

# Bioprospecting for bioactive polysaccharides from marine algae endemic to South Africa

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UNIVERSITY *of the*  
WESTERN CAPE

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A thesis submitted in partial fulfilment of the requirements for the degree of *Magister Scientiae* (MSc) in the Faculty of Natural Sciences, Department of Biotechnology, University of the Western Cape

4<sup>th</sup> January 2016

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Co-supervisor: Dr. Renè Naidoo

# **Bioprospecting for bioactive polysaccharides from marine algae endemic to South Africa**

Grant Garren January

## **Keywords (10)**

Polysaccharide

Fucoidan

Heterogeneity

Extractions

Chemical composition

Fractionation

Purification

Bioactivity

Anti-oxidant

Anti-cancer



## Plagiarism Declaration

I, *Grant Garren January*, hereby declare that “*Bioprospecting for bioactive polysaccharides from marine algae endemic to South Africa*” is my own original work, that it has not been submitted before for any degree or examination in any other university, and that all sources I have used or quoted have been accurately reported and acknowledged by means of complete references.

Full name: Grant Garren January

Date: 4<sup>th</sup> January 2016

Signed: .....



Witness: .....



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## **Dedication**

This thesis is dedicated to my parents: Jonathan and Christina January, who instilled in me from a very young age the love for lifelong-learning, the value and power of knowledge, and amongst trying circumstances afforded me the opportunity to pursue my dreams through education.

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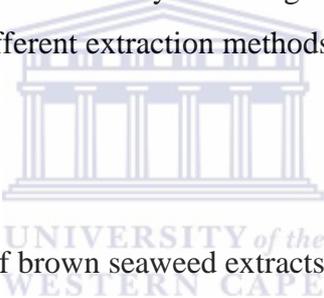


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## List of Nomenclature

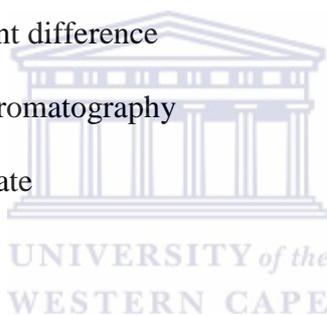
### *Acronyms and abbreviations*

°C	Degrees Celsius
$\alpha$	Lowercase alpha
$\beta$	Uppercase beta
$\mu$	Micro
$\mu\text{g/mL}$	Microgram per millilitre
$\mu\text{L}$	Microlitre
€	Euro
x g	Relative centrifugal force
ANOVA	Analysis of variance
(AP)-1	Activator protein
Apaf-1	Apoptotic protease activating factor 1
ATIII	Anti-thrombin
ATP	Adenosine triphosphate
BaCl <sub>2</sub>	Barium Chloride
BHA	Butylates hydroxyanisole
BHT	Butylatedhydroxytoluene
Bid	Bcl-2 interacting protein
BSA	Bovine serum albumin
C <sub>12</sub> H <sub>9</sub> N	Carbazole
CaCl <sub>2</sub>	Calcium chloride



CHCA	$\alpha$ -Cyano-4-hydroxycinnamic acid
CO <sub>2</sub>	Carbon dioxide
CTAB	Hexadecyltrimethylammoniumbromide
CUPRAC	Cupric Reducing Antioxidant Capacity
Da	Dalton
DCs	Dendritic cells
ddH <sub>2</sub> O	Distilled deionized water
DHB	2,5-dihydroxybenzoic acid
DMEM/F12	Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptors
ERK	Extracellular regulated kinase
ESI	Electrospray ionization
EtOH	Ethanol
FAO	The Food and Agriculture Organization of the United Nations
FADD	Fas-associated death domain
FBS	fetal bovine serum
FCSPs	Fucose-containing–sulphated-polysaccharides
FGF	Fibroblast growth factor
g	gram
$\gamma$ -irradiation	Gamma-irradiation

GC-MS	Gas chromatography mass spectroscopy
H <sub>2</sub> SO <sub>4</sub>	Sulphuric acid
HCl	Hydrochloric acid
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
HMEC	Human mammalian epithelial cells
HMW	High molecular weight
HMWF	High Molecular Weight Fucoidan
HSD	Honest significant difference
IEC	Ion exchange chromatography
K <sub>2</sub> SO <sub>4</sub>	Potassium sulphate
kDa	Kilodalton
kGy	Kilogray
kV	Kilovolt
L	Litre
LMW	Low molecular weight
LMWF	Low molecular weight fucoidan
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MAPK	Mitogen activated protein kinase
Mbp	Mega base pairs
MEK	Mitogen activated kinase/ERK-kinase
m	Metre
mg/mL	Milligram per millilitre



min	minutes
mL	Millilitre
MMWF	Medium molecular weight fucoidan
MNPs	Marine natural products
MPA	Marine protected areas
MWCO	Molecular weight cut off
M	Molar
MS	Mass spectroscopy
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
N	Normal solution
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub>	Sodium tetraborate
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaI	Sodium Iodide
NaOH	Sodium hydroxide
NH <sub>4</sub> Ac	Ammonium acetate
nm	Nanometre
NPs	Natural products
NGS	Next generation sequencing
NK	Natural killer cells
NMR	Nuclear magnetic resonance
PARP	Poly ADP-ribose polymerase
PBS	Phosphate-buffered saline
PDGFR	Platelet-derived growth factor receptor
PENSTREP	Penicillin-streptomycin



pmol	Picomole
RNA	Ribonucleic acid
rpm	Revolutions per minute
ROS	Reactive oxygen species
RT	Room temperature
RTKs	Receptor tyrosine kinases
SA	Sinapinic acid
SD	Standard deviation
SE	Standard error
SI	Selectivity index
SO <sub>4</sub> <sup>2-</sup>	Sulphate ion
t	Metric tonnes
tBid	Truncated Bid
TBHQ	Butyl hydroquinone
TFA	Trifluoroacetic acid
TFPI	Tissue factor pathway inhibitor
THAP	2,4,6-trihydroxyacetophenone
TNFR-1	Tumour necrosis factor receptor-1
USD	United States Dollar
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
vol	Volume
v/v	Volume per volume
w/v	Weight per volume
WST	Water-soluble tetrazolium salts



WST-1

4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate



## Research outputs

### Publications

The following articles are in preparation for publication based on the research derived from this study:

**January G.G.**, Naidoo, R.K., Kirby, B., and Bauer, R. 2016. Assessing the effect of extraction method on fucoidan extraction from brown macroalage of South Africa.

**January G.G.**, Naidoo, R.K., Kirby, B., and Bauer, R. 2016. Fractionation, purification, and bioactivity assessment of low molecular weight fucoidan from South African brown macroalgae.



### Presentations

**January G.G.**, Naidoo, R.K., and Bauer, R. Bioprospecting for bioactive polysaccharides from marine brown algae. *28<sup>th</sup> Congress of the Phycological Society of Southern Africa, Western Cape, South Africa, 13-17 January 2014.*

**January G.G.**, Naidoo, R.K., and Bauer, R. Bioprospecting for bioactive polysaccharides from marine brown algae. *5<sup>th</sup> International Symposium of Applied Phycology, Sydney, Australia, 22-27 June 2014.*

**January G.G.**, Naidoo, R.K., and Bauer, R. Anti-cancer potential of fucoidan isolated from South African brown seaweeds. *24th biennial congress of the South African Society for Biochemistry and Molecular Biology, Western Cape, South Africa, 6-9 July 2014.*

## Abstract

Bioprospecting for bioactive polysaccharides from marine algae endemic to South Africa

G.G. January

MSc Thesis, Faculty of Natural Sciences, Department of Biotechnology, University of the Western Cape, South Africa

Fucoidan is a marine-derived sulphated polysaccharide with bioactive properties ideal for the food, chemical and pharmaceutical industries. The polysaccharide consists largely of L-fucose, has a highly heterogeneous structure and is of diverse origin. Fucoidan was extracted from *Ecklonia maxima*, *Laminaria pallida* and *Splachnidium rugosum* and the effect of different extraction methods on fucoidan heterogeneity was assessed. Extraction methods employed hot water, hydrochloric acid or calcium chloride salt. Fucoidan yield and purity were determined by various colorimetric assays. Highest fucoidan yield was obtained with the hot water extraction method as seen by highest L-fucose content. *Splachnidium rugosum* extracts contained ~5 times more L-fucose than *Ecklonia maxima* and *Laminaria pallida* extracts. The salt extraction method yielded extracts free of contaminants, however L-fucose content in all extracts was >20 times lower. Acid extraction yielded highest levels of uronic acid contamination and liberated sulphate from the fucoidan polysaccharide. The fucose-to-sulphate ratio for *Ecklonia maxima* was approximately 1:5, whilst the ratios for *Splachnidium rugosum* and *Laminaria pallida* were approximately 1:1 and 1:2, respectively. The acid and salt extraction methods removed all traces of protein contaminants, while the hot water method retained very low levels of protein. The extraction method used to isolate fucoidan was a determining factor in yield and purity.

Chemical compositional analyses of hot water extracts were assessed by gas chromatography mass spectroscopy. *Splachnidium rugosum* and *Laminaria pallida* extracts consisted largely of L-fucose, while *Ecklonia maxima* fucoidan was characterized with high glucose abundance. Crude hot water and acid extracts from *Splachnidium rugosum* tissue were fractionated and purified by (anionic) ion exchange chromatography as bioactivity has been correlated to lower molecular weight forms. In water extracts, ion exchange chromatography resulted in close to 90% decrease in L-fucose, sulphate and uronic acid, while protein content

increased by 57%. Similar results were reported for acid extracts; however protein content did not change significantly. These results show that method of extraction may affect the composition of fucoidan post-purification. Hot water extraction is recommended due to higher fucoidan yield, as reflected by L-fucose content, and higher sulphate-to-fucose ratio. High protein content after ion exchange chromatography was however of concern. Since mucilage in *Splachnidium rugosum* thallus was free of protein, fucoidan was precipitated from mucilage with ethanol. Fucoidan yield of mucilage was >15-fold higher than content in purified hot water extracts with a sulphate-to-fucose ratio of ~1:1. The average molecular weight of native fucoidan in mucilage was estimated at 2367 kDa. The polysaccharide was hydrolysed by gamma-irradiation levels of 10-50 kGy to fractions ranging between 60 and 15.5 kDa.

Hot water crude fucoidan extracts from *Ecklonia maxima*, *Laminaria pallida*, and *Splachnidium rugosum* were assessed for anti-oxidant activity by measuring the ability to scavenge free radicals and the capacity to reduce copper ions with 2,2-Diphenyl-1-picrylhydrazyl and Cupric Reducing Anti-oxidant Capacity assays, respectively. *Ecklonia maxima* crude fucoidan displayed highest anti-oxidant activity and capacity, having the potential to scavenge reactive oxygen species as well as the capacity to reduce copper to less toxic forms in mammalian systems. *Splachnidium rugosum* showed weakest anti-oxidant activity and lowest reducing capacity. The anti-cancer activity of crude and purified hot water *Splachnidium rugosum* extracts, as well as non-irradiated (native) and gamma-irradiated fucoidan, and commercially procured fucoidan were assessed for anti-cancer activity against MCF-7 breast cancer cells. *Splachnidium rugosum* crude and purified fucoidan displayed a half maximal inhibitory concentration of 0.7 mg/mL and 0.029 mg/mL, respectively. Low cytotoxicity of crude and purified *Splachnidium rugosum* fucoidan against non-cancerous breast epithelial cell line MCF-12A was observed, as seen by half maximal inhibitory concentration values of 2 mg/mL and 0.663 mg/mL, respectively. The cancer specific selectivity of purified *Splachnidium rugosum* fucoidan was therefore much higher as reflected by 10-fold higher selectivity index than that of crude fucoidan. Native and low molecular weight gamma-irradiated fucoidan also showed bioactive properties including anti-cancer activity as seen by the reduction of cell proliferation *in vitro*, whereas crude fucoidan showed the ability to scavenge free radicals, and the capacity to reduce copper ions.

# Chapter 1. General introduction and project aims

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## 1.1) Introduction

Marine macroalgae (seaweeds) are a unique group of photosynthetic organisms that appear similar to land plants, but are molecularly distinct (Yoon *et al.*, 2004). Seaweeds are crucial species in marine ecosystems as they provide food, and shelter for fish and invertebrates, and act as substrata for epiphytic smaller algae (Connell, 2003). Seaweeds are comprised of photosynthetic primary producers, which are pivotal to the food supply chain in marine environments, as well as giant under-water kelp forests which offer protection and habitat to marine life (Reed and Foster, 1984; Tegner and Dayton, 2000). Moreover, they contribute approximately 70-80% of oxygen in the atmosphere required by terrestrial organisms. Macroalgae can be broadly categorised into brown, green, and red seaweeds, and possess a plethora of natural products (NPs), vitamins, and minerals (De Clerck *et al.*, 2013). Brown macroalgae are particularly interesting as they possess unique characteristics which have evolved over time leading to the development of novel bioactive compounds (Cock *et al.*, 2010). One such compound is fucoidan, a sulphated polysaccharide found within the mucilage of brown seaweeds. Fucoidan has been shown to exhibit several bioactivities in mammalian cells including anti-oxidant and anti-cancer activity (Jiao *et al.*, 2011).

South Africa possesses the third highest biodiversity globally with 80% of its flora not being found anywhere else in the world (Griffiths *et al.*, 2010). This is due mainly to its unique geographic location and physical features (Bolton *et al.*, 2009). In this study our interest lies with three seaweed species found along the South Western coast of South Africa. *Ecklonia maxima* and *Laminaria pallida* are two kelp species endemic to South Africa with large industrial and agro-economical value (Troell *et al.*, 2006; Rothman *et al.*, 2015). *Splachnidium rugosum* is a unique species of brown seaweed that possesses internal and external mucilage that could potentially consist of high yields of fucoidan (Clayton, 1985; Harden *et al.*, 2009). *S. rugosum* is found only in parts of the Southern Hemisphere, with

most species studied originating on the Australian or New Zealand coastline (Clayton, 1987; Hurd *et al.*, 2004). These three algae species hold enormous commercial importance as a source of fucoidan, however they have not been well studied or characterised with respect to fucoidan content or composition.

Fucoidan compounds are difficult to extract and characterize but remains of interest due to several potent bioactivities that it presents in mammalian systems (Ale *et al.*, 2013). The pharmaceutical industry is particularly interested because of its organic nature, intense bioactivity and low cytotoxicity, as well as opportunities for sustainable harvesting from seaweeds (Fitton *et al.*, 2015). Fucoidan compounds have a proposed common structure consisting of an  $\alpha$ -L-fucose backbone, with varying degrees of sulphation, glycosidic linkages, minor monosaccharides, short discrete uronic acid branches, proteins and other macromolecule contaminants (e.g. lipids, carbohydrates, acetates, acids, & polysaccharides) (Fitton *et al.*, 2011). The common structure of fucoidan is similar at a Phylum ranking but becomes more complex at species level. The fucoidan extraction method adds significantly to diversity, introducing a certain amount of bias which can lead to changes in structure, charge, molecular weight and chemical composition (Ale and Meyer, 2013). This makes research on each individually isolated fucoidan molecule unique. Currently a standardised fucoidan extraction technique is currently not available. The establishment of a simple, cost-effective, standardised procedure would enable the exploitation of fucoidan providing an opportunity for the development of a commercially viable algae industry in South Africa.

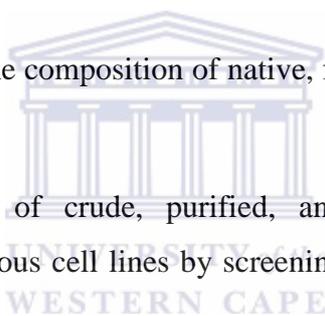
Today, breast cancer is the most common cancer affecting women worldwide (Siegel *et al.*, 2015) and despite tremendous advances in screening, diagnosis, and treatment, the causes of this disease remain elusive and complex (Hanahan, 2014). Teas *et al.* (2013) has presented a proof of principle manuscript wherein they correlate the consumption of (daily) seaweed rich diets, in Japan, to lower postmenopausal breast cancer incidence and mortality rates. Research has shown that fucoidan possess anti-cancer activity by inhibiting proliferation of cancerous cells *in vitro* (Yamasaki-Miyamoto *et al.*, 2009) and *in vivo* (Kwak *et al.*, 2014), with a few clinical trials in humans showing potential as an adjunct to current cancer therapy (Ikeguchi *et al.*, 2011). However, fucoidan molecules are generally large, heterogeneous, complex compounds (Choi and Kim, 2013). The purification and/or fractionation of the compound (for increased absorption and drug delivery) is therefore required for enhanced efficacy. However, no study has evaluated fucoidan from highly diverse, brown seaweeds of

South Africa. Thus, this study will assess the feasibility of isolating fucoidan from endemic species for potential use as a cancer therapeutic drug.

## **1.2) Project aims**

This research project aims to:

- (I) Isolate fucoidan compounds from three species of brown macroalgae from South Africa using three independent extraction methods, and assess yield and purity of crude extracts;
- (II) Develop a novel, effective, purification and fractionation technology for fucoidan compounds;
- (III) Chemically characterise the composition of native, fractionated, and purified fucoidan compounds;
- (IV) Evaluate the bioactivity of crude, purified, and fractionated fucoidan against cancerous and non-cancerous cell lines by screening for anti-oxidant and anti-cancer properties.



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# Chapter 2. Literature review

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## 2.1) Marine resources

Aquaculture is the aquatic component of agriculture, wherein freshwater and marine organisms are farmed for food or industrial uses (Alexander *et al.*, 2015; Martínez-Espiñeira *et al.*, 2015). The global aquaculture industry has increased exponentially over the last half-century, with the production of 52.5 million metric tonnes (t) of marine derived products in 2008, at an estimated value of USD 98.5 billion (Bostock *et al.*, 2010). Marine resources are not limited to nutrients, animal feed, or food supply (accounting for 50% of the global fish food supply), but also include pharmaceutical, cosmetics, and industrial compounds derived from the ocean (Martinez-Porchas and Martinez-Cordova, 2012). The Food and Agricultural Organization of the United Nations (FAO) strictly manages these marine resources. Globally, aquaculture production has steadily increased over the last decade (Table 2.1).

**Table 2.1. Global aquaculture production of aquatic algae by continent over the last decade (million tonnes).**

Location	Year										
	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Africa	0.08	0.12	0.11	0.08	0.08	0.09	0.10	0.12	0.11	0.14	0.14
Americas	0.07	0.07	0.04	0.02	0.02	0.04	0.03	0.03	0.09	0.01	0.02
Asia	9.55	10.40	11.25	12.56	13.41	13.95	14.87	15.73	17.14	18.84	20.80
Europe	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oceania	0.01	0.01	0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01
Total	9.7	10.6	11.4	12.7	13.5	14.1	15.0	15.9	17.4	19.0	21.0
Year-on-year growth rate	4.4%	9.1%	7.6%	11.1%	6.7%	4.2%	6.4%	5.9%	9.3%	9.5%	10.4%

\*Adapted from FAO Department of Aquaculture and Fisheries.

Marine aquaculture, or mariculture, is a specific branch of aquaculture reserved for the farming of marine organisms such as marine algae, fish, and plants (Troell *et al.* 2003; Edwards, 2015). According to the FAO Fisheries and Aquaculture Department's global statistics (2011), farmed aquatic algae production reached 21 million t with total revenue of USD 5.5 billion. Seaweed mariculture has a long history of importance in Asian countries where they are used for food and medicinal purposes (Zaneveld, 1959). The farming of seaweed originated in Japan and today produces more than 92% of the market demand (Chopin and Sawhney, 2009). Recently Europe has taken a much greater interest in seaweed mariculture as the prospect of using algae for biofuels, bioremediation, and commercially valuable marine natural products (MNPs) gains favour (Bixler and Porse, 2011; Handå *et al.*, 2013).

