxima (summer)	5.44	0.1425	18.27	0.0408	4.52
(winter)	4.80	0.1539	19.74	0.0594	8.18

To determine the uronic acid content, glucuronic acid stock solutions were made in pure water at a concentration of 0, 5, 10, 20, 40, 50 and 100 µg/ml quantities and used for the

generation of a calibration curve. The curve was used for calculating the uronic acid content of the polysaccharide samples.

HM/E/1(summer) 0.0408= 0.008x + 0.011   x= 4.52
HM/E/2 (winter) 0.0594= 0.008x + 0.011   x= 8.18

The sensitivity for glucuronic acid in such a reaction is approximately 1μg and 2 μg for complex polysaccharides according to the conventional assay (Bitter & Muir, 1962). There is a linear function of glucuronic acid concentration between 1 μg and 100 μg. However, presence of glucuronic acid has already been reported in fucoidan from brown seaweeds (Adhikari, *et al*., 2006; Mandal, *et al*., 2007). Therefore, HM/E/1, HM/E/2 is essentially a fucoidan that contains a high number of sulfate groups and a small percentage of the uronic acid.

A number of carbohydrates can be detected by means of a reaction using carbazole assay. The reaction of uronic acid with carbazole is the most satisfactory method of measuring its presence (Cesaretti, *et al*., 2003). Primarily, it is based on the principle that when carbohydrates are treated with H_2SO_4 or HCl, they yield mixtures of products that react with carbazole to give colors (Bitter & Muir, 1962).

3.3.4 Neutral sugar composition

The monosaccharide compositions were determined after hydrolysis, reduction, and acetylation to give a mixture of alditol acetates that were analysed by GC and GC-MS. The retention times (in minutes) of the alditol of standard sugars were: rhamnitol acetate 2.329; fucitol acetate 3.375; arabinitol acetate 4.257; xylitol acetate 5.177; mannitol acetate 9.357; galactitol acetate 10.190 and glucitol acetate 11.15 (Figure 24a). The percentage of all neutral sugars of the fractions samples were summarized in Table 27 section 3.3.6. The content of uronic acid was less than 10% in the fractions as shown in Table 26 section 3.3.3.2. The experiment

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served as confirmatory test of the identity of the neutral sugars. Overall, the total retention time chromatogram of this analysis is shown in Figure 24, along with identities of the components. Consequently, the sugar residues in the samples were identified by comparing their relative retention time with those of the respective standard sugar alditol acetates.
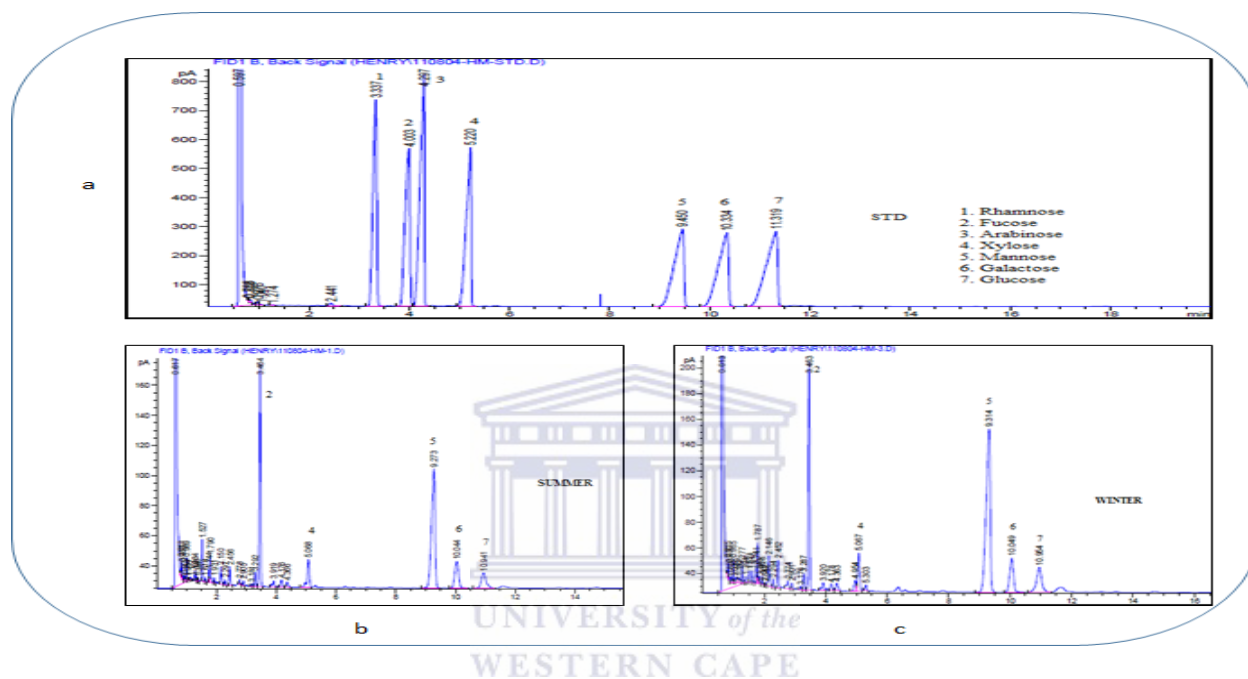
Figure 24: GC analysis peaks of the a) standard sugars (b) summer (c) and winter collections

Interestingly, similar peaks were detected in both the summer and winter collections as shown in Figure 24 b and c. These gave an indicator that time of collection did not influence presence of the neutral sugars in the samples. Individual polysaccharides were showing a high concentration of fucose, xylose, mannose, galactose and glucose but traces of other sugars as determined by initial concentrations used. Specific sugars were further confirmed by use of GC-MS. The GC-MS of fraction 5 and the results of its monosaccharide composition analysis are represented in Figure 25 and were compared to data obtained from standards extracted by similar methods.
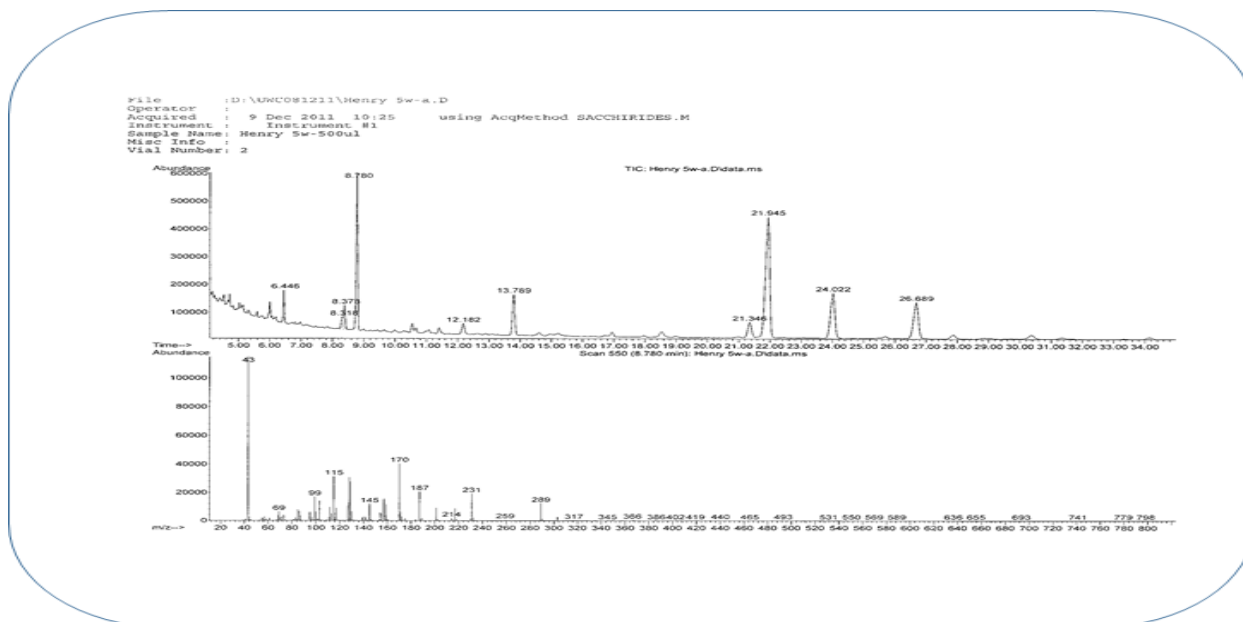
Figure 25: GC-MS of fraction 5 winter collection

The confirmatory test was done on one of the fractions collected after an anion permeation experiment. The retention time at 8.780 gave a perfect match of those spectral peaks relative to fucitol pentaacetate (**21**). Identity of the alditol acetate was based on electron-impact MS and co-elution on GC with authentic standards.
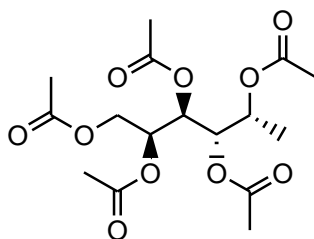
21

This analysis showed the principal monosaccharide unit in *Ecklonia maxima* was fucose. Accounts of other sugars; rhamnose, xylose, mannose, galactose, glucose, and indeterminate methyl hexoses were also present in vanishingly small amounts. Note that the alditol acetates have been assumed to be derived from aldoses rather than ketoses, but this was later verified for

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fucose by methylation analysis.

3.3.5 Separation of standard PMP-sugars and PMP-hydrolyzed fucoidan sugars

The PMP derivatization method was selected among a number of reported methods for labeling carbohydrates since it involves quantitative yields, rapid reactions and simple clean-up procedures (Bilan, *et al*., 2002). Preliminary work was carried out on characterization of PMP derivatives of small sugars prior to studying derivatives of larger oligosaccharides obtained from this alga.

Analysis of standard PMP-sugars by HPLC Figure 26 shows the elution pattern of PMP derivatives of small sugars. Furthermore, a good separation of all the PMP-sugars was achieved. The PMP labelled sugar peaks were all sharp and baseline resolved. The deoxy-hexose (fucose) eluted later than its parent compound mannose. The PMP reagent appeared as a minor peak at a retention time of 4.560 min, which indicated that PMP can be almost completely removed by the extraction procedure. Our results showed that a conspicous initial mobile phase concentration peak and a shallow gradient could provide good quality separation of the reagents and PMP-labelled products. A HPLC profile was developed to be validated for the qualitative and quantitative determination of standard PMP derivatives.
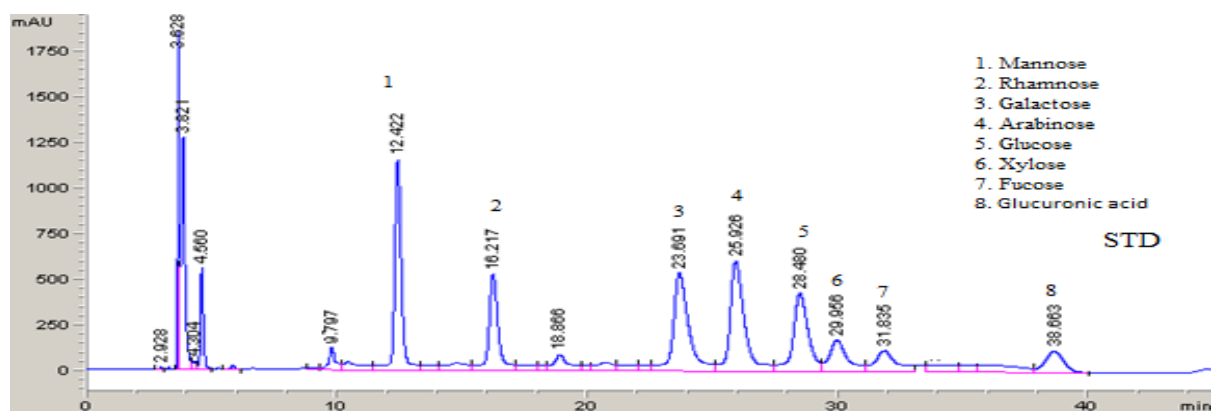
Figure26: Reversed-phase HPLC chromatogram of the PMP derivatives of small sugars.
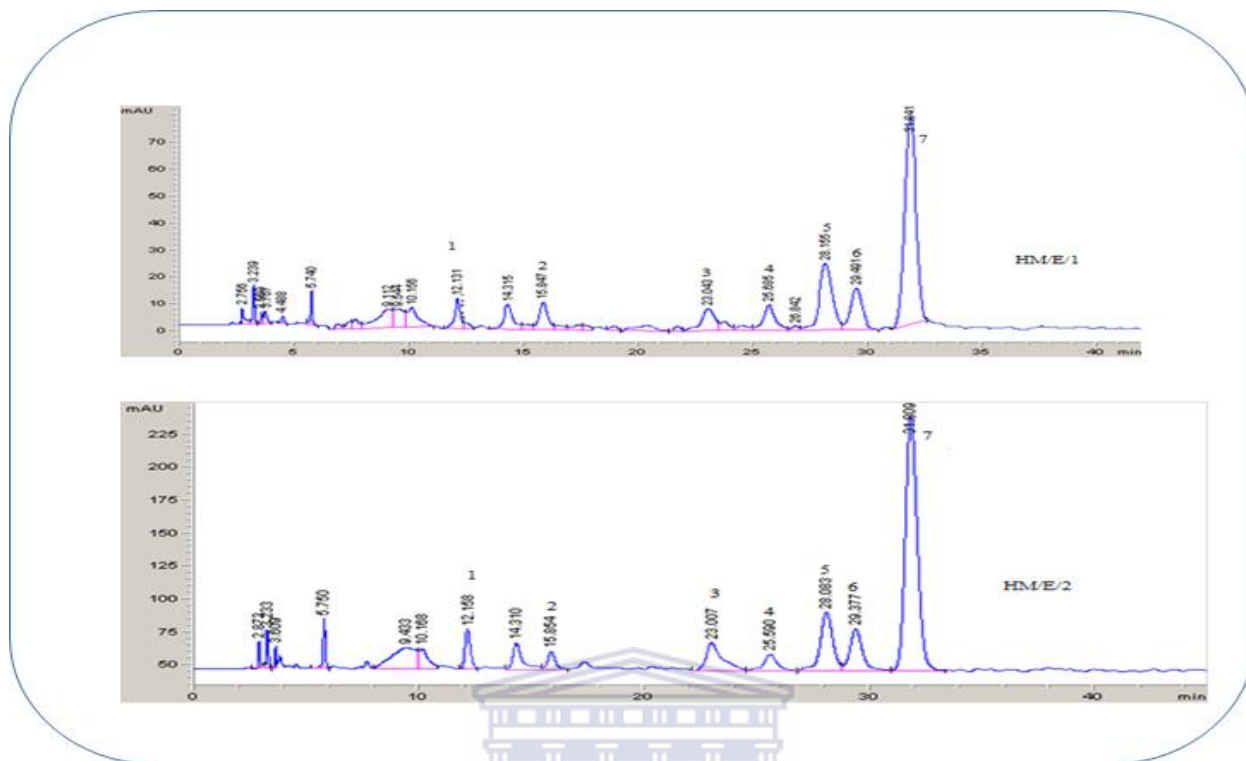
Figure 27: Monosaccharide composition analysis of HM/E/1 and HM/E/2 by HPLC

The initial broad reagent peak was well separated from all the PMP-sugars. The following monosaccharides were detected in the alga; Mannose, Rhamnose, Galactose, arabinose, glucose, xylose and fucose respectively. The separation of the PMP-degraded monosaccharides from the fucoidan summer and winter are illustrated in Figure 27. There were few additional peaks detected in the sample peaks between major ones. We hypothesized that these represented mannuronic acid and glucuronic acid formed by degradation of alginate, an impurity.

Compositions of the two collections were similar upon comparing their retention times. However, the biomass of winter collection was higher on calculating the percentage yield of each monosaccharide as previously reported. A reproducible standard curve was generated under the set column conditions that will be validated on carrying out absolute quantification procedure.

3.3.6 Fractionation of polysaccharides

Preparations of crude polysaccharides HM/E1 (summer) and HM/E2 (winter) were fractionated by anion-exchange chromatography on DEAE-sepharose using aqueous sodium chloride as eluent. As a result, various fractions from both collections were obtained. Absorbances of the sampled individual test tubes were plotted against the fraction number. Four fractions of sulfated polysaccharides were obtained: EM1, EM2, EM3 and EM4 from the summer and F1 to F5 for the winter samples respectively in a stepwise gradient from using water, 0.1M-1M NaCl elution on a Sepharose CL-6B column. The yields and compositions of these fractions are shown in Table 27. No polysaccharide was detected after using 1.0M NaCl. The chromatogram showed that majority of crude fucoidan applied to the DEAE sepharose gel was eluted more at a concentration of between 0.4M and 0.6M NaCl. The tubes with phenol–sulfuric acid positive reaction were pooled together; Figure 28 shows the summer and winter profiles forming four and five main symmetrical peaks respectively. All test tube fractions under the peaks were combined, dialyzed and lyophilized.
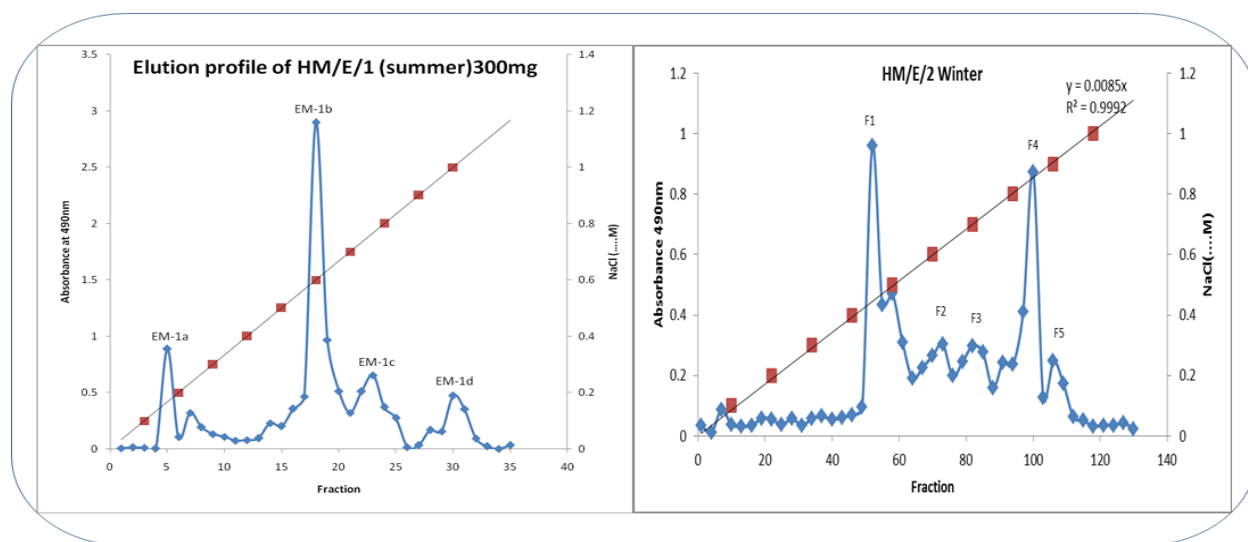
Figure 28: Elution profiles of HM/E/I (summer) and HM/E/2 (winter) on DEAE-sepharose CL-6B

Purified polysaccharides subjected to ion exchange work on the principle of ionic centers. The less charged oligosaccharides flow out earlier; heavily charged ionic centers elute much later. Neutral oligosaccharide molecules are known to elute early in relation to acidic ones. The results showed a major fraction from the summer collection EM-1b eluted at a concentration of 0.6M NaCl while for the winter sample, the first fraction eluted at 0.4M NaCl and another at 0.8M NaCl. The linear line shows NaCl concentration. The fractions were dialyzed for 48hours, evaporated to a small volume, and then freeze-dried for further chemical analysis. The total monosaccharide sugar and sulfate content of each fraction was detected qualitatively by phenol-sulfuric acid and ion chromatography respectively. Consequently, sugar compositions were determined by GC of alditol acetates as described under the materials and methods section.

Table 27: Composition and yield of purified fucoidan fractions

Fraction	% Yield	Neutral monosaccharides (%)							Sulfate (SO_4^{2-}) (%)
		Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	
EM-1a	21.2	3.8	42.8	nd	11.4	19.4	14.4	8.2	37.47
F1	11.2	4.9	29.4	nd	14.5	20.8	13.0	17.4	38.64
EM-1b	65.4	5.8	9.4	nd	42.1	42.7	nd	nd	20.05
F2	48.8	1.7	5.5	nd	40.8	52.1	nd	nd	45.79
EM-1c	29.8	9.5	23.3	nd	27.8	39.3	nd	nd	53.09
F3	9.6	5.9	12.4	nd	38.0	43.7	nd	nd	73.01
EM-1d	9.2	6.2	40.0	nd	16.3	14.9	14.0	8.8	90.70
F4	28.5	5.2	17.5	nd	24.4	32.1	3.6	17.2	37.00
F5	11.4	3.4	19.5	nd	17.0	35.4	11.1	13.3	74.1

nd- not determined

Invariably, ion exchange chromatography using the diethylaminoethyl (DEAE)-sepharose is suitable for separating neutral and acidic polysaccharides. The former pass through the column without binding, whereas the latter, because of their negative charge, are retained on the column and can be eventually eluted with buffers of increasing ionic strength or pH. Affinity chromatography, based on specific non-covalent interaction between the binding ligand attached to the column and polysaccharides, is one of the most powerful techniques for purification of any certain carbohydrate polymers. Fractionation of fucoidan by anion-exchange chromatography yields small fractions by chromatography on a DEAE Sepharose CL-6B column. Different fractions were eluted out according to the varying concentrations of NaCl. The fractions with high sulfate combination were a reflection of richness in fucoidan. Anionic groups such as sulfate are the main contributors to the overall negative charge on fucoidan (Ponce, *et al*., 2003).

Once crude fucoidan is introduced into a column, branched sulfate groups automatically get drawn to the positively charged ion-exchange groups of DEAE. Only the use of strong ionic solvents like NaCl will release the sulfate groups from the gel. In this case study, there was an increase in sulfate content from F1 to F5. As the concentration of NaCl increased in the mobile phase, more sulphate groups were released. Nevertheless, it is worth noting that previous studies using the same type of gel but a different species of seaweed required a concentration of 3.5M NaCl solution to wash out all the remaining fucoidan in the gel, whereas this study only required a 1M concentration. This implied that fucoidan from different species of brown seaweed not only have varying amounts of constituents in them, but also varying levels of ionic strengths (Ly, *et al*., 2005).

The pooled fractions were analyzed for their neutral monosaccharides specifically targeting fucose and sulfate content, and they gave varying results from each other. A trend was

observed where the fraction eluted with a lower NaCl concentration (0.1M) had fewer sulfate groups and lower in fucose content. In contrast, the fraction eluted with higher NaCl concentration (1M) was higher in sulfate groups and higher in the fucose. A similar trend was previously reported using the alga *S. swartzii* harvested in Vietnam that was fractionated on a DEAE Sephadex A-25 column (Ly, *et al*., 2005). Consequently, the sulfate: fucose ratio increased as the molarity of NaCl eluting solvents increased. The fucoidan fractions and crude samples showed no significant changes in fucose content (Ly, *et al*., 2005; Skriptsova, *et al*., 2009).

3.3.7 Molecular weight determination

To determine the response of the eluents to the molecular weights of sugars from some selected fractions, a sample was subjected to molecular weight fractionation by gel permeation chromatography, and several fractions, 2 ml volumes, of narrow size range were collected. The collected fractions were analyzed for sugar content by means of the phenol-sulfuric test. Fraction 2 W appeared as a single and symmetrical sharp peak after the gel permeation chromatography (Fig. 29a). The gel permeation chromatographic column was calibrated by means of five dextran standards of known average molecular weights. Figure 29b shows the resulting calibration graph with an equation obtained through semi-logarithmic regression analysis (log molecular weight versus volume), and the correlation coefficient.
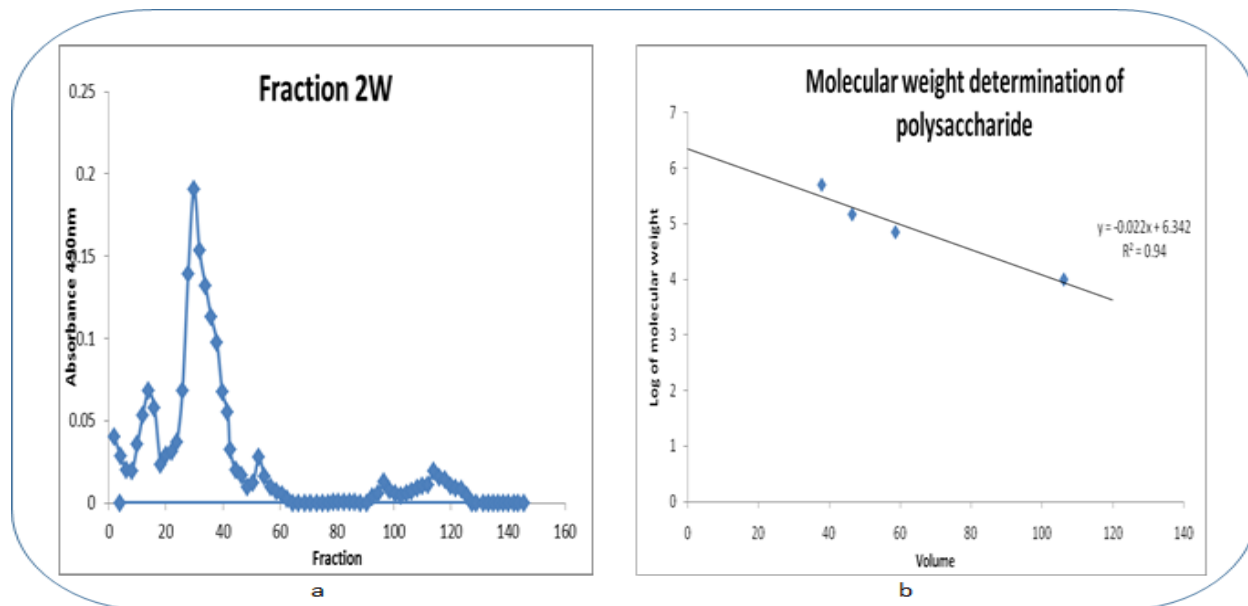
Figure 29: a) Elution profile of fraction 2W from Sephacryl S-400 column and b) Dextrans calibration graph from the gel permeation column.

The average molecular weight of sugars from 2W were between 25×10^3 Da and 250×10^4 Da by reference to the calibration curve ($y = -0.022x + 6.3424$, $R^2 = 0.94$) made from Dextran standards of known molecular weight (10,000, 70,000, 110,000, 150,000, 500,000). Such a range of molecular weights is a representation of effectiveness of this seaweed to various biological activities. Furthermore, the molecular weight of fucoidan is closely related to their biological action that requires a sugar-chain long enough to proteins, such that a certain minimum weight is required to achieve an activity. Studies have shown this biological activities are not only related to the sugar composition (Nishino, *et al*., 1989; Li, *et al*., 2008) but has been speculated that the influence of activity is rather the sulfate groups attached to them. Moreover, uronic acid composition although not necessary for biological activity, improves the actions by enhancing the sugar chain's flexibility (Li, *et al*., 2008).

3.3.8 Ion chromatograph
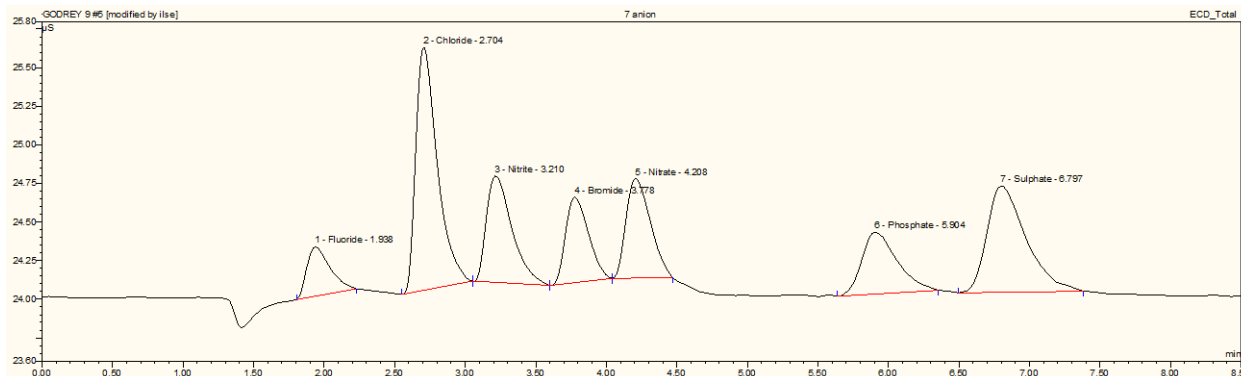
Figure 30: A chromatogram of *F⁻*, *Cl⁻*, *NO⁻₂*, *Br⁻*, *NO⁻₃*, *PO₄⁻³*, *SO₄⁻²* standard

Table 28: Minimum concentrations of *F⁻*, *Cl⁻*, *NO⁻₂*, *Br⁻*, *NO⁻₃*, *PO₄⁻³*, and *SO₄⁻²* at 1000ppm

Sample fraction	Fluoride	Chloride%	Nitrite%	Bromide	Nitrate%	Sulphate%
EM/1a	-	32.53	-	-	-	67.47
EM/1b	-	52.87	27.07	-	-	20.05
EM/1c	-	46.91	-	-	-	53.09
EM/1d	-	6.42	-	-	2.88	90.70
F1	-	41.36	-	-	-	58.64
F2	-	32.62	21.59	-	-	45.79
F3	-	-	26.99	-	-	73.01
F4	-	46.23	16.66	-	-	37.00
F5	-	25.95	-	-	-	74.05

Figure 30 shows a standard separation profile of typical anions found in the commercial standard solution. In turn, Table 28 contains the average percentages of the anions found in the polysaccharide fractions of the alga under study, based on 4 replicate injections. The results showed high percentages of sulfate in all fractions, a characteristic of fucoidans; chlorides were due to the fact that as marine algae, they have an adaptation towards growing under an oceanic

environment with high chloride concentrations. Invariably, this application note demonstrates that IC with electrolytic eluent generation provides a convenient and reliable method for the simultaneous determination of sulfate, and other anionic ingredients in purified fractions of *Eckonia maxima*. The results were a confirmation of sulfate presence reflected by the IR report.

3.3.9 FT-IR spectroscopy

Further analytical data was provided by the FTIR spectrum which showed typical absorption bands of fucoidans. Of particular interest for structural investigation were the bands for sulfate groups. Fucoidan from the different collection times were analyzed to determine if their infrared absorption properties were similar to the previously reported fucoidan IR absorption data (Patankar, *et al*., 1993). The spectra of the HM/E/1 (summer) and HM/E/2 (winter) samples were scanned between wavenumbers 4000 and 380 cm^{-1} (Figure 31) where both exhibited a major broad band of absorption between 3600 cm^{-1} and 3200 cm^{-1}, which was due to the hydroxyl groups stretching vibration. The bands in the region of 2940 cm^{-1} were characteristic of C-H anti-symmetrical stretching vibration. Bands around 1750 cm^{-1} and 1676 cm^{-1} represented the ester carbonyl groups (C=O) and carboxylate (COO-) stretching band (Gnanasambanda and Proctor, 2000), respectively, indicating that there were esterified and free carboxyl groups present in the polysaccharides. This validated the presence of uronic acids. The peak absorption around 1380 cm^{-1} and 1460 cm^{-1} which was most intense in HM/E/2 could be attributable to scissoring vibration of CH_2 (galactose, mannose) and asymmetric bending vibration of CH_3 (fucose, *O*-acetyls) as suggested previously for absorption at around 1455cm^{-1} by Synytsya, *et al*., (2010).

The absorption bands region from 1300 cm^{-1} to 800 cm^{-1}, is considered the "finger print" region for carbohydrates, which allows the identification of major chemical groups in

polysaccharides (Cerna`, *et al*., 2003; Kalapathy and Proctor, 2001). Primarily, this is related to conformation and surface structure of molecules. The absorption band at 1229 cm^{-1} observed for both samples, but being particularly prominent in the winter sample, was assigned as S=O stretching vibration, indicating the presence of esterified sulfate. This absorption band confirmed the significant amount of sulfate in the polysaccharide. A similar signal pattern at around 816 cm^{-1} was corresponding to a primary (equatorial) ester sulphate (C-*O*-S) characteristic of fucoidans, an indication of most sulfate groups located at positions 2 and/or 3 (Patankar, *et al*., 1993).

IR absorption at 840 cm^{-1} has also been reported to be for sulfate groups at the axial C-4 position; the observed absorption bands at 820-840 cm^{-1} were interpreted as being indicative of sulfate groups. This band suggests a complex pattern of substitution, primarily at C-4 position (axial C-4 substitution of α-linked L-fucopyranose) (Tako, *et al*., 1999; Marais and Joseleau., 2001) with other substitution at C-2 and/or C-3 (equatorial positions) in lower amounts. One strong band at 1030 cm^{-1} for (C-O, C-C) common to all sulfated polysaccharides, and other minor bands related to sugar cycles were detected. Consequently, from the spectra, it was proposed that the absorbance between 1000 cm^{-1} and 1200 cm^{-1} was due to the pyranose ring. The S=O, C-*O*-S stretching absorption bands shows sulfur to be present as a sulfate ester (Stancioff, *et al*., 1969; Asare, 1980; Mathlouthi and Koenig, 1986). These results supported outcomes from the sulfate content assay through ion chromatography (IC), an indicator that the polysaccharide samples were a sulfated type.
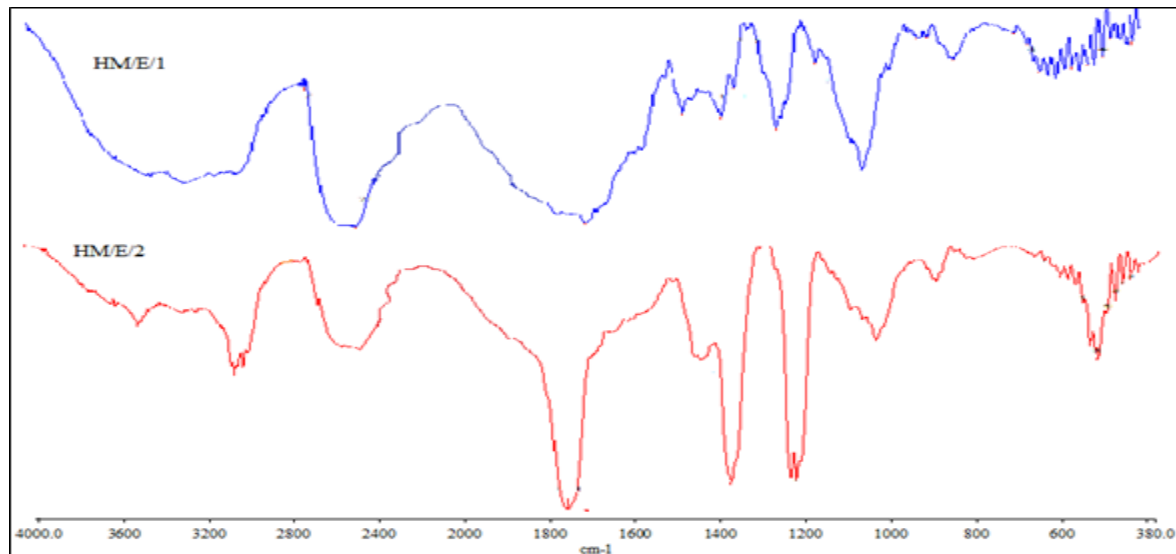
Figure 31: FT-IR analysis of HM/E/1 (summer) and HM/E/2 (winter) scanned between 4000 and 380 cm^{-1}

3.3.10 NMR spectrum

NMR spectroscopy is a convenient method that gives valuable structural information of polysaccharides. However, NMR spectra of fucoidan are very complex, giving different linkages due to; sulfation and branching patterns, presence of other monosaccharides, consequently producing many signals that are hard to distinguish from the noise (Pereira and Mulloy, 1999). Like many other brown algal fucoidans, the polysaccharide from *Ecklonia maxima* had rather a very complex ^{1}H NMR spectrum (Figure 32) with broad signals. This illustrated the heterogeneity of the algal fucoidan regarding the position of glycosidic linkage and the sites of sulfation on the fucose unit. At least separate spin systems attributable to anomeric protons of L-fucopyranose residues and D-galactose residues were distinguishable in the spectrum. It contained resonances characteristic of L-fucopyranosyl such as signals from ring protons H-2 to H-5 between δ 3.6 and δ 4.2 ppm, and intense signals from the methyl protons H-6, a major envelope of signals at around 1.3 ppm. A separate broad spin system ranging between δ 5.2 and δ

5.6 ppm was attributable to anomeric protons of α-fucose residues distinguishable in the spectrum of the pure fucoidan from anomeric proton (H-1).

Primarily, the appearance of a large number of anomeric signals suggest that the environment of the fucose residues are different and this may arise from varied sulfation patterns, different glycosidic linkage positions or diverse sequence of monosaccharide residues. The signals between δ 3.6 and δ 4.2 ppm were attributed to ring protons of β-linked sugars signals from H-4 of 4-*O*-sulfated residue. The high proportion of xylose and galactose residues must be responsible for these signals in the spectrum, but it was not possible to assign any particular signals to these residues. Furthermore, methyl signals appear at around δ 1.35-1.40 ppm only for →1)-L-fucopyranosyl-(4→ residues (Pereira and Mulloy, 1999; Alves, *et al.*, 1997). Consequently, upfield peaks were probably assignable to the methyl groups of fucose bearing another fucose at C-6. There were several intense and narrow signals at 2.10-2.40 ppm which have been attributed to CH$_3$ protons of *O*-acetyl groups (Tako, *et al.*, 2000). Therefore, it was apparent that the NMR spectra of this fucoidan was complex as observed for sulfated polysaccharide from other sources (Adhikari, *et al.*, 2006; Mandal, *et al.*, 2007; Bilan, *et al.*, 2004; Chevolot, *et al.*, 2001).
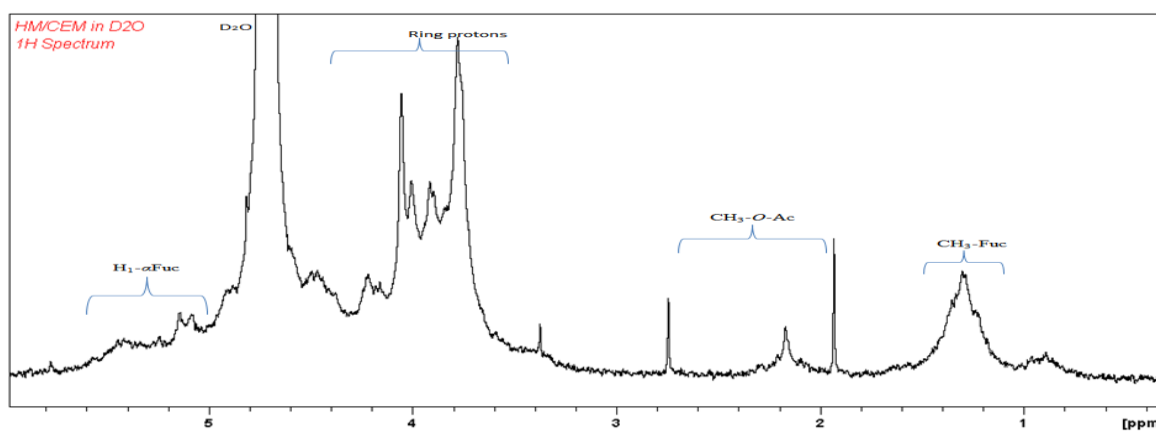
Figure 32: ^{1}H NMR spectrum of the fucoidan fraction 4 isolated from *E. maxima*

3.3.11 Methylation analysis

The treated *Ecklonia maxima* was pre-methylated, hydrolyzed, reduced, and acetylated to give a mixture of partially methylated alditol acetates (PMAA's), each of which bears a methyl substituent at sites which were un-substituted in the original sample, and acetates at the sites which were originally substituted. In this way, one can determine the linkage positions and ring sizes, but not always unambiguously, for each of the monosaccharides present in the sample through separation and identification of PMAA's by GC-MS. The reduction of partially methylated monosaccharides may be accomplished with either sodium borohydride or sodium borodeuteride, but the latter is advantageous because all even-electron fragment ions which contain C-1 (the former anomeric carbon) will have even mass, whereas those fragments from the other end of the molecule all have odd mass (Cui, 2005). The deuterium atom in a PMAA gives diagnostic fragments in the mass spectrum. This simplifies the determination of the substitution pattern of the PMAA in a case where computerized spectral matching is limited or unavailable. Invariably, the deuterated alditol-D1's help resolve ambiguities in substitution arising from certain cases of symmetry of the un-deuterated PMAA's (Cui, 2005).

A sample of the partially methylated alditol acetates of the alga was subjected to GC-MS analysis. The respective gas chromatogram is shown in Figure 33. From the sample, nine compounds were identified applying rules of fragmentation for methyl and acetyl derivatives of saccharides. However, these did not distinguish the stereochemistry of the compounds under investigation. The manual interpretation was used as a base for the evaluation of the results obtained (Sassaki, *et al*., 2005) and also applying the computer library search method, using an available mass spectra library under http://www.ccrc.uga.edu/databases/index.php; one specially created for such purposes. Table 29 summarizes the results of interpretation.
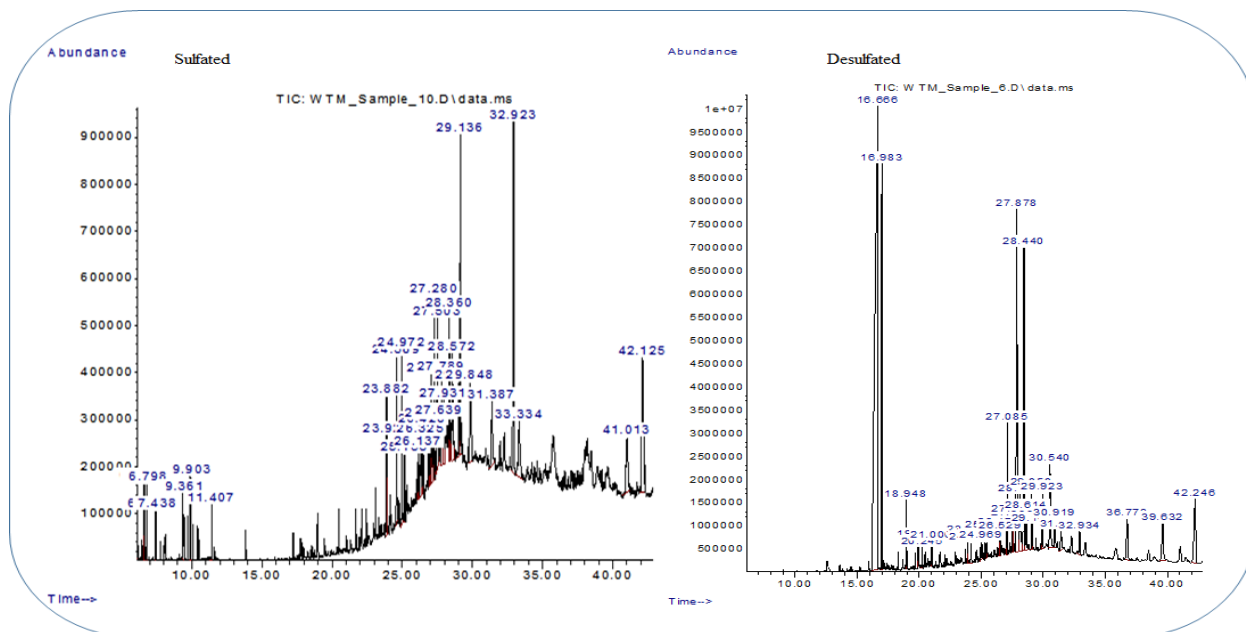
Figure 33: GC-MS analysis of sulfated and desulfated fucoidan of *Ecklonia maxima*

Table 29: Methylation linkage analyses of native and desulfated *Ecklonia maxima*.

Glycosyl residue	Position of the *O*-methyl group	Deduced linkage pattern	Sulfated (mol %)	Desulfated (mol %)
Xylosyl	2,3,4	Terminal Xyl(1→	4.0	4.1
Fucosyl	2,3,4	TerminalFuc*p*(1→	2.7	2.8
	2,3	→4) Fuc*p*(1→	1.3	4.1
	2,4	→3) Fuc*p*(1→	2.2	5.2
	3,4	→2) Fuc*p*(1→	2.1	0.4
	2	→3, 4) Fuc*p*(1→	5.9	1.5
	3	→2, 4) Fuc*p*(1→	1.8	3.5
	4	→2, 3) Fuc*p*(1→	3.8	5.8
Galactosyl	2, 3	→4, 6) Gal*p*(1→	4.2	5.0

The methylation analysis of this fucose-containing sulfated polysaccharide *Ecklonia maxima*, showed the presence of 3-linked and 3, 4-disubstituted fucopyranosyl residues in addition to small proportions of non-reducing, terminal fucofuranosyl and fucopyranosyl groups. The 2-*O*- and 4-*O*- fucopyranosyl were the abundant products of the methylation analysis of the native fucoidan. Furthermore, there was 4-linked, 2, 3-di-substituted galactopyranosyl with glycosidic linkages and a 2, 3, 4-tri-*O* fucopyranosyl terminal residues respectively. Methylation analysis of the desulfated sample showed that the proportion of 3-*O*-linked and 2, 4-di-*O*-fucosyl residues increased while that of 3, 4-*O*-disubstituted decreased significantly compared to the native sample. A significant decrease of 2-*O*-methyl fucitol residues suggested that the sulfate esters when present are located at C-4 of the (1→3)-linked fucosyl residues. Linkage analysis of the neutral oligosaccharide fractions showed the presence of mainly (1→3) in addition to small proportions of (1→4) and (1→2) linkages (Nishino, *et al.* 1991). Most of the desulfated methyl groups were linked to *O*-4 of the 2, 4-*O*-linked fucosyl residues. Therefore, these results suggested a highly branched, type of fucan sulfate containing a backbone of (1→3)-linked L-fucosyl residues having sulfate groups mainly attached to C-4. This polysaccharide resembles a literature related *Eklonia kurome* (Nishino, *et al.* 1991) in some respects that it is highly sulfated, and contains (1→3)-linked residues of fucose- 4-sulfate.

Invariably, the use of these searches led to provisional proposed structural results. The number and the position of substituents in partially methylated alditol acetates or the number of carbons in compounds could not be fully established. Thus, the library search method provided only proposed identification of components yielding structures more or less the same as the correct ones. Nonetheless, regarding the use of a library search, dissatisfactory results may be caused by some factors like, errors in nomenclature, inexact intensities of peaks in the reference

spectra, incorrect rounding-off of *m/z* values in the measured spectra, incompleteness of the reference spectra, insufficient purity of compounds the reference spectra of which are comprised in the libraries, or influence of background (Durindová, *et al*., 1992). Where necessary, the structurally not significant peaks in the part of the spectra were omitted. Consequently, as a rule, the most intensive peak in acetylated compounds (*m/z*=43) is that of the CH_3CO^+ ions of acetyl (Ac) groups (Biermann, and Ginnis, 1989), and the intensities of fragments originating from the skeleton of the molecule may be relatively low as compared to that peak.

3.4 Conclusion.

This part of research was carried out to determine the organic compounds and fucoidan composition of *Ecklonia maxima* growing in South African waters. Experiments on this main alga of interest led to the isolation of phlorotannins phloroglucinol (**22**), eckol (**23**), 7-phloroeckol (**24**), 2-phloroeckol (**25**) and the sterol, 24-ethylidine cholesterol (**26**), a fucosterol. The tetramers are reported from this alga for the first time. It was also critical to examine the antioxidant activity of the organic isolates, crude or pure in order to promote their use in the cosmeceutical and pharmaceutical industry. The antioxidant activities of the crude organic extracts and pure samples were compared. Ethyl acetate crude extract showed the strongest activity, an indicator that it possessed most of the active principles.

Experimental and quantum chemical studies on the antioxidant radical scavenging properties of phlorotannin derivatives was subsequently performed by isolating, characterizing and assaying the radical scavenging activity to provide an explanation for the difference in the radical scavenging activity of the isolated compounds. The property of these compounds was measured using Oxygen radical absorption capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC), the results followed the order **22**< **23** < **25** < **24.** Compound **24** was found to

exert significant inhibitory effects than even the commercial antioxidant, ascorbic acid, indicating that **24** could be a good candidate for food, cosmeceutical and pharmaceutical applications.

Theoretical studies on the neutral and radical species of the studied compounds were meant to identify the preferred geometries of the species and also identify the factors influencing the values of the BDE, which determines the hydrogen atom transfer mechanism, and the values of adiabatic IP, which determines the electron transfer mechanism. The stabilization energy (ΔE_{iso}) was also investigated as it provides information on the ability of antioxidants to scavenge free radical species of phenolic derivatives. The conformational studies indicated that the lowest-energy conformer of **23**, **24** and **25** are stabilized by intra-molecular hydrogen bonds and electron delocalization throughout the rings. Overall, the results suggested that the position of substitution of phloroglucinol unit on eckol (**23**) played a significant role in determining the radical scavenging ability of the resulting eckol derivatives.