Mariculture in South Africa is important in the agro-economic sector, with tons of cultivated seaweed (e.g. *Ulva*, *Gracilaria*, and *Ecklonia*) being produced as abalone (*Haliotis midae*) feed annually (Bolton *et al.*, 2009). South Africa is the second largest producer of cultivated abalone outside of Asia and this herbivorous marine mollusc is considered a delicacy in many Asian countries. In 2002, over 22 600 t of abalone were produced globally, of which 8600 t were farmed to a value of USD 0.8 billion (Troell *et al.*, 2006). Commercial companies such as Afrikelp<sup>®</sup>, Kelpak, and Dermikelp<sup>®</sup> (Lamicare Health) use extracts of South African kelp for cosmetic (e.g. dermatological conditions) and agricultural purposes (e.g. fertilizer and plant growth regulators). Since the 1950's beach-cast *Ecklonia maxima* has been shipped to Europe and North America for alginate production (Anderson, 1989). Thus, maintaining and cultivating macroalgae in South Africa is important for sustainable and profitable mariculture.

## **2.2) Taxonomy of the brown seaweed**

Macroalgae are a unique group of aquatic organisms, evolutionary diverse from land plants but with distinct phenotypical similarities (Yoon *et al.*, 2004). They can exist in both fresh water and marine environments (Simons, 1994; Cole *et al.*, 2013; and Lawton *et al.*, 2013). Macroalgae can be taxonomically classified into three major Classes, namely, the Phaeophyta (brown algae), the Rhodophyta (red algae), and the Chlorophyta (green algae) (Rindi *et al.*, 2012; De Clerck *et al.*, 2013). Macroalgae are not taxonomically classified by their

pigmentation but rather by variations at a molecular and physiological level, including flagella morphology and anatomy; reproductive cell ultrastructure and motility; deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) analysis, as well as plastid origin and endosymbiotic properties (Martin *et al.*, 1992; Bhattacharya and Medlin, 1998). These traits are evolutionarily more conserved compared to the vegetative cell classifications used in the past (Bhattacharya and Medlin, 1998).

The brown macroalgae, or Phaeophyceae, are a Class of macroalgae that belong to the Phylum Stramenopiles (Heterokonts) (Charrier *et al.*, 2008). These seaweeds are mostly found within marine ecosystems, with less than 1% of its species found in freshwater environments (McCauley and Wehr, 2007; Tonon *et al.*, 2011). The Phaeophyceae have developed complex multicellularity over the course of evolution via secondary endosymbioses and horizontal gene transfer (Bhattacharya and Medlin, 1998; Raymond and Blankenship, 2003). Taxonomic classification of these species were further validated and expanded through the advancement of the whole genome sequencing of a model brown macroalgal species, *Ectocarpus siliculosus* (Cock *et al.*, 2010). The data revealed fascinating genetic detail, such as light-acquiring and pigment biosynthesis gene clusters, signal transduction genes and receptor kinases, halogen metabolising complexes, and an integrated DNA viral genome (Cock *et al.*, 2010).

### **2.3) Anatomy and physiology**

The Phaeophyceae are complex multicellular seaweeds that are much more diverse than their red and green seaweed relatives, with respect to biochemical, genetic, anatomical, and physiological profile (Hajibabaei *et al.*, 2007; Rodrigues *et al.*, 2015; Zardi *et al.*, 2015). The brown colour of these algae are partially accounted for by the pigments they carry, such as chlorophyll a and c, and fucoxanthin, but their habitat and the physics of light absorption and surface chemistry play a major role too (Branch and Branch, 1981; Branch *et al.*, 1994; Stegenga *et al.*, 1994). Fucoxanthin is found exclusively in brown seaweeds and other Heterokonts. Morphologically brown algae are extremely diverse, consisting of large kelp forests, to hanging wracks, and polysaccharide filled “dead man’s fingers” (Branch and Branch, 1981; Branch *et al.*, 1994; Stegenga *et al.*, 1994).

Brown algae are mostly found in rocky tidal pools or closely related intertidal regions, when not washed up on shore (Tonon *et al.*, 2011). Consequently, they are constantly exposed to a wide range of environmental stresses and have evolved molecular mechanisms of survival (Cock *et al.*, 2010; Silberfeld *et al.*, 2010). These stressors include fluctuations in abiotic factors such as light intensity, pH, salinity, temperature and wave action (Hurd, 2000). Biotic stresses are caused by epibiotic organisms and herbivorous marine life, and can often lead to infections and fatal diseases (Hurd, 2000). The sequencing of the *E. siliculosus* genome – the first model brown macroalgae - in 2010 was a significant development as it allowed researchers to elucidate physiologic adaptations from a molecular evolutionary perspective for the first time (Cock *et al.*, 2010).

The genome of *E. siliculosus* revealed that the Phaeophyceae adapt to changes in light intensities with the aid of light harvesting gene clusters and processes white light into a carbon source via photosynthesis by complex pigment biosynthesis genes and their resultant proteins (Cock *et al.*, 2010). This seaweed overcomes damage caused by ultraviolet (UV) light and reactive oxygen species (ROS) by using phenolic compounds and anti-oxidant enzymes, respectively (Rozema *et al.*, 2002; Cock *et al.*, 2010). Halide metabolism genes were also found to help in warding off halogenated defensive compounds secreted by other brown macroalgae, allowing *E. siliculosus* to symbiotically grow on these larger kelp species (Russell, 1983a; Russell, 1983b; and La Barre *et al.*, 2010; Thomas *et al.*, 2011). Physiological adaptations to mechanical stress caused by wave action include the incorporation of polysaccharides, such as fucose-containing-sulphated-polysaccharides (FCSPs) and alginates, in the cell wall of these algae (Kloareg and Quatrano, 1988; Ale and Meyer, 2013). These polysaccharides aid in structural rigidity of the algae as well as protection against predators (Skriptsova *et al.*, 2015).

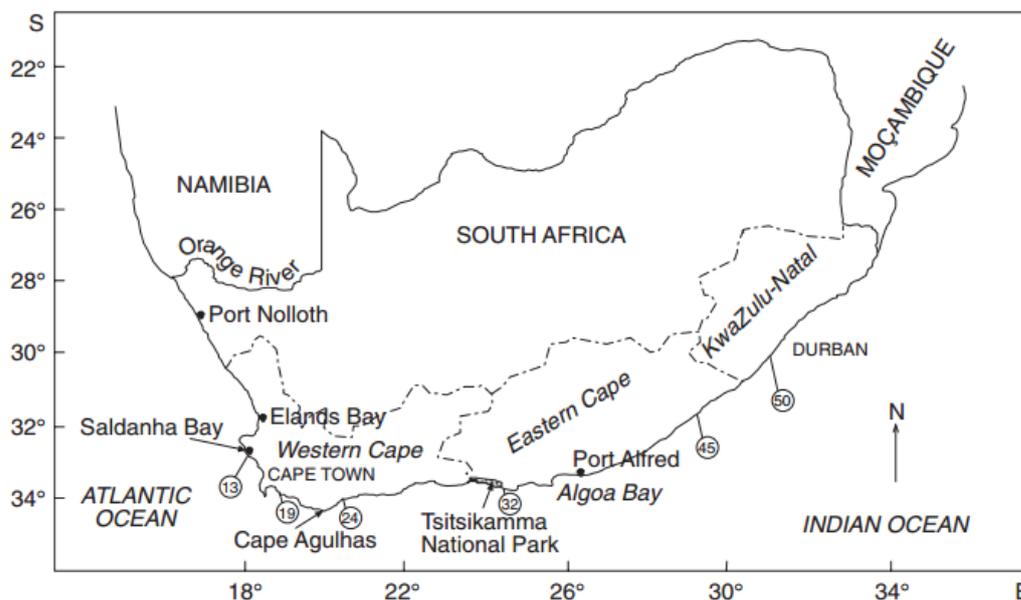
#### **2.4) Distribution and habitat of brown macroalgae**

The brown macroalgae's geographical diversity extends into marine ecosystems in the Northern and Southern Hemispheres (Kantachumpoo *et al.*, 2014; Neiva *et al.*, 2015; Yesson *et al.*, 2015). The Phaeophyceae of the south include the kelps, wracks, *Sargassums*, Dictyota, Zonaria, and other unique macroalgae (Bolton and Stegenga, 2002; Mattio *et al.*, 2015; Rothman *et al.*, 2015). Species that have some form of commercial or

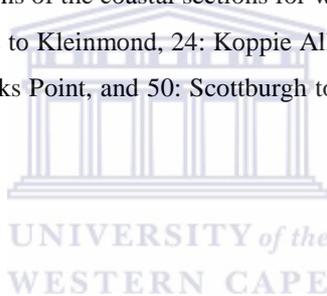
eco-conservational importance include: *S. rugosum* (brown macroalgae), *E. maxima* (kelp), *L. pallida* (kelp), *Sargassum* species, *Macrocystis angustifolia* (kelp), *Ecklonia biruncinata* (kelp), *Bifucaria brassicaeformis* (wrack), *Dictyopteris* species, and *Pandina boryana* (Zonaria group). Most of the research conducted on macroalgae inhabiting the coast of South Africa has been limited to conservation studies (Griffiths *et al.*, 2010) and there is a need for more molecular biology and biotechnological studies to explore the biotechnological potential of these macroalgae (Zardi *et al.*, 2015).

## 2.5) South African brown macroalgae

Seaweeds of South Africa are extremely diverse containing some of the richest global diversity of seaweed flora (Bolton and Stegenga, 2002). The South African coastline consists of approximately 800 species, with the South Coast containing the highest species diversity between 250-300 species per section, and the West Coast boasting the second highest diversity with 140 species per section (Figure 2.1). The commercial use of South African seaweeds began amidst the Second World War, when the export of agar from Japan to Britain ceased (Rothman *et al.*, 2006). This led to a boom of the South African seaweed industry which started in the 1950's and died down around 1980's. Several species of brown, green, and red seaweeds are cultivated in South Africa and are used both locally and exported abroad (Bolton *et al.*, 2009). The average dry seaweed biomass exported annually varies between 100-1500 t, however South Africa has observed a sharp decline in exports with the highest recorded export amount of 4000 t per year reported between 1972 to 1977 (Troell *et al.*, 2006). In South Africa, seaweeds have been collected and exported for use as feeds, or raw materials for the extractions of agricultural (e.g. fertilizer), food (e.g. stabilizing agents), industrial (e.g. alginate extractions), pharmaceutical (e.g. capsule formulations and bioactive substrates) and cosmetic agents (e.g. dermatological creams) (Griffiths *et al.*, 2010; Mwangi *et al.*, 2013). Bolton and Stegenga (2002) reported that brown seaweeds along the South African coastline consist of 101 known species. Locally, brown seaweeds are currently being used as feedstock for mariculture and in the pharmaceutical and research industries for extraction of bioactive compounds (Davies-Coleman, 2010).



**Fig. 2.1. Map of South Africa showing the coasts of which algal species have been observed and studied.** The numbers on the map indicate positions of the coastal sections for which detailed species data were analysed; 13: Langebaan to Postberg, 19: Rooi Els to Kleinmond, 24: Koppie Alleen to Cape Infanta, 32: Storms River to Skuinsklip, 45: Mncwasa River, to Sharks Point, and 50: Scottburgh to Ispingo Beach (Taken from Bolton and Stegenga, 2002).



### 2.5.1) *Ecklonia maxima*

The order Laminariales contains brown macroalgae that are more generally referred to as kelps. Kelps are the most efficient primary producers in the coastal inshore ecosystems and play an important role in the food-web of this ecosystem (Troell *et al.*, 2006; Rothman *et al.*, 2015). Kelps can grow up to 50 m in length, forming beds or underwater-forests that act as both habitats and food sources for numerous marine organisms. *E. maxima* (also known as sea bamboo) is a split fan kelp of the Southern Hemisphere (Figure 2.2). *Ecklonia* species are abundant in the Northern Hemisphere; with *E. maxima* and *Ecklonia radiata* being the only two endemic species in South Africa (Lane *et al.*, 2006; Rothman *et al.*, 2015). *E. maxima* have a strap resembling frond that has been reported to grow a stipe (stem) up to 9 m in length (Troell *et al.*, 2006). These fronds hold beneath them a gas containing bulb, which keeps the kelp buoyant, making the fronds visible from above the water surface. *E. maxima* have been observed from Cape Agulhas to Cape Columbine, being the dominant inshore kelp species in this environment. *E. maxima* play an important role in the South African

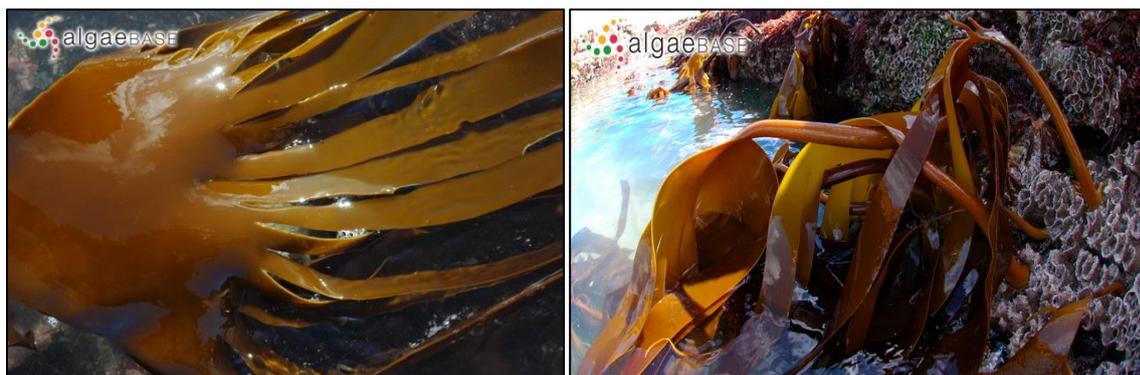
mariculture sector, as it is exported to Europe, North America and Asia for alginate extraction. Locally, it is sustainably cultivated and used as abalone feed, an agricultural fertilizer, and as an active ingredient in dermatological products.



**Fig. 2.2.** Adult sporophyte of *Ecklonia maxima* in the wild. (Source: [www.algaebase.org](http://www.algaebase.org))

### 2.5.2) *Laminaria pallida*

*L. pallida* is the other dominant kelp second to *E. maxima* in the inshore coastline environment of South Africa (Troell *et al.*, 2006). The morphology of the adult *L. pallida* is phenotypically represented by a digitate frond, a hollow stipe that can grow up to 2 m in length, and the absence of the hollow bulb which is characteristic of *E. maxima* (Figure 2.3). *L. pallida* also differs morphologically from that of *E. maxima*, as it has warty stipes and *E. maxima* has smooth stipes. The split-fan-like blades of *L. pallida* can grow up to 5 m in length, and droop over in the deeper waters, sweeping the seafloor. *L. pallida* has been found between the Agulhas and Columbine region. In the deeper inshore environment (north of Columbine) *L. pallida* appears as a sub canopy and gradually starts to replace *E. maxima* as the dominant inshore kelp species. Unlike *E. maxima* which appears at the water-surface *L. pallida* is submerged beneath the surface, often forming extensive kelp-beds in deeper waters (up to 30 m). Beach-cast *L. pallida* has been harvested since 1950's and exported for use in the food and chemical industries. *L. pallida* is also harvested locally for abalone feed, where it is either mixed with *E. maxima* or used by itself, and fed to the abalone in dry or in pellet form.



**Fig. 2.3. Adult sporophyte of *Laminaria pallida* in the wild.** (Source: [www.algaebase.org](http://www.algaebase.org))

### **2.5.3) *Splachnidium rugosum***

*S. rugosum* is a brown macroalgal species with unique characteristics. This alga is the sole species in its genus as well as the sole in its family (Clayton, 1985). There have been no molecular systematic studies on this species and as such its taxonomic ranking has not been confirmed. *S. rugosum* occurs only in the Southern Hemisphere and has been observed on the coastlines of Australia, South Africa, and New Zealand (Hurd *et al.*, 2004; Mwangi, 2014). It has a distinctive morphology when compared to other brown seaweeds as it does not possess a stipe-like holdfast characteristic of many kelp species (Figure 2.4) (Price and Ducker, 1966). Instead *S. rugosum* has a disc-like holdfast which is attached to rocks or limpets, and from this holdfast monopodial thalli branch. The alga consists of finger-like thalli filled with sticky mucilage and it is from this phenotypic trait that the alga derives its common name “dead man’s fingers” (Clayton and Shankly, 1985). It is postulated that this mucilage keeps the alga buoyant as well as providing structural rigidity (e.g. from harsh intertidal wave action) (Skriptsova *et al.*, 2015). The sticky mucilage inside and around the brown algae’s thallus is thought to mostly consist of a sulphated polysaccharide commonly known as fucoidan (Black, 1954). Fucoidans play a role in protecting brown algae from harsh intertidal wave actions and other extrinsic environmental stressors such as fluctuations in salinity and heavy metals, UV-radiation, and predation (Skriptsova, 2015).



**Fig. 2.4. Adult sporophyte of *Splachnidium rugosum* in the wild.**

## **2.6) Marine sources of bioactive compounds**

The Earth's surface is covered by approximately 70% water, of which 95% are considered to be marine environments (Ellis, 2001; Jimeno *et al.*, 2004). These statistics alone highlight the extent to which marine ecosystems overshadow terrestrial environments. However, it was only during the late 20<sup>th</sup> century that aquatic-exploration-technologies such as deep-sea diving and scuba diving paved the way for economically and scientifically feasible marine exploration studies (Ramirez-Llodra *et al.*, 2011). These technological advances allowed biological resources from marine ecosystems to be more readily accessed.

Marine organisms outnumber their terrestrial counterparts in terms of diversity mainly due to their ability to adapt to constantly changing aquatic habitats. This has allowed for better proliferation and altered biochemistry through molecular adaptations over the course of evolution which ensures growth and survival (Pörtner *et al.*, 2001; Pörtner *et al.*, 2002; Wu, 2002; Pörtner 2006; Bichego *et al.*, 2007; Tomanek, 2014; Jebbar *et al.*, 2015; Yancey and Siebenaller, 2015). Thus, for marine organisms water essentially equals life. Mankind, being

the resourceful species that we are, have since exploited these aquatic mechanics for our own gain — giving birth to the field of marine biotechnology.

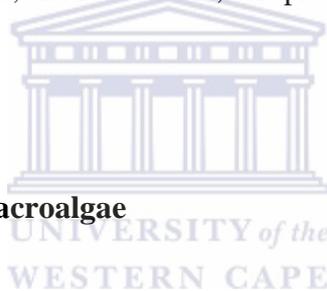
### **2.6.1) Marine biotechnology: Bioprospecting for marine natural products**

Marine biotechnology entails the exploitation of biological systems from marine and other aquatic ecosystems for commercial gain (Burgess, 2012). Since the late 1980's the marine environment has been the leading biological resource for the discovery of novel genes, antibiotics, and other biotechnologically important compounds (Hu *et al.*, 2011; Imhoff *et al.*, 2011). Marine biotechnology is not a new scientific discipline but has garnered research interest, and an increase in commercial funding recently, as modern technologies for bioprospecting have improved (Ritchie *et al.*, 2013). Enhancements in technologies such as metagenomic and next generation sequencing (NGS) are showcasing the true biodiversity from these extreme environments; however a large amount of this biodiversity remains largely untapped (Kennedy *et al.*, 2010). Marine biodiversity, a causative effect of a history of molecular adaptations to biotic and abiotic stresses, gave rise to unique organisms from which novel bioactive compounds could be extracted, modelled against, or co-synthesised from (Tomanek, 2014). Other driving forces that are boosting the marine biotechnology industry include the need for novel pharmaceuticals and sustainable fuel, food, and chemical products (Zemke-White and Ohno, 1999; Bixler and Porse, 2011; Freitas *et al.*, 2012). Research conducted on marine organisms has migrated away from the traditional taxonomy and conservation studies and more focus has been placed on elucidating the mechanisms behind defensive strategies of these deep-sea organisms (Ritter *et al.*, 2008; Thomas *et al.*, 2011; Tonon *et al.*, 2011; Goulitquer *et al.*, 2012). Marine or “Blue” biotechnology involves the search and extraction of commercially valuable compounds from aquatic organisms residing in the sea (Burgess, 2012). Many highly valuable MNPs used in the pharmaceutical, food, cosmetic, and chemical industries have already been extracted from marine sources (Blunt *et al.*, 2013). The majority of MNPs are bioactive compounds which display useful properties in mammalian systems (Gerwick and Moore, 2012; Blunt *et al.*, 2013). These bioactive compounds can be isolated from organisms such as invertebrates, algae, and microbial associated symbionts (Proksch *et al.*, 2003; Jimeno *et al.*, 2004). Several of the currently identified bioactivities from MNPs include: anti-cancer, anti-viral, anti-coagulant,

anti-microbial, anti-oxidant, anti-bacterial, anti-protozoal, anti-inflammatory and anti-metastatic properties, as well as lifestyle associated diseases (Bhakuni and Rawat, 2005).