The isolated compounds were further subjected to antitumor testing. Their effects on metastasis was evaluated on human cancer cervical (*HeLa*), lung (H157) and breast (MCF7) cells. The protective effects of the compounds against the cancer cells were evaluated via MTT assay. The bioactivity of them towards these types of cell lines was generated by the OH groups in the phlorotannin structures. Among the isolated phlorotannins, compound **23**, an eckol showed prominent inhibitory activity against metastasis and effectively reduced induced cell damages. This compound thus appeared to be a potent cancer-preventative agent. The results have indicated that the compound can be used as an effective natural source to make cosmeceutical or pharmaceutical products.

In chapter 2, technique on extraction of fucoidan was carried out considering seasonality. The study proved that from the different seasons, fucoidan varied only in terms of yield of fucose, sulfate and uronic acid content. In order to investigate the best time of harvesting, winter collection had increased biomass hence possessing increased fucoidan content. In terms of the chemical composition of fucoidan from both times, there was no difference. Fucoidan fractions collected from ion exchange and gel permeation varied significantly in terms of sulfate content and molecular weight respectively, a factor that may influence biological activity. It is clear that some of these activities have diverse structural specificities. Invariably, biological activities could be improved by modifying these attributes (Cho, *et al*., 2011; Koyanagi, *et al*., 2003).

Principally, fucoidan is known to have various biological activities. Further investigations are necessary to determine the average chain length and their branching. Since low molecular weight fucans are usually reported to have biological activities, fractions from *Ecklonia maxima* have been of most interest to study. Additional research is necessary to improve the structural comprehension of this polysaccharide because it has not yet been documented. The polysaccharide under our study has demonstrated to possess a backbone of alternating $1\rightarrow3$-linked and $1\rightarrow4$ linked fucopyranosyl residues.

CHAPTER 4

4.0 *Splachnidium rugosum*

4.1 Introduction

The brown alga *Splachnidium rugosum* so far is known to be the sole representative of the genus *Splachnidium* (Greville, 1830). The life history of this brown alga has been studied in culture (Price and Ducker, 1966). The anatomy of the alga has been examined by several researchers (Hopkins, 1950; Skottsberg, 1920; Roe, 1916; Mitchell, *et al*., 1892), but the life history has seldom been studied (Hopkins, 1948, 1950; Lerner, 1949). The genus occurs only in the Southern Hemisphere and has been recorded from South Africa (Linnaeus, 1771, p. 311), southern Australia (Turner, 1811, p. 118) and New Zealand amongst other few places of the coastal regions of the world. It was first described by Linneaus as *Ulva rugosa* but the genus *Splachnidium* was afterwards founded for its reception by Greville, and it still remains the only species of the genus (Greville, 1830). This brown seaweed is interestingly common on the coastal line and it grows a foot or two above the lower water mark. It seems to be an annual alga as its specimens are very scarce during the winter months. It occurs at the Cape of Good Hope, on the open rocky coastline at Kommetjie where there are strong to extreme wave actions. *S. rugosum* is found in this part of the Southern hemisphere and contains sulfated fucan (Miller and Blunt, 2003). The alga develops on a wide variety of rock types including granite, basalt, and various sedimentary rocks. Although it usually exhibits marked seasonal development, it is frequently a conspicuous and important constituent of the littoral flora, commonly occurring in the mid-littoral zone, but occasionally in the upper-littoral zone (Price and Ducker, 1966). Much as the anatomy of the alga has been examined, its phytochemistry has not been studied.

4.1.1 Morphology

The macroscopic seaweed consists of monopodially branched, primary axes arising from a small discoid holdfast (Price and Ducker, 1966). The crosswise branches are initiated at the apex of their primary axes. Morphologically and anatomically, all branches are similar. They are cylindrical, constricted at the base and have the central part filled with transparent viscous mucilage (Tanaka, *et al*., 2007).

Plate 3: *Splachnidium rugosum* (Source: www.algaebase.org)

Along the shores, the algae at maturity are commonly 8 to 10 cm high, although they may grow up to 20 cm. When the alga develops in close proximity to one another, the holdfasts often fuse, making it impossible to delimit the individual plants. Along this coastline, a dense band of *Splachnidium* is present during the warmer months of the year. Within the band, the alga commonly grows up on barnacles. Interestingly, most of this alga disappears during winter and in spring, with juveniles appearing usually on barnacles. This juveniles are often found upon the

branches of the alga in intimate association with a filamentous phase (Price and Ducker, 1966). Modern taxonomists have classified *Splachnidium* either in the order Chordariales (Levring, 1941; Papenfuss, 1951, 1955) or in the order Ectocarpales (Fritsch, 1945), depending upon the rank which they give to certain assemblages within the Phaeophyta. The examination of the alga shows that the vegetative structure is allied to some extent with the *Fucaceae* but on the other hand in its reproduction, it is connected with the *Laminariaceae*. The sum of its characters, however, is such as to expressly exclude it from any existing natural order. They therefore proposed to establish the order *Splachnidiaceae*.

4.2. Collection and treatment of material

4.2.1. Algal material

Sporophytes of *Splachnidium rugosum* were collected on 22nd February 2012 at Kommetjie, south of Cape Town near the Cape of Good Hope, South Africa. The algal material was washed with running tap-water to remove sea water salts. The seaweed material was kept in a freezer until use.

4.2.2 Treatment of seaweed

Frozen seaweed was thawed and washed under running tap water. Visible debris was carefully removed with slight agitation, while the holdfast and any degraded areas of the seaweed were removed by hand. The sporophylls were milled with a food blender (Krups 75 blender) to obtain a fine paste. The milled seaweed paste was transferred into metallic plates, lyophilized in a freeze-drier for several days. The dried seaweed sample was weighed and kept at 4 ^{0}C until further use. The rest of the experimental procedures outlined in chapter 2 were followed for detailed analysis from section 2.1.2.2.

4.3 Results and discussion

4.3.1 Physico-chemical data of isolated compounds from *S. rugosum* organic extract

4.3.1.1: 24-ethylidine cholesterol (26).

White needles; ES-MS data; m/z 411.3584 [M - H]$^+$ (calcd for $C_{29}H_{48}O_1$, 411.3216); ^{1}H NMR (600 MHz, CDCl$_3$) same as that outlined in section 3.2.3.1.5.

4.3.1.2: 1, 3-Dicapryloyl-2-oleoylglycerol (27)

Colourless oil; ES-MS, m/z 609.0397 [M + H]$^+$, (calcd, for $C_{37}H_{68}O_6$, 609.0403). ^{1}H NMR (600 MHz, CDCl$_3$) δ 5.35 (*d*, *J* = 4.2 Hz, 1H), δ 5.27 (*td*, *J* = 5.8, 2.6 Hz, 1H), δ 4.31 (*dd*, *J* = 11.9, 4.3 Hz, 2H), δ 4.16 (*dd*, *J* = 11.9, 5.9 Hz, 2H), δ 2.32 (*td*, *J* = 7.6, 3.7 Hz, 6H), δ 2.02 (*tt*, *J* = 6.3, 3.5 Hz, 3H), δ 1.62 (*m*), 1.15-1.4 (*m*, 58H), δ 0.89 (*t*, *J* = 7.0 Hz, 9H). ^{13}C NMR (150 MHz, CDCl$_3$); δ 173.3 (C-1″); δ 172.9 (C-1′,C-1‴); δ 129.8-130.0 (C-9″,C-10″); δ 68.9 (C-2), δ 62.2 (C-1, C-3); δ 34.1(C-2″); δ 31.9 (C-2′, C-2″,C-6′, C-6‴, C-16″); δ 30.0 (C-7″,C-12″); δ 29.7 (C-6″, C-13″, C14″); δ 29.3-29.7 (C-4′, C-4″, C-4‴, C-5′, C-5″,C-5‴, C-15″); δ 27.2 (C-8″, C-11″); δ 24.9 (C-3′, C-3″, C-3‴); δ 22.7 (C-7′, C-7″, C7‴); δ 14.1 (C-8′, C-8″, C-8‴).

4.3.1.3: *E*-3, 7, 11, 15-Tetramethylhexadec-2-en-1-ol (28)

Colourless oil; EI-MS, m/z 296.0351 [M+H]$^+$ (calcd for $C_{20}H_{40}O$, 296.0331) ^{1}H-NMR (600 MHz, CDCl$_3$): δ 5.42 (*m*, 1H, H-2), δ 4.16 (*d*, 2H, *J* = 7.14, H-1), δ 1.99 (*t*, 2H, *J* = 7.55, H-4), δ 1.67 (*s*, 3H, H-20), δ 1.0-1.6 (br *m*, 19H, H-5-15), δ 0.8 (*m*, 12H, H-16, 17-19). ^{13}C-NMR (150 MHz): δ 140.4 (C-3), δ 123.3 (C-2), δ 59.5 (C-1), δ 39.8 (C-4), δ 39.4 (C-14), δ 37.4 (C-10), δ 37.4 (C-8), δ 37.3 (C-12), δ 36.7 (C-6), δ 32.8 (C-11), δ 32.7 (C-7), δ 29.7 (C15), δ 25.1 (C-5), δ 24.8 (C-13), δ 24.5 (C-9), δ 22.7 (C-17), δ 22.6 (C-16), δ 19.8 (C-19), δ 19.7 (C-18), δ 16.2 (C-20).

4.3.2 Structural elucidation and characterization of compounds

The filtrate of methanol extract extract of *Splachnidium rugosum* was suspended in distilled water, and partitioned with hexane, dichloromethane and ethyl acetate (Scheme 1). The hexane fraction (6.0 g) was subjected to chromatography on a silica gel column, with 500 ml volumes of hexane: DCM (10:1-10) as eluent, yielding 16 sub-fractions (H1-H16). The fractions were subjected to TLC analysis using DCM-MeOH (9.8:0.2) ratio. Pooling of fractions with similar TLC profiles was done. Fractions 2 and 3 of the hexane extract yielded compound **27** as a colourless oil (189.1 mg) that gave a positive colour test for oils with vanillin-sulfuric acid. A preparative TLC of combined fractions 6 to 8 from the same extract yielded compound **26** (32.2 mg) and compound **28** (12.6mg) that gave a positive colour test for steroids with vanillin-sulfuric acid.

4.3.2.1: Compound 26 (24-ethylidine cholesterol)

Details were similar to a compound characterized under section 3.2.4.5.

4.3.2.2: **Compound 27** (**1, 3-Dicapryloyl-2-oleoylglycerol**)

Column chromatography of combined fractions 2 and 3 of the hexane extract from *S. rugosum* yielded **27** as a colourless oil (189.1 mg) giving a positive colour test for oils with vanillin-sulfuric acid.
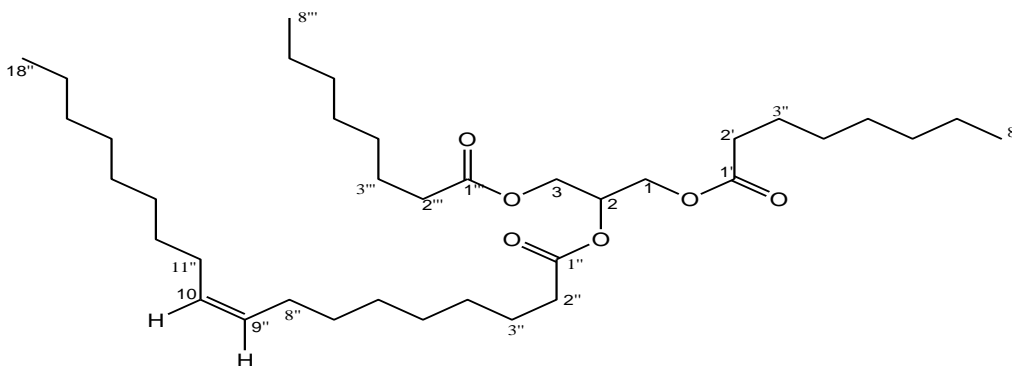
27

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The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and APT) and 2D-NMR experiments (HMQC, COSY and HMBC), (Table 30) plus MS data. A molecular ion peak at m/z 609 (M+H)$^+$ in FAB-MS along with ^{1}H- and ^{13}C-NMR spectral data showed its molecular formula to be $C_{37}H_{68}O_6$. The signals at δ 173.3 (C-1″), 172.9 (C-1′, C-1‴) in ^{13}C-NMR spectrum suggested the presence of three ester carbonyls in **27**. The proton signals at δ 4.16 (2H, *dd*, H-1/ H-3), δ 4.31 (2H, *dd*, H-1/ H-3) and δ 5.27 (1H, *m*, H-2), and the carbon signals at δ 62.2 (C-1, C-3) and 68.9 (C-2) along with MS fragments at m/z 265 ($C_{18}H_{33}O$) and 127 ($C_8H_{15}O$) confirmed that **27** is a triglyceride with C_{18} and C_8 fatty acid ester; a 9-Octodecenoic acid (9Z)-, 2-[(1-oxooctyl) oxy]-1-[[(1-oxooctyl) oxy] methyl] ethyl ester.

In HMBC spectrum, the carbonyl carbon signal at δ 173.3 (C-1″) and δ 172.9 (C-1′, C-1‴) was showing correlation with proton signals at δ 2.33 (2H, *t*, H-2″) and δ 1.62 (6H, *m*, H-3′, 3″, 3‴), also confirming that **27** is a triglyceride with long chain fatty acid ester. The proton signals at δ 2.02 (4H, *m*, H-8′, H-11″) showing correlation with carbon signals at δ 129.8-130.0 (C-9″, C-10″), confirmed the presence of an olefinic bond in **27**. Important HMBC's are represented as in Figure 34. The proton signals at δ 5.35 (2H, *d*, H-9″, H-10″) correlating with signals at δ 129.8-130.0 (C-9″, C-10″) in the HMQC spectrum also indicated the presence of olefinic bond in **27**. A closely related compound was isolated by Swaroop, *et al* 2005 from an ethanol extract of *Hippophae rhamnoides*. However, this compound **27** had only one olefinic bond giving a new triglyceride compound isolated from this seaweed for the first time. The peak at m/z 265 ($C_{18}H_{33}O$)$^+$ in the MS confirmed the presence of oleic acid moiety in **27**. The position of the oleic acid moiety was confirmed on the basis of ^{13}C-NMR. The presence of only two carbon signals for the three ester carbonyls at δ 173.3 (C-1″) and 172.9 (C-1′, C-1‴) suggested it as a symmetrical triglyceride, (Ramsewak, *et al*., 2001; Momin, *et al*., 2000), hence confirming

the position of oleic acid moiety at C-2. This is a structured triglyceride containing one long

chain fatty acid (oleic acid, C18:1) and one short chain saturated fatty acid (caprylic acid, 8:0).

Invariably, such structured triglycerides have been reported to have significantly reduced blood

cholesterol levels in hypercholesterolemic hamsters (Wilson, *et al*., 2006).

Table 30: ^{1}H (600MHz), ^{13}C NMR (150 MHz) and HMBC spectral data of 27 in CDCl$_3$

Atom	^{1}H (δ ppm, *J*=Hz)	^{13}C(δ ppm)	HMBC
1	4.16 (*dd*, *J*=11.9,4.9 Hz, 2H)	62.2	C-1′, C-2
2	5.27 (*td*, *J*=5.8, 2.6 Hz, 1H)	68.9	C-1, C-3, C-1″
3	4.31 (*dd*, *J*=11.9, 4.3Hz, 2H)	62.2	C-2, C-1‴
1″	-	173.3	
1′, 1‴	-	172.9	
2″	2.33 (*t*)	34.1	C-1″
2′, 2‴	2.32 (*td*, *J*=7.6, 3.7Hz)	31.9	C-1′,C-1‴
3′, 3″, 3‴	1.62 (*m*)	24.9	C-1′,C-1″,C-1‴
4′,4″,4‴,5′,5″,5‴,15″	1.26 (*m*)	29.3-29.7	
6′,6‴	1.26 (*m*)	31.9	
6″,13″,14″	1.26 (*m*)	29.7	
7′,17″,7‴	1.22 (*m*)	22.7	
7″,12″	1.28 (*m*)	30.0	
8″,11″	2.02 (*m*, 4H)	27.2	C-9″,C-10″
9″,10″	5.35 (*d*, *J*=4.2Hz, 1H)	129.8-130.0	C-8″,C-11″
16″	1.26 (*m*)	31.9	
8′,18″,8″	0.89 (*m*, 9H)	14.1	

C-1 and C-3 are interchangeable
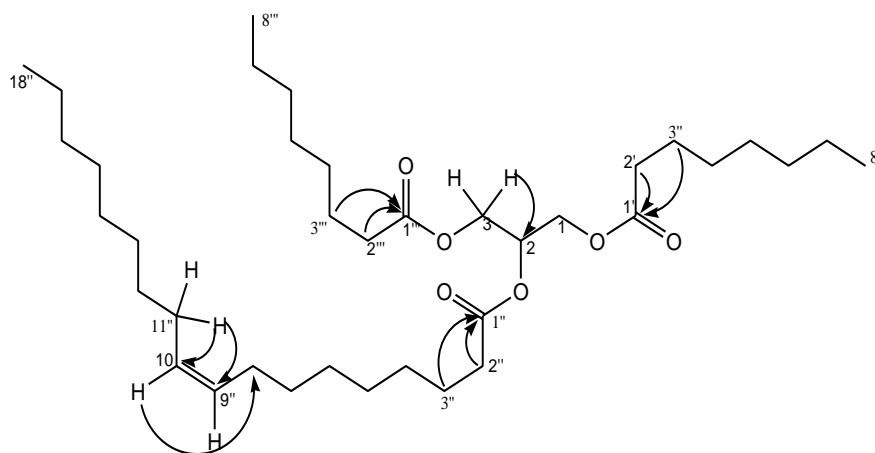
Figure 34: Important correlations observed in HMBC (H↔C) of 27

4.3.2.3: Compound 28 (*E*-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol)

A column chromatography of fraction 7 of the DCM extract from *S. rugosum* yielded **28** as a yellowish oil (12.6 mg) that gave a positive colour test for oils with vanillin-sulfuric acid.
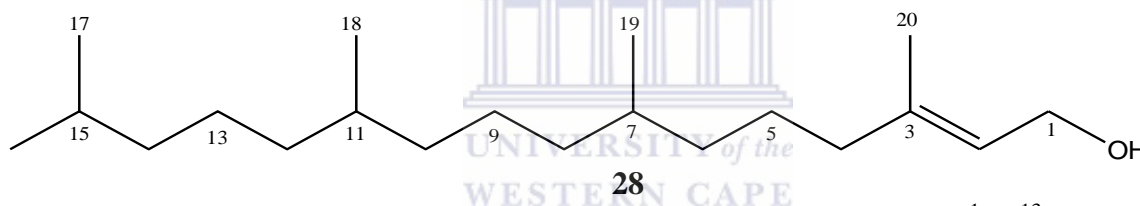
28

The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and APT) and 2D-NMR experiments (HMQC, COSY and HMBC), (Table 31) plus MS data. A molecular ion peak at *m/z* 296 (M+H)$^+$ in FAB-MS along with ^{1}H- and ^{13}C-NMR spectral data showed its molecular formula to be $C_{20}H_{40}O_6$. A proton-decoupled natural-abundance ^{13}C NMR spectrum of **28** is shown by the aliphatic structure. The carbon peaks are numbered consecutively, starting downfield. The resonances and their assignments are represented in Table 31 including the chemical shifts. The spectrum indicated resonances for this compound as follows: an olefinic proton at δ 5.42 (1 H, *m*), carbinyl protons at δ 4.16 (2H, *d*, *J* = 6.95 Hz),four methyl protons at δ 0.84, 0.85 (6H, *d*, *J*= 6.4, H-18, 19) and δ 0.87 (6H, *d*, *J*= 6.4, H-16, 17); an allylic methyl singlet at δ 1.67 (3H, *s,* H-20); and allylic methylene protons at δ 1.99 (2H, *m*, H-4). Other

resonances in the shielded region δ 0.99-1.6 (19H, *br m*, H-5-15) were attributed to other methylene and methine protons in the molecule.

The ^{13}C NMR spectral data of **28** indicated resonances for twenty carbons for the major constituents, suggesting a diterpene. Consequently, the following functionalities were deduced: two olefinic carbons at δ 140.4 and δ 123.3, a carbinyl carbon at δ 59.4 and seventeen shielded resonances (δ 39.8-16.2) for methyl, methylene and methine carbons. The secondary methyl at δ 1.67 was showing correlation with an olefinic proton δ 5.42 *m* in H-H COSY experiment, suggesting the presence of a double bond between C-2 and C-3. In the HMBC spectrum, there was a correlation signal between δ 4.16 (2H, H-1) and C-2, (δ 123.3) and C-3 (δ 140.4). Literature search revealed that **28** is phytol as evidenced by similar ^{13}C NMR spectral data (Goodman, *et al*. 1973). However, this compound is being reported for the first time from this alga. Such compounds are used in the fragrance industry for making cosmetics, shampoos, toilet soaps, household cleaners, and detergents. Phytol is widespread in nature, especially because it occurs ubiquitously as a component of chlorophyll (Vetter, *et al*., 2012; McGinty, *et al*., 2010).

Table 31: 1D and 2D ^{1}H (600 MHz) and ^{13}C NMR (150 MHz), CDCl$_3$ data for compound 28

Position	δ ^{13}C	δ ^{1}H	Phytol (Lit)
1	59.5	4.16 (*d*, *J*= 6.95)	59.4
2	123.3	5.42 (*m*)	123.1
3	140.4	-	140.2
4	39.8	1.99 (*t*)	39.9
5	25.1	1.36 (*m*)	25.1
6	36.7	1.19 (*m*)	36.7
7	32.7	1.32 (*m*)	32.7
8	37.4	1.17 (*m*)	37.4
9	24.5	1.21 (*m*)	24.5
10	37.4	1.17 (*m*)	37.4
11	32.8	1.38 (*m*)	32.8
12	37.3	1.15 (*m*)	37.3
13	24.8	1.25 (*m*)	24.8
14	39.4	1.13 (*m*)	39.4
15	29.7	1.54 (*m*)	27.9
16	22.6	0.87 (*d*, *J*= 6.4)	22.6
17	22.7	0.87 (*d*, *J*= 6.4)	22.7
18	19.7	0.85 (*d*, *J*= 6.4)	19.7
19	19.8	0.84 (*d*, *J*= 6.4)	19.7
20	16.2	1.67 (*s*)	16.1

4.3.3 Chemical composition of the crude of polysaccharide

Upon obtaining 58.4% yield of the freeze-dried material, 10 g of the sample was used to determine the chemical compositions of the crude polysaccharide as a preliminary analysis. The results obtained from the brown alga are reported in Table 32.

Table 32: % composition of the crude *S. rugosum* polysaccharide

	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
S. rugosum	0.9	86.9	0.9	1.2	6.4	1.9	1.7

Fucose was a predominant neutral sugar constituent of this fucoidan present at 86.9% in comparison to respective amounts of other units.

4.3.4 Fucose content determination

The fucose mg/g dry weight from the chemical analysis of ethanol treated fucoidan derived from the algae was recorded in table 33.

Table 33: Fucose mg/g dry weight of *S. rugosum*

Seaweed	Yield (g)	Absorbance	Total fucose (mg/g)
S. rugosum	3.86	0.1180	178.8

$0.1180/0.0033 = 35.76\mu g$

$(35.76/200) \times 100 = 17.88\%,$

$17.88 \times 3.86g = 0.6902g$

In mg/g $(0.6902 \times 1000)/3.86 = 178.8mg/g$

However, it is worth noting that the sugar rhamnose is also a methyl pentose (or a deoxy-hexose) and may also contribute to the total amount of fucose estimated as reported in section 3.3.3.1 even if present as a minor component.

4.3.5 Sulfate and uronic acid determination

Same standard absorbance calibration curves for colorimetric determination of sulfate content using potassium sulfate and uronic acid were obtained as shown in Figure 23 section

3.3.3.2. The results in Table 34 represent a summary of the percentage contents of the sulfate and uronic acid present in the seaweed.

Table 34: Percentage sulfate and uronic acid contents in *S. rugosum*

Seaweed	Yield (g)	Absorbance	% sulfate	Absorbance	% uronic acid
S. rugosum	3.86	0.1042	13.36	0.0677	7.09

S. rugosum 0.1042/0.0039 = 26.72μg

(26.72/200) x100 = 13.36% sulfate

Subsequently, curve b in section 3.3.3.2 was used for calculating the uronic acid content of the polysaccharide sample.

0.0677= 0.008x + 0.011   x= 7.09

Presence of glucuronic acid has been reported in literature on fucoidan from brown seaweeds (Adhikari, *et al*., 2006; Mandal, *et al*., 2007). Therefore, *S. rugosum* is essentially a fucoidan that contains sulfate groups and uronic acid.

4.3.6 Separation of PMP-hydrolysed fucoidan sugars

Analysis of hydrolysed *S. rugosum* PMP-sugars by HPLC Figure 35 shows the elution pattern of the derivatives of small sugars. Furthermore, a good separation of the PMP-sugars was achieved. The PMP labeled sugar peaks were all sharp and baseline resolved. The results showed a conspicuous initial mobile phase concentration peak and a shallow gradient providing good quality separation of the PMP-labeled products. A HPLC profile of standard PMP derivatives was developed subject to validation for the qualitative and quantitative determination as outlined in section 3.3.5.
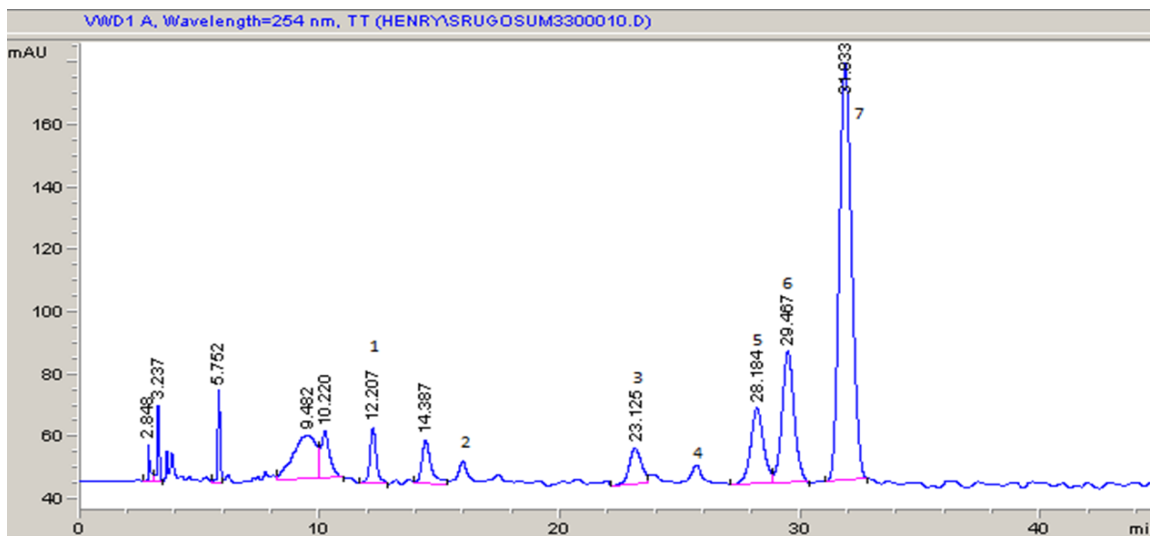
Figure 35: Monosaccharide composition analysis by HPLC of PMP-derivatized *S. rugosum*

The monosaccharides detected in the alga were mannose, rhamnose, galactose, arabinose, glucose, xylose and fucose respectively. Figure 35 shows the separation of the PMP-degraded monosaccharides from the fucoidan. There were few additional peaks detected in the sample peaks between major ones and the hypothesis is that they represented mannuronic acid and glucuronic acid formed by degradation of alginate.

4.3.7 Fractionation of polysaccharide from *S. rugosum*

From the anion-exchange chromatography elution profile of crude fucoidan HM/SR Figure 36, the absorbance of the sampled individual test tubes were plotted against the fraction number. Five fractions were obtained: SR1, SR2, SR3, SR4 and SR5 in a stepwise gradient from using water, 0.1M until 1M NaCl elution on a Sepharose CL-6B column, respectively. No polysaccharide was detected after using 1M NaCl. The tubes with phenol-sulfuric acid positive reaction were pooled together; Figure 36 showing the elution profile of HM/SR with five symmetrical peaks obtained. Fractions from respective peaks were combined, dialyzed and lyophilized. Consequently, the extraction yield of total carbohydrate content and sugar composit-

ions are shown in Table 35.
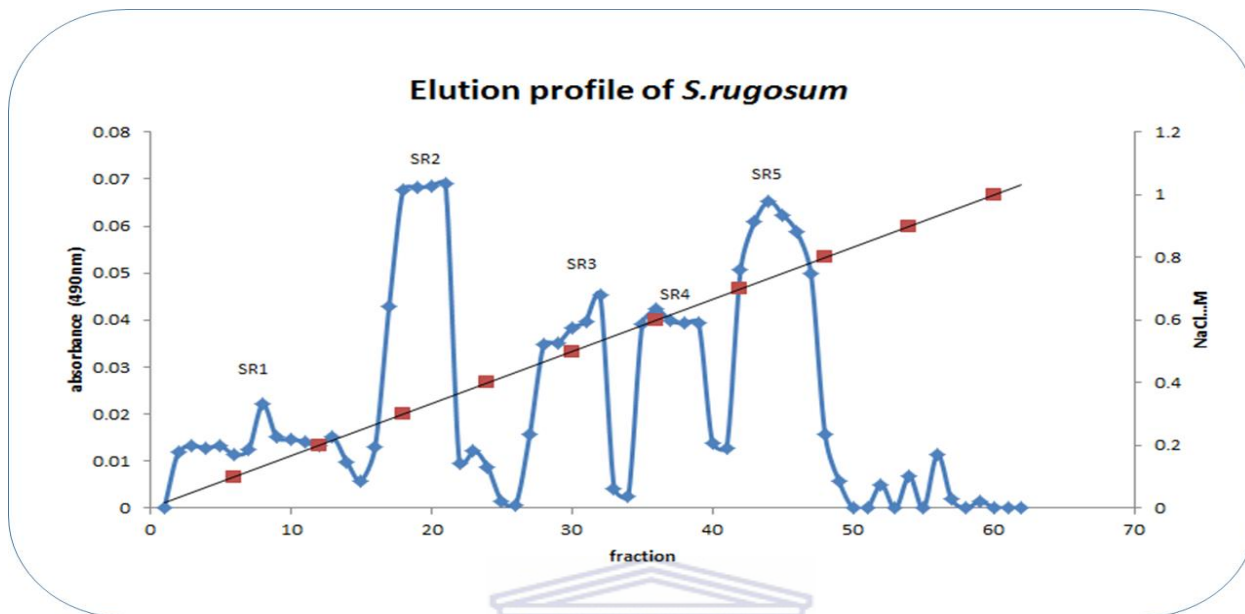
Figure 36: Elution profile of HM/SR on DEAE-sepharose CL-6B

Invariably, purified polysaccharides subjected to ion exchange work on the same principle of ionic centers. Therefore, less charged oligosaccharides flow out earlier; and heavily charged ones much later. The 5 ml fractions were collected and checked by phenol-sulfuric acid at 490 nm. The fractions were dialyzed for 2 days, evaporated then freeze-dried for subsequent chemical analysis. The qualitative monosaccharide sugar and sulfate content of each fraction was determined by phenol-sulfuric acid and ion chromatography, respectively. The sugar compositions were determined by GC after conversion into their alditol acetates as described under the materials and methods section 2.7.2.1.

Table 35: Composition and yield of purified fucoidan in *S. rugosum* fractions

Fraction	%Yield	Neutral monosaccharides (%)							Sulfate (SO_4^{2-}) (%)
		Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	
F1	10.82	9.2	20.1	12.0	15.7	15.8	15.1	12.0	55.23
F2	21.2	10.8	22.8	13.3	16.3	24.1	17.5	16.9	61.88
F3	20.4	8.3	40.3	8.3	9.5	12.2	9.4	12.1	86.72
F4	34.8	5.4	36.0	7.2	10.4	16.7	9.3	14.9	95.04

Fucose was the main constituent of the alga and as the concentration of NaCl increased in the mobile phase, more sulfate groups were released. However, fucoidans from different species of brown seaweed have varying amounts of neutral sugars and sulfate contents, but also have varying levels of ionic strengths caused by those constituents (Ly, *et al*., 2005).

Primarily, the GC results of the alditol acetates of the purified polysaccharide, the congruence of retention time of each of their peaks, were compared with those of known standards in order to identify the monosaccharide unit while the area or height of the chromatographic peak was used for quantification. Furthermore, *S. rugosum* as brown seaweed had a molar percentage of neutral sugar fucose among others dominating as a major unit. Other neutral sugars, rhamnose, arabinose, xylose, mannose, and galactose were only in varying proportions.

4.3.8 Molecular weight determination

Selected fraction 2 from S. rugosum (1 ml, 3mg) was fractionated into different ranges of molecular weights by gel permeation chromatography, using a 400 x 16 mmcolumn, Sephacryl

S-400 HR as stationary phase, and 1M NaCl as eluent, at a flow rate of 0.5 ml/ min. Subsequently, 2 ml volumes of the purified polysaccharide were collected. A profile of molecular weight determination was plotted as shown by Figure 37. Dextrans of known molecular weight were used as markers. The gel permeation chromatographic column was calibrated by means of five dextran standards of known average molecular weights as reported earlier in section 3.3.7. Collected fractions were analyzed for p-glucan content by means of the Phenol-sulfuric acid.
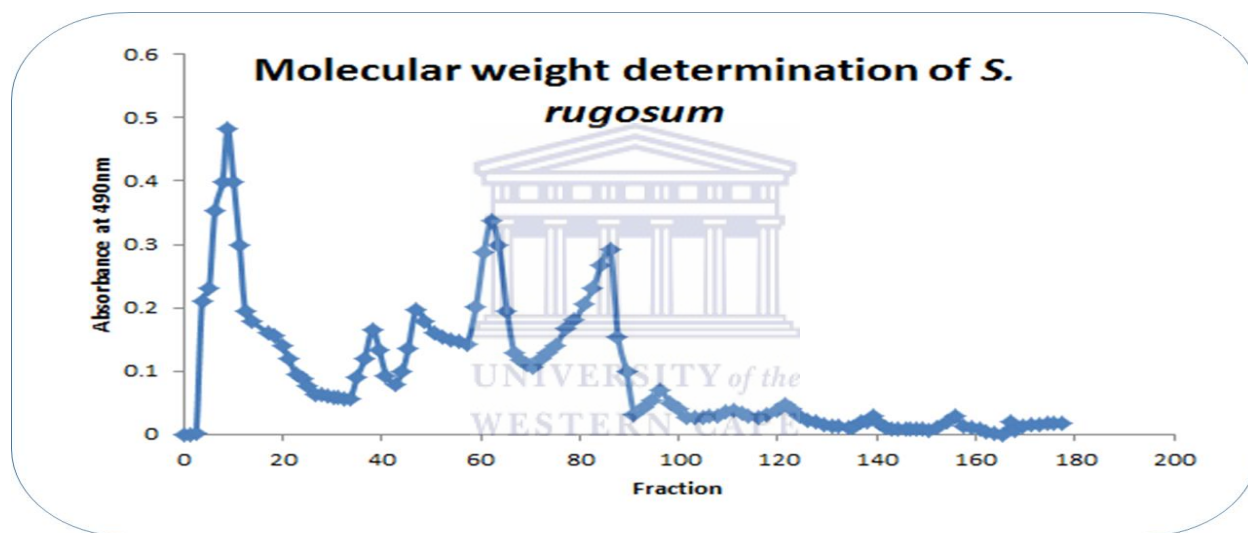
Figure 37: An elution profile of fraction 2 from Sephacryl S-400 column

The average molecular weight of sugars from the alga were between 25×10^3 Da and 250×10^4 Da by reference to the calibration curve (y = -0.022x + 6.3424, R^2 = 0.94) made from Dextran standards of known molecular weight (10,000, 70,000, 110,000, 150,000, 500,000). Invariably, such a range of molecular weights is a representation of effectiveness of this seaweed to various biological activities thereof.

4.3.9 Ion chromatographic results

Sulfate presence was determined again by ion chromatography (Miskaki, *et al*., 2007).

After carrying out the anion-exchange purification procedure through DEAE-Sepharose column, fractions pooled together had the following ions composition. The purified oligosaccharides after hydrolysis were extracted with water to remove ions from their surfaces in order to obtain an aqueous solution for analysis. These results were recorded as shown in Table 36

Table 36: Minimum concentrations of F^-, Cl^-, NO^-_2, Br^-, NO^-_3, PO_4^{-3}, and SO_4^{-2} at 1000 ppm

Fraction	Fluoride%	Chloride%	Nitrite%	Bromide%	Nitrate%	Sulphate%
SR/1	-	4.77	-	-	-	75.23
SR/2	-	38.12	-	-	-	61.88
SR/3	-	3.25	-	-	-	96.75
SR/4	-	24.96	-	-	-	95.04
SR/5	-	12.91	-	-	-	87.09

The data shows the average percentages of the anions found in the fractions of the alga, based on 4 replicate injections. The results represented high percentages of sulfate in all fractions, a characteristic of fucoidans; chlorides were due to the the adaptation of the alga growing under saline sea environment. Invariably, the application of IC demonstrated electrolytic eluent generation providing as a convenient and reliable method for the simultaneous determination of sulfate and other anionic ingredients in purified fractions. The results served as justification of presence of sulfate reflected by the IR report.

4.3.10 Infrared spectrum of *S. rugosum*

To investigate the functional groups of *S. rugosum*, the FTIR spectrum was recorded at the absorbance mode from the range of 4000-450 cm⁻¹ as shown in Figure 38.
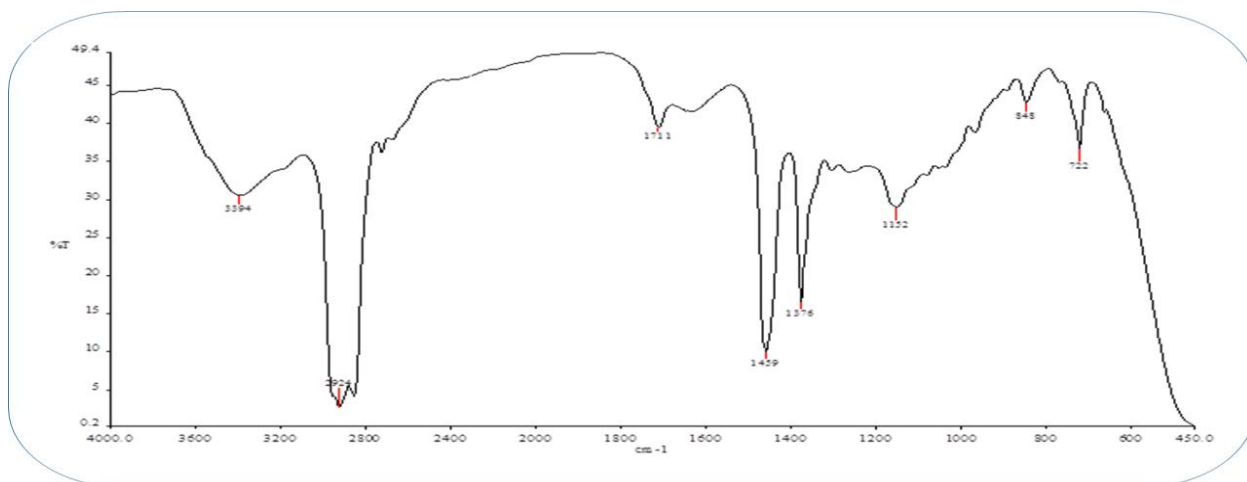
Figure 38: IR spectrum of *S. rugosum* indicating characteristic bands

Common to most brown seaweeds, the spectrum displayed a broad band of absorption between 3600 cm^{-1} and 3200 cm^{-1}, which was due to the hydroxyl groups stretching vibration. The bands in the region of 2924 cm^{-1} were a characteristic of C-H anti-symmetrical stretching vibration. Bands around 1711 cm^{-1} represented the ester carbonyl groups (C=O) and carboxylate (COO-) stretching band (Leal *et al*., 2008; Gnanasambanda and Proctor, 2000), indicating esterification and free carboxyl groups present in the polysaccharide containing some of uronic acids. The peak absorption around 1459 cm^{-1} could be attributed to CH$_3$ asymmetric bending (fucose) and *O*-acetyls. This was assigned due to deformation vibration with contribution of *O*-C-*O* symmetric stretching (Synytsya, *et al*., 2010; Gómez-Ordóñez and Rupérez, 2011).

The absorption bands region from 1300 cm^{-1} to 800 cm^{-1}, as the "finger print" region for carbohydrates, allowed the identification of major chemical groups in polysaccharides (Cerna` *et al*., 2003; Kalapathy and Proctor, 2001). This is related to conformation and surface structure of molecules. The spectrum showed a signal at 1376cm^{-1} attributable to scissoring vibration of CH$_2$ (glucose and mannose) and, a signal at 842cm^{-1} corresponding to a primary (equatorial) ester sulphate (C-*O*-S) characteristic of fucoidans, an indication of most sulfate groups located at

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positions 2 or 3; one strong band at 1152cm^{-1} for (C-O, C-C) stretching vibrations of pyranose ring common to all sulfated polysaccharides, and other minor bands related to sugar cycles. The anomeric region of fingerprint (950-720 cm^{-1}) showed characteristic absorption bands of alginate polysaccharides. The band around 960 cm^{-1} was assigned to the C-O stretching vibration of uronic acid residues, and the one at 722 may be assigned to the C$_1$-H deformation vibrations of residues (Chandía, *et al*., 2004).

4.3.11 NMR spectroscopy

NMR spectroscopy as a convenient method to obtain valuable structural information about polysaccharides was performed. Invariably, like many other native algal fucoidans, *S. rugosum* sample had a complex ^{1}H NMR spectrum, which was difficult to interpret completely (Fig. 39). It contained some intense signals in the anomeric and up-field regions, which are typical of L-fucopyranosides. This ^{1}H NMR spectrum contained poorly resolved signals in the α-anomeric region around 5.0-5.4 ppm but up-field signals between 1-1.5 ppm regions being particularly prominent. Such high-field region signals have been assigned to a C-6 methyl proton group of L-fucopyranose (Synytsya, *et al*., 2010). The small signals at about 2.2 ppm confirmed the presence of small amount of O-acetyl groups.
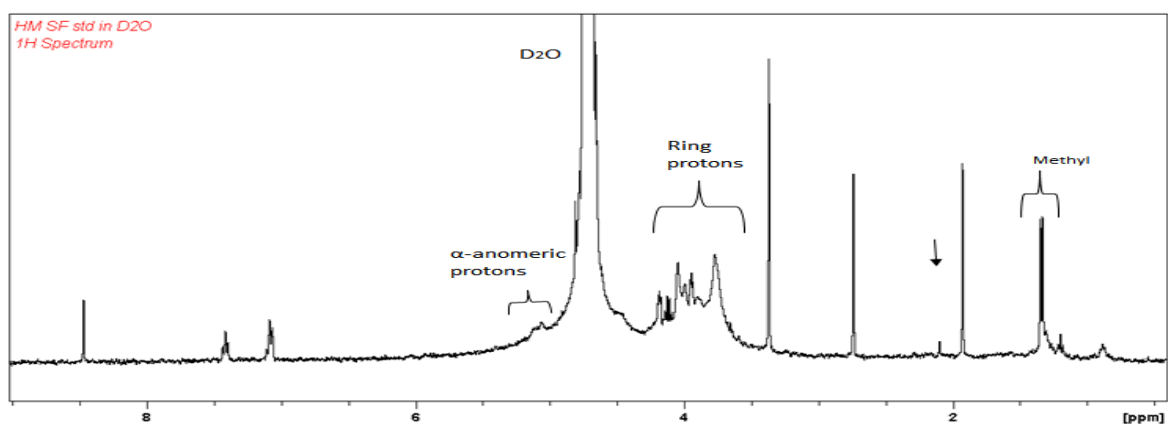
Figure 39: ^{1}H NMR spectrum of the fucoidan isolated from *S. rugosum*

4.3.12 Methylation analysis of *S. rugosum*

Sugars in this fucoidan sample were identified by comparing their respective retention times and mass spectra to the authentic standards of PMAAs. Consequently, most of the major peaks were identified within the 10-42 min chromatographic window, while several unknown peaks appeared at greater than 42 min as shown in Fig. 40 (a) sulfated and (b) desulfated. Invariably, several of the methylated monosaccharides were identified comprising hexoses and a few pentoses. However, there were unidentified compounds eluting between 10 and 42 min of the integrated area. Therefore, based on their characteristic fragments, their structures could not be fully resolved.
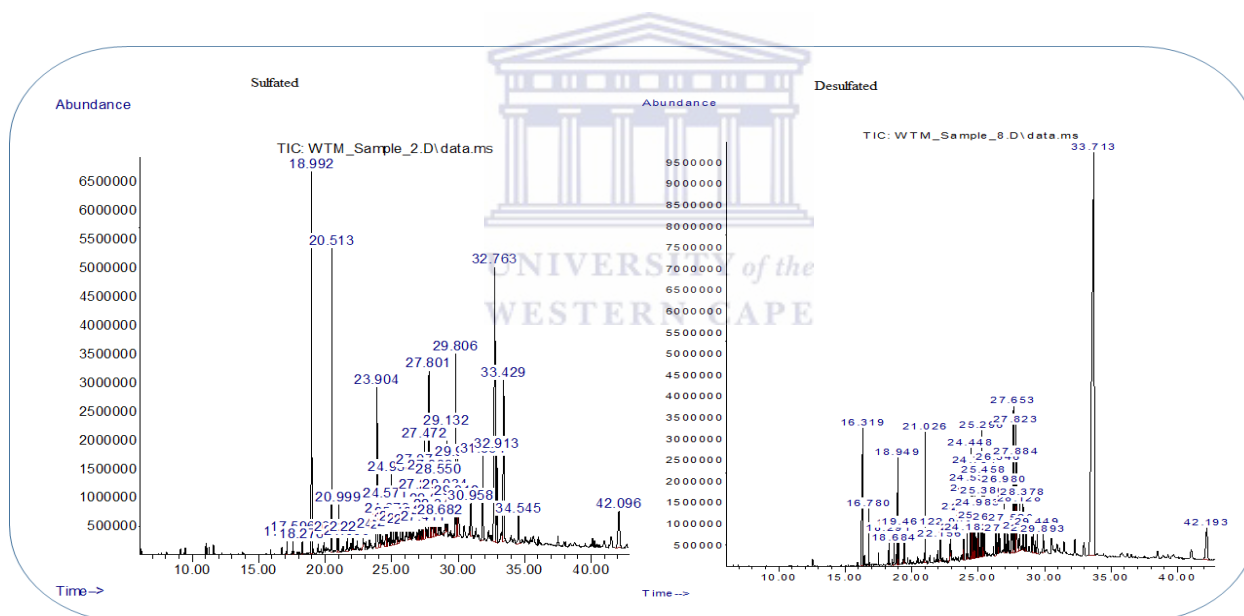
Figure 40: GC-MS analysis of PMAA of *S. rugosum*

Table 37: PMAAs derived linkage analysis from native and desulfated *S. rugosum*

Methylation product	Deduced position of substitution	Sulfated (mol %)	Desulfated (mol %)
2,3,4- Me₃-Xylitol	Terminal Xyl(1→	5.3	5.8
2,3,4-Me₃-Fucitol	TerminalFuc*p*(1→	8.8	8.5
2,3-Me₂-Fucitol	→4) Fuc*p*(1→	6.4	6.9
2,4 Me₂-Fucitol	→3) Fuc*p*(1→	9.4	18.8
3,4-Me₂-Fucitol	→2) Fuc*p*(1→	9	Nd
2-Me-Fucitol	→3, 4) Fuc*p*(1→	19.2	4.5
3-Me-Fucitol	→2, 4) Fuc*p*(1→	8.5	8.2
4-Me-Fucitol	→2, 3) Fuc*p*(1→	17.2	16
2,4,6- Me₃-Galactitol	→3) Gal*p*(1→	7.8	5.1

Glycosyl linkage analysis of the desulfated fucan gave, 2, 3, 4-tri-*O*-methylfucitol, 2, 4-di-*O*-methylfucitol and 4-*O*-methylfucitol residues. This suggested that the fucan sulfate has a (1→3)-linked backbone together with branches at C-2. The presence of a terminal xylopyranosyl residue was indicated. Furthermore, linkage analysis of the native fucan sulfate yielded a variety of mono-methylated, di-methylated and tri-methylated products (Table 37) indicating that the structure of this polysaccharide was highly complex. Invariably, the 2-*O*-methyl fucitol and 4-*O*-methyl fucitol were the abundant products of the methylation analysis of the native fucoidan. Subsequently, there was an increase in the proportions of the 2, 4-di-*O*-methyl fucitol after desulfation. The results of decreased proportions of 2-*O*-methyl fucitol residues suggesting that the sulfate esters when present are located at C-4 of the (1→3)-linked fucosyl residues. The results were in agreement with the predictions made by IR analysis that sulfate groups are

located at C-4 of fucosyl residues. Nonetheless, small amounts of (1→4)-linked fucopyranosyl residues was also determined (Mandal, *et al.*, 1991).