Algal biotechnology dates back as early as the 18<sup>th</sup> and 19<sup>th</sup> centuries where kelp were burnt for iodine, potash, and soda, as well as being used as a fertiliser (Tseng, 2001). Algae contain numerous bioactive active compounds, or NPs, that includes polyunsaturated fatty acids (PUFAs), B-vitamins, carotenoid, polyphenols, dietary fibre, polysaccharides, proteins, plant growth hormones auxins, gibberellins, and cytokinins.

Macroalgal biotechnology is of enormous industrial importance because of their phycocolloid substances such as agar, alginate and carrageen, which are harvested at large scale for commercial exploits (Michalak and Chojnacka, 2015). Similarly, microalgal biotechnology is a multibillion dollar industry, where microalgae are cultured and harvested at an industrial scale (Wijffels *et al.*, 2013). The biomass is refined through downstream processes and sold as food and dietary supplements, nutraceuticals, or processed as pigments or speciality chemicals.



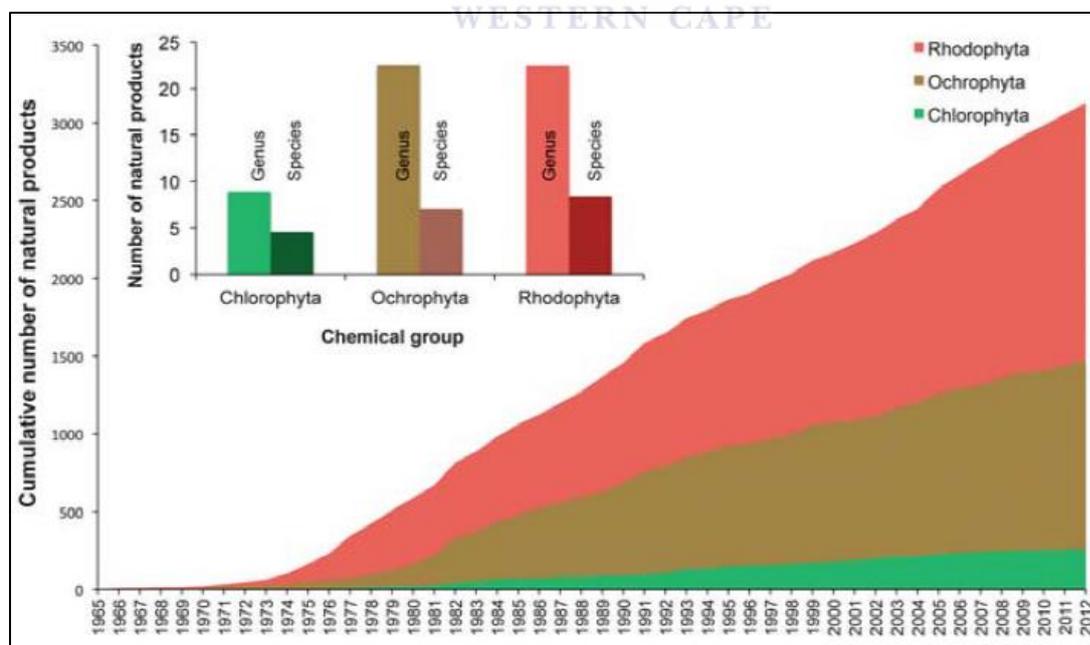
### **2.6.2) Bioprospecting marine macroalgae**

In contrast to land plants and microalgae, macroalgae (seaweed) are unique in terms of their anatomy, physiology and metabolism (La Barre *et al.*, 2010). Seaweed encounter numerous environmental stresses including varying light intensities, high salinity, rapid fluctuations in pH and osmotic pressure, and harsh intertidal ocean currents (Hurd, 2000; Cock *et al.*, 2010). Macroalgae also encounter biotic attacks, such as those by pathogenic microorganisms and larger predators, which are successfully evaded by immune responses and chemical defences (Küpper 2002; Küpper 2006; Thomas *et al.*, 2011). The course of evolution has led to adaptive mechanisms of survival, which include natural products such as halogenated compounds, secondary metabolites, and sulphated polysaccharides (La Barre *et al.*, 2010; Shalaby, 2011). The macroalgae are particularly interesting because of the sulphated fucans and galactan polymers found within their cell walls, known as FCSPs (Ale *et al.*, 2011). These compounds display several bioactivities against microbial pathogens; viruses; systemic disorders; and anti-proliferative effects against numerous cancers and tumours in mammalian cells (Ale *et al.*, 2011). The major FCSPs isolated from macroalgae are fucoidans, which are

mainly found in brown algae, hypothesised to play a role in the structural rigidity and survival of the seaweed (Pomin and Mourão, 2008; Skriptsova *et al.*, 2015).

### 2.6.3) Marine bioactive compounds

The bioactive compounds found within macroalgal extracts are as diverse as the species they originate from. These compounds produced by brown, red, and green algae include polysaccharides, pigments, anti-oxidant compounds, plant growth promoting substances/hormones and a range of other bioactive compounds (Michalak and Chojnacka, 2015). Macroalgae are an important source of MNPs as discussed by Leal *et al* (2013). They reported that between 1965 and 2012 red, brown, and green seaweeds have been the source of 3129 MNPs (reds, 53% of the total; browns, 39%; green, 8%) (Figure 2.5). Historically, MNPs from macroalgae have been significant in marine drug progression, with 28% of novel MNPs originating between 1970 and 1980. Therapeutically, macroalgae have been used in traditional and folk medicine for hundreds of years.



\*Ochrophyta referred to in this study is synonymous with Phaeophyta (brown seaweed)

**Fig. 2.5. The total number of new marine natural products from macroalgal phyla over four decades (1965-2012).** (Taken from Leal *et al.*, 2013).

According to the *Dictionary of Marine Natural Products* (Blunt and Munro, 2008), MNPs are categorised into the following groups: aliphatic, alkaloids, simple aromatic, amino acids and peptides, carbohydrates, oxygen heterocycles, polyketide, polypyrroles, steroids, and terpenoids. One of the categories of brown macroalgal MNPs that has received a considerable amount of interest are sulphated polysaccharides (e.g. fucans, laminarans, galactans, sargassans) (Table 2.2).

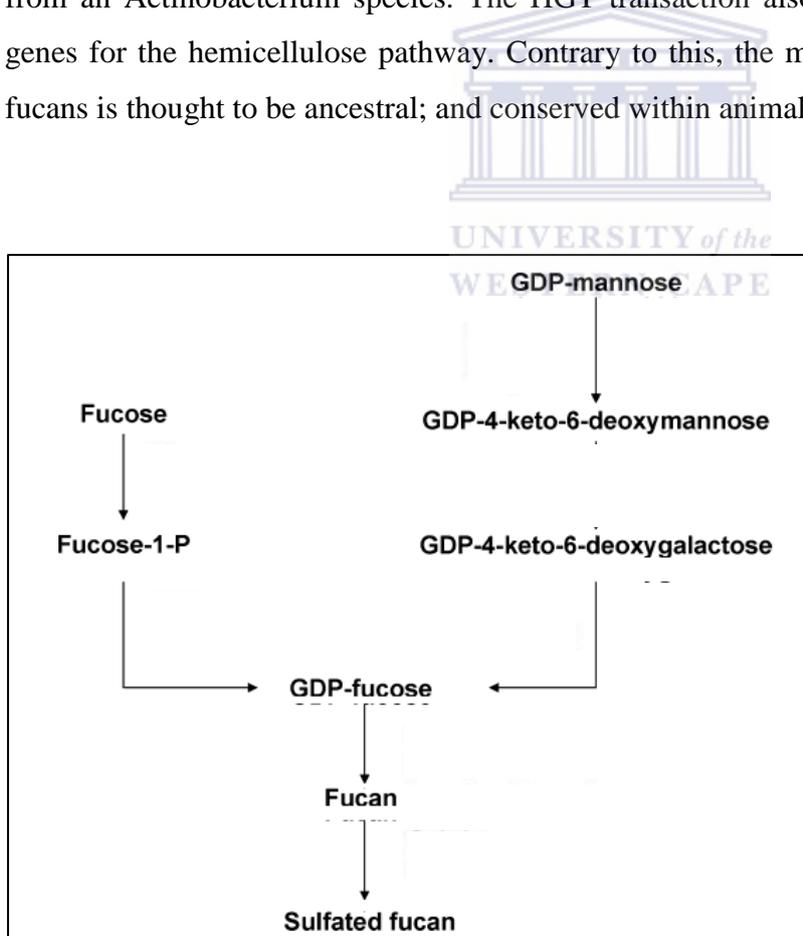
**Table 2.2. Bioactive polysaccharides from marine algae.** (Adapted from Michalak and Chojnacka, 2015).

	<b>Brown algae (Phaeophyta)</b>	<b>Red algae (Rhodophyta)</b>	<b>Green algae (Chlorophyta)</b>
<b>Polysaccharides</b>	Alginate	Agar	Amylose
	Cellulose	Carrageenan	Amylopectin
	Fucoidan	Cellulose	Cellulose
	Laminarin	Fucellaran	Inulin
	Sargassan	Mannan	Mannan
		Porphyran	Pectin
		Xylan	Xylan
			Ulvan

#### 2.6.4) Genomic approaches to elucidating marine bioactive compounds

*E. siliculosus* represents the first model organism for the brown algae and has helped to elucidate the underlying genetics that govern the production of compounds of interest (Michel *et al.*, 2010). *E. siliculosus* was chosen based on a multifactorial set of characteristics such as an extensive history of experimental research (diversity and taxonomy studies), complex multicellularity, global distribution, compact genetic profile (214 Mbp), physical size, high fertility and growth rates, and extensive knowledge of its life cycle (which can be completed *in vitro* in three months) (Charrier *et al.*, 2008). *E. siliculosus* genome data allowed researchers to hypothesise how molecular adaptations to intertidal environments and evolution of multicellularity arose (Michel *et al.*, 2010). The genomic data also delivered a detailed interpretation of the carbohydrate metabolism of brown algae. Michel *et al.* (2010), reported that cell walls of brown algae share components with terrestrial plants (e.g.

cellulose) and with animals (e.g. sulphated fucans), but also contain exclusive polysaccharides such as alginates. Analysis of the *Ectocarpus* genome allowed researchers to gain insight into molecular basis of these metabolic pathways. Bioinformatic tools were used to determine which enzymes are involved in the biogenesis and remodelling of alginates, cellulose, and fucans and a phylogenetic tree of the key enzymes involved was constructed. They discovered that *Ectocarpus* metabolic networks are lacking known families of cellulases, and expansins, which are enzymes involved in cell wall expansion that are commonly found in metabolic pathways of terrestrial plants. Thus, it can be inferred that novel mechanisms and/or proteins must exist in this alga for cell wall expansion. This data describes a multifaceted evolutionary history of the cell wall components of brown algae, many of which are bioactive compounds of interest. Furthermore, it was determined that cellulose synthesis was passed along from a red algal endo-symbiont, and that the final steps of the alginate biosynthesis pathway were acquired through horizontal gene transfer (HGT) from an Actinobacterium species. The HGT transaction also resulted in the acquisition of genes for the hemicellulose pathway. Contrary to this, the metabolic pathway for sulphated fucans is thought to be ancestral; and conserved within animals (Figure 2.6).



**Fig. 2.6. Diagram of the metabolic network of the sulphated fucans of *Ectocarpus siliculosus*.** (Taken from Michel *et al.*, 2010). FK, L-fucokinase; GFPP, GDP-fucose pyrophosphorylase; GFS, GDP-L-fucose synthetase; GM46D, GDP mannose4,6-dehydratase.

## 2.7) Fucoidan: Sulphated polysaccharides from the seas

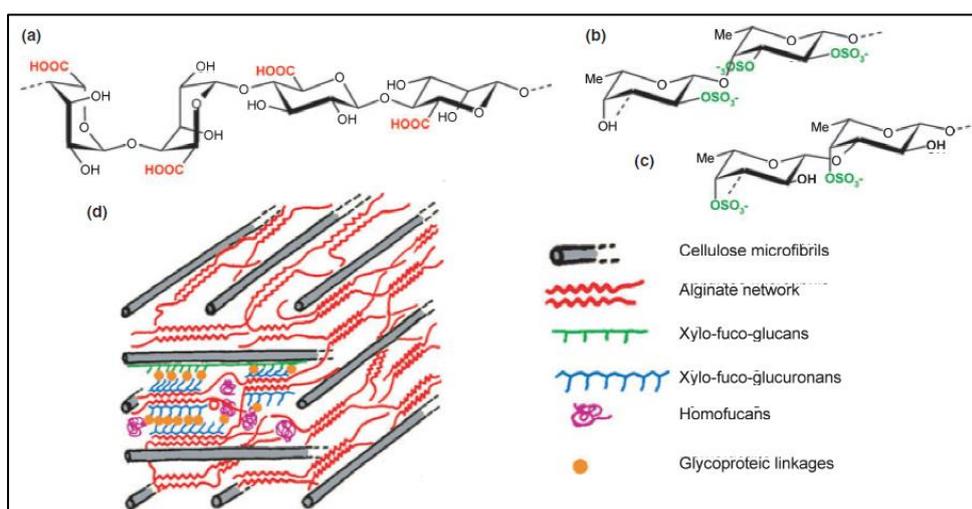
### 2.7.1) Structure and chemistry

The largest constituent of algal polysaccharides are found within their cell walls, thereby aiding in structural rigidity, and offering protection against mechanical stress caused by intertidal wave currents (Skriptsova *et al.*, 2015). Similar to green plants, brown algae also produce crystalline cellulose (fibres) but this accounts for only 1-8% of the dry weight of the thallus (Michel *et al.*, 2010). The main cell wall components are anionic polysaccharides such as alginate and fucoidan. These polysaccharides (alginate:fucoidan:cellulose) are found at an average weight ratio of 3:1:1 in mature brown macroalgae (Figure 2.7). Most of the polysaccharides within brown marine algae are sulphated, such as fucoidan, which consists of a pentose sugar backbone comprising of (1-3) and (1-4)  $\alpha$ -L- fucopyranose residues and also contain side chains of sulphate and/or acetyl groups (Ale *et al.*, 2011). Monosaccharides sugars such as mannose, galactose, xylose and rhamnose, as well as protein and uronic acid moieties complete the structure. *Sargassum* species are slightly different as their polysaccharides are composed of sulphated galactofucans known as 'sargassans', and consist of (1-6)- $\beta$ -D-galactose and/or (1-4)- $\alpha$ -D-glucuronic acid, ending in either  $\beta$ -D-xylose or (1-4)- $\alpha$ -D-glucose (Duarte *et al.*, 2001; Ale *et al.*, 2011a).

Marine derived sulphated polysaccharides of algal origin, such as fucans and galactans, are often heterogeneous, displaying different chemical structures' at species level (Pomin and Mourão, 2008). However, these chemical compounds show a degree of conserved structural similarity at the Phylum level (Pomin and Mourão, 2008). Fucoidans from brown algae differ from fucoidans isolated from marine invertebrates, such as sea urchins and sea cucumbers, which display a regularity in structure with respect to linearity of sugar backbone; degree of sulphation and lacks branching, even when isolated from different species (Berteau and Mulloy, 2003). Brown algal fucoidans display extremely branched fucoidans ranging from high uronic acid, low sulphate containing polysaccharides, with noteworthy amounts of D-xylose, D-galactose, and D-mannose (xylofuco-glucuronan and xylo-fuco-glucans) to highly sulphated homofucan compounds (Michel *et al.*, 2010). The sulphated fucans from the order Fucales contain extensive repeat units of the disaccharide (  $\rightarrow$  4)- $\alpha$ -L-fucose-2,3-disulfate-(1  $\rightarrow$  3)- $\alpha$ -L-fucose-2-sulfate-(1  $\rightarrow$  ) (Figure 1.7b). Whereas, the sulphated fucans

from the order Laminariales and Ectocarpales exhibit a structure primarily consisting of 3-linked  $\alpha$ -l-fucose residues mostly sulphated at C-4 position. (Figure 2.7c). As illustrated below, brown macroalgal fucoidans have a common structure, but show a large level of structural diversity at the species level (Table 2.3) (Ale *et al.*, 2013).

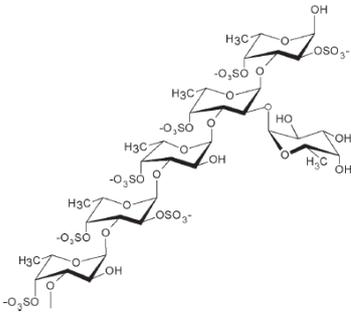
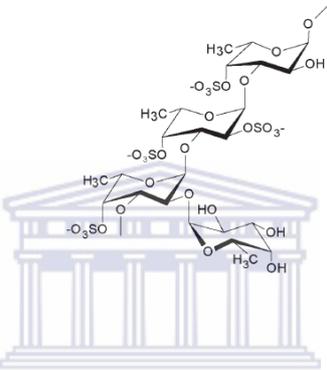
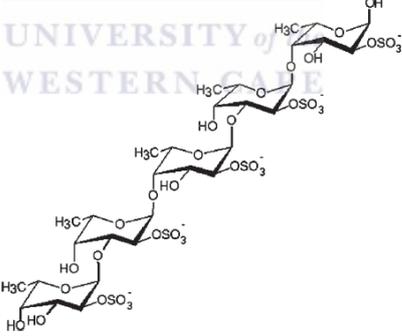
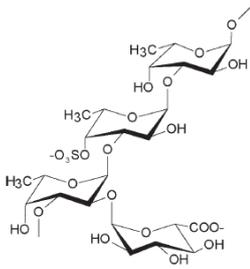
The chemical composition of the extracted sulphated polysaccharide may be subject to bias due to factors such as method of extraction, origin of the compound (e.g., species or geographical location), seasonal or climatic conditions, and/or maturity of the seaweed (e.g., juvenile or adult life cycle). Purification of sulphated fucans, such as fucoidan, is proposed to be necessary for optimal bioactivity of the compound. The heterogeneity of these highly complex polysaccharides are problematic and are hypothesised to be caused by fucose branch lengths; scattered sulphation around the core structure; different glycosidic bonds and other chemical characteristics such as acetylation, methylation, and pyruvilation (Pomin and Mourão, 2008). Techniques such as partial acid hydrolysis, desulphation, or deacetylation have been utilised prior to chemical analysis by mass spectroscopy (MS), matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF), or nuclear magnetic resonance (NMR), in attempt to resolve the complex heterogeneity of these compounds (Bilan *et al.*, 2002; Berteau and Mulloy, 2003; Bilan *et al.*, 2006; and Anastyuk *et al.*, 2009). However, with every pre-treatment step employed the original structure of the compound is altered (Table 2.4).



**Fig. 2.7. Structures of the main polysaccharides typical of brown algae: (a) alginate; (b) sulphated fucan from Fucales; (c) sulphated fucan from Ectocarpales. (d) Hypothetical model of the biochemical organization of cell walls of brown algae. (Taken from Michel *et al.*, 2010).**

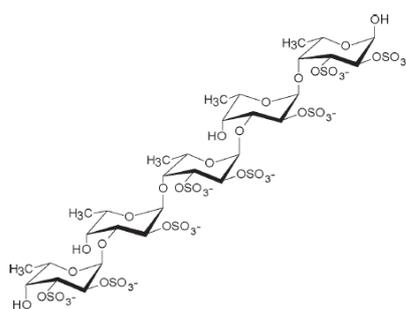
**Table 2.3. Brown algal fucoidans from different species displaying the compounds heterogeneity.**

(Adapted from Ale *et al.*, 2013).