4.4 Conclusion

Studies on the organic extracts of *Splachnidium rugosum* were performed by isolating and characterizing some pure compounds. Three compounds; 24-ethylidine cholesterol (**26**), 1, 3-Dicapryloyl-2-oleoylglycerol (**27**), and *E*-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol (**28**) were isolated for the first time from this alga.

Although there are various methods of extracting best quality fucoidan and stated advantages of the methods over others, it was important to consider the one where the solvent used to solubilize fucoidan in the seaweed gave better yield. Fucoidan content from the sporophylls parts of the seaweed was determined. These parts of the alga produced a good quality fucoidan in terms of percentage yield (58.4% dry weight), and the fucose and sulfate content. The sulfate content was reported high from the fractions.

Crude polysaccharide was fractionated using ion-exchange chromatography. Chemical compositions of both the crude and purified types were compared. The average molecular weights of the fucoidan and/or its fractions were also determined using gel permeation chromatography. Most reported studies have revealed molecular weight estimates which average 25,000 - 800,000 Daltons (Li, *et al.*, 2006; Zvyagintseva, *et al.*, 2003). Monosaccharide content of the fucoidan was determined using gas-chromatography (GC) method.

The application of nuclear magnetic resonance (NMR) spectroscopy was very useful in order to obtain structural information on fucoidan by identifying the residues present and how they are linked together. The side chains were determined through methylation analysis. The results suggested that the sulfate esters present in the seaweed were located at C-4 of the (1→3)-

linked fucosyl residues. The results were in agreement with the predictions made by IR analysis that sulfate groups are located at C-4 of fucosyl residues. Nonetheless, small amounts of (1→4)-linked fucopyranosyl residues were also detected (Mandal, *et al.*, 1991).

CHAPTER 5

5.0 *Macrocystis angustifolia*

5.1 Introduction

Kelp beds and forests represent some of the most conspicuous and well-studied marine habitats. As it might be expected, these diverse and productive systems derive most of their habitat structure and available energy from the kelps, a relatively diverse order of large brown algae comprising Phaeophyceae and Laminariales (Rengasamy, *et al*., 2013; Graham, *et al*., 2007). Kelps and their associated communities are conspicuous features of temperate coasts worldwide (Coyer, *et al*., 2001; Lüning, 1990), including all of the continents except Antarctica (Moe and Silva 1977; Foster, *et al*., 1985), and the proximity of such species-rich marine systems to large coastal human populations has subsequently resulted in substantial extractive and non-extractive industries (Leet, *et al*. 2001).

It is therefore not surprising that the basic and applied scientific research and literature on kelps is extensive. Present understanding of the ecology of kelp taxa is not uniform, as the giant kelp *Macrocystis* has received the greatest attention. *Macrocystis* is the most widely distributed kelp genus in the world, forming dense forests in both the Northern and Southern hemispheres (Guiry and Guiry, 2013). The floating canopies of the adult sporophytes have great structural complexity and high rates of primary productivity (Demes *et al.* 2009; North, 1994; Mann, 1973; Jackson, 1977). Furthermore, although *Macrocystis* primary production can fuel secondary productivity through direct grazing, most fixed carbon probably enters the food web through complex pathways or is exported from the system (Harrold, *et al*., 1998; Graham, 2004). In some regions, such habitat and energy provision support to over 275 common species of the flora and fauna (Vásquez, *et al*., 2006; Graham 2004; Beckley and Branch, 1992).

Populations of this marine giant kelp *Macrocystis*, forms extensive natural beds along the South African coast. The alga is often described as the fastest growing plant in the world. Furthermore, it is of commercial value as a raw material for the production of alginic acid, and recent interest has developed in extending the utilization of this plant to large scale food and energy farms (Rengasamy, *et al*., 2013; Wilcox 1975, Bryce 1977, Hall 1978, Neushul 1978). One of the principal reasons for present commercial utilization and increasing maricultural interest in *Macrocystis* is the high productivity of their population. Production, growth rate and the standing crop of the plant present at any time, is a matter of continuing concern to commercial harvesters (Ryan, 2008; Neushul, 1978).

The Giant Kelp (genus *Macrocystis*) or `String Kelp' are large, canopy forming plants which grow in dense beds along the inshore sub-tidal reefs of South-west coast of Cape Province, from the Olifants R. to Cape Point in Cape Town, South Africa within the Benguela currents. Invariably, they are among the most beautiful and biologically productive habitats in the marine environment. The distribution of Giant Kelp is greatly influenced by physical factors, particularly the influence of nutrients, currents which provide cool and nutrient rich waters, water temperature and the frequency of storm events (Macaya and Zuccarello, 2010).

Specifically, they require a hard surface for attachment, high nutrient concentration, moderate water motion, and cool, clear and clean ocean water for growth. Individual alga grows up to 30m tall, forming tall spectacular forests with the fronds providing a dense canopy which shade and modify under-storey reef communities (http://www.kelp watch.tas.gov.au/). The characteristics of this family include branched stipes, each branch terminating in a single blade, and blades that split at an intercalary meristem. Some of the genera, like *Macrocystis*, have gas-filled float called pneumatocysts that hold the plant erect in the water. In South Africa, one

major species is found as *Macrocystis angustifolia* that occurs along the coast of Kommetjie, to the sheltered side from the low tide level to 10 m deep. Primarily, these Giant Kelp forests are habitats of outstanding ecological and economic significance, representing areas of high biodiversity and productivity, providing key habitats for the abalone (Rengasamy, *et al*., 2013).

Macrocystis angustifolia (Plate 4) is not used in the alginate industry, although it has been reported to have a higher alginate quality than *L. pallida*; the natural populations of this species are too small to warrant commercial harvesting in South Africa (Neushul 1978). There are, however, a number of scientific studies underway on the feasibility of cultivating species of this kelp specifically for alginate extraction. Marine cultivation, or mariculture as it is termed, could be the answer to a sustainable high-grade alginate quality. Consequently, the purpose of this study is to examine the phytochemistry and structural characteristics of adult alga in the sea.
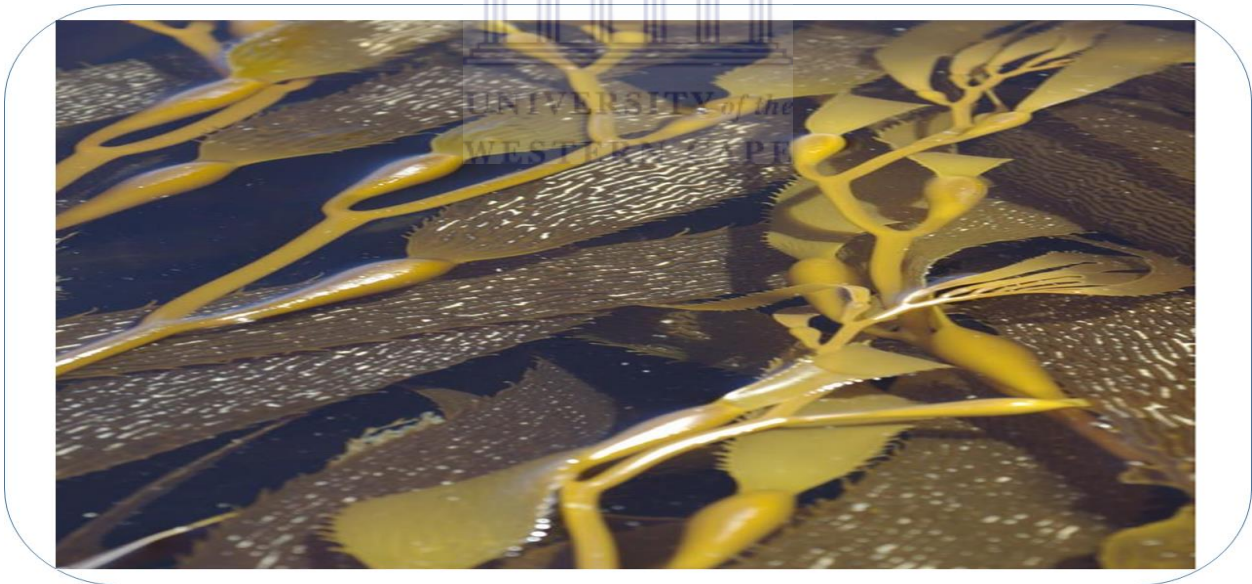
Plate 4: *M. angustifolia* (Source: www.algaebase.org)

However, studies have recognized three species; *Macrocystis pyrifera, Macrocystis angustifolia* and *Macrocystis integrifolia*, based on holdfast morphology (Womersley, 1954). *M. angustifolia* is found only off South Africa and south of Australia; a southern California population was

determined on the basis of the morphology to be a subspecies of *Macrocystis pyrifera* (Neushul, 1971; Brostoff, 1977). A fourth species, *Macrocystis laevis* has been located at Marion Island in the Indian Ocean, south of South Africa, based on the characteristic of smooth blades (Hay, 1986). In addition, the large kelp plants themselves represent a major ecological key to new species, influencing the hydrological and light environment, and also, the recruitment of rocky inshore fish and invertebrates (Hay, 1986).

5.2 Morphology and growth

This Kelp has the distinction of being the largest marine seaweed in the world; with the largest attached alga recorded at 65m long (Bronstoff, 1977; Neushul, 1963; Towle and Pearse, 1973). It has a root-like holdfast that fixes to rocky surfaces with a slender stalk or stipe; and long, leaf-like blades or fronds, which are the major site of photosynthetic activity. It is supported in the water by gas-filled bladders on each frond called pneumatocysts. The holdfast is cone-shaped and can grow up to 60 cm in diameter for large plants. This world's largest algae, produces a surface canopy of numerous vegetative fronds that arise from a single holdfast. Vegetative fronds constitute most of the biomass of the plant and account for approximately 95% of the organic production (Neushul, 1963; Towle and Pearse, 1973).

The forests undergo regular seasonal growth, with peak growth in spring and regular dieback of plants during the winter months. Sporophylls which are the reproductive blades grow from the base of each frond near the holdfast, with the number of sporophylls per frond and the number of fronds per plant being highly variable (Lobban, 1978; Reed, 1987), some plants can have greater than 200 sporophylls (Reed, *et al.,* 1996). The holdfast is never rhizomatous or creeping, but forms domed or conical masses. Lamina is never corrugated and the solitary vesicular blades in the canopy are commonly sporogenous.

5.3 Algal material

Algal material of *Macrocystis angustifolia* were collected on the 22nd February 2012 at Kommetjie, south of Cape Town near the Cape of Good Hope, South Africa. The material was washed with running tap-water to remove sea water salts. This was kept in a freezer until use.

5.3.1 Treatment of seaweed

The frozen seaweed was thawed and washed under running tap water. Any visible debris was carefully removed with slight agitation, while the stipes and fronds with any degraded areas of the seaweed were removed by hand. Subsequently, the stipes and long, leaf-like blades were milled with a food blender (Krups 75 blender) to obtain a fine paste. The milled seaweed paste was transferred into metallic plates, lyophilized in a freeze-drier for several days. The dried seaweed sample weighed and kept at 4 ^{0}C until further use. Consequently, some of the experimental procedures outlined in chapter 2 were followed for further analysis, section 2.1.2.2.

5.4 Results and discussion

5.4.1 Physico-chemical data of isolated compound from *M. angustifolia*

5.4.1.1: 24-ethylidine cholesterol (26)

White needles; ES-MS data; *m/z* 411.3584 [M - H]$^+$ (calcd for $C_{29}H_{48}O_1$, 411.3216); ^{1}H NMR (600 MHz, CDCl$_3$) similar to the one outlined in section 3.2.3.1.5.

5.4.2 Structural elucidation and characterization of a compound

5.4.2.1: Compound 26 (24-ethylidine cholesterol)

The filtrate of methanol extract of *Macrocystis angustifolia* was suspended in distilled water, and partitioned with hexane, dichloromethane and ethyl acetate (Scheme 1). The hexane fraction (7.0 g) was subjected to chromatography on a silica gel column, eluted with 500 ml volumes of hexane: DCM (10:1-10) , yielding 5 sub-fractions (H1-H5). The fractions were

subjected to TLC analysis using DCM-MeOH (9.8:0.2) ratio. Pooling of fractions with similar TLC profiles was performed. Fraction 10 of the hexane extract was subjected to a preparative TLC and this yielded compound **26** (18.5 mg) that gave a positive colour test for steroids with vanillin-sulfuric acid. This compound was similar in structure to another isolated from *Ecklonia maxima* described earlier in chapter 3 section 3.2.4.5. Other compounds could not be characterized due to insufficient experimental data.

5.4.3 Chemical composition of the crude of polysaccharide

A 49.8% polysaccharide yield of the freeze-dried material was obtained. The chemical compositions of this crude material from preliminary analysis were as shown in Table 38.

Table 38: % composition of the crude *M. angustifolia* polysaccharide

	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
M. angustifolia	0.6	77.1	1.3	11.5	5.7	3.7	5.3

Neutral sugar fucose was a predominant at 77.1% of this fucoidan in comparison to respective amounts of other units.

5.4.4 Fucose content determination

The fucose mg/g dry weight from the chemical analysis of ethanol treated fucoidan derived from this alga was recorded in table 39.

Table 39: Fucose mg/g dry weight of *M.angustifolia*

Seaweed	Yield (g)	Absorbance	Total fucose (mg/g)
M. angustifolia	3.88	0.1180	178.8

0.1180/0.0033 = 35.76µg

(35.76/200) x100 = 17.88%,

17.88% x 3.88g = 0.6937 g

In mg/g (0.6937x1000)/3.88 = 178.8 mg/g

Furthermore, the sugar rhamnose as a methyl pentose (or a deoxy-hexose) may have contributed to the total amount of fucose estimated as reported in section 3.3.3.1 even if present as a minor component.

5.4.5 Sulfate and uronic acid determination

The standard absorbance calibration curves as outlined earlier in Figure 23 section 3.3.3.2 for colorimetric determination of sulfate content using potassium sulfate and uronic acid analysis were used. The results in Table 40 represent a summary of the percentage contents of the both present in this alga respectively.

Table 40: Percentage sulfate and uronic acid contents

Seaweed	Yield (g)	Absorbance	% sulfate	Absorbance	% uronic acid
M. angustifolia	3.88	0.1753	22.48	0.1140	12.88

M. angustifolia 0.1753/0.0039 = 44.95 µg

(44.95/200) x100 = 22.48% sulfate

Furthermore, curve b represented in section 3.3.3.2 was used for calculating the uronic acid content of the polysaccharide sample.

0.1140= 0.008x + 0.011   x= 12.88

The presence of glucuronic acid has been reported in literature in fucoidan from brown seaweeds (Adhikari, *et al*., 2006; Mandal, *et al*., 2007). *Macrocystis angustifolia* possesses fucoidan that comprises sulfate groups and uronic acid.

5.4.6 Separation of PMP-hydrolysed fucoidan sugars of *M. angustifolia*

Analysis of hydrolysed *M. angustifolia* PMP-sugars by HPLC in Figure 41 shows the elution pattern of the derivatives of small sugars. The sugar peaks were all sharp and baseline resolved. A profile of standard derivatives was developed subject to validation for the qualitative and quantitative determination as outlined in section 3.3.5. Subsequent to this, a good quality separation of the PMP-labeled products was achieved.
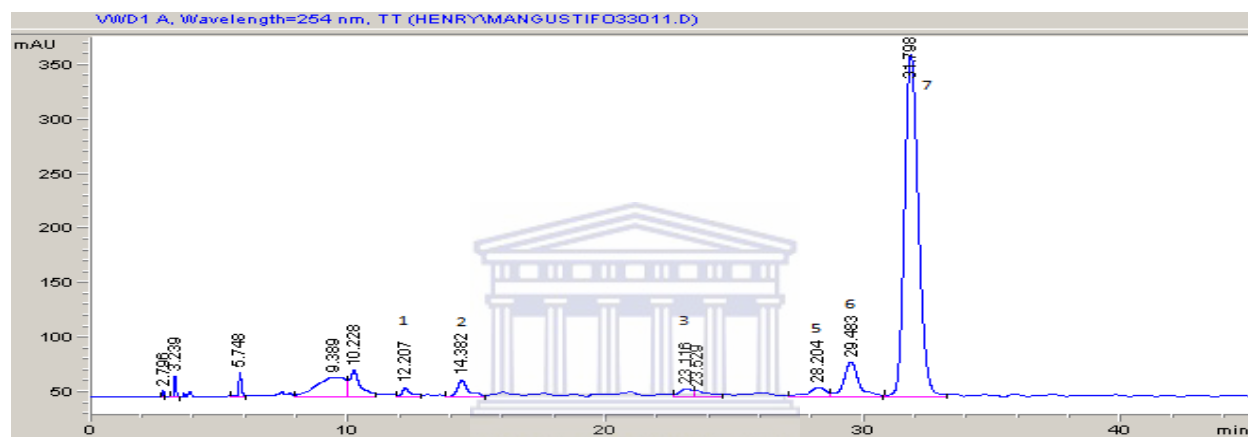
Figure 41: Monosaccharide composition analysis by HPLC of PMP-derivatized *M. angustifolia*

Consequently, the monosaccharides detected in the alga were mannose, rhamnose, galactose, glucose, xylose and fucose respectively. The profile in Figure 41 shows the separation of the PMP-degraded monosaccharides from the fucoidan. Furthermore, a few additional peaks detected between major peaks were hypothesized to represent mannuronic acid and glucuronic acid formed by degradation of alginate.

5.4.7 Fractionation of polysaccharide from *M. angustifolia*

Following the anion-exchange chromatography elution profile of the crude fucoidan HM/Ma (Figure 42), absorbances of the sampled individual test tubes were plotted against the fraction number. Four fractions were obtained: Ma1, Ma2, Ma3 and Ma4 in a stepwise gradient

from using water, 0.1M until 1M NaCl elution on a Sepharose CL-6B column, respectively. All tubes were tested with phenol-sulfuric acid for positive reaction. Subsequently, the fractions from respective positive peaks were combined, dialyzed against distilled water and lyophilized. The neutral sugar yield of total carbohydrate content chemical compositions are shown in Table 41.
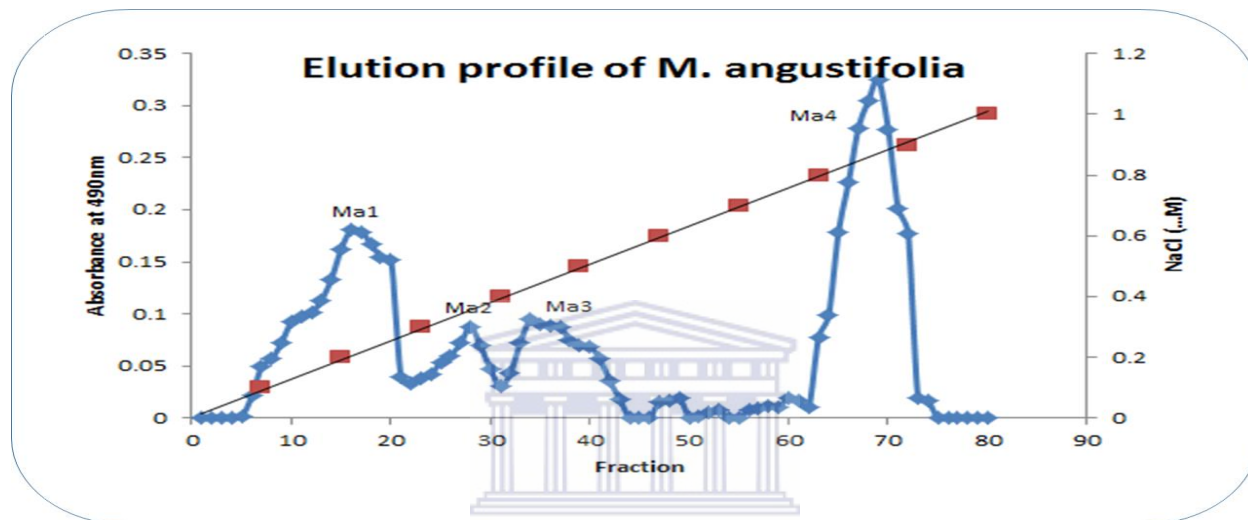
Figure 42: Elution profile of the MA1-purified fucoidan on the DEAE- Sepharose CL-6B column. The straight line shows NaCl concentration

Table 41: Composition and yield of purified fucoidan fractions

| Fraction | % Yield | Neutral monosaccharides (%) | | | | | | | Sulfate (SO_4^{2-}) (%) |
		Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	
F1	41.2	4.9	29.4	3,5	14.5	20.8	13.0	17.4	8.32
F2	26.8	1.7	5.5	4.1	40.8	52.1	11.6	nd	28.43
F3	7.6	3.8	25.2	2.5	54.3	40.1	10.9	nd	37.0
F4	22.5	4.4	28.4	1.9	73.4	2.3	3.4	2.2	54.0

nd- not determined

F1 which made an earlier through elution out of DEAE-Sepharose contained 8.32 % of ester sulfate. Thus, it appeared to be a fucoidan fraction tightly-bonded to the residue. All fractions contained fucose as a major component sugar followed by xylose, then mannose, glucose, galactose, rhamnose and arabinose as minors, but the yields of these sugar components varied from one fraction to another. On the other hand, the fraction which was eluted with higher concentration of NaCl had a higher sulfate. These results indicated that crude fucoidan extracted from this alga was heterogeneous with respect to not only molecular weight, sulfate and uronic acid contents, but also to sugar constituents.

5.4.8 Molecular weight determination

Following a selection of purified fraction 1 from *M. angustifolia* (3 mg), it was fractionated into different ranges of molecular weights by gel permeation chromatography, using a similar Sephacryl S-400 HR column as described in section 2.7.9. 1M NaCl was used as the eluent, at a flow rate of 0.5 ml/min. collecting 2 ml volumes of the purified polysaccharide. A profile of molecular weight determination was plotted as shown in Figure 43. The gel permeation chromatographic column was calibrated using five dextran standards of known average molecular weights as done previously in section 3.3.7. Collected fractions were analyzed for p-glucan content by means of the Phenol-sulfuric acid.
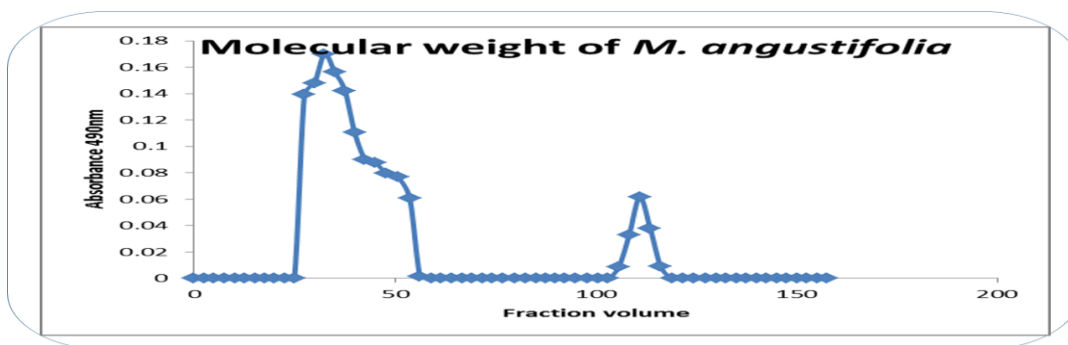
Figure 43: An elution profile of fraction 1 from Sephacryl S-400 column

Average molecular weight of sugars from the alga were between 25×10^3 Da and 250×10^4 Da with reference to the calibration curve ($y = -0.022x + 6.3424$, $R^2 = 0.94$) made from Dextran standards of known molecular weight. Such a range of molecular weights is a representation of effectiveness of this seaweed to various biological activities. Some studies have speculated that biological activities are related to the sulfate groups attached to those sugars (Nishino, *et al*., 1989; Li, *et al*., 2008).

5.4.9 Ion chromatographic results

Qualitative sulfate ions presence was determined by ion chromatography technique (Miskaki, *et al*., 2007). Subsequent to carrying out the anion-exchange purification procedure through DEAE-Sepharose column, fractions ions composition was determined. Furthermore, the purified oligosaccharides after hydrolysis were extracted with water to remove ions from their surfaces in order to obtain an aqueous solution for analysis. The results were recorded as shown in Table 42
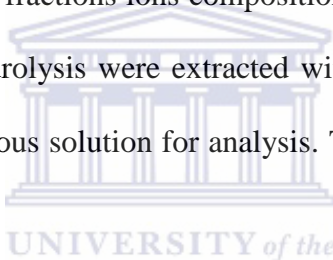
Table 42: Minimum concentrations of F^-, Cl^-, NO^-_2, Br^-, NO^-_3, PO_4^{-3}, and SO_4^{-2} at 1000 ppm

Fraction	Fluoride %	Chloride %	Nitrite %	Bromide %	Nitrate %	Sulfate%
Ma/1	1.11	51.39	39.18	-	-	8.32
SR/2	3.53	3.98	64.06	-	-	28.43
SR/3	nd	nd	nd	-	-	nd
SR/4	-	3.69	4.41	-	-	91.90

nd = not determined

Table 42 shows the average percentages of the anions found in the fractions of the alga, based on 4 replicate injections. Fraction 3 was not enough for the analysis hence its ions composition were not determined. The results showed high percentage of sulfate particularly in fraction 4.

Primarily, the application of IC demonstrates electrolytic eluent generation providing a convenient and reliable method for the simultaneous determination of sulfate and other anionic ingredients in purified fractions.

5.4.10 Infrared spectrum of *M. angustifolia*

On the basis of FT-IR analyses, crude fucoidan spectrum of various species are usually similar but some differences in minor peak patterns get observed (Virginia, *et al.*, 2012; Kong, J. and Yu, S. 2007).

Figure 44: IR spectrum of *M.angustifolia* indicating characteristic bands

Common to all polysaccharides are bands in the region 4000-3200 cm^{-1}; Figure 44 had a broadband at around 3303 cm^{-1} which was assigned to the OH stretching vibrations. The band in the region of 2916 cm^{-1} was characteristic of C-H anti-symmetrical stretching vibrations. It is noteworthy that an absorption band at around 1602 cm^{-1} assigned to C=O stretching vibration represented the ester carbonyl groups and/or carboxylate (COO-) stretching band of uronic acid (Leal, *et al.*, 2008; Gnanasambanda and Proctor, 2000), indicating esterification and free carboxyl groups present in the polysaccharide. This was revealing the presence of some *O*-acetyl groups in crude fucoidan, similar to that found in fucoidan from *Fucus distichus* (Bilan, *et al.*

2004). This is a remarkable finding since previous isolation and formulation of such an acetyl fucoidan from other brown alga have wide uses due to their numerous biological activities (Tako, 2002). Furthermore, a recent study by Teruya *et al* (2009) suggested that the acetyl-fucoidan could be used as an efficient immune stimulant.

The same peak at 1602 cm^{-1} was coherent with the presence of uronic acids (Tako, *et al.*2002). The band at 1419 cm^{-1} was also observed and was assigned to scissoring vibration of CH$_2$. The bands around this region vary in their position and intensities reflecting differences in composition and substitution of monomeric units that can be explained by the change in Gal/Fuc ratio (Kim *et al*., 2010). An absorption band at 1241 cm^{-1} associated to S=O stretching vibration of the sulfate group was also observed and was correlated with the amount of sulfate evidenced by the chemical analyses. An additional sulfate absorption band at 822 cm^{-1} (C-*O*-S, secondary axial sulfate) indicated that the majority of sulfate groups are located at C-4 and to a lesser extent at C-2 and/or C-3 of fucopyranose residues (Chizhov, *et al*. 1999).

This configuration is in agreement with the previous model reported by Patankar *et al* (1993) for the only commercially available fucoidan extracted from *Fucus vesiculosus.* As mentioned before, the position of sulfate groups is related to the biological activities of sulfated polysaccharides. Thus, sulfate located at C-4 of fucopyranosyl units seems to be very important for fucoidan antiviral activity (Mandal, *et al.,* 2007), whereas Silva *et al*. (2005) reported that sulfation at C-3 in fucoidan is responsible for the anticoagulant activity. Furthermore, studies using other techniques such as NMR spectroscopy are needed to confirm on such preliminary results.

5.4.11 NMR spectroscopy

NMR spectroscopy is one convenient method to obtain valuable structural information about polysaccharides. Invariably, like many other native algal fucoidans, *M. angustifolia* sample had a complex ^{1}H NMR spectrum, which was difficult to interpret completely (Figure 45).
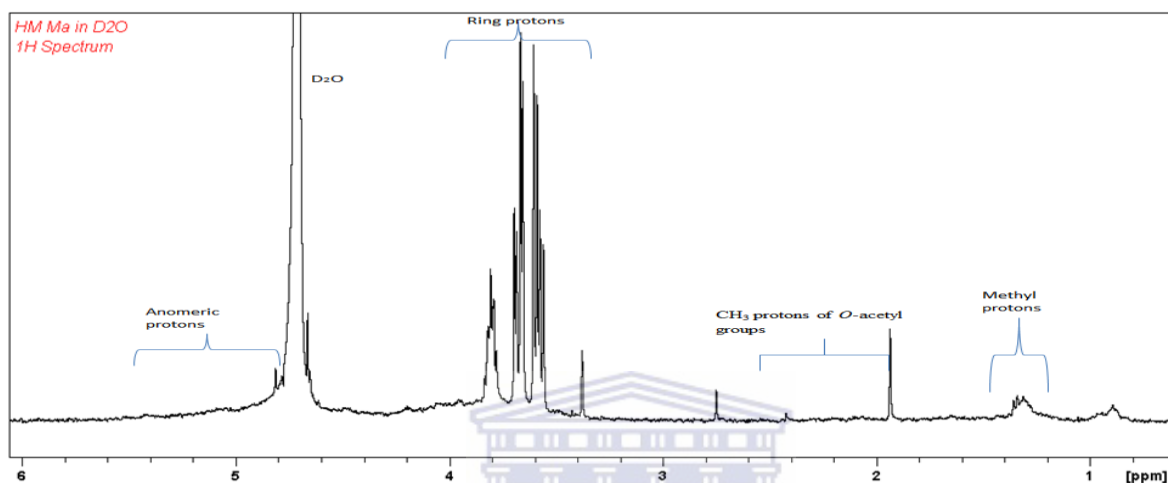
Figure 45: ^{1}H NMR spectrum of the fucoidan isolated from *M. angustifolia*

The ^{1}H NMR spectrum of the sample showed a band at around 1.4 ppm due to 6-deoxy-sugar methyl protons, corresponding to the main neutral sugar in alga, fucose. The intensity of the peaks in the spectrum was proportional to the number of protons in the sample. Apparently, there was a mixture of *α*-and *β*-glycosidic linkages in the polysaccharide, as inferred from the downfield intensity bands at about 4.8 and 5.4 ppm distinguishable in the spectrum of the pure sulfated fucan due to anomeric protons *α*-and *β*-glycosidic configuration, respectively. Furthermore, it included resonances characteristic of sulfated α-fucans, such as intense signals from ring protons H-2 to H-5 between 3.4 and 3.9 ppm, and signals from the methyl protons H-6 at around 1.2-1.4 ppm. The residues with H-6 signals at around 1.4 ppm may be 3-linked (Kariya, *et al*., 2004). The signals appearing around 3.8 ppm are attributed to the H-4 of 4-*O*-sulfated residues (Bilan, *et al*., 2004; Kariya, *et al*., 2004; Pereira, *et al*., 1999). Therefore, it is

apparent that the NMR spectrum of this polysaccharide was complex, as observed for sulfated fucans from other marine brown algae (Kariya, *et al.,* 2004; Mourao, *et al.,* 1996; Patankar, *et al.*, 1993; Pereira, *et al.*, 1999; Vilela-Silva, *et al.*, 2002).

5.4.12 Methylation analysis of *M. angustifolia*

Identity of most polysaccharides cannot be ascertained without determining the glycosyl linkage composition. In this research, the glycosyl linkage composition was determined by methylation analysis. At times, polysaccharides have a difficulty to dissolve in dimethyl sulfoxide. Therefore, this results to undermethylation that is very common in large polymers. Methylation of a polysaccharide at first may result to some of the sample not dissolving well in the first stage of the polysaccharide permethylation. Therefore, a second stage of the process is crucial.

The positions of *O*-acetyl and *O*-methyl groups on the PMAA polysaccharide derivatives were determined by GC-MS. The electron-impact fragmentation patterns of the mass spectra of PMAA derivatives were obtained. Consequently, the retention times and the *m/z* values of the main fragments in GC-MS of glycosyl residues of the polysaccharide, sulfated/desulfated were obtained. The possible fragmentation patterns of the glycosyl residues were generated from a chromatogram shown in Fig. 46a and b. They match the *m/z* values of standards GC-MS, suggesting all the given types of glycosyl linkages in a sample.
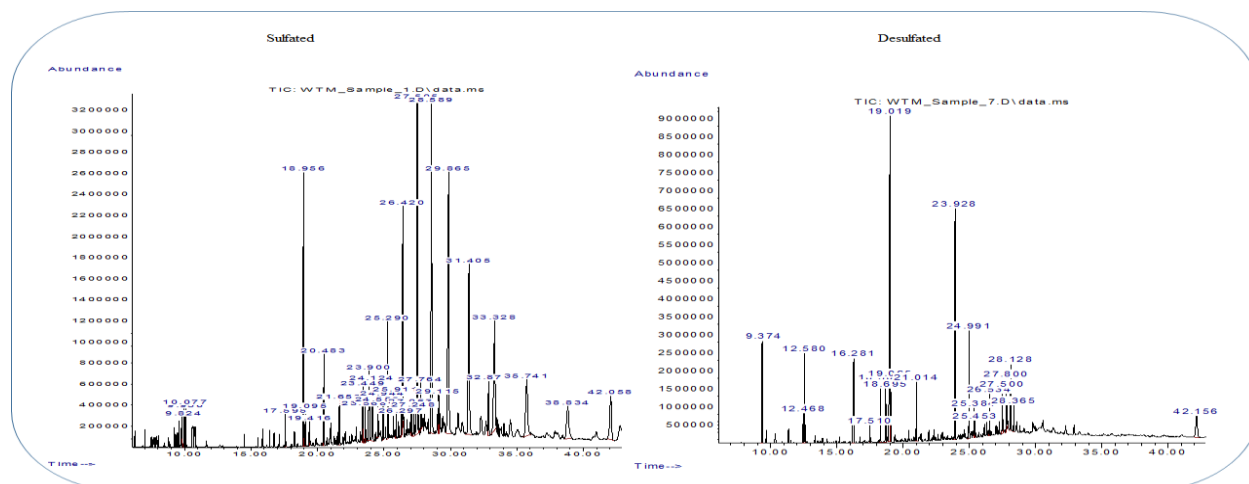
Figure 46: GC-MS analysis of PMAA of *M. angustifolia*

Table 43: Methylation linkage analysis of native and desulfated *M. angustifolia*

Glycosyl residue	Position of the *O*-methyl group	Deduced position of substitution	Sulfated (mol %)	Desulfated (mol %)
Xylosyl	2,3,4	Terminal Xyl(1→	9.0	9.0
	2,3	→4) Xyl*p*(1→	4.0	4.1
Fucosyl	2,3,4	TerminalFuc*p*(1→	10.1	10.0
	2,3	→4) Fuc*p*(1→	11.2	11.9
	2,4	→3) Fuc*p*(1→	10.4	18.3
	3,4	→2) Fuc*p*(1→	2	1.9
	2	→3, 4) Fuc*p*(1→	19.2	3.5
	3	→2, 4) Fuc*p*(1→	6.3	6.5
Galactosyl	2,4	→3, 6) Gal*p*(1→	4.4	4.9
	2, 3	→4, 6) Gal*p*(1→	4.2	5.0

The results of the methylation linkage analysis of sulfated and desulfated *M. angustifolia* are shown in Table 43. The methylated derivatives obtained suggested the presence of a central

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core composed of 3- or 4-linked glycosidic bonds with minor amounts of 3- or 4-linked galactose and xylose units. Furthermore, more than 50% of 3-linked glycosyl units are branched at C-2. The branches of galactoses may be at C-2, C-3 or C-4 on di-substituted galactose. Furthermore, the fucose chains are made up of 3- and 4-linked fucose and in addition, minor amounts of 4-linked fucose are proposed to be branched at C-2 with chains of xylose and/or fucose.

Desulfation eliminated significant amounts of the sulfate groups in the sample. The 3, 4-disubstituted fucosyl residues almost disappeared in the desulfated polysaccharide, suggesting that most were sulfated at C-3, in agreement with IR spectrum results. The high content of non-reducing fucose and minor xylopyranose terminal residues indicated that *M. angustifolia* is a highly branched polymer. Therefore, the glycosyl-composition and linkage composition analyses were sufficient to propose the identity of the polysaccharide. However, they were not enough to determine the complete primary structure of the polysaccharide. This can be accomplished by application of other techniques (Lindberg and Lönngren, 1978).

5.5 Conclusion

From the organic extracts of *Macrocystis angustifolia,* a pure compound, 24-ethylidene cholesterol (**26**) was isolated, a result which is reported for the first time for this alga. The same compound has been reported from other two seaweeds in this report. Other compounds could not be characterized due to insufficient experimental data.

Subsequently, fucoidan was isolated from the sporophylls/blades of this a seaweed and further examined by separation using anion chromatography and spectroscopic (FT-IR, NMR) methods. This polysaccharide is a sulfated fucan containing fucose (77.1%) as a predominant sugar in addition to others which occurred at varying percentages. Uronic acid level was very low.

High percentage of sulfate content was reported from various fractions. Gel permeation chromatography was employed which gave an estimate of the average molecular weight of the fucoidan. Application of nuclear magnetic resonance (NMR) spectroscopy was useful in order to provide structural information by identifying the residues present and polysaccharide linkage. The fucoidan contained significant amounts of *O*-acetyl groups as suggested by IR data.

The methylation analysis results suggested the presence of a central core composed of fucosyl 3- or 4-linked glycosidic bonds with minor amounts of 3- or 4-linked galactose and xylose units. Additional research is required to realize a complete structural analysis of this polysaccharide.

CHAPTER 6

6.0 *Aeodes orbitosa*

6.1 Introduction

The red algae, or Rhodophyta, are one of the oldest groups of eukaryotic algae, and also one of the largest, with about 5,000-6,000 species of mostly multicellular, marine algae, including many notable seaweeds (Lee, 2008; Thomas, 2002). They form a distinct group characterized by attributes like eukaryotic cells without flagella and centrioles, using floridean polysaccharides as food reserves, with phycobiliproteins as accessory pigments giving them their red color, and with chloroplasts containing unstacked thylakoids (Woelkerling, 1990). Primarily, most red algae are also multicellular, macroscopic, marine, and have sexual reproduction. Furthermore, red algae such as *Palmaria palmate* is a traditional part of European and Asian cuisines and is used to make other products such as agar, carrageenan and other food additives (Guiry, 2007).

In the system of Adl, *et al*. 2005, the algae are classified in the Archaeplastida where the name Rhodophyceae is used for the red algae. From the morphology, red algae have double cell walls. The outer layers are usually composed of "pectic substances", from which agar can be manufactured. The internal walls are mostly cellulose and with their reproduction, they display alternation of generation (Kohlmeyer, 1975). Several species are used as food, for example *Palmaria palmate* and *Porphyra* are the best known in the British Isles. In the East and South-east Asia, agar is most commonly produced from *Gelidium amansii*. Rhodophytes are important sources of food, such as *nori*. The high vitamin and protein content of this food makes it attractive, as does the relative simplicity of cultivation, which began in Japan more than 300 years ago.

The red algae are seaweeds that are distinguished from others due to the unique red and blue pigments; phycocyanin and phycoerythrin, in addition to green chlorophyll a. The former can absorb blue-green light in deep water, passing the energy to chlorophyll for food production by photosynthesis, thus, an advantage over other seaweeds (Branch and Branch, 1981). Rhodophyceae can live in high shore and at great depths where deepwater algae have more red pigments, while those in the intertidal may be reddish brown, yellowish or black. As a result many red algae are confused with brown algae (Phylum *Phaeophyta*) that are yellow-brown in colour (Branch and Branch, 1981).

Primarily, the chemistry of marine organisms is fascinating and many compounds have been isolated from them. Many of the isolated compounds have been found to be bioactive inhibiting the growth of bacteria, fungi, viruses, etc. Red algae are very palatable and provide the staple diet for many of the marine herbivores, such as molluscs, crustaceans and fish. The algae are also a source of agar, a gelling substance, of economic importance in confectionery and as a growth medium for bacteriological studies. Rhodophyceae are known to metabolize a starch known as floridean as their food reserve material (Nagashima, *et al*., 1969). There are many different species of red algae with a diverse range of plant forms and a variety of complex life cycles (Stegenga, *et al*., 1994). Examples of common red seaweeds of high shores to intertidal zones are; *Porphyra capensis*, a fast-growing, membranous seaweed that looks like crumpled black plastic when dried, slippery orbits, *Aeodes orbitosa*, a slippery, yellowish-brown with tough blades, *Iridea capensis*, spotted iridea, with brown strap-shaped blades and dark spots, *Notogenia stiriata*, hedgehog seaweed, a very dark blackish-brown that occurs on sheltered rocks. Red seaweeds have been known to produce carrageenans which are sulfated cell-wall polysaccharides and they are distributed among several families (Witvrouvw, *et al*., 1997).

Because of their available biomass in nature, and their high yield of phycocolloids of commercial importance, species belonging to the order Gigartinales have been the most studied (Craigie 1990).

6.2 Polysaccharides in red algae

Most red algae contain cellulose as the main structural polysaccharide (Park, *et al*., 2000), although species of *Rhodeminea* contain β-1, 3 and β-1, 4 xylans for this purpose (Park, *et al*., 1997a). Primarily, the mucilage polysaccharide of red algae is 1→3-linked and 1→4-linked sulfated galactan. The major polysaccharides are agar, carrageenan and porphyran (Park, *et al*., 2000). Agar is a mucilage polysaccharide used widely for commercial purposes as a gelling agent in foods and in media for culturing microbial organisms (Fuse and Goto, 1971). It is composed of agarose which is approximately 70% and agaropectin about 30%. The former is made of a 1→4-linked 3, 6-anhydro-L-galactose and 1→3-linked D-galactose (about 56%). The latter is essentially agarose with sulfate ester and D-glucuronic acid side groups (Park, *et al*., 1997a; Park, *et al*., 2000). Properties of agar as gel forming or gel strength depends on the ratio of agarose to agaropectin, the sulfate content, harvesting region and season and the extraction method (Fuse and Goto, 1971; Park, *et al*., 1997a; Park, *et al*., 2000).

Carrageenan is another sulfate-containing polysaccharide abundant in red algae (Park, *et al*., 1997a; Park, *et al*., 2000). It consists of D-galactose and 3, 6-anhydro-D-galactose sulfate esters. However, the gelling ability of carrageenan is lower, while its viscosity is higher than that of agar (Park, *et al*., 1997a). According to Campo, *et al*., (2009), carrageenan are widely used in the food industry because of their physical properties such as thickening, gelling and stabilizing abilities. These properties are useful to control the texture and viscosity of dairy products, and are utilized for binders and stabilizers in the meat-processing industry such as sausages and low-

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fat hamburgers.

The taxonomy of the alga under study is; Kingdom: *Plantae*; Phylum: *Rhodophyta*; Class *Florideophyceae*; Order: *Halymeniales*; Family: *Halymeniaceae;* Genus: *Aeodes;* Species: *Aeodes orbitosa.* It grows prolifically around and along south-west coast of Cape Province, from Kommetjie Cape Point in Cape Town, South Africa within the Benguela currents, and also to the south of Luderitz, but north of Ichaboe Island along the Namibian coastline (Molloy, 1990). The intertidal seaweed communities within this overlap region of Cape point tend to show affinities with the Benguela rather than the Agulhas region (Leliaert, *et al*., 2000). This is with the exception that those in the warmer regions of the partially enclosed False Bay are similar to geographically distant sites within warmer sea water temperature regimes (Bolton and Anderson 1990). It fluorishes in the intertidal zone that largely overlaps the 'Porphyra zone'. It grows better in sheltered habitats where it can attain a frond surface area of over $1m^2$. Under exposed conditions, the thalli are small and stunted. A change in floristic composition of sub-tidal algal communities around the Cape Peninsula is principally related to sea water temperature and wave exposure. These are carrageenophytes whose commercial interests would lead industries that easily could convert them into production of carrageenan.

Commonly known as 'slippery orbits', it has a tough, flat, slippery, leaf-like structure. Color varies from yellow-brown to reddish-brown (Plate 5). It occurs on rocky shores from the cold seas. However, although it is widespread along most temperate oceanic coastlines, the recorded distribution of individual species tends to be more limited, and generally considered to be confined to clearly demarcated geographical regions (http://sacoast.wcape.gov. za).
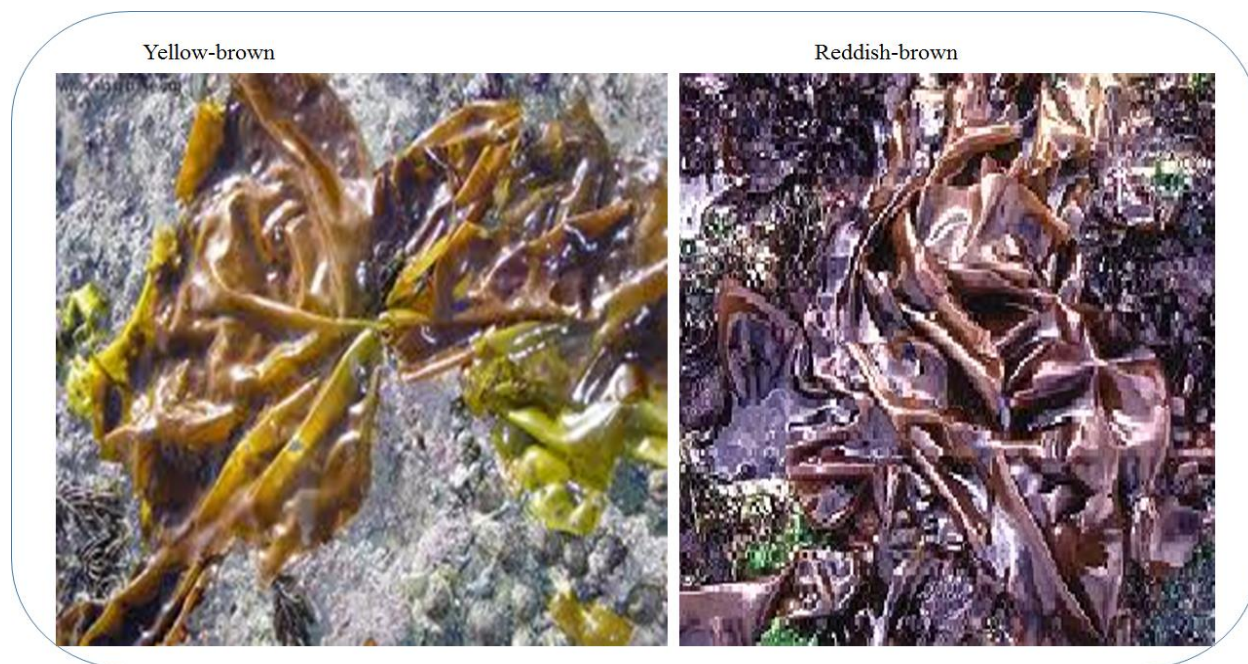
Plate 5: *A. orbitosa* **(Source: www.algaebase.org)**

6.3 Algal material

Aeodes orbitosa was collected on 22nd February 2012 at Kommetjie, south of Cape Town near the Cape of Good Hope, South Africa. The algal material was washed with running tap-water to remove sea water salts. The seaweed material was kept in a freezer until use.

6.3.1 Treatment of seaweed

Frozen seaweed was thawed and washed under running tap water. Visible debris was carefully removed with slight agitation, while the blades and any degraded areas of the seaweed were removed by hand. These blades were milled with a food blender to obtain a fine paste. The milled seaweed paste was transferred into metallic plates, lyophilized in a freeze-drier for several days. The dried seaweed sample weighed and kept at $4\ ^0C$ until use. Extraction, isolation and further experiments of the sporophyte were performed as outlined in some procedures of section 2.1.2.2.

6.4 Results and discussion

6.4.1 Physico-chemical data of isolated compound from *A. orbitosa* organic extract

6.4.1.1: *E*-3, 7, 11, 15-Tetramethylhexadec-2-en-1-ol (28)

Colourless oil; EI-MS, *m/z* 296.0322 [M+H]$^+$ (calcd for $C_{20}H_{40}O$, 296.0321) ^{1}H-NMR (600 MHz, CDCl$_3$): same data as that outlined in section 4.3.1.2.