Seaweed species	Order	Fucoidan structural element	Characteristics
<i>Chorda filum</i>	Laminariales		Contains a poly-a(1A3)-fucopyranose backbone with a high degree of branching, mainly of a(1A2)-linked single units. Sulphated at O-4 (mainly) and O-2 positions. Some a(1A3)-linked fucose residues are 2-O-acetylated.
<i>Laminaria saccharina</i>	Laminariales		Mainly made up of 4-sulfated a(1A3)-linked a-L-fucopyranose residues, some of which are additionally 2-sulfated or carry a 2-O-a-L-fucopyranosyl substituent.
<i>Fucus serratus L.</i>	Fucales		Contains a backbone built of alternating 3- and 4-linked a-L-fucopyranose moieties, with about half of the 3-linked residues being substituted at C-4 by trifucoside units a-L-Fucp-(1A4)-a-L-Fucp-(1A3)-a-L-Fucp-(1A) (not shown)). Sulphate groups have been shown to be substituted mainly on C-2 and sometimes C-4, although some terminal fucose residues may be non-sulphated.
<i>Cladosiphono kamuranus</i>	Chordariales		The main structure is a linear backbone of a(1A3)-fucopyranose with half of the sulphate substitutions at C-4, and a portion of the fucose residues are O-acetylated. The a-glucuronic acid residues are linked to the C-2 positions of the fucose residues which are not substituted by a sulphate group.

*Ascophyllum  
nodosum*

Fucales



An  $\alpha(1A3)$ -linked fucose backbone and a high proportion of  $\alpha(1A4)$  linkages mainly sulphated at O-2, to a lesser extent at O-3, and only slightly at O-4, and fucose 2, 3-O-disulfate residues were observed. Anticoagulant activity appears related to 2-O-sulfation and 2,3-O-disulfation levels.

**Table 2.4. Chemical compositional analysis of algal extracts from brown seaweeds produced using different extraction methods.** (Adapted from Ale *et al.*, 2013).

Seaweed species	Order	Extraction method	Composition
<i>Cladosiphono kamuranus</i>	Chordariales	Seaweed-H <sub>2</sub> O suspension was treated with 30% HCl (pH 3) at 100 °C for 15 min. The supernatant was neutralized with NaOH, precipitated with CaCl <sub>2</sub> and ethanol for 20 h at 4 °C, precipitate was dissolved with H <sub>2</sub> O and subsequently dried.	Fucose, glucose, uronic acid and sulphate.
<i>Sargassum stenophyllum</i>	Fucales	Extracted with water 7% (w/v), 12 h, 3x. Precipitated with ethanol and CaCl <sub>2</sub> and cetylpyridiniumchloride. Soluble fraction (SF) was then fractionated (F1-F6).	Fucose, xylose, mannose, galactose, glucose, sulphate and uronic acid.
<i>Fucus evanescens</i> ; <i>Fucus distichus</i>	Fucales	Pre-treatment: MeOH-CHCl <sub>3</sub> -H <sub>2</sub> O (4 : 2 : 1), then extracted with 2% CaCl <sub>2</sub> for 5 hr at 85 °C, precipitated and the precipitate was washed with water, stirred with 20% ethanolic solution and dissolved with water.	Fucose, xylose, galactose, uronic acid and sulphate.

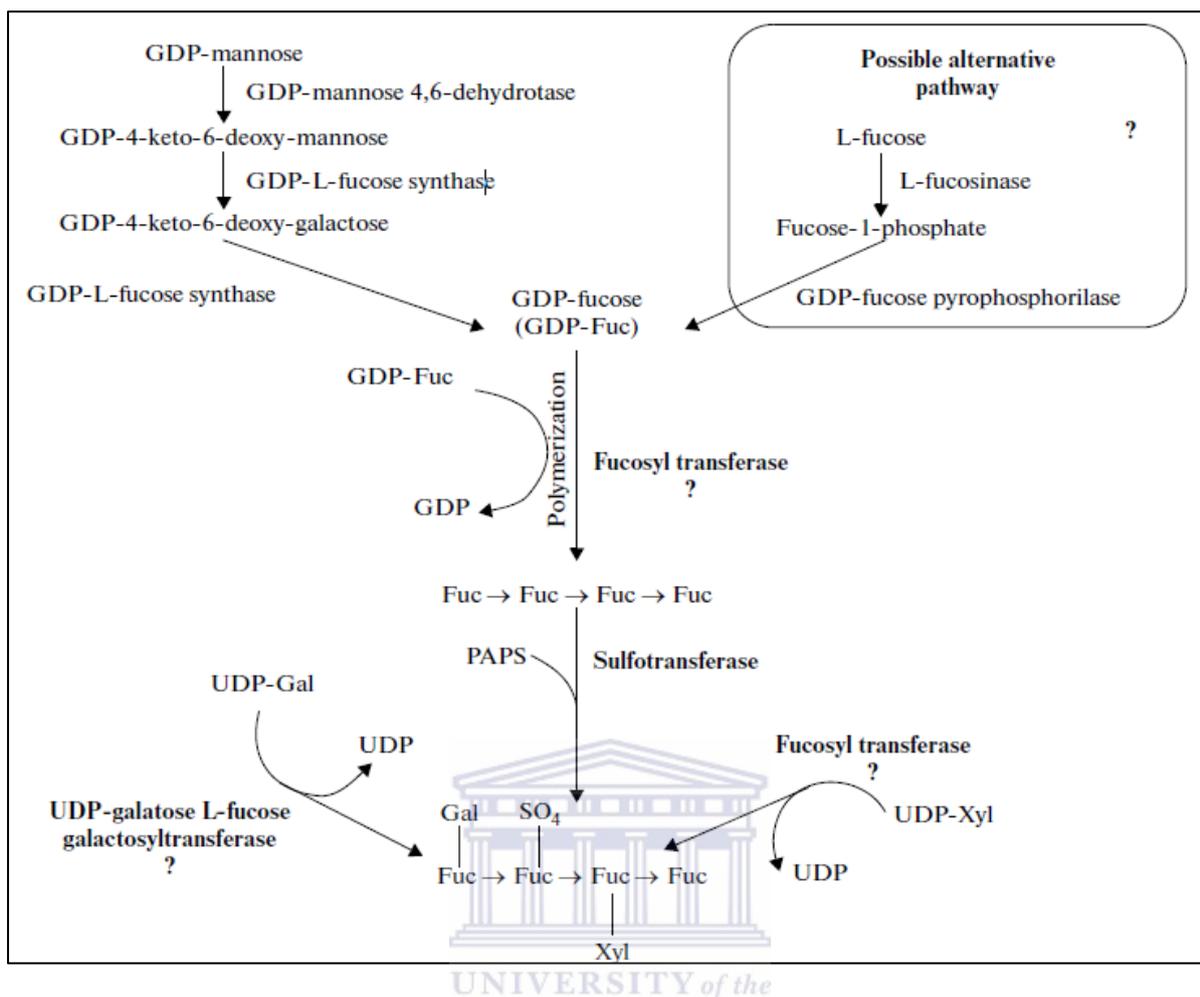
<i>Fucus serratus</i>	Fucales	Pre-treatment: MeOH-CHCl <sub>3</sub> -H <sub>2</sub> O(4 : 2 : 1), then extracted with 2% CaCl <sub>2</sub> for 5 hr at 85 °C, the extracts were collected by centrifugation, combined, dialyzed and lyophilized.	Fucose, xylose, mannose, glucose, galactose, uronic acid and sulphate.
<i>Chorda filum</i>	Laminariales	Extracted with CHCl <sub>3</sub> -MeOH-H <sub>2</sub> O (2 : 4 : 1) followed by 80% ethanol, then extracted successively with 2% CaCl <sub>2</sub> at 20 and 70 °C, then with HCl (pH 2) and 3% Na <sub>2</sub> CO <sub>3</sub> , precipitated with calcium salt.	Fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate.
<i>Adenocystis utricularis</i>	Ectocapales	80% ethanol, 24 hr, 70 °C pre-treatment, then extracted with water (or 2% CaCl <sub>2</sub> ; or HCl) for 7 hr, RT, followed by exhaustive extraction at 70 °C.	Fucose, xylose, mannose, glucose, galactose, uronic acid and sulfate.
<i>Undaria pinnatifida</i>	Laminariales	Ground seaweed extracted twice at RT for 6 hr with 1% H <sub>2</sub> SO <sub>4</sub> , neutralized with 10% NaOH and lyophilized.	Fucose, mannose, xylose, rhamnose, galactose, glucose and sulfate.
<i>Laminaria religiosa</i>	Laminariales	Water extraction at boiling temperature for 4 hr, fucoidan fraction was obtained by using 0.09 HCl at 4 °C for 2 hr, then precipitated with 85% ethanol and dried.	Fucose, mannose, xylose, rhamnose, galactose, glucose and sulfate.

### 2.7.2) Biological function in macroalgae

The elusive nature of fucoidan is not only limited to its bioactive properties in mammalian systems but also to the physiology of its host. The function of fucoidan in marine organisms has only been proposed to date. It has been deduced that fucoidan plays a role in protection

against desiccation, as the amount of fucoïdan found in intertidal algal species is higher than that found in sublittoral species (Skriptsova *et al.*, 2012). Another hypothesis is that fucoïdan plays a role in the structural rigidity of the cell wall of algae as seasonal shifts in fucoïdan content mirrors the seasonal shift from winter to summer which results in more sunlight exposure. Sulphated fucans of brown macroalgae are biologically synthesised in the dictyosomes of Golgi bodies, and this includes the synthesis of fucoïdan chains (Skriptsova *et al.*, 2015). The common, active form of L-fucose, GDP-fucose is regarded as the fucan precursor. GDP-fucose is synthesised in most bacteria, mammals, and plants produced from GDP-mannose by a *de novo* pathway (Figure 2.8). Thereafter, the precursor GDP-mannose is enzymatically converted into GDP-4-keto-6-deoxymannose (Figure 2.8). This keto-intermediate is then converted into GDP-fucose in two steps, catalysed by a bifunctional epimerase/reductase, GDP-1-fucose synthetase which has been found in the *E. siliculosus*. There is an alternate proposed pathway in brown macroalgae whereby GDP-fucose is synthesised from L-fucose through fucose-1-phosphate (Figure 2.8). This is achieved when cytosolic fucose is phosphorylated by L-fucokinase, followed by the enzymatic reversible reaction of fucose-1-phosphate with GDP to form GDP-fucose. However, these enzymes have not been found in brown algae so this pathway remains putative.

Skriptsova *et al.* (2015) established that fucoïdan compounds are involved in construction of cell walls and physical support of alga structure. Other significant physiological roles of fucoïdan in brown algae include release of spores and gametes from their reproductive organs as well as the morphogenesis of zygotes of fucoïd algae. Fucoïdan also serves to facilitate the polarity of the cell, as well as fixing the cell division axis, which is responsible for the development of the rhizoidal and apical poles of juvenile thalli. The sulphated nature of fucoïdan compounds are also vital in capturing divalent ions such as  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$  ions from the ecosystem. This form of ion exchange with its environment allows for the adaption of algae to environmental stressors such as rapid fluctuations in pH, salinity, and lethal heavy metal compounds.



**Fig. 2.8. Metabolic map of the biosynthesis of sulphated fucans in brown algae.** Fuc, fucose; Gal, galactose; Xyl, xylose; UDP, uridine diphosphate; GDP, guanosine diphosphate. Enzymes whose existence remains unconfirmed are highlighted in bold italics. (Taken from Skriptsova *et al.*, 2015)

### 2.7.3) Bioactive properties

Fucoidan isolated from brown macroalgae have been shown both *in vitro* and *in vivo* to have bioactive properties in mammalian systems, with some compounds currently undergoing clinical trials (Kwak, 2014; Fitton *et al.*, 2015). These bioactive properties include: anti-viral; anti-microbial; anti-cancer; anti-metastatic; immunomodulatory; anti-thrombic; anti-inflammatory; and anti-oxidant activity. The precise mechanisms of action of these compounds in mammalian systems are not fully understood. However, partial elucidation of the structure of these compounds has led researchers to infer that the following are key contributors: molecular weight, structural make-up, sulphation pattern and content, charge

density, purity, and monosaccharide composition (Haroun-Bouhedja *et al.*, 2000; Pomin and Mourão, 2008; Usov and Bilan, 2009; Jiao *et al.*, 2011; Senthilkumar *et al.*, 2013).

Previous studies have reported that the bioactivity of fucoidan in mammalian systems is both size- and dose-dependent (Yamasaki-Miyamoto *et al.*, 2009; Cho *et al.*, 2010; Lee *et al.*, 2012; Morya *et al.*, 2012; Kimura *et al.*, 2013; Zhang *et al.*, 2013; Kwak, 2014). The average reported size of fucoidan is ~20 kDa with low molecular weight fucoidan (LMWF) ranging in size from <10 kDa, medium molecular weight fucoidan (MMWF) between 10-10,000 kDa, and high molecular weight fucoidan (HMWF) is categorised as >10,000 kDa (Senthilkumar *et al.*, 2013).

LMWF can be produced by several experimental techniques including enzymatic, gamma-irradiation, acidolysis, ultrasound, electronic beam and mechanical shearing (Choi *et al.*, 2009; Cho *et al.*, 2011; and Jo and Choi, 2014). LMWF has been found to have anti-thrombic (Chabut *et al.*, 2003; Chabut *et al.*, 2004), anti-cancer (Ozawa *et al.*, 2006; Choi and Kim, 2013; Kimura *et al.*, 2013; Mak *et al.*, 2014), and anti-oxidant (Wang *et al.*, 2010; Mak *et al.*, 2013; Lim *et al.*, 2015) bioactivities (Morya *et al.*, 2012). LMWF also show endothelial cell migration (Matsubara *et al.*, 2005; Lake *et al.*, 2006), and 3D culture stimulant abilities (Changotade *et al.*, 2008), as well as alloimmune injury protective activity (Alkhatib *et al.* 2006), and cardiovascular disease therapeutic activities (Bachelet *et al.*, 2009; Yu *et al.*, 2014).

Similarly, HMWF could be quite useful as an anti-coagulant or anti-thrombic compound in mammalian systems (Jiao *et al.*, 2011). The structure of HMWF fucoidan is similar to that of heparin, an anti-coagulant, which binds to blood coagulating protein thrombin. Both fucoidan and heparin are heavily sulphated and contain high molecular weight sugar chains. The exact mechanism of this bioactivity is not fully understood, but several investigations have reported that direct and indirect inhibition of thrombin by the activation of thrombin inhibitors such as anti-thrombin (ATIII) and heparin cofactor II could potentially be its mechanism of action (Grauffel *et al.*, 1989; Mauray *et al.*, 1995; Kuznetsova *et al.*, 2003; Ustyuzhanina *et al.*, 2015). Fucoidan has also been shown to promote coagulation by obstructing tissue factor pathway inhibitor (TFPI) (Zhang *et al.*, 2015).

Sulphated polysaccharides, such as fucoidan, exhibit immunomodulatory properties that could potentially trigger the immune response or control cellular immune activity which alleviates undesirable effects such as inflammation (Chen *et al.*, 2008). Fucoidans may have

several targets in both immune and inflammatory systems which can affect the progression of diseases as well as the development of tumours and metastasis (Groth *et al.*, 2009). The immune related activities of algal fucoidan include its ability to interfere with the movement of leukocytes to sites of inflammation.

Fucoidan has been reported to show anti-viral activity against enveloped viruses, including human cytomegalovirus (HCMV), human immunodeficiency virus (HIV), herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2) and tobacco mosaic virus, a plant pathogen (Ponce *et al.*, 2003; Usov and Bilan, 2009). Active extracts have been isolated from brown algal species such as *Undaria pinnatifida*, *Adenocystis utricularis*, *Sargassum horneri*, *Sargassum patens*, *Stoechospermum marginatum* and *Cystoseira indica* (Ponce *et al.*, 2003; Hayashi *et al.*, 2008; Ermakova *et al.*, 2011). Researchers have postulated that the anti-viral activity can be linked to the degree of sulphation, with increasing degrees of sulphation having higher efficacies against viral penetration and adsorption to host cells (Harden *et al.*, 2009; Wozniak *et al.*, 2015). Although other researchers have reported that an alternative mode of action involves the early blocking of viral entry, as shown in the case of shielding off the positively charged amino acids present in the viral envelope glycoprotein gp120 of HIV (Thuy *et al.*, 2015).

Fucoidans have also exhibited several anti-microbial activities against pathogenic bacteria, such as *Pseudomonas aeruginosa*. Fucoidan extracts prohibited biofilm formation, inhibited toxic metabolites, inhibited secretion of virulence factors, suppressed expression of quorum sensing genes, and motility in *P. aeruginosa* (Kandasamy *et al.*, 2015). These FCSPs also protected *Caenorhabditis elegans* from *P. aeruginosa* infection by altering immune gene expression in the presence of the pathogen and by altering functional pathways in the presence of the pathogen.

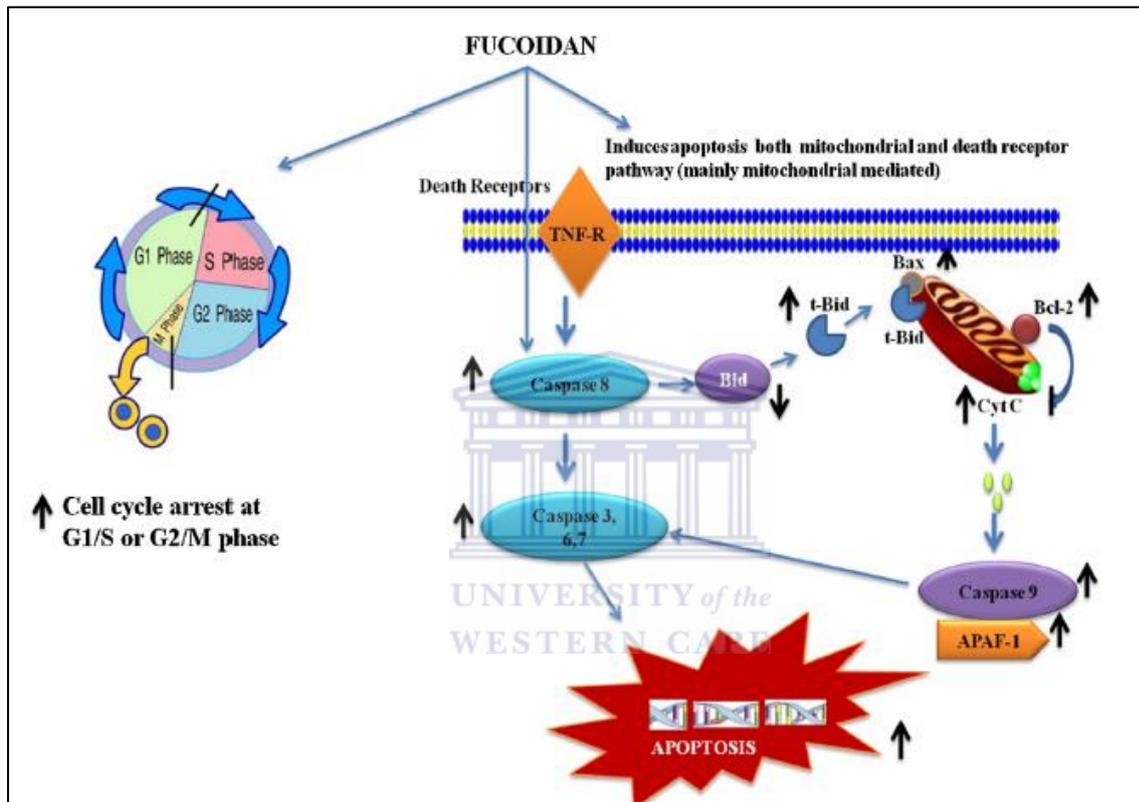
#### **2.7.4) Fucoidan and cancer: specificity and efficacy**

Cancerous cells or tumours are the uncontrolled growth or proliferation of abnormal cells that should have been destroyed by cellular regulation mechanisms such as apoptosis or other immune responses but have evaded these cellular mechanisms (Hanahan and Weinberg, 2000). The economic cost of cancer is a pandemic problem with the estimated cost on the European economy being approximately more than 50 billion euros (€) in care, treatment,

and lost productivity (Hanahan, and Weinberg, 2011). The hunt for cancer therapeutics have led to a vast range of therapies, ranging from chemical, radiation treatment, surgery, NPs, and holistic therapies. However, every cancer is different in terms of growth, mechanism of proliferation, and metastasis (Weinberg, 2007). Finding a cancer therapy that is effective in destroying tumours, possesses a low cytotoxicity (not affecting non-cancerous cells), and selectively only targeting the cancerous growth has focussed research toward MNPs (Hanahan, 2014). Fucoidan, the sulphated polysaccharide, is one such bioactive compound possessing these unique abilities. For years fucoidan has been sold as a dietary or nutraceutical product with various therapeutic properties (Kwak *et al.*, 2014). Fucoidan or FCSPs have advantages such as low toxicity, oral bioavailability, and multiple mechanisms of action. Therefore, in Asian countries such as China, Japan, and Korea, fucoidan derived foods and drinks are administered to cancer patients as an oral adjunct. Fucoidan has several pharmacological actions which affect carcinogenesis, inflammation, oxidative stress, and vascular physiology (Fitton *et al.*, 2011). While the underlying mechanism of action of fucoidan as an anti-cancer agent has not been fully elucidated, it has been proven that it includes cytotoxicity and apoptosis of cancer cells. Fucoidan can also act as an anti-angiogenic agent preventing the metastasis of cancer by suppressing signalling molecules such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Ye *et al.*, 2005; Ustyuzhanina *et al.*, 2015). Additionally, fucoidan can stimulate dendritic cells (DCs) (Jin *et al.*, 2009; Cheng *et al.*, 2010) and natural killer cells (NK) (Ale *et al.*, 2011b; Azuma *et al.*, 2012) improving anti-cancer immunity by means of immune cell activation and the production of anti-cancer cytokines.