6.4.1.2: 17-(5-Ethyl-6-methylheptan-2-yl)-10, 13-dimethyl-2, 3, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol (29)

White needles; ES-MS data; *m/z* 414.3584 [M +H]$^+$ (calcd for $C_{29}H_{50}O_1$, 414.3216); ^{1}H NMR (600 MHz, CDCl$_3$) δ 1.11 (1H, *m*, H-1*ax*); δ 1.88 (1H, *m*, H-1*eq*); δ 1.55 (1H, *m*, H-2*ax*); δ 1.88 (1H, *m*, H-2*eq*); δ 3.54 (1H, *m*, H-3); δ 2.05 (1H, *m*, H-4*ax*); δ 2.29 (1H, *m*, H-4*eq*); δ 5.34 (1H, *m*, H-6); δ 1.81 (1H, *m*, H-7*ax*); δ 2.02 (1H, *m*, H-7*eq*); δ 1.49 (1H, *m*, H-8); δ 0.96 (1H, *m*, H-9); δ 0.93 (1H, *m*, H-11*ax*); δ 1.43 (1H, *m*, H-11*eq*); δ 1.08 (1H, *m*, H-12*ax*); δ 1.18 (1H, *m*, H-12*eq*); δ 1.12 (1H, *m*, H-14); δ 1.37 (1H, *m*, H-15*ax*); δ 1.61 (1H, *m*, H-15*eq*); δ 1.80 (1H, *m*, H-16*ax*); δ 1.87 (1H, *m*, H-16*eq*); δ 1.02 (1H, *m*, H-17); δ 0.67 (3H, *s*,H-18); δ 1.04 (3H, *s*, H-19); δ 1.79 (1H, *m*, H-20); δ 0.96 (3H, *m,* H-21); δ 1.10 (2H, *m*, H-22); δ 1.87 (2H, *m*, H-23); δ 1.54 (1H, *m*, H-24); δ 2.02 (1H, *m*, H-25); δ 0.90 (3H,*m,* H-26); δ 0.85 (3H,*m,* H-27); δ 1.18 (1H, *m*, H-28*ax*); δ 1.37 (1H, *m*, H-28*eq*); δ 0.71 (3H, *s*, H-29). ^{13}C NMR (150 MHz, CDCl$_3$); δ 37.3 (C-1); δ 31.7 (C-2); δ 71.8 (C-3); δ 42.3 (C-4); δ 140.5 (C-5); δ 121.6 (C-6); δ 31.9 (C-7); δ 31.9 (C-8); δ 50.1 (C-9); δ 36.5 (C-10); δ 21.1 (C-11); δ 39.5 (C-12); δ 42.3 (C-13); δ 56.2 (C-14); δ 24.3 (C-15); δ 28.3 (C-16); δ 56.8 (C-17); δ 12.0 (C-18); δ 19.4 (C-19); δ 36.4 (C-20); δ 18.8 (C-21); δ 35.2 (C-22); δ 25.8 (C-23); δ 41.3 (C-24); δ 31.9 (C-25); δ 22.8 (C-26); δ 22.6 (C-27); δ 23.8 (C-28); δ 12.0 (C-29).

6.4.2 Structural elucidation and characterization of the compound

Filtrate of methanol extract of *A. orbitosa* was suspended in distilled water, and partitioned with hexane, dichloromethane and ethyl acetate (Scheme 1). The hexane fraction (13.0 g) was subjected to a silica gel column chromatography and using 400 ml volumes of hexane: DCM (10:1-10) as eluent, yielded 10 sub-fractions (A1-A10). The fractions were subjected to TLC analysis using DCM-MeOH (9.8:0.2) ratio. A preparative TLC of fraction 3 DCM-MeOH (9.8:0.2) from the same extract yielded compound **28** (7.5 mg) that gave a positive colour test for oils with vanillin-sulfuric acid. Subsequently, fraction 4 was subjected to a repeated silica gel column chromatography using DCM-EtOAc (8:2) that lead to the isolation of compound **29** as white needles (48.0 mg) that gave a positive colour test for a terpene with vanillin-sulfuric acid.

6.4.2.1: Compound 28 (*E*-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol)

This compound was similar in structure to another one isolated from *Splachnidium rugosum* described earlier in chapter 4 section 4.3.2.3.

6.4.2.2: Compound 29 (17-(5-Ethyl-6-methylheptan-2-yl)-10, 13-dimethyl-2, 3, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17- dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol)

Repeated column chromatography (SiO$_2$, DCM-EtOAc) of a 4th fraction of the hexane extract from *Aeodes orbitosa* yielded compound **29** as a white solid that gave a positive colour test with vanillin-sulfuric acid spray.
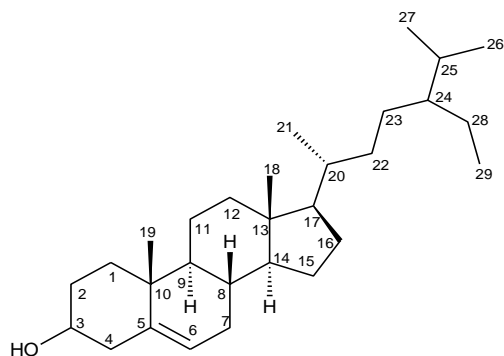
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The structure was established on the basis of 1D (^{1}H, ^{13}C and APT) and 2D-NMR experiments (HMQC, COSY and HMBC), (Table 44) plus MS data. The steroidal nature of **29** was deduced from a combination of ^{13}C, HMQC, and HMBC NMR data, that revealed 29 carbons (6 x CH$_3$, 11 x CH$_2$, 9 x CH and 3 quaternary centers). ^{1}H-NMR data revealed 33 signals between δ 0.6 and 2.3 and only two protons with high chemical shifts downfield; δ 5.34 (1H, *d*, *J*= 2.36Hz), characteristic of H-6 in sterols and δ 3.54 (1H, *m*, H-3*ax*), suggesting a 3-*β*-hydroxyl group (Goad, 1991; Morales, *et al*., 2003;De-Eknamkul and Potduang, 2003). These signals were well separated and excited selectively in one-dimensional experiments. Furthermore, the neighbours of excited protons could be identified even if the resonances appeared in a crowded chemical shift region. The signal at δ 71.8 (C-3) confirmed the presence of a secondary hydroxyl group (Morales, *et al*., 2003; Abrantes, *et al*., 2010; De-Eknamkul and Potduang, 2003; Jongaramruong and Kongkam, 2007).

COSY cross peaks between H-3*ax* (δ 3.54) and H-4*eq* (δ 2.29), H-4*ax* (δ 2.05), H-2*ax* (δ 1.55) and H-2*eq* (δ 1.87) confirmed 3*β*-hydroxyl orientation. H-19 (δ 1.04) showed long range HMBC to C-5 (δ 140.5) suggesting the olefinic bond at C-5/C-6 in ring B of the steroid. Three-bond HMBCs were also observed between H-19 and C-1 (δ 37.3) and C-9 (δ 50.1). The HMBCs observed between H-6 (δ 5.34) and C-4 (δ 42.3) as well as C-7 (δ 31.9) confirmed the position of

the double bond at C-5/C-6. The downfield chemical shift of the 3α-methine proton (δ 3.54) is consistent with the de-shielding of the axial proton double bond (Forgo and Kover, 2004).

COSY spectrum of compound **29** confirmed the presence of the four almost isolated ^{1}H spin systems, one spin system for each of the four rings A-D of the triterpene skeleton, ring A: H-1*ax* (δ 1.11) H-1*eq* (δ 1.88); H-2*ax* (δ 1.55); H-2*eq* (δ1.87); H-3 (δ 3.54); H-4*ax* (δ 2.05) *m*,); H-4*eq* (δ 2.29); Ring B: H-6 (δ 5.34); H-7*ax* (δ 1.81); H-7*eq* (δ 2.02); Ring C: H-8 (δ 1.49); H-9 (δ 0.96); H-11*ax* (δ 0.93); H-11*eq* (δ 1.43); H-12*ax* (δ1.08); H-12*eq* (δ 1.18) and Ring D: H-14 (δ 1.12); H-15*ax* (δ 1.37); H-15*eq* (δ 1.61); H-16*ax* (δ 1.80); H-16*eq* (δ 1.87);H-17 (δ 1.02). The orientation of H-7*ax* (δ 1.81) was deduced from COSY cross peaks with H-6 (δ 5.34), which in turn showed COSY interaction with H-8*eq* (δ 1.49). The magnitude of the proton-proton coupling constants in the B ring indicated that the ring orientation was influenced by the C-5/C-6 double bond. The data confirmed that H-6, H-7*eq* and H-8*eq* are on the same face of the steroid ring. Some important HMBCs (Figure 47) and COSY data are summarized in Table 44.
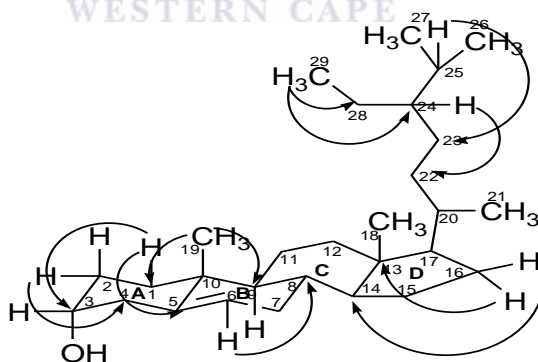
Figure 47: Important HMBCs for compound 29

Table 44: 1D and 2D ^{1}H (600MHz) and ^{13}C NMR (150 MHz), CDCl₃ data for compound 29

Position	δ ^{13}C	δ ^{1}H	COSY	HSQC	HMBC	β-sitosterol
1	37.3	ax, 1.11, eq, 1.88 (2H, m)	H-2eq	1.11, 1.88	C-2, C-3, C-5	37.3
2	31.7	ax, 1.55, eq, 1.87 (2H, m)	H-1eq, H-3ax	1.55, 1.87	C-3, C-4	31.6
3	71.8	3.54 (1H, m)	H-2ax, H-2eq, H-4ax, H-4eq	3.54	C-2, C-4	71.7
4	42.3	ax, 2.05, eq, 2.29 (2H, m)	H-3ax	1.99. 2.05	C-6, C-5, C-3	42.2
5	140.5	-		-		140.8
6	121.6	5.34 (1H, d, J= 2.36Hz)	H-7eq	5.34	C-4, C-8, C-10	121.6
7	31.9	ax, 1.81, eq, 2.02 (2H, m)	H-6	1.81, 2.02		31.9
8	31.9	1.49 (1H, m)		1.49		31.9
9	50.1	0.96 (1H, m)		0.96	C-12	50.2
10	36.5	-		-		36.5
11	21.1	ax, 0.93, eq, 1.43 (2H, m)		0.93, 1.43	C-9	21.1
12	39.5	ax, 1.08, eq, 1.18 (2H, m)		1.08, 1.18	C-13	39.8
13	42.3	-		-		42.3
14	56.2	1.12 (1H, m)		1.12		56.8
15	24.3	ax, 1.37, eq, 1.61 (2H, m)		1.37, 1.61		24.3
16	28.3	ax, 1.80, eq, 1.87 (2H, m)		1.80, 1.87	C-13, C-14	28.3
17	56.8	1.02 (1H, m)		1.02	C-20	56.1
18	12.0	0.67 (3H, s)		0.67		11.9
19	19.4	1.04 (3H, s)		1.04	C-1, C-5	19.4
20	36.4	1.79 (1H, m)		1.79	C-21	36.2
21	18.8	0.96 (3H, m)		0.96		18.8
22	35.2	1.10 (2H, m)		1.10		33.9
23	25.8	1.87 (2H, m)		1.87		26.1
24	41.3	1.54 (1H, m)		1.54	C-28	46.9
25	31.9	2.02 (1H, m)		2.02		29.2
26	22.8	0.90 (3H, m)		0.90	C-27	19.8
27	22.6	0.85 (3H, m)		0.85	C-26	19.1
28	23.8	ax, 1.18, eq, 1.37 (2H, m)	H-29	1.18, 1.37	C-29	23.1
29	12.0	0.71 (3H, s)	H-28	0.71	C-28	12.3

The ES-MS gave the molecular ion at m/z 414^+ corresponding to the formula $C_{29}H_{50}O$. There were some prominent peaks seen at m/z 396 $[M-H_2O]^+$, 329 $[M-C_7H_{11}O]^+$, 273 M-$C_{10}H_{21}]^+$ and 255 $[M-C_{10}H_{22}O]^+$ that were consistent with the proposed compound **29**. All these data confirmed that the elucidated compound was in consistence with literature (Morales, *et al*., 2003; De-Eknamkul and Potduang, 2003) values of *β*-sitosterol. However, this is the first report on the isolation of compound **29** from *A. orbitosa* namely; **(17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-decahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol**.

6.4.3 Chemical composition of the crude of polysaccharide

A yield of 49.2 % of the freeze-dried material of the alga was obtained and the chemical compositions of the crude material for preliminary analysis were reported in Table 45.

Table 45: % composition of the crude polysaccharide

	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
A. orbitosa	5.6	-	4.9	1.1	33.5	47.1	7.8

6.4.4 Sulfate and uronic acid determination

The standard absorbance calibration curves for colorimetric determination of sulfate content using potassium sulfate and uronic acid analysis were obtained as shown in Figure 23 section 3.3.3.2. The results in Table 46 represent a summary of the percentage contents of the sulfate and uronic acid present from this alga.

Table 46: Percentage sulfate and uronic acid contents of *A. orbitosa*

Seaweed	Yield (g)	Absorbance	% sulfate	Absorbance	% uronic acid
A. orbitosa	2.82	0.0615	7.885	0.0798	8.60

0.0615/0.0039 = 15.77 µg

(15.77/200) x100 = 7.885% sulfate

Invariably, curve b represented in section 3.3.3.2 was used for calculating the uronic acid content of the polysaccharide sample.

0.0798= 0.008x + 0.011   x= 8.60

The presence of glucuronic acid has been reported in literature on red seaweeds (Mandal, *et al*., 2008).

6.4.5 Separation of PMP-hydrolysed fucoidan sugars of *A. orbitosa*

Analysis of hydrolysed *A. orbitosa* PMP-sugars by HPLC in Figure 48 shows the elution pattern of PMP derivatives from small sugars. The labeled sugar peaks were all sharp and baseline resolved. The monosaccharides detected from the alga were mannose, rhamnose, galactose, glucose and xylose respectively. Galactose had a prominent peak, a characteristic of red seaweed.
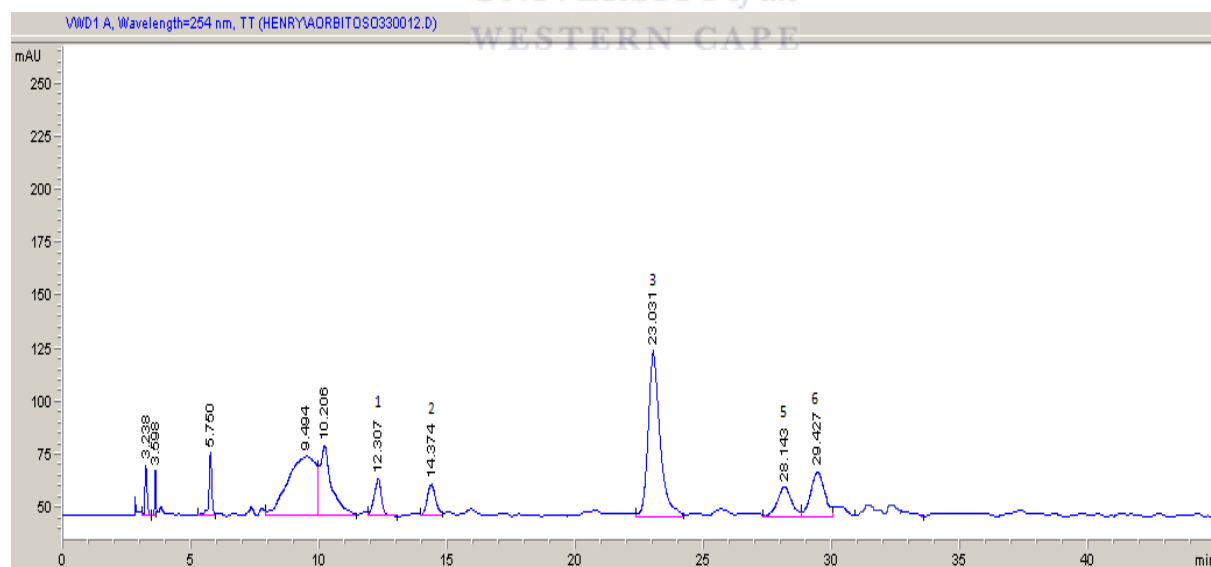
Figure 48: Monosaccharide composition analysis by HPLC of PMP-derivatized *A. orbitosa*

6.4.6 Fractionation of polysaccharide from *A. orbitosa*

From the anion-exchange chromatography elution profile of *A. orbitosa* Figure 49, absorbance of the sampled individual test tubes were plotted against the fraction number. Six fractions were obtained: Ao1, Ao2, Ao3, Ao4, Ao5 and Ao6 using a stepwise gradient from water then 0.1M-1M NaCl on a Sepharose CL-6B column, respectively. 1M NaCl concentration was allowed to elute until no positive sugar test was detected. The chromatogram showed fractions eluting best from 0.2M. The major fraction (Ao1) amounted to 41% of the total polymers recovered from the column and contained mostly galactose and mannose residues (Table 47). Galactose accounted for 65.1% of the neutral sugars of fraction Ao1 which also contained 19.95% (w/w) of sulfate groups. The tubes with positive phenol-sulfuric acid reaction were pooled together, dialyzed and lyophilized.
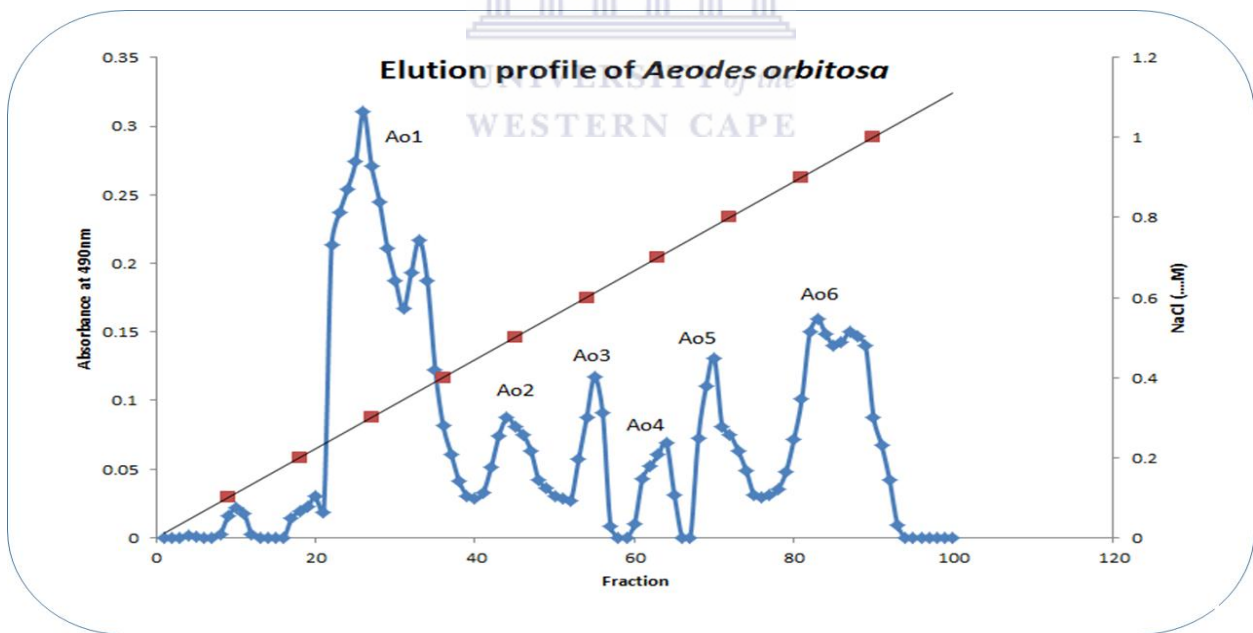
Figure 49: Elution profiles of HM/AO on DEAE-sepharose CL-6B

Table 47: Composition and yield of purified *A. orbitosa* polysaccharide fractions

| Fraction | % Yield | Neutral monosaccharides (%) | | | | | | | Sulfate (SO$_4^{2-}$) (%) |
		Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	
F1	41.0	1.6	1.0	0.9	3.6	26.0	65.1	1.7	19.95
F2	26.8	1.4	0.7	0.5	8.4	35.3	53.5	nd	13.95
F3	17.6	3.8	nd	2.5	10.9	40.1	54.3	nd	11.37
F4	22.5	2.0	nd	0.3	4.2	30.0	53.3	9.3	2.23
F5	17,3	0.9	0.4	0.4	1.0	13.0	82.6	1.7	37.08
F6	12.0	1.9	1.1	1.0	2.0	42.1	50.9	1.0	33.91

nd**-** not determined

All fractions contained galactose as a component sugar with the highest percentage along with mannose (Table 47) but rhamnose, glucose, xylose, and arabinose as minor units. Nevertheless, the proportions of these sugar components varied from one fraction to another. On the other hand, the fractions contained very little or no fucose at all. These types of galactopyrans are usually readily soluble in aqueous solvents (Mandal, *et al*., 2008).

6.4.7 Molecular weight determination

Following a selection of purified fraction 1 from *A. orbitosa* (2 mg), the amount was fractionated into different ranges of molecular weights by gel permeation chromatography, using a similar Sephacryl S-400 HR column as described in section 2.7.9. 1M NaCl was used as the eluent, at a flow rate of 0.5 ml/min. Subsequently, an elution profile of molecular weight determination was plotted as shown by Figure 50.
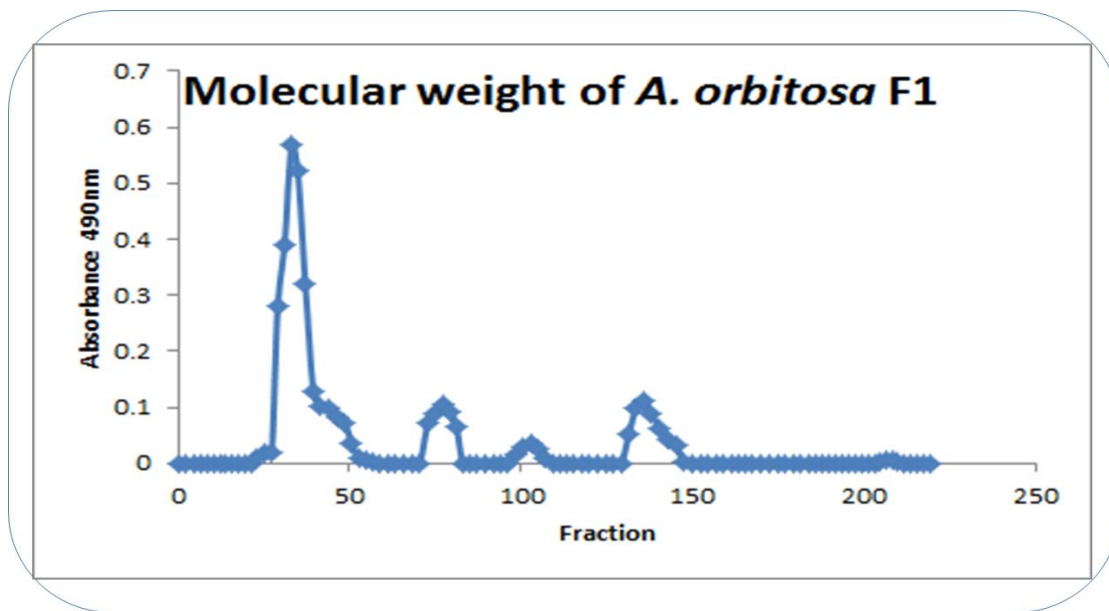
Figure 50: An elution profile of fraction 1 from Sephacryl S-400 column

Dextrans of known molecular weight were used as markers and calibrated as outlined previously in section 3.3.7. On the basis of this, the apparent average molecular weight of sugars were between 10×10^3 Da and 500×10^4 Da by reference to the calibration curve ($y = -0.022x + 6.3424$, $R^2 = 0.94$). Such a range is a representation of effectiveness of this sulfated red seaweed to various biological activities (Pomin, 2009). Sulfate groups attached influenced this activities.