The central processes of development of the cell cycle and of apoptosis involve the complex interaction of several co-ordinated cascades of protein families (e.g. the caspases) in a systematic manner (Kwak *et al.*, 2014). These processes are related and are key factors during the communication of malignant cells to chemotherapeutic treatments. The cell cycle is a large energy requiring process by which cells act to divide. If the series of phases in the cell cycle are not ordered, or are hindered in some way, the duplication and segregation of that organism's genome will be incorrectly inherited by future generations. The cell cycle's ordered series of events starts with quiescence (G0 phase) and progresses to proliferation (G1, S, G2, and M phases) and back to quiescence (Norbury and Nurse, 1992). With the advancement of knowledge of cell cycle deregulation in cancers, research into marine compounds as anti-cancer agents has been accelerated. Studies have shown that MNPs can

modulate signalling pathways leading to cell cycle regulation or directly modify cell cycle regulatory molecules in cancer therapies (Figure 2.9) (Kwak *et al.*, 2014). Fucoidan has been reported to dose-dependently increase G0/G1-phase in a hepatocarcinoma cell line (Huh7) accompanied by a decrease in the S phase, which inferably suggests that fucoidan may cause the cell cycle arrest at the G0/G1 phase (Senthilkumar *et al.*, 2003; Nagamine *et al.*, 2009).

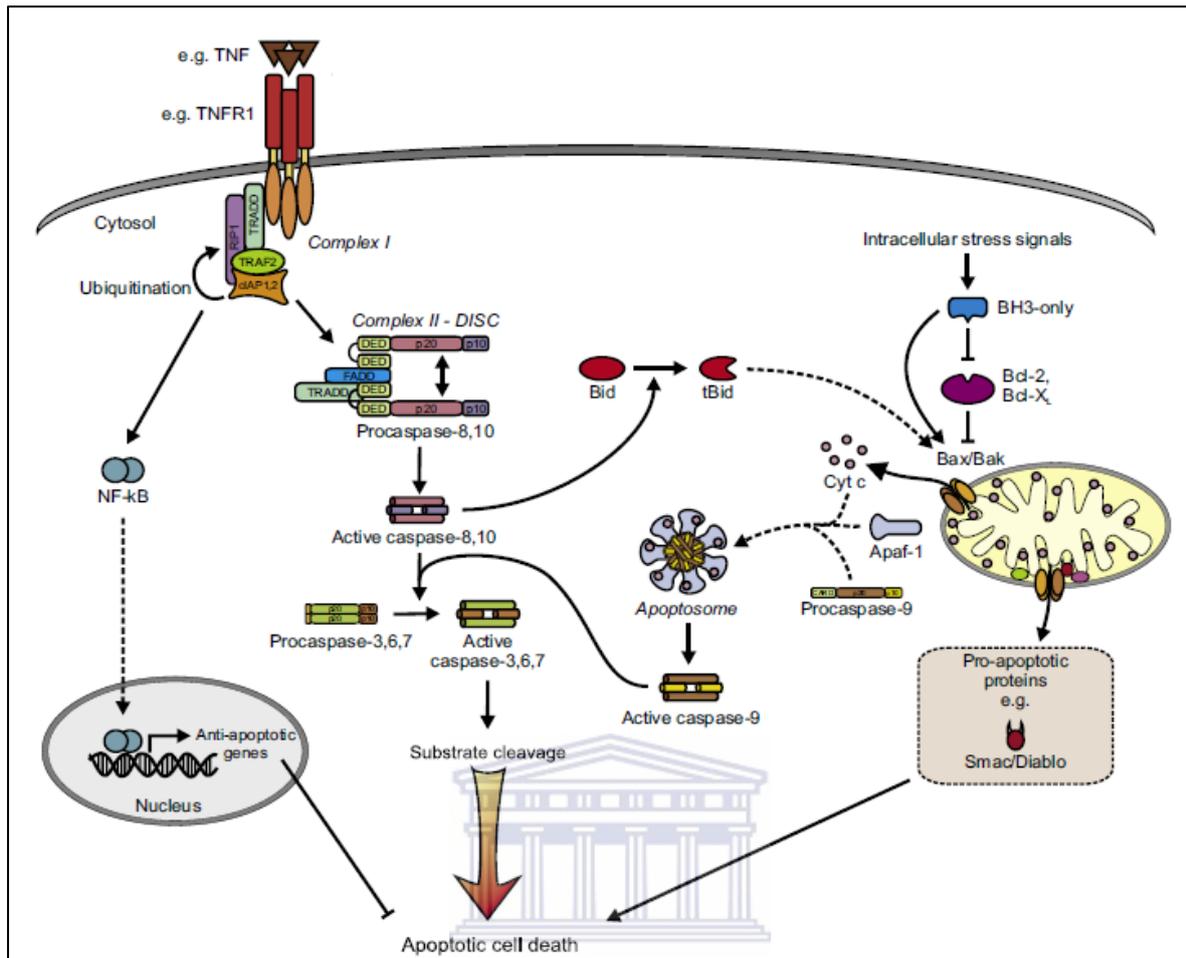


**Fig. 2.9. Representation of fucoidan mechanisms of action against cancer by cell cycle arrest and apoptosis.** Fucoidan arrest cell cycle at G1/S or G2/M phase. Apoptosis occurs via extrinsic and intrinsic pathways. Fucoidan can activate both pathway mediated apoptosis processes. Black colour small arrow indicates downregulation and upper arrow indicates upregulation of protein activation by fucoidan. (Taken from Senthilkumar *et al.*, 2003).

Programmed cell death, or apoptosis is facilitated through two major pathways, i.e. the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (Sprick *et al.*, 2004; Duprez *et al.*, 2009). In the extrinsic pathway stimulation of the death receptors Fas and tumour necrosis factor receptor-1 (TNFR-1), leads to the gathering and formation of a death inducing signalling complex, which includes the adaptor protein Fas-associated death

domain (FADD) and initiator caspases, caspase-8 (Figure 2.10). Once caspase-8 is activated, a cascade of downstream events occur, which include activation of effector caspases, caspase-3 and caspase-7 (Ashkenazi and Dixit, 1998) (Figure 2.10). Caspase-8 also functions to cleave Bcl-2 interacting protein (Bid) into truncated Bid (tBid) and interacts with pro-apoptotic protein Bax (Figure 2.10). The build-up of Bax in the mitochondria of the cell aids in the release of cytochrome c into the cytosol (Desagher *et al.*, 1999; Eskes *et al.*, 2000; Li and Yuan, 2008). In the intrinsic pathway the death receptors communicate death signals to mitochondria, consequently releasing several mitochondrial intermembrane space proteins such as cytochrome c (Figure 2.10). These mitochondrial intermembrane space proteins then associate with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 (intact enzyme) to form the apoptosome (Figure 2.10). Once caspase-9 is activated (by enzymatic cleavage) it can fulfil its function to cleave and activate effector caspases, caspase-3 and caspase-7 (Hengartner, 2000).

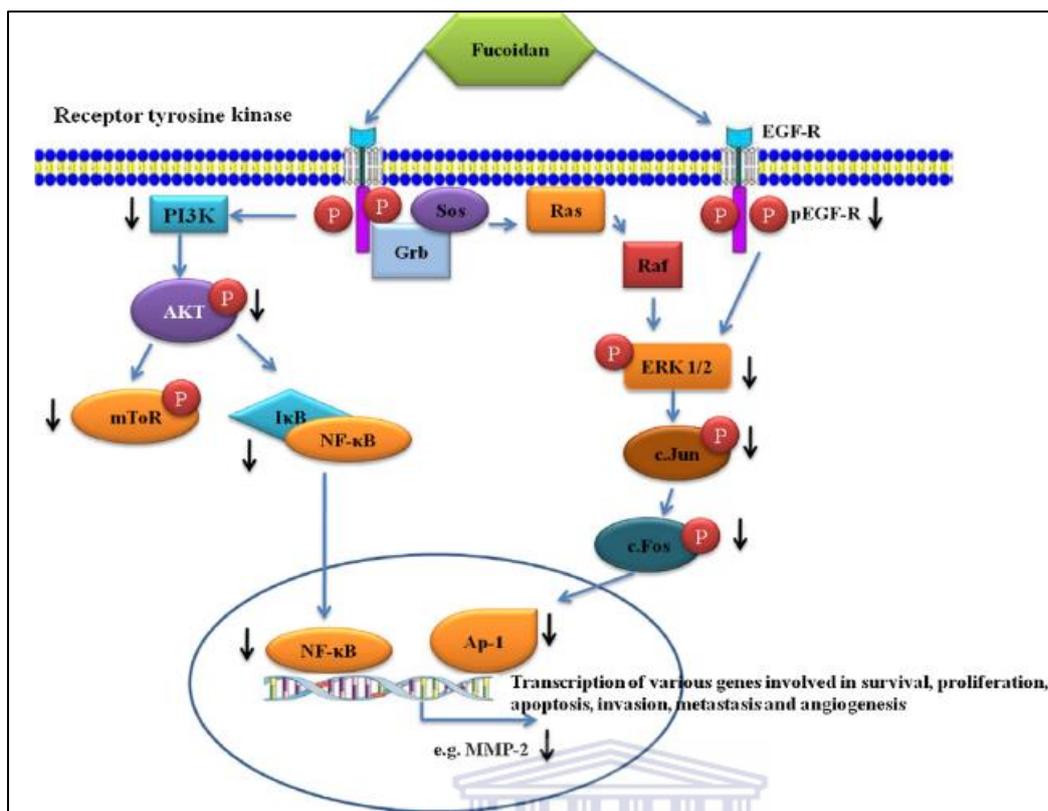
Research of brown algal fucoidan as an anti-cancer agent has shown strong anti-proliferative and apoptotic effects on human breast cancer cell line MCF-7 in a dose-dependent manner (Yamasaki-Miyamoto *et al.*, 2009). However, this fucoidan showed no cytotoxicity to human mammalian epithelial cells (HMEC). Apoptotic cell death has unique characteristics such as induction of chromatin condensation, fragmentation of nuclei and DNA; and cleavage of apoptotic specific proteins. Fucoidan triggered clustering of sub-G1 population, chromatin condensation, and internucleosomal fragmentation of DNA. The effector caspases caspase-3 and caspase-7 activate deoxyribonuclease (DNase), which results in the cleavage of DNA in response to apoptotic stimuli. Interestingly, MCF-7 cells do not express functional caspase-3 but does express caspase-7. Caspase-7 is an executioner caspase which cleaves poly ADP-ribose polymerase (PARP) which is a hallmark of apoptotic cell death in the MCF-7 cell line (Kaufmann *et al.*, 1993; Liang *et al.*, 2001). Another study showed that PARP was cleaved and caspase-7 activated in MCF-7 cells treated with fucoidan and fucoidan-induced apoptosis can be inhibited in the presence of caspase-7 inhibitor z-DEVD-fmk (Aisa *et al.*, 2005). A hallmark of fucoidan-induced apoptosis is the activation of caspase 3 (Teruya *et al.*, 2007). Thus, caspase-7 is required for fucoidan-induced apoptosis in MCF-7 cell lines but not caspase-3. Mitochondrial depolarization is another hallmark of apoptosis in MCF-7 cells, and Zhang *et al.* (2011) showed that a fucoidan extract increased mitochondrial depolarization by up-regulating the expression of pro-apoptotic proteins Bax and Bad, and down-regulating the expression of anti-apoptotic proteins Bcl-2 and Bcl-x.



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Fig. 2.10. Extrinsic and intrinsic pathways of apoptosis. (Taken from Duprez *et al.*, 2009).

MNPs have been shown to have chemopreventive and chemotherapeutic properties by the inhibition of phosphorylation of membrane receptors such as receptor tyrosine kinases (RTKs), epidermal growth factor receptors (EGFR) and platelet-derived growth factor receptor (PDGFR) (Senthilkumar *et al.*, 2013). These membrane receptors are involved in the transduction of mitogenic signals across the plasma membrane and in the facilitation of cell growth and propagation. Certain MNPs, such as fucoidan, effectively disrupt growth factor stimulated pathways, generally initiating a pathway consisting of Ras to Raf extracellular regulated kinase (ERK) to mitogen activated kinase/ERK-kinase (MEK) to activator protein (AP)-1 pathway (Figure 2.11). Mitogen activated protein kinase (MAPK) pathways are associated with in cellular propagation, differentiation, and apoptosis (Chang and Karin, 2001; Wada and Penninger, 2004). Whereas, the ERK1/2 pathway is implicated in the invasive or migratory behaviour of several malignancies.



**Fig. 2.11. Fucoidan action in growth signalling molecules and their pathways in cancer.** PI3K/AKT mediated pathway and EGF-R pathways are important in cancer progression. Fucoidan controls growth signalling molecules involved in the cell existence, propagation, apoptosis, invasion, metastasis and angiogenesis. Downward black colour arrow indicates that decreased activity of the molecules caused by fucoidan. Fucoidan (Taken from Senthilkumar *et al.*, 2013).

### 2.7.5) Fucoidan as a natural anti-oxidant

One of fucoidans most extensively researched bioactive properties is its ability to scavenge free radicals. Fucoidan acts as an anti-oxidant by neutralising ROS (Mak *et al.*, 2013). The food and pharmaceutical industry are constantly seeking natural anti-oxidants that can act as preservatives as well as therapeutic agents (Wang *et al.*, 2010) as chemically synthesised anti-oxidants are not stable and have potential environmental and health risks (Ito *et al.*, 1989). The consumption of fruits and vegetables that contain natural anti-oxidants has also been correlated to a lower risk of cardiovascular diseases and cancer (Thaipong *et al.*, 2006).

Rupérez *et al.* (2002) showed that fucoidan extracts from *Fucus vesiculosus* displayed significant ferric iron reducing anti-oxidant action, whereas de Souza *et al.* (2007) showed

that *F. vesiculosus* fucoidan exhibited superoxide radical scavenging ability. Fucan fractions from *Laminaria japonica* exhibited anti-oxidant properties in superoxide radical and hydroxyl radical scavenging assays (Wang *et al.*, 2009; Wang *et al.*, 2010; Zhao *et al.*, 2012). An investigation of the properties of fucoidan compounds which make it an effective anti-oxidant revealed that the sulphate content of the polysaccharide was positively correlated with superoxide radical scavenging activity (de Souza *et al.*, 2007; Wang *et al.*, 2010). Polysaccharide extracts from red and green seaweeds such as carrageenans (de Souza *et al.*, 2007) and ulvans (Zhang *et al.*, 2012) also display anti-oxidant properties that are related to their sulphate content. Other polysaccharide specific anti-oxidant properties such as metal chelating, free radical, and hydroxyl radical scavenging activities appear to be linked to sulphate:fucoase ratios (Jiao *et al.*, 2011).

In normal cells, ROS acts as secondary cell signalling messengers for several regular biological processes. However, under cellular homeostasis, physiologically produced ROS is eliminated through free radical scavenging systems. A hallmark of cancerous cell growth is the increase in the rate of production of ROS and a modified redox environment compared to cells in homeostasis (Glasauer and Chandel, 2014). Additionally, redox regulation and redox signalling have a pivotal part in the formation of tumours (tumorigenesis) and in the response to anti-cancer drugs. In the biology of cancer, ROS takes on two-part, contradictory roles. These implications for tumorigenesis are significant, especially with respect to cancer therapeutics that aim to modulate cellular redox levels. ROS has a fundamental role in cancer development and progression by inducing genetic mutations, genome stability, and abnormal pro-tumorigenic signalling. Conversely, high levels of ROS can also be toxic to cancer cells and may destroy cancer cells. To bring the state of oxidative stress to equilibrium, cancer cells intensify their anti-oxidant capacity, which suggests that high ROS levels may have the potential to block tumorigenesis. The two-sided role (a double-edged sword) of ROS in cancer development is seen when ROS contributes to cancer initiation, progression and metastasis, through instigation and modulation of signalling pathways, which control cell proliferation, survival, angiogenesis and metastasis (Weinberg *et al.*, 2010; Wallace, 2012). This pro-tumorigenic cell signalling cascade is the oncogenic role of ROS. However, the anti-cancer role is seen when excessive amounts of ROS in cancer cells induce cell death signalling, senescence, and cell cycle arrest. Wang *et al.* (2015) reported on the anti-oxidant properties of aqueous and ethanolic fucoidan extracts from *Sargassum cristaefolium* and how

it showed potential anti-cancer activity against HT-29 (human colon cancer cell line) cells by scavenging ROS, an important determinant in cancer progression.



## **2.8) Concluding Remarks: Towards isolating and chemically characterising an endemic fucoidan molecule**

Fucoidans are natural compounds from brown seaweeds that possess several potent bioactivities. However, these bioactive properties have not been elucidated due to the heterogeneity of the compounds. These polysaccharides differ from species to species, and the variability in structure and chemical composition is subject to several biases. Age, life-cycle, sexual maturity and geochemistry are intrinsic factors that affect the complex structural identity and complexity of these compounds. Extrinsic factors include extraction method, temperature, pH and time. Numerous studies have explored optimal extraction strategies for fucoidan from species from Europe, Asia, Australia, and New Zealand of commercial, environmental, and agronomical importance. However, to the best of our knowledge this is the first study investigating the extraction and characterisation of fucoidan from endemic brown seaweeds of South Africa. In order to possibly characterize heterogeneous fucoidan compounds a unified approach is required to holistically study these polysaccharides. A standardised extraction technique is needed to account for biodiversity of fucoidan from different seaweeds and to retain structural features, which are the key to understanding fucoidans bioactivities. Therefore, this study aims to identify a standardised fucoidan extraction technique, establish an effective purification and fractionation platform, and assess the bioactivity of these fucose-containing-sulphated-polysaccharides with emphasis on anti-cancer and cytotoxicity screening against human cancerous cell lines. This study will also assess the variability of fucoidan with respect to extraction method; comparatively analyse the chemical composition between crude and fractionated, and purified fucoidan; and assess if these factors influence overall bioactivity.

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# Chapter 3. Assessing the effect of extraction method on fucoidan heterogeneity

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## Abstract

Fucoidan is a marine-derived sulphated polysaccharide with useful properties ideal for food, chemical and pharmaceutical industries. The polysaccharide consists largely of L-fucose, has a highly heterogeneous structure and is of diverse origin. In this study different extraction methods were assessed for effect on fucoidan heterogeneity from *Ecklonia maxima*, *Laminaria pallida* and *Splachnidium rugosum*. Extraction methods employed hot water, hydrochloric acid or calcium chloride. Extracts were assessed for fucoidan yield and purity by colorimetric assays. The highest fucoidan yield was obtained with the hot water method as seen by highest L-fucose content. *Splachnidium rugosum* extracts contained ~5 times more L-fucose ( $265 \pm 49.2 \mu\text{g/mL}$ ) than *Ecklonia maxima* and *Laminaria pallida* extracts. The salt method yielded extracts free of contaminants, however L-fucose content in all extracts was >20 times lower. Acid extraction yielded the highest levels of uronic acid contamination and liberated sulphate from the fucoidan polysaccharide. The fucose-to-sulphate ratio for *Ecklonia maxima* was approximately 1:5, whilst the ratios for *Splachnidium rugosum* and *Laminaria pallida* were approximately 1:1 and 1:2, respectively. The acid and salt method removed all traces of protein contaminants, while the hot water method retained very low levels of protein ( $<40 \mu\text{g/mL}$ ). The extraction method used to isolate fucoidan was a determining factor in yield and purity. Chemical compositional analyses of hot water extracts were assessed by gas chromatography mass spectroscopy. *Splachnidium rugosum* and *Laminaria pallida* extracts consisted largely of L-fucose, while *Ecklonia maxima* fucoidan was characterized by an abundance of glucose. Other neutral sugars present at low amounts were galactose, mannose, rhamnose, xylose and fructose. The L-fucose ratio in crude and purified fucoidan extracts was highest in *Splachnidium rugosum* (79-98%) which was comparable to the ratio determined for the commercial *Fucus vesiculosus* control (86%).

### 3.1) Introduction

Fucoidan, laminarin, and alginic acid are the most concentrated water-soluble polysaccharides in brown macroalgae (seaweeds) (Skriptsova *et al.*, 2012). Unique rheological properties offer commercial value to medical, biotechnological, agricultural and food industries. Caution is required when isolating these polysaccharides from brown macroalgae as more abundant polysaccharides (or even unwanted contaminants) with similar properties are often co-extracted (Ale *et al.*, 2012).

Over the past century, fucoidan and other sulphated polysaccharides have been extracted from brown macroalgae with a myriad of extraction techniques (Ale *et al.*, 2011). Fucoidan compounds have been shown to possess enormous commercial and pharmaceutical value (Michalak and Chojnacka, 2015). However, to the best of our knowledge, fucoidan compounds from indigenous South African brown macroalgae have not been characterised or studied. The South African coastline is characterized by high biodiversity (Griffiths *et al.*, 2010) which has been largely untapped, primarily because of the large number of pristine marine protected areas (MPA). The pharmaceutical and biotechnology sector could benefit greatly by exploiting marine algal resources, which are currently mostly being used for mariculture (e.g. abalone feed). Moreover, abundant amounts of seaweed wash onto the shore where it decays. Thus, seaweed species are ideal candidates for bioprospecting studies geared towards the identification of bioactive compounds such as fucoidan.

The method used for algal polysaccharide extraction should be governed by the application of the end-point use of the compound (e.g. application in food industry or as a potential therapeutic agent). For example, the food industry requires good emulsifying agents, whereas in the pharmaceutical industry natural product derived drug targets should be of high purity, high bioactivity, bioavailability and have low cytotoxicity (Tseng, 2001; Pulz and Gross, 2004; Fitton *et al.*, 2015).

Fucoidan compounds have a high degree of structural diversity, with studies showing inter-compositional variation between different species of brown macroalgae, as well as intra-compositional variations of fucoidan within the same species (Lee *et al.*, 2006; Ale *et al.*, 2013). The fucoidan extraction method adds significantly to its diversity, introducing a certain amount of bias which can lead to changes in structure, charge, molecular weight and

chemical composition (Ale and Meyer, 2013). Extraction methods typically employ hot water, hydrochloric acid (HCl) or sulphuric acid (H<sub>2</sub>SO<sub>4</sub>), or salt such as calcium chloride (CaCl<sub>2</sub>) (Ale *et al.*, 2011). A consensus has yet to be reached as to which extraction method yields the highest quality fucoidan, while retaining the compound's natural chemistry and structural traits. This chapter will assess the effect of various extraction methods on the variability of fucoidan extracts from South African brown algae.

### **3.1.1) Aim and objectives of this chapter**

The aim of this Chapter was to identify an extraction method for the isolation of high quality fucoidan from brown macroalgal species of South Africa. Factors to be considered when extracting good quality fucoidan are fucoidan yield, purity, and desired chemical characteristics. The aim was broken down into two objectives:

1. Assessment of water, acid, and salt extraction methods on fucoidan yield, as well as analyses of sulphate, protein, and uronic acid content using spectrophotometric assays.
2. Determination of the chemical composition of fucoidan extracts by gas chromatography mass spectroscopy (GC-MS) and spectrophotometric analysis.

## 3.2) Methods and materials

### 3.2.1) Sampling and processing

Brown macroalgae species *E. maxima*, *L. pallida* and *S. rugosum* were harvested from three locations in the Western Cape, South Africa: Betty's Bay (34° 21' 20.16 "S; 18° 55' 42.96"E), Rooi Els (34° 18'05"S; 18° 48' 59"E) and Oudekraal (33°59'0"S; 18° 21'0"E) (Figure 3.1). *S. rugosum* was harvested from rocks outside the intertidal zone (Figure 3.2 A). *E. maxima* and *L. pallida* were collected by scuba diving a few metres offshore from intertidal zones (Figure 3.2 B and C). Three biological replicates of sporophytes from each species were collected and processed in the laboratory.

Seaweed was rinsed with distilled water and left to dry at room temperature (RT). Samples to be processed immediately were further dried at 45°C for 3 days and the remainder was frozen at -80°C. Dried samples were milled to a fine powder by grinding with a mortar and pestle.



**Fig. 3.1.** Sampling sites of brown macroalgae in the Western Cape, South Africa. **A.** Betty's Bay; **B.** Rooi Els; **C.** Oudekraal.



**Fig. 3.2.** Species of brown macroalgae harvested from South African coastline. **A.** *Splachnidium rugosum*; **B.** *Ecklonia maxima*; **C.** *Laminaria pallida*. Seaweed was collected and immediately transported to the laboratory for processing. Seaweed species were identified by Mark Rothman and Chris Boothroyd of the Department of Agricultural, Forestry, and Fisheries (DAFF).

### 3.2.2) Fucoïdan extraction methods

#### 3.2.2.1) Water extraction

Fucoïdan was extracted from dried seaweed samples using the hot water extraction method described by Lee *et al.* (2012) with slight modifications. Dried biomass (0.5 g) from three biological replicates were dissolved in 50 mL distilled deionized water (ddH<sub>2</sub>O), respectively. Samples were incubated overnight at 70°C with agitation, filtered through Whatman® Filter Paper 42 (Sigma-Aldrich), frozen at -80°C, and lyophilised to a dry powder (Figure 3.3).



**Fig. 3.3.** Lyophilised *Splachnidium rugosum* fucoïdan after hot water extraction.

### 3.2.2.2) Acid extraction

Fucoidan was extracted from *S. rugosum*, *L. pallida* and *E. maxima* using an acid extraction method as described by Lee *et al.* (2004) with slight modifications. Dried biomass from three biological replicas was washed by dissolving 0.5 g in 0.15M HCl at 65°C for 2 hours. Samples were centrifuged at 7,500 x *g* for 40 min at RT. The supernatant was neutralized with 3M sodium hydroxide (NaOH) (Merck) and stored at 4°C. Fucoidan was precipitated from solution by the addition of 4 volumes (vol) absolute ethanol (EtOH) (Kimix Chemicals & Laboratory Suppliers) at 4°C, overnight. Polysaccharides were recovered by centrifugation at 7,500 x *g* for 40 min at RT. Fucoidan pellets were washed with absolute EtOH, dried overnight at 40°C, and milled by grinding in a mortar and pestle to a fine powder (Figure 3.4). Dried fucoidan samples were stored at RT in conical tubes (SDL).

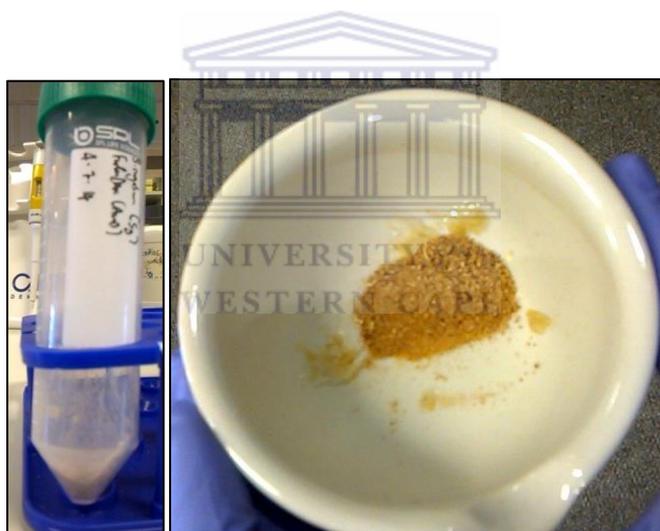


Fig. 3.4. *Splachnidium rugosum* fucoidan after acid extraction.

### 3.2.2.3) Salt extraction

Fucoidan was extracted from *S. rugosum*, *L. pallida* and *E. maxima* using a modified salt extraction method as described by Bilan *et al.* (2002) and Mak *et al.* (2013). Dried biomass (0.5 g) from three biological replicates were incubated overnight at RT in 5 mL methanol-chloroform-water (4:2:1), with regular stirring, to remove macromolecule contaminants such as pigments, lipids, and proteins. Supernatants were decanted and defatted

biomass was washed with acetone and dried overnight in a fume hood. Dried biomass was mixed with 5 mL of 2% (w/v)  $\text{CaCl}_2$  and incubated at  $85^\circ\text{C}$  for 5 hours with mechanical stirring to extract polysaccharides. The supernatant was mixed with 1.5 mL of 10% (w/v) hexadecyltrimethylammoniumbromide (CTAB) (Merck) and stored at  $4^\circ\text{C}$  overnight to precipitate fucoidan. The CTAB-supernatant complex was centrifuged at  $7,500 \times g$  for 40 min at RT, rinsed with  $\text{ddH}_2\text{O}$ , and washed with 10 mL 20% ethanolic sodium iodide (NaI) (Sigma-Aldrich). Residual CTAB was removed by extending the washing step to 3 days at RT with mechanical stirring. Solutions were centrifuged at  $7,500 \times g$  for 40 min at RT and the resulting pellets were washed with EtOH to remove NaI. Samples were lyophilised to obtain a water soluble white powder (Figure 3.5).



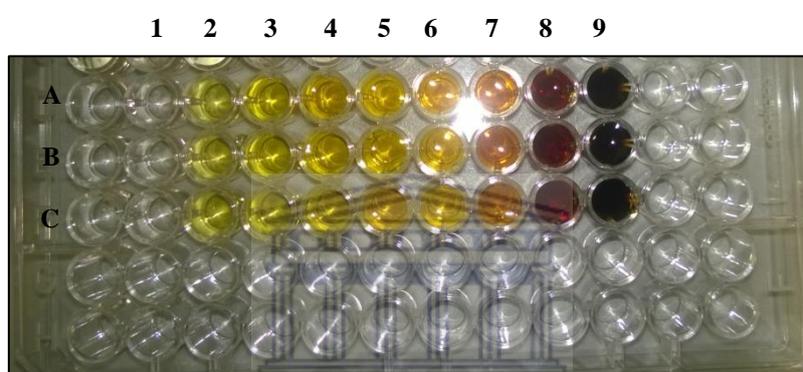
**Fig. 3.5** Lyophilised *Splachnidium rugosum* fucoidan after salt extraction.

### **3.2.3) Chemical composition of fucoidan**

#### **3.2.3.1) L-fucose assay**

Fucoidan was assessed for L-fucose oligosaccharide content by a method developed by Dische and Shettles (1948). This method was modified by down-scaling the assay to microtitre plate volumes. Firstly,  $200 \mu\text{L}$  of  $1 \text{ mg/mL}$  fucoidan sample was added to  $900 \mu\text{L}$  of diluted  $\text{H}_2\text{SO}_4$  (95-98%) (1:7 in  $\text{ddH}_2\text{O}$ ). Acid hydrolysis was performed in triplicate. Samples were boiled at  $100^\circ\text{C}$  for 10 min, followed by cooling on ice for 5 min. Once samples reached RT,  $50 \mu\text{L}$  3% (w/v) L-cysteine HCl (Sigma-Aldrich) solution was added.

Each experimental sample had a sample blank without L-cysteine HCL that was used to calibrate the spectrophotometer (POLARstar Omega, BMG Labtech) before measuring the absorbance readings of experimental samples. Absorbance readings were calculated using the following equation:  $Abs\ 396\ nm - Abs\ 427\ nm$  to correct for the presence of hexoses, as methylpentoses absorbance values were the objective. Commercially procured crude fucoidan from *F. vesiculosus* (Sigma-Aldrich) was prepared in the same manner as the experimental samples and used as a positive control. A standard curve was constructed with L-fucose (Sigma-Aldrich) as a standard with a concentration range between 0.01-0.1 mg/mL (Figure 3.6).

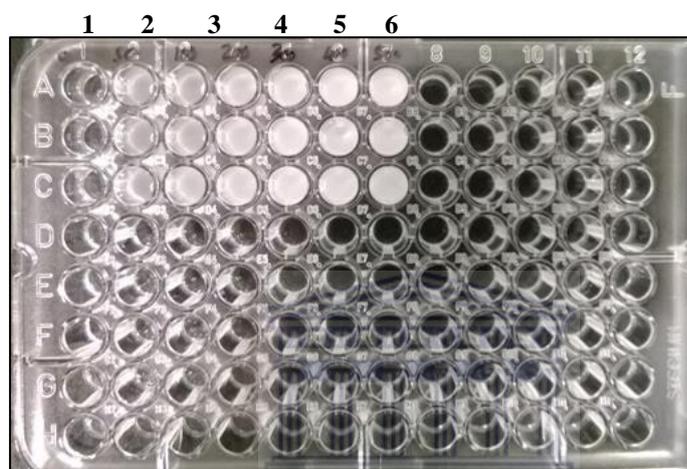


**Fig. 3.6. Microtitre plate assay for L-fucose content.** Lane A1-C1: 10  $\mu\text{g/mL}$ ; Lane A2-C2: 20  $\mu\text{g/mL}$ ; Lane A3-C3:40  $\mu\text{g/mL}$ ; Lane A4-C4: 60  $\mu\text{g/mL}$ ; Lane A5-C5: 80  $\mu\text{g/mL}$ ; Lane A6-C6: 100  $\mu\text{g/mL}$ ; Lane A7-C7: 120  $\mu\text{g/mL}$ ; Lane A78-C8: 140  $\mu\text{g/mL}$ ; Lane A9-C9: 160  $\mu\text{g/mL}$ .

### 3.2.3.2) Sulphate assay

Fucoidan was assessed for sulphate ( $\text{SO}_4^{2-}$ ) content by a method developed by Dodgson and Price (1962). This method was modified by performing the assay in microtitre plate volumes. Stock solutions included a conditioning reagent, 1 mg/mL potassium sulphate ( $\text{K}_2\text{SO}_4$ ) (Sigma-Aldrich) and 3% (w/v) barium chloride dihydrate ( $\text{BaCl}_2$ ) (Sigma-Aldrich). The conditioning reagent consisted of 50 mL glycerol (Sigma-Aldrich), 30 mL HCl 32% (v/v) (Merck), 100 mL 2-propanol (Merck) and 250 mL 5M sodium chloride (Merck) made up to 1 L with ddH<sub>2</sub>O. Fucoidan stock solutions of 1 mg/mL were prepared in ddH<sub>2</sub>O. Serial dilutions of  $\text{K}_2\text{SO}_4$  standards and fucoidan samples were prepared in a 96-well flat bottom microtitre plate (Greiner). Standards and samples were loaded in triplicate (final volume of

250  $\mu\text{L}$ ) as follows:  $\text{K}_2\text{SO}_4$  standards (0-500  $\mu\text{g}/\text{mL}$ ), containing 12.5  $\mu\text{L}$  of conditioning reagent and 25  $\mu\text{L}$  of 3% (w/v)  $\text{BaCl}_2$  stock solution; 125  $\mu\text{L}$  of fucoidan stock (1mg/mL) with 12.5  $\mu\text{L}$  of conditioning reagent, 87.5  $\mu\text{L}$  of ddH<sub>2</sub>O and 25  $\mu\text{L}$  of 3% (w/v)  $\text{BaCl}_2$ . The microtitre plate was agitated for 1 min at RT so that a precipitate could form (Figure 3.7). The resulting suspension was measured spectrophotometrically at a wavelength of 420 nm using a Nano spectrophotometer (BMG LABTECH). A water blank was prepared and treated in the same manner and was subtracted from the raw data.

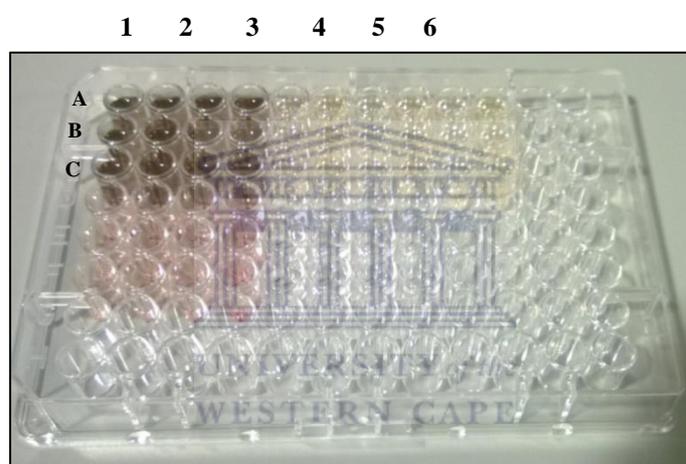


**Fig. 3.7. Microtitre plate assay for sulphate content.** Lane A1-C1: 0  $\mu\text{g}/\text{mL}$ ; Lane A2-C2: 50  $\mu\text{g}/\text{mL}$ ; Lane A3-C3; 100  $\mu\text{g}/\text{mL}$ ; Lane A4-C4: 200  $\mu\text{g}/\text{mL}$ ; Lane A5-C5: 300  $\mu\text{g}/\text{mL}$ ; Lane A6 C6: 400  $\mu\text{g}/\text{mL}$ ; Lane A7-C7: 500  $\mu\text{g}/\text{mL}$ .