6.4.8 Ion chromatographic results

Sulfate ions presence was determined qualitatively by ion chromatography technique (Miskaki, *et al*., 2007). Subsequent to carrying out the anion-exchange purification procedure through DEAE-Sepharose column, fractions ions composition was determined. The purified hydrolyzed oligosaccharides were extracted with water to remove ions from their surfaces in order to obtain an aqueous solution for analysis. The results were recorded as shown in Table 48

Table 48: Minimum concentrations of *F⁻, Cl⁻, NO⁻₂, Br⁻, NO⁻₃, PO₄⁻³, and SO₄⁻²* at 1000 ppm

Fraction	Fluoride%	Chloride%	Nitrite%	Bromide%	Nitrate%	Sulfate%
Ao1	0.29	1.92	77.84	-	-	19.95
Ao2	-	17.70	68.34	-	-	13.95
Ao3	-	0.94	87.69	-	-	11.37
Ao4	-	-	97.77	-	-	2.23
Ao5	1.78	24.16	36.98	-	-	37.08
Ao6	-	35.01	31.08	-	-	33.91

Table 48 shows the average percentages of the anions found in the fractions of the alga, based on 4 replicate injections. However, the amount of nitrite was high in all fractions compared to the rest, an indicator of protein materials in the sample. The results showed high percentage of sulfate particularly in fraction 5 and 6 where elution was by using the highest NaCl concentration. Primarily, the application of IC was to demonstrate electrolytic eluent generation providing a convenient and reliable method for the simultaneous determination of sulfate and other anionic ingredients in purified fractions.

6.4.9 Infrared spectrum of *A. orbitosa*

The FT-IR spectrum of crude algal powder (Fig. 51), the sample collected showed characteristic absorption bands at 1265 cm⁻¹ and 1228 cm⁻¹. These were assigned to the asymmetric stretching vibration of sulfate group and a small hump signal at 817 cm⁻¹ was indicative of a sulfate group attached to a primary hydroxyl group. These data suggested that the sulfate functional groups occur at C-6 of the galactose residues (Brasch, *et al*., 1983).
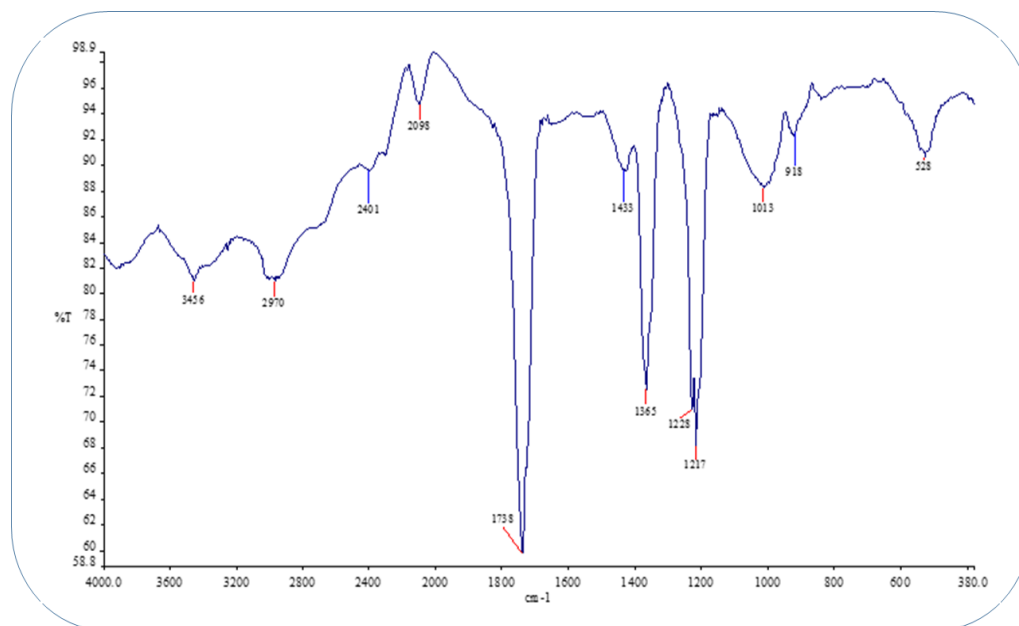
Figure 51: IR spectrum of *A. orbitosa* showing characteristic bands

The portion of the spectrum around 1010 cm^{-1} was not clearly resolved. . Furthermore, that absorption band at around 1738 cm^{-1} would be assigned to C=O stretching vibration representing the ester carbonyl groups and carboxylate (COO-) stretching band, indicating esterification and free carboxyl groups of uronic acid present in the polysaccharide.

6.4.10 NMR spectroscopy

The ^{1}H NMR spectroscopy as a convenient method for valuable structural information about polysaccharides was applied. The alga was analyzed by proton NMR to examine the anomeric configuration and sulfation pattern. Presence of a number of broad signals in the anomeric region of the proton NMR spectra suggested that their structures were very complex. The polysaccharide showed two anomeric resonances, one at around 5.20 ppm and the other at 4.80 ppm (Figure 52). The anomeric configuration of galactose and mannose residues of a structurally related sulfated galactopyrans isolated from other red algae have been reported to be *α*- and *β*-, respectively (Mandal, *et al*., 2009; Matulewicz and, Cerezo, 1987). Therefore, signals

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at around these chemical shifts were tentatively assigned to anomeric protons of α-(1→3)-linked galactopyranosyl and β-linked terminal mannose and/or xylose residues, respectively. The relative proportion of galactose residues as estimated from the integral of these anomeric signals also fits well with the results obtained from sugar compositional analyses. There was also the inclusion of such signals from ring protons H-2 to H-6 between 3.40 and 4.30 ppm. Therefore, NMR analyses confirmed the results of the methylation analyses and indicated the presence of α-linked galactopyranosyl and β-linked terminal xylopyranosyl residues.
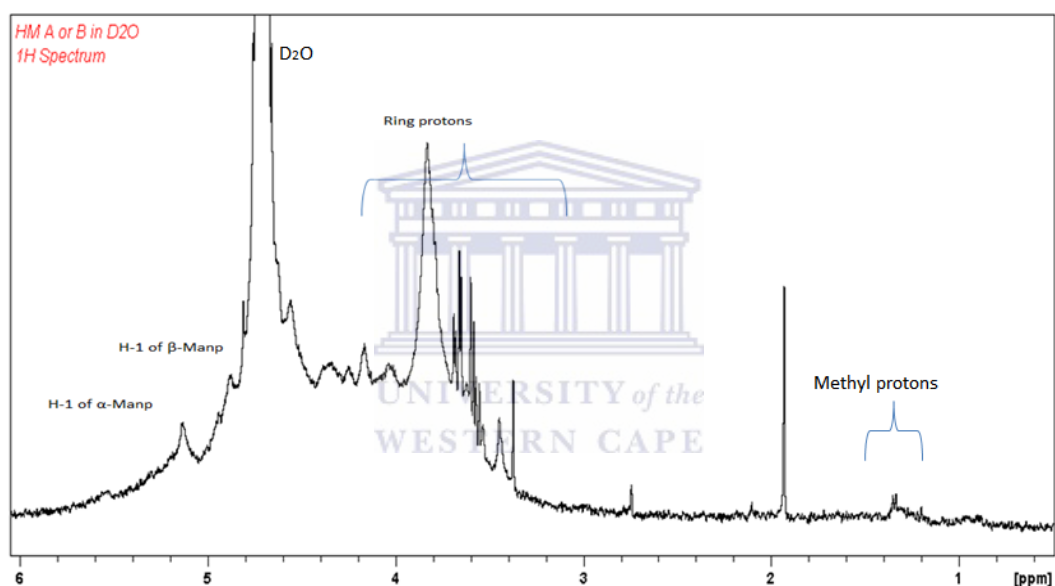
Figure 52: ^{1}H NMR spectrum at 400 MHz of the sulfated polysaccharide of *A. orbitosa*

6.4.11 Methylation analysis of *A. orbitosa*

To study the structural features of the polysaccharide isolated from *Aeodes orbitosa* (Ao), methods of sugar composition analysis, methylation and IR were used to characterize them. In order to determine glycosyl linkage positions and identify sulfate group location on this alga, methylation was performed before and after desulfation. The results on methylation of native and desulfated polysaccharide were in agreement with the composition analysis by gas

chromatography. Primarily, the major contents for 3-linked galactose units originate from D-galactose plus 6-*O*-methyl D-galactose and major content of 4-linked are related to L-anhydrogalactose and L-galactose-6-sulfate. There were significant changes in the profiles of Ao_{sul} and Ao_{des} contents before and after desulfation.

The linkage analyses of the sulfated alga are shown in Figure 53a and b. To determine the positions of sulfate esters, analysis of their desulfated polysaccharide were performed and the results are shown in Figure 53b and summarized in Table 49.
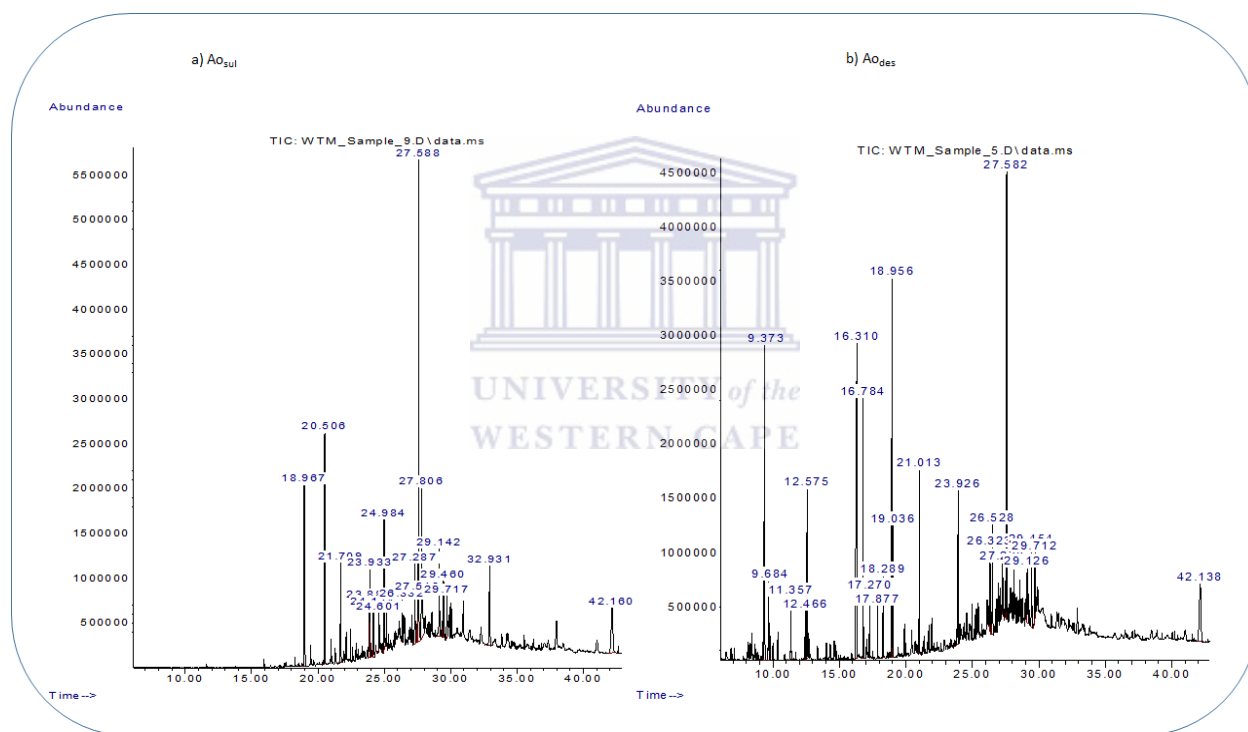
Figure 53: **GC-MS analysis of PMAA of** *A. orbitosa*

Table 49: Linkage analysis of Ao$_{sul}$ and Ao$_{des}$

	A$_{Osul}$ Mol %	A$_{Odes}$ Mol %	Deduced linkage
2, 3, 4-tri Me- Xyl	0.9	1.5	Terminal Xyl(1→
2, 3, 4, 6- tetra-Me- Gal	1.8	-	TerminalGal*p*(1→
2, 4, 6-tri Me-Gal	4.3	18.2	→3) Gal*p*(1→
2, 3, 6-tri Me-Gal	2.1	15.9	→4) Gal*p*(1→
2, 3, 6-tri Me-Glc	1. 2	0. 3	→4) Glc*p*(1→
2, 6-di Me-Gal	8.9	3.3	→3, 4) Gal*p*(1→
4, 6-di Me-Gal	22.1	3.2	→2, 3) Gal*p*(1→
3, 6-di Me-Gal	1.5	0. 4	→2, 4) Gal*p*(1→
2, 3-di Me-Gal	1.3	4.2	→4, 6) Gal*p*(1→
2, 4-di Me-Gal	0. 3	0. 10	→3, 6) Gal*p*(1→

The linkage analysis revealed the presence of 2, 3, 6-tri-*O*-methyl-D-galactose, 4, 6-di-*O*-methyl-D-galactose and 2, 4, 6-tri-*O*-methyl-D-galactose as the main components with 2, 6-di-*O*-methyl-D-galactoses and 2, 3, 4, 6-tetra-*O*-methyl-D-galactose present in small proportions. Furthermore, the presence of 2, 4, 6-tri-*O*-methyl-D-galactose provided additional strong evidence for the presence of (1→3) linkages, whereas the presence of the 2, 3, 6-isomer confirmed the presence of (1→4) linkages in the molecule. Furthermore, 2, 3, 4, 6-tetra-*O*-methyl-D-galactose, 2, 3, 6- and 2, 4, 6-tri-*O*-methyl-D-galactoses, 2, 6-di-*O*-methyl-D-galactoses, and 2, 3, 4-*O*-methyl-xylose were isolated. The main option for the location of xylose was as a branch residue attached to the 4-position of 3-linked galactosyl residues. However, in addition to the above sugars, 2, 4-di-*O*-methylgalactose was detected in the linkage analysis.

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Nonetheless, all methylation results must be treated with some caution because of the uncertainty of the extent of the process. A possible appearance of 4, 6-di-*O*-methyl-D-galactose suggested further the presence of (1→3)-linked units sulfated at position 2.

The detection of 2, 4, 6-tri-*O*-methyl-D-galactose appeared to indicate that not all of the (1→3)-linked D-galactose residues were sulfated. Subsequently, 2, 6-di-*O*-methyl-D-galactose may have arisen from (1→3)-linked units sulfated at position 4, evidenced by an asymmetric stretching vibration of sulfate group with the IR having a small hump signal at 817 cm^{-1} that was indicative of such attached to a primary hydroxyl group. However, this may also represent a genuine (1→4)-linkage in case of under-methylation. Therefore, the main linkage model of this polysaccharide was a (1→3) and (1→4), branching points located at *O*-2 and *O*-6 of galactose residues. Sulfate groups may have been located at 2 and/or 6 position of (1→4)-linked galactose residue and 2 position of (1→3)-linked galactose residue.

There was a decrease in 2, 6-di-*O*-methylgalactitol contents in the methylated product after desulfation and minor amounts of 3-linked D-galactose-4-sulfate residues. Consequently, there was a change in 2, 4, 6-tri-*O*-methylgalactitol content suggesting that a fraction of (1→4) linked Galactitol units were sulfated at C-2 and C-6 atoms as reported in λ carrageenan (Guibet *et al.* 2006).

At present, there is no unique structure that can be proposed for *A.orbitosa*. It is fairly clear, however, that the molecule is composed of D-galactose residues linked (1→3) and (1→4), with, the former appearing in greater concentrations. This polysaccharide resembles the λ carrageenan (Guibet, *et al.* 2006) in some respects that is highly sulfated, and contains (1→3)-linked residues of D-galactose 4-sulfate. However, most of the (1→4)-linked D-galactose units

are free from sulfates and therefore a lower proportion of alkali labile sulfate. Further studies on this property can be researched in future.

6.5 Conclusion

Following chromatographic fractionation of the organic extracts, two compounds; phytol (**28**) and *β*-sitosterol (**29**) were isolated for the first time from this alga.

Determination of the sugar composition revealed the presence of galactose as a dominant monosaccharide. Anion exchange chromatography showed six polysaccharide fractions (F1-F6) which contained varying amounts of sulfate. Size exclusion chromatography of fraction F1 on Sephacryl 400 column indicated that the average molecular weight was in the range between 10×10^3 D and 500×10^4 Da.

From the NMR data, signals at around 4.8 and 5.2 ppm were tentatively assigned to anomeric protons of α-(1→3) linked galactopyranosyl and β-linked terminal residues. However, due to the complexity of the ^{1}H NMR spectrum, it was difficult to fully account for all the signals present. From methylation analysis, *A. orbitosa* polysaccharide was found to consist of alternating (1→3) linked 2-sulfated-galactosyl residues, (1→4) linked 2, 3-disulfated-galactosyl and significant amounts of (1→4) linked 2, 6-disulfated-galactosyl residues.

CHAPTER 7

7.0 Conclusion and recommendations

7.1 Conclusion

Kommetjie, near Simon's town South Africa, has become a lucrative source of seaweeds and it is a highly conducive environment for the growth of several species including those reported in this thesis. These algae are beneficial to marine organisms such as mussels and fish, as they can soak up nutrients out of the water (Wright, 2012). For pharmaceutical or cosmeceutical applications, their blades or sporophylls may be minced into a gel rich in phlorotannins and fucoidan that have biologically active constituents.

Following the investigations conducted in this study, four seaweed species were found to provide interesting results from each other. A detailed investigation on the chemistry of the organic and polysaccharide composition was conducted. This led to the isolation of both small and complex structures of the algae. Great focus was on *Ecklonia maxima* as the main alga of interest that resulted in the isolation of phlorotannins phloroglucinol (**22**), eckol (**23**), 7-phloroeckol (**24**), 2-phloroeckol (**25**) and the sterol, 24-ethylidine cholesterol (**26**), a fucosterol. It was also critical to examine the antioxidant activity of organic extracts in order to promote and/or support the use of the seaweed in the pharmaceutical and cosmeceutical industries. Antioxidant activities of crude extracts and isolated compounds, were compared to some commercial antioxidants. Furthermore, purified isolates showed the strongest antioxidant property than the crude extracts. The phlorotannins activities varied significantly and appeared to be dependent on hydroxyl groups content and positioning. It was clear that some of these activities have diverse structural specificities.

Experimental and computational quantum chemical studies on the antioxidant radical scavenging properties of phlorotannin derivatives were performed in an attempt to provide an explanation for the differences in the antioxidant activities of the isolated compounds. Theoretical studies on the neutral and radical species of the target compounds facilitated the identification of the preferred geometries for the different species as well as the factors which influence the values of their BDE. This in turn determines hydrogen atom transfer mechanism (HAT), and the values of adiabatic IP that influences the electron transfer mechanism (ET). The stabilization energy (ΔE_{iso}) was also investigated and it provided information on the ability of antioxidants to scavenge free radical species of phenolic derivatives (Mwangi, *et al*., 2013).

Additional compounds were isolated from other seaweeds as follows; *Splachnidium rugosum*: 24-ethylidine cholesterol (**26**), 1, 3-Dicapryloyl-2-oleoylglycerol (**27**), *E*-3,7,11,15-tetramethylhexadec-2-en-1-ol (phytol) (**28**), *Macrocystis angustifolia*: 24-ethylidine cholesterol (**26**), and *Aeodes orbitosa*: *E*-3,7,11,15-tetramethylhexadec-2-en-1-ol (phytol) (**28**) and (17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H* cycl openta[*a*]phenanthren-3-ol) (*β*-sitosterol) (**29**). Notably, some of the isolates characterized were obtained from more than one of the seaweeds investigated.

Investigations of the polysaccharide structures of these algae provided some similarities and differences. The important differences observed were their molecular weight, fucose, sulfate and uronic acid contents, all of these being important for the various biological activities. Further investigations will be necessary to determine the average chain lengths and the branching of fucoidans. Invariably, low molecular weight fucans are usually reported to be more important for biological activity compared to their higher M_W associates.

In the research work, it was important to investigate the best time to harvest *Ecklonia maxima* for its fucoidan content. Experiments were carried out by extracting fucoidan from winter and summer collections, during the normal course of growth of the alga. The optimal levels of sulfate content were determined accordingly. Such functional groups have been reported to be related to the bioactivity of fucoidan (Li, *et al*., 2008). The chemical changes within the cell walls of the alga are likely to be caused by the maturation and synthesis of the sporophylls (Skriptsova, *et al*., 2009). The yields of fucoidan was found to be higher in winter on comparing the two seasons. This observation served to illustrate the influence of environmental factors on the production of fucoidan. It was attributed to the fact that, the alga generates its biomass reserve after the rapid growth phase in spring in order to survive the winter where hardly any photosynthesis occurs (Painter, 1983).

From the time of collection of the respective seaweed extracts, several physical techniques were applied. This comprised extraction of the sulfated crude polysaccharide through to the precipitation and purification process. Subsequently, determination of the fucose, sulfate and uronic acid percentages. Hydrolysis process and initial derivatization of the crude material to determine the chemical compositions was performed. This was further supported by HPLC for the determination of the small monosaccharide content as PMP-derivatives. The technique was sensitive to HPLC separation of saccharides (Lattova and Perreault, 2009).

Anion exchange using DEAE sepharose was applied for purposes of purifying the crude samples for further chemical analysis. Use of gel permeation chromatographic system was applied for the determination of the average molecular mass of the fucoidan and its fractions. The method was employed in order to give an estimate of the average molecular weight of the fucoidans. Mainly, most studies have used gel chromatography to estimate molecular masses of

fucoidans (Choosawad, *et al*., 2005; Li, *et al*., 2006; Patankar, *et al*., 1993; Zvyagintseva, *et al*., 2003). Chemical treatment method followed for methylation analysis process which helped determine the tentative linkage patterns.

The three brown algae studied were shown by methylation analysis to be composed of a fucose backbone, characterized by (1→3) and (1→4) linkages with sulfate groups at *O*-2 and *O*-2, 3 positions. Earlier work on similar structures had shown them to contain primarily (1→2) linked 4-*O*-sulfated fucopyranose residues (Patankar., *et al* 1993; Chevolot *et al*., 2001). However, 3-linked fucose units with 4-*O*-sulfate groups were subsequently reported present among similar algae (Chevolot *et al*., 2001). Additionally, their chain was shown to carry branches at every 2 to 3 fucose residues. The only red seaweed investigated, *Aeodes orbitosa* was observed to contain D-galactose and 2-*O*-methyl-D-galactose. Both the native and desulfated polysaccharide of this red alga were considered and evidence presented the presence of (1→3) - and (1→4)-glycosidic linkages with sulfate on positions 2 and 6.

The protective effects of some of the isolated compounds from *E. maxima* against three cancer cell lines were evaluated via the MTT assay. Among the isolated phlorotannins, compound **23**, an eckol showed prominent inhibitory activity against some selected cancer cell lines, and may serve as potential anticancer drug for cancer treatment. It would contribute in inhibiting metastasis by effectively reducing induced cell damage. These biological activities may be improved by modifying various attributes (Cho, *et al*., 2011; Koyanagi, *et al*., 2003). The present findings indicated that these compounds may be used as natural antioxidants and antitumors in the nutraceutical, cosmeceutical and pharmaceutical industries. The production of phlorotannins and fucoidan from these South African seaweeds may be a good selling point to the national and international markets.

7.2 Future recommendations

Studies on phlorotannins have on many occasions shown that these compounds have some capacity to inhibit tumor growth (Ko and Joo, 2011; Synytsya, *et al*., 2010; Maruyama, *et al*., 2006). Hence, testing the antitumor activity and apoptotic effects of these phlorotannins as reported herein and on other cell lines would be of interest. Furthermore, they have a great potential to be used as natural chemotherapy drugs on other human cancer cells in future.

Further research is necessary to improve on the structural comprehension of the polysaccharides in addition to what has now been documented. The differences reported on each polysaccharide from the different sources are likely to have varying influences on their properties. Primarily, their functionalities are dependent on the structure, the seaweed species, the extraction protocol and the harvesting period. The study of these characteristics for fucoidan and alginate has been particularly interesting because few researchers have been able to identify the link between the structure, deformation and flow behavior of the polysaccharides.

Consequently, it would be of great research interest to further elucidate and characterize fully the structures of fucoidan from these South African seaweeds. Furthermore, sulfate content has been linked with fucoidan's biological activity hence, increasing the degree of sulfation can be a key aspect in enhancing their bioactivity (Teruya, *et al*., 2007). Previous literature has shown that bioactivity increases with increased sulfate content (Cho, *et al*., 2011; Teruya *et al*., 2007). Further experiments in producing more sulfated polysaccharides would be interesting in order to enhance their bioactivity by subjecting them to more assays.

In-depth application of nuclear magnetic resonance (NMR) spectroscopy would be very useful to obtain more structural information. Molecular weight of any fucoidan can also be more accurately determined using LC-MS, and/or MALDI-TOF-MS systems. Since their bioactivity

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are related to molecular weight as shown in other studies (Zvyagintseva, *et al*., 2003; Choosawad, *et al*., 2005; Patankar, *et al*., 1993), the information in this study would be equally critical in their discussion.

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