### 3.2.3.3) Uronic acid assay

Fucoidan was assessed for uronic acid contamination by a method that was first developed by Dische (1947) and later optimised by Cesaretti *et al.* (2003). This method was further modified by boiling the samples in microcentrifuge tubes (Eppendorf) before transferring to microtitre plates. Reagents included 25mM sodium tetraborate reagent ( $\text{Na}_2\text{B}_4\text{O}_7$ ) prepared in concentrated  $\text{H}_2\text{SO}_4$  (Sigma-Aldrich) and 0.125% (w/v) carbazole ( $\text{C}_{12}\text{H}_9\text{N}$ ) reagent prepared in absolute ethanol. D-Glucuronic acid (Sigma-Aldrich) standards were prepared from a 1 mg/mL stock solution in 2 mL heat-resistant microcentrifuge tubes (Eppendorf) to concentrations ranging from 25-150  $\mu\text{g}/\text{mL}$  in a final volume of 50  $\mu\text{L}$ . This was performed

in triplicate. Ten-fold serial dilutions were prepared from 1 mg/mL fucoidan samples, to a final volume of 50  $\mu\text{L}$ , in 2 mL heat-sensitive microcentrifuge tubes. This was conducted in triplicate and followed by the addition of 200  $\mu\text{L}$  of 25 mM  $\text{Na}_2\text{B}_4\text{O}_7$ . Samples were boiled at 100°C for 10 min. After cooling on ice for 10 min, 50  $\mu\text{L}$  of  $\text{C}_{12}\text{H}_9\text{N}$  was added and the samples were boiled at 100°C for 10 min. After cooling on ice, 250  $\mu\text{L}$  aliquots of the standards, samples and blanks were transferred to a 96-well flat bottom microtitre plate (Greiner) and measured spectrophotometrically (POLARstar Omega, BMG Labtech) at a wavelength of 550 nm (Figure 3.8). Blanks contained ddH<sub>2</sub>O instead of fucoidan sample and were used to zero the spectrophotometer before measuring the absorbance readings of the experimental samples.



**Fig. 3.8. Microtitre plate assay for uronic acid content.** Lane A1-C1: 0  $\mu\text{g/mL}$ ; Lane A2-C2: 25 $\mu\text{g/mL}$ ; Lane A3-C3; 50  $\mu\text{g/mL}$ ; Lane A4-C4: 75  $\mu\text{g/mL}$ ; Lane A5-C5: 100  $\mu\text{g/mL}$ ; Lane A6-C6: 125  $\mu\text{g/mL}$ ; and Lane A7-C7: 150  $\mu\text{g/mL}$ .

#### 3.2.3.4) Protein assay

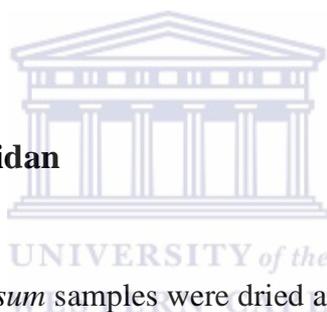
The Bradford Assay (Bradford, 1976) was used to quantify protein concentration (mg/mL) in 96-well microtitre plates. Protein standards were prepared using Bovine serum albumin (BSA) (Sigma-Aldrich) over a concentration range of 0.2-1.2 mg/mL. Samples of unknown concentration were prepared within an approximate range of the protein standards. A volume of 5  $\mu\text{L}$  of protein standard, blank, or unknown sample was added to respective wells, followed by the addition of 250  $\mu\text{L}$  Bradford Reagent (Sigma-Aldrich). Samples were mixed

and incubated at RT for 30 min and the absorbance determined at a wavelength of 595 nm. The net absorbance versus the protein concentration of the standards was plotted and the protein concentration of the unknown samples was determined by correlating the net Abs 595 nm against the standard curve (Figure 3.9).



**Fig. 3.9. Microtitre plate assay for protein content.** Lane A1-C1: 0  $\mu\text{g/mL}$ ; Lane A2-C2: 200  $\mu\text{g/mL}$ ; Lane A3-C3: 400  $\mu\text{g/mL}$ ; Lane A4-C4: 600  $\mu\text{g/mL}$ ; Lane A5-C5: 800  $\mu\text{g/mL}$ ; Lane A6-C6: 1000  $\mu\text{g/mL}$ ; Lane A7-C7: 1200  $\mu\text{g/mL}$ .

### 3.2.4) Acid hydrolysis of fucoidan



*E. maxima*, *L. pallida* and *S. rugosum* samples were dried at 40°C for 2-3 days. Fucoidan was extracted with a hot water method (Section 3.2.2.1). A 1 mg/mL stock of fucoidan was prepared by dissolving the lyophilised biomass in ddH<sub>2</sub>O. Fucoidan extracts were acid hydrolysed for 2 hours at 100°C in 0.5 mL of 5M HCl. Samples were reconstituted in ddH<sub>2</sub>O to a concentration of 1 mg/mL. Native and acid-hydrolysed samples were assayed for sulphate content by the turbidimetric precipitation in BaCl<sub>2</sub> as previously described (Section 3.2.3.2). For sulphate content analysis, 0.5 mL of 1 mg/mL fucoidan stock was used. A water-blank was prepared and treated in the same manner by adding 0.5 mL of ddH<sub>2</sub>O to 0.5 mL of 5M HCl, and incubated at 100°C for 2 hours.

### 3.2.5) High Performance Liquid Chromatography analysis of fucoidan

Molecular weight of crude fucoidan tissue extracts and irradiated fucoidan precipitated from mucilage were run as a service and determined by High Performance Liquid

Chromatography (HPLC) on an Agilent 1200 series HPLC instrument. A volume of 50  $\mu\text{L}$  of 1 mg/mL fucoidan samples were injected into the HPLC, and run on Shodex 804/805 columns in series for 30 min with a flow rate of 1 mL/min. The buffer used for HPLC, 10 mM phosphate buffered saline (PBS), was equilibrated to pH 7.0.

### **3.2.6) Gas chromatography mass spectrometry**

#### **3.2.6.1) Sample preparation for gas chromatography mass spectrometry analysis**

Chemical characterization of crude and purified fucoidan was conducted by GC-MS that was run as a service, with specifications and parameters used described below. Polysaccharides were hydrolysed into monomeric units before being subjected to GC-MS analysis by a modified method described by Goubet *et al.* (2002). Internal standard was prepared by resuspending/dissolving 20 ng/mL of adonitol (ribitol) (Sigma-Aldrich) in 70% (v/v) aqueous methanol (Sigma-Aldrich). Approximately 0.2-1 mg of polysaccharide was deposited into 2 mL screw cap tubes (Eppendorf) and resuspended into 1 mL of 70% (v/v) methanolic adonitol solution and vortexed until homogeneity. Monosaccharide sugars used as reference standards were prepared in the same manner but were not subjected to hydrolysis. Monomeric sugar standards (Sigma-Aldrich) included L-fucose, galactose, glucose, fructose, mannose, rhamnose, and xylose were prepared to 1mg/mL in UV-treated ddH<sub>2</sub>O. Screw caps were pierced before hydrolysis to ensure that the tubes did not explode during built up pressure. Polysaccharides were hydrolysed by boiling at 100°C in 5M trifluoroacetic acid (TFA) (Sigma-Aldrich) for 2 hours. Hydrolysates were dried in a SpeedVac (Labconco) and washed with 0.5 mL methanol (MeOH). After vigorous mixing by vortex, samples were dried in the SpeedVac and the washing step repeated.

Hydrolysates were derivatized by adding 80  $\mu\text{L}$  of 20 mg/mL methoxyamine hydrochloride in pyridine for 90 min at 30 °C. Samples were silyated by adding 80  $\mu\text{L}$  N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) (Sigma-Aldrich) for 30 min at 37°C. Samples were injected into a Agilent DB5ms column of a mass spectrometer (Agilent Technologies Network Gas Chromatograph 7890) and run under the following parameters: helium gas mobile phase, constant flow of 1.3928 mL/min; temperature gradient of 70 °C for

0.5 min; ramp at 30 °C/min to 150 °C; ramp at 5 °C/min to 220 °C; run time of 32.267 min. Mass spectrometric scans were acquired as single quadrupole scans between 0 and 1000 m/z (mass to charge ratio) on an Agilent 7000A triple quadrupole mass spectrometer.

### **3.2.7) Statistical analysis**

Significant differences between polysaccharide extraction techniques were determined by one-way analysis of variance (ANOVA). Statistically significant differences were assessed using Tukey's honest significant difference (HSD) or Student *t*-test. ANOVA and Student *t*-test were performed with Microsoft® Office Excel 2010 Version 14.0.7153.500 and Tukey's HSD by GraphPad Prism 6 Version 6.01.



### 3.3) Results

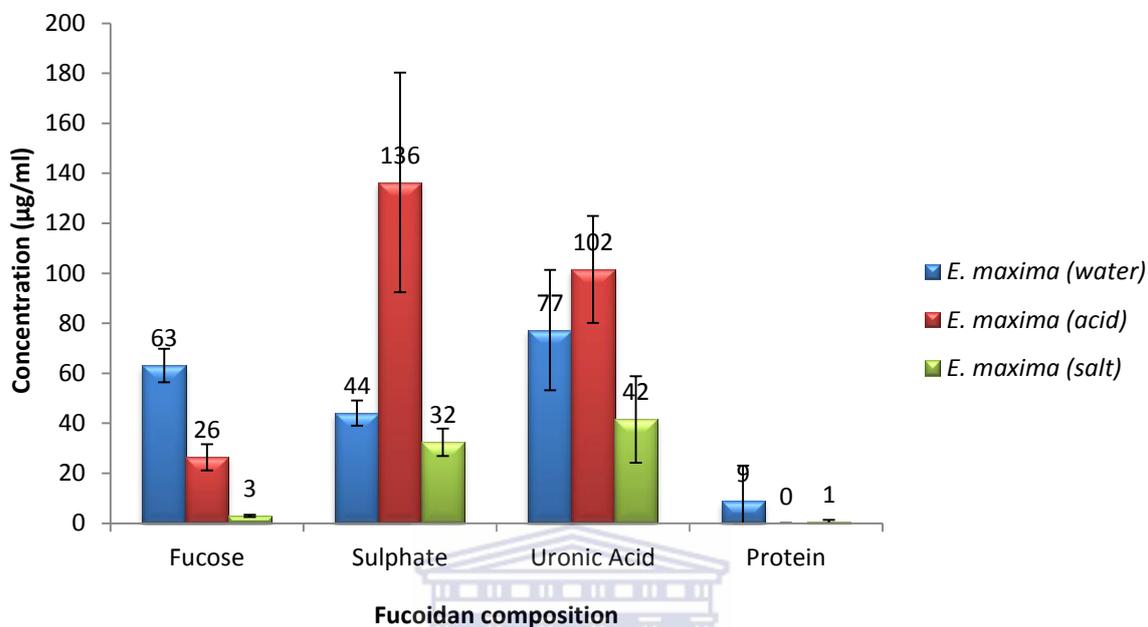
#### 3.3.1) Colorimetric analyses

Fucoidan extracts were assessed by several colorimetric tests to determine yield, basic chemical composition and purity. Fucoidan was hydrolysed into oligosaccharide units and assayed for L-fucose content. L-fucose is the building block of the skeletal structure of fucoidan (Dische and Shettles, 1948). Sulphate content was assessed by the turbidimetric precipitation of  $\text{SO}_4^{2-}$  ions in a  $\text{BaCl}_2$  solution (Dodgson and Price, 1962). Hydrolysed and native fucoidan were assessed for  $\text{SO}_4^{2-}$  content. Fucoidan is a sulphated polysaccharide that contains sulphate groups on varying positions of the L-fucose backbone. The positioning and concentration of  $\text{SO}_4^{2-}$  have a direct correlation to the compound's bioactivity (Jiao *et al.*, 2011). Purity of fucoidan was assessed by determining uronic acid and protein content. Uronic acid is a major component of alginate (alginic acid) which is abundant in brown macroalgae and is easily co-extracted with fucoidan (Ale *et al.*, 2013). Uronic acid contamination was assayed by the carbazole sulphuric acid reaction (Cesaretti *et al.*, 2003). Protein content was determined with the Bradford (1976) assay.

##### 3.3.1.1) Fucoidan extraction from *Ecklonia maxima*

Three methods were employed to assess the suitability of extraction technique of fucoidan from *Ecklonia maxima*. The hot water extraction technique yielded highest ( $p < 0.05$ ) concentration of L-fucose ( $63 \pm 6.7 \mu\text{g/mL}$ ), followed by extraction with HCl ( $26 \pm 5.2 \mu\text{g/mL}$ ) and salt (i.e.  $\text{CaCl}_2$ ) ( $3 \pm 0.4 \mu\text{g/mL}$ ) (Figure 3.10, Table 3.1). Acid extraction with HCl yielded highest ( $p < 0.05$ ) sulphate content ( $136 \pm 43.9 \mu\text{g/mL}$ ), followed by extraction with water ( $44 \pm 5.0 \mu\text{g/mL}$ ) and salt ( $32 \pm 5.5 \mu\text{g/mL}$ ). Acid and salt extraction techniques removed all protein, while the hot water extraction method removed most protein with only  $9 \pm 14.2 \mu\text{g/mL}$  protein remaining after extraction. Uronic acid contamination was assessed spectrophotometrically in a 96-well microtitre plate. The salt extraction technique yielded samples with the lowest ( $p < 0.05$ ) uronic acid content ( $42 \pm 13.3 \mu\text{g/mL}$ ), followed by extraction with water ( $77 \pm 24.1 \mu\text{g/mL}$ ) and acid ( $102 \pm 21.3 \mu\text{g/mL}$ ). The molecular weight

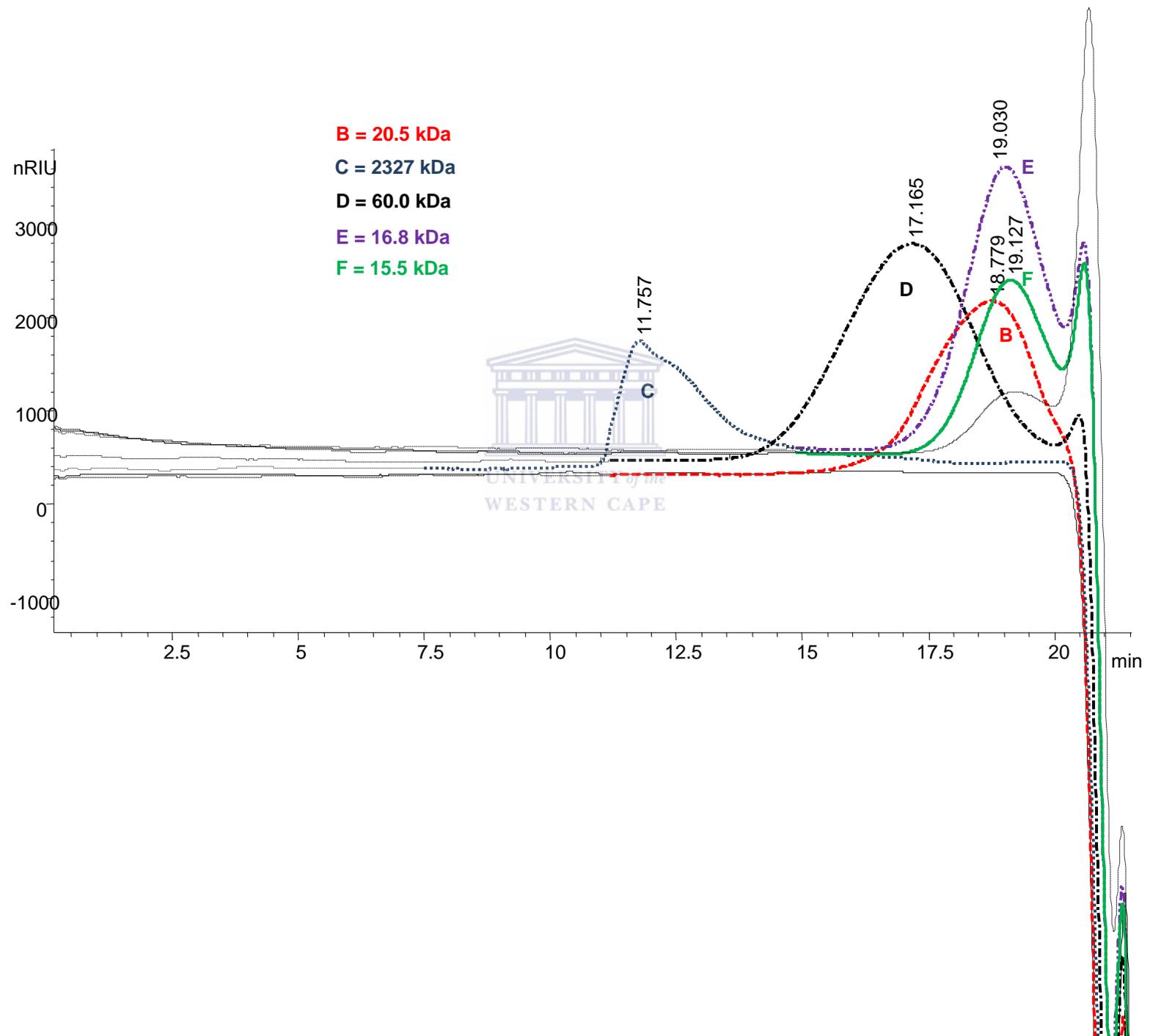
of *E. maxima* fucoidan was estimated by HPLC (Section 3.2.5) to be approximately 470 kDa (Figure 3.11).

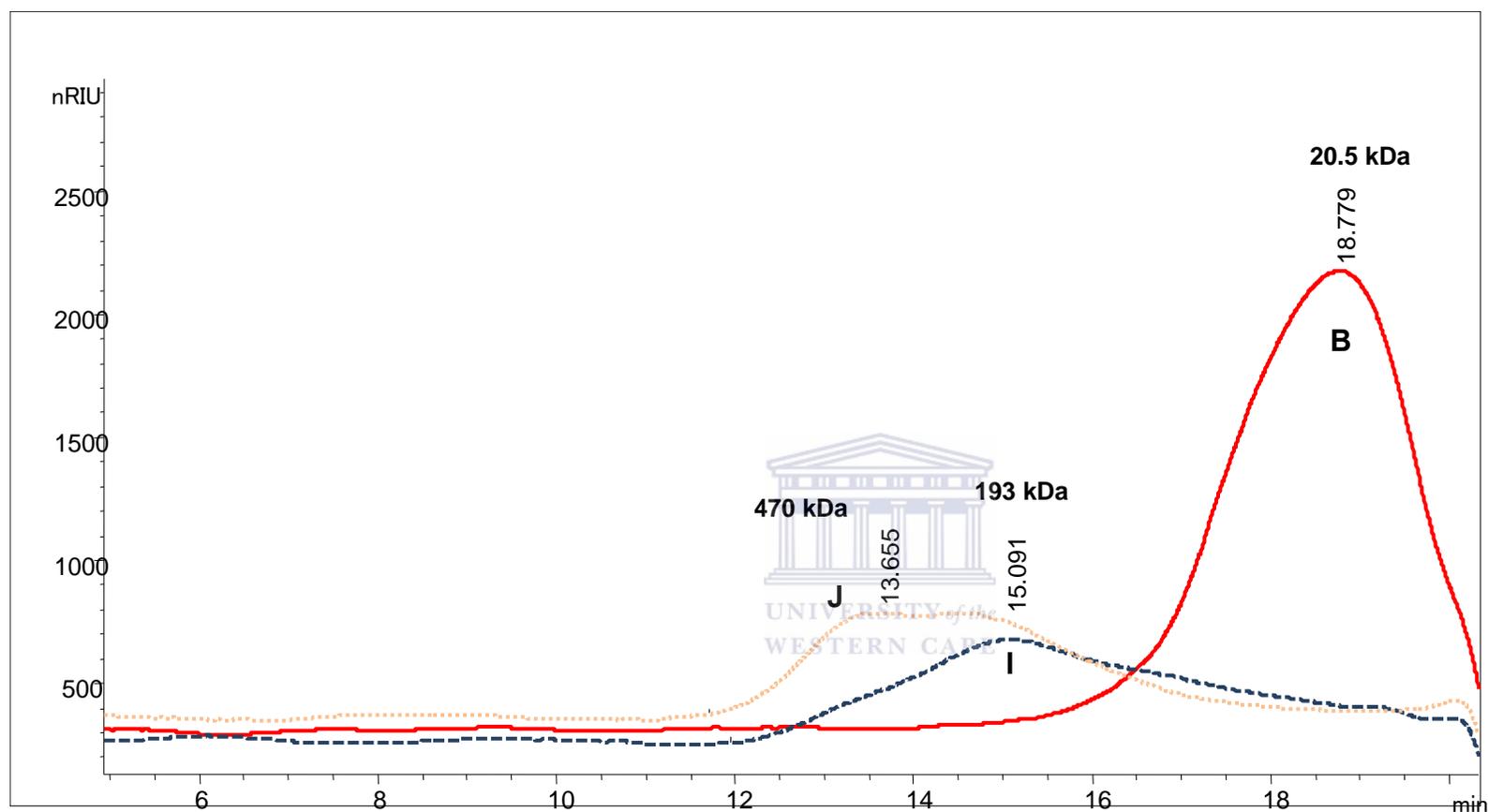


**Fig. 3.10.** Chemical profile of fucoidan extracted from *Ecklonia maxima* using hot water, acid or salt. Fucoidan compounds were assessed for L-fucose, sulphate, uronic acid and protein content. Mean  $\pm$  standard error (SE) ( $n = 3$ ).

**Table 3.1.** Fucoidan profile of brown seaweed extracts.

Species	Extraction method	L-fucose (µg/mL)	Sulphate (µg/mL)	Uronic acid (µg/mL)	Protein (µg/mL)
<i>E. maxima</i>	Hot water	63 $\pm$ 6.7	44 $\pm$ 5.0	77 $\pm$ 24.1	9 $\pm$ 14.2
	Acid	26 $\pm$ 5.2	136 $\pm$ 43.9	102 $\pm$ 21.3	0
	Salt	3 $\pm$ 0.4	32 $\pm$ 5.5	42 $\pm$ 13.3	0
<i>L. pallida</i>	Hot water	51 $\pm$ 5.5	29 $\pm$ 9.5	22 $\pm$ 7.1	8 $\pm$ 2.5
	Acid	33 $\pm$ 5.9	55 $\pm$ 17.2	69 $\pm$ 27.7	0
	Salt	2 $\pm$ 0.7	15 $\pm$ 3.2	32 $\pm$ 2.8	0
<i>S. rugosum</i>	Hot water	265 $\pm$ 49.2	157 $\pm$ 6.7	50 $\pm$ 9.6	36 $\pm$ 0.4
	Acid	220 $\pm$ 13.4	226 $\pm$ 40.2	83 $\pm$ 14.7	0
	Salt	12 $\pm$ 4.8	96 $\pm$ 29.9	19 $\pm$ 8.1	0

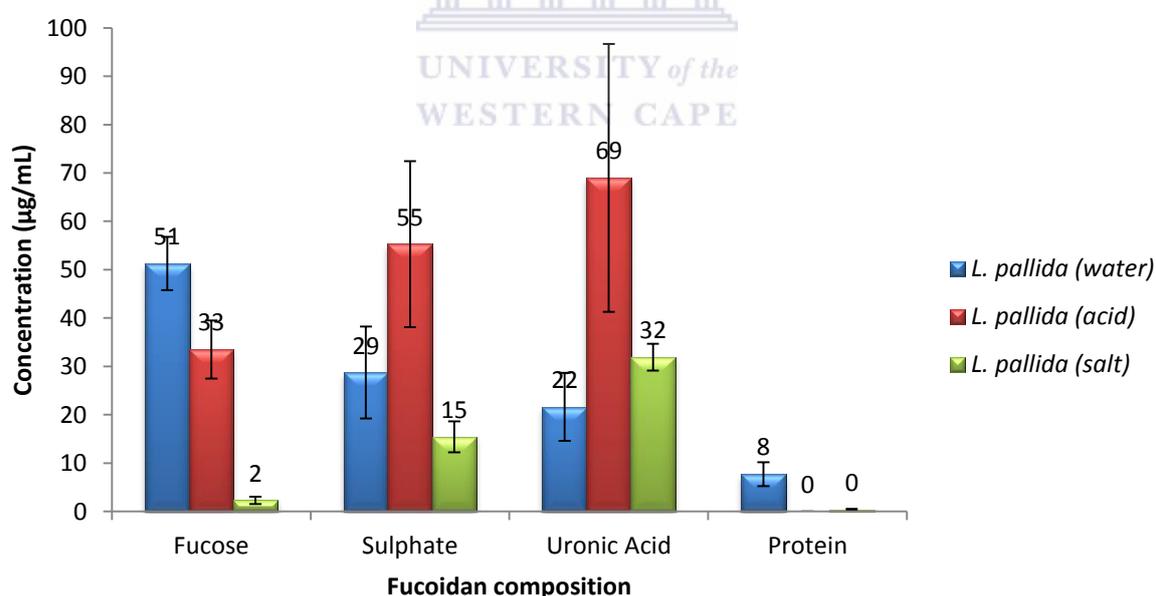




**Fig. 3.11. Chromatogram of molecular weight estimation of intact and fractionated fucoidan compounds analysed by HPLC (Section 3.2.5).** **B.** Commercially procured fucoidan from *F. vesiculosus* (Sigma-Aldrich) **C.** Non-irradiated untreated control (0 kGy) of *S. rugosum* fucoidan **D.** *S. rugosum* fucoidan  $\gamma$ -irradiated at 10 kGy **E.** *S. rugosum* fucoidan  $\gamma$ -irradiated at 30 kGy **F.** *S. rugosum* fucoidan  $\gamma$ -irradiated at 50 kGy. **I.** *E. maxima* crude hot water extracted fucoidan **J.** *L. pallida* crude hot water extracted fucoidan. Fucoidan was prepared to 1 mg/mL in ddH<sub>2</sub>O before being run on HPLC.

### 3.3.1.2) Fucoïdan extraction from *Laminaria pallida*

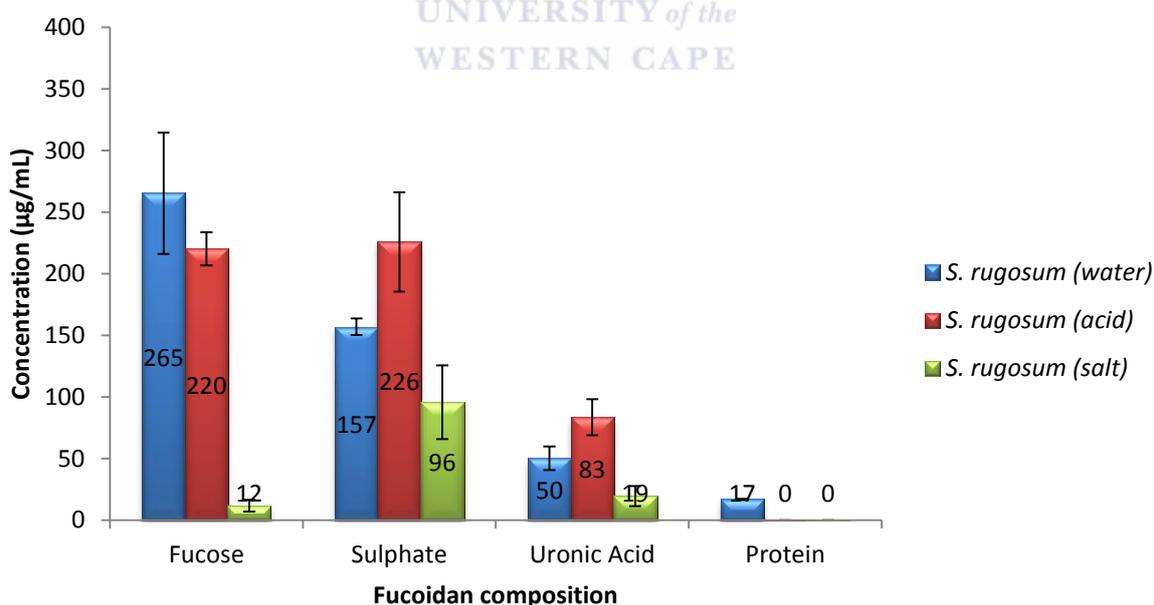
Three methods were assessed to extract fucoïdan from *Laminaria pallida*. The hot water extraction technique yielded highest ( $p < 0.05$ ) amounts of L-fucose with  $51 \pm 5.5 \mu\text{g/mL}$ , followed by the HCl extraction technique with  $33 \pm 5.9 \mu\text{g/mL}$ , and the  $\text{CaCl}_2$  salt extraction technique with the lowest yield at  $2 \pm 0.7 \mu\text{g/mL}$  (Figure 3.3; Table 3.1). The hot water extraction technique also yielded lowest ( $p < 0.05$ ) uronic acid at  $22 \pm 7.1 \mu\text{g/mL}$ , followed by the salt extraction technique with  $32 \pm 2.8 \mu\text{g/mL}$ , and the acid extraction technique with  $69 \pm 27.7 \mu\text{g/mL}$ . The HCl method yielded highest ( $p < 0.05$ ) sulphate content  $55 \pm 17.2 \mu\text{g/mL}$ , followed by water method at  $29 \pm 9.5 \mu\text{g/mL}$ , with the salt method yielding the lowest at  $15 \pm 3.2 \mu\text{g/mL}$  (Figure 3.12). The acid and salt methods removed all protein, while the hot water extraction technique yielded a low protein content of  $8 \pm 2.5 \mu\text{g/mL}$  (Figure 3.12, Table 3.1). The molecular weight of *L. pallida* was estimated by HPLC (Section 3.2.5) to be approximately 197 kDa (Figure 3.11).



**Fig. 3.12.** Chemical profile of fucoïdan isolated from *Laminaria pallida* using hot water, acid, or salt. Fucoïdan compounds were assessed for L-fucose, sulphate, uronic acid and protein. Mean  $\pm$  SE ( $n = 3$ ).

### 3.3.1.3 Fucoidan extraction from *Splachnidium rugosum*

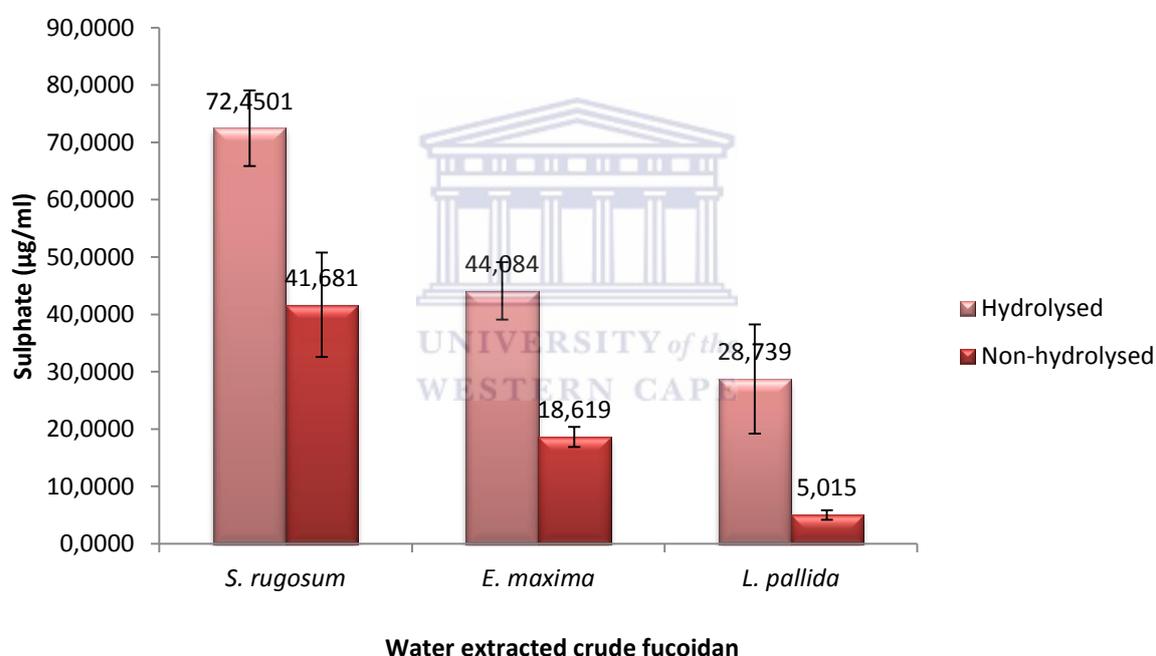
Three methods were assessed to extract fucoidan from *S. rugosum*. The hot water extraction technique yielded the highest ( $p < 0.05$ ) amount of L-fucose with  $265 \pm 49.2 \mu\text{g/mL}$ , followed by the HCl extraction technique with  $220 \pm 13.4 \mu\text{g/mL}$ , and the  $\text{CaCl}_2$  salt extraction technique had the lowest yield at  $12 \pm 4.8 \mu\text{g/mL}$  (Figure 3.13; Table 3.1). The acid extraction technique yielded the highest ( $p < 0.05$ ) sulphate content of  $226 \pm 40.2 \mu\text{g/mL}$ , followed by the hot water extraction technique with  $157 \pm 6.7 \mu\text{g/mL}$ , and the salt extraction technique with the lowest yield of  $96 \pm 29.9 \mu\text{g/mL}$ . The salt extraction technique produced the lowest content of uronic acid with  $19 \pm 8.1 \mu\text{g/mL}$ , followed by the hot water extraction technique with  $50 \pm 9.6 \mu\text{g/mL}$ , and the acid extraction technique with  $83 \pm 14.7 \mu\text{g/mL}$ . The HCl and  $\text{CaCl}_2$  salt technique completely removed protein from *S. rugosum* fucoidan, whereas the hot water extraction technique yielded a low protein content of  $36 \pm 0.4 \mu\text{g/mL}$ . Molecular weight of *S. rugosum* could not be determined by HPLC and was not displayed on chromatogram (Sample A, Figure 3.11).



**Fig. 3.13.** Chemical profile of fucoidan isolated from *Splachnidium rugosum* using hot water, acid, or salt. Fucoidan compounds were assessed for L-fucose, sulphate, uronic acid protein. Mean  $\pm$  SE ( $n = 3$ ).

### 3.3.1.4) Assessing acid hydrolysed vs non-hydrolysed brown macroalgal fucoïdan

Fucoïdan was extracted from seaweed samples with the hot water method and analysed for sulphate content before and after acid hydrolysis with HCl (Figure 3.14). The highest sulphate content was measured in *S. rugosum* polysaccharide extracts,  $42 \pm 9.1 \mu\text{g/mL}$  and  $72 \pm 6.6 \mu\text{g/mL}$ , respectively, in native and hydrolysed samples. *E. maxima* extracts and hydrolysates contained  $19 \pm 1.9 \mu\text{g/mL}$  and  $44 \pm 5.0 \mu\text{g/mL}$  sulphate, respectively. Sulphate content in *L. pallida* was the lowest at  $5 \pm 0.8 \mu\text{g/mL}$  and  $29 \pm 9.5 \mu\text{g/mL}$ , respectively, in native and hydrolysed samples.



**Fig. 3.14. Sulphate content of native and acid hydrolysed fucoïdan from several brown macroalage.** Fucoïdan compounds were extracted from *S. rugosum*, *E. maxima*, and *L. pallida* tissue using a hot water extraction method. Fucoïdan compounds were assessed for sulphate content by turbidimetric barium chloride method before and after hydrolysis with HCl. Mean  $\pm$  SE ( $n = 3$ ).

### 3.3.2) Fucoidan compositional analysis by gas chromatography mass spectroscopy

Fucoidan was extracted from several samples of brown seaweeds using a hot water extraction method and analysed by GC-MS. Samples included commercially procured fucoidan from *F. vesiculosus* as well as crude and purified extracts from *S. rugosum*, *E. maxima* and *L. pallida* (Table 3.2). *S. rugosum* crude extracts consisted mainly of L-fucose and galactose, with trace amounts of glucose, mannose and xylose. *E. maxima* crude fucoidan extracts consisted mainly of L-fucose and glucose, with trace amounts of xylose, mannose, galactose and rhamnose. *L. pallida* extracts consisted mainly of L-fucose, a fair amount of galactose, and small amounts of xylose, mannose, rhamnose and glucose. The *F. vesiculosus* positive control fucoidan consisted mainly of L-fucose, a fair amount of galactose, and trace amounts of xylose, mannose and rhamnose.



**Table 3.2. Monosaccharide composition of hot water fucoidan extracts by GC-MS analysis.<sup>a</sup>**

Sample code	Species	Quality	Biological replicate	<sup>b</sup> Fucose %	<sup>b</sup> Fructose %	<sup>b</sup> Galactose %	<sup>b</sup> Glucose %	<sup>b</sup> Mannose %	<sup>b</sup> Rhamnose %	<sup>b</sup> Xylose %
A	<i>S. rugosum</i>	Crude	1	79.39	-	18.86	0.87	0.87	-	-
B	<i>S. rugosum</i>	Crude	2	92.83	-	0.98	4.24	-	-	1.94
C	<i>S. rugosum</i>	Crude	3	97.74	-	-	1.03	-	-	1.23
D	<i>S. rugosum</i>	Purified	1	78.20	13.55	-	1.40	-	-	6.85
E	<i>S. rugosum</i>	Purified	2	79.37	15.04	-	1.95	-	0.85	0.85
F	<i>S. rugosum</i>	Purified	3	84.85	6.58	-	-	-	-	8.58
G	<sup>c</sup> <i>F. vesiculosus</i>	Crude	1	86.18	-	1.95	-	5.08	2.27	4.52
H	<i>L. pallida</i>	Crude	1	73.72	-	11.86	2.66	4.69	1.24	5.83
I	<i>E. maxima</i>	Crude	1	26.36	5.32	3.25	60.35	3.77	-	0.95

<sup>a</sup>Fucoidan samples extracted from several samples of brown seaweeds were analysed by GC-MS. The values listed above represent baseline corrected peak area (converted to %): the total number of ions that hit the detector during the time the peak was eluting, minus the baseline count.

<sup>b</sup>Monosaccharide constituent found within the sample and expressed as % abundance.

<sup>c</sup>Commercially procured fucoidan from Sigma-Aldrich used as a control in this study.

## 3.4) Discussion

### 3.4.1) Fucoidan extraction from brown seaweeds

Fucoidan has been extracted from seaweeds with a myriad of different techniques, most of which use water, mild to concentrated acids, or salts. Good quality fucoidan consists of high levels of L-fucose, a high degree of sulphation, and low levels of contaminants such as uronic acid and protein (Ale *et al.*, 2011; Ale *et al.*, 2013). Moreover, it is important to retain the native structure of fucoidan as it is often structural characteristics that confer bioactive properties in higher biological systems. The extraction methodology is pivotal to preserving the structural integrity of fucoidan. Other factors are linked to the host, which include species biogeography, sampling season, climate and age of organism when sampled.

Fucoidan was extracted from South African brown seaweed species, *S. rugosum*, *E. maxima* and *L. pallida*, using hot water, mild HCl solution, or CaCl<sub>2</sub>. Extracts were assessed by colorimetric assays to determine fucoidan yield, sulphate content and purity (Table 3.1). Samples were hydrolysed into oligosaccharide units and assayed for L-fucose content. The hot water method delivered extracts with highest concentration of L-fucose, suggesting highest fucoidan yield. L-fucose is the building block of the skeletal structure of fucoidan (Dische and Shettles, 1948). Highest sulphate content was obtained with HCl extraction compared to methods using hot water and salt. This was not surprising as more sulphate may be liberated from fucoidan polysaccharides with acid hydrolysis. Fucoidan is a sulphated polysaccharide that contains sulphate groups at varying positions of the L-fucose backbone. The positioning and concentration of SO<sub>4</sub><sup>2-</sup> have a direct correlation to the compound's bioactivity (Jiao *et al.*, 2011). Fucoidan has been extracted using acetic acid, HCl, and H<sub>2</sub>SO<sub>4</sub> ranging from dilute, mild, to highly acidic conditions (Ale *et al.*, 2011). HCl is preferred since H<sub>2</sub>SO<sub>4</sub> may interfere with sulphate analysis. Purity of fucoidan extracts were assessed by determining uronic acid and protein content. Acid extraction resulted in highest uronic acid contamination. Uronic acid is a major component of alginate (alginic acid) which is abundant in brown macroalgae and is easily co-extracted with fucoidan (Ale *et al.*, 2013). HCl and CaCl<sub>2</sub> methods yielded extracts free of protein; hot water extracts contained very low levels 0-9 µg/mL) which was considered pure, with the exception of *S. rugosum* extract that contained 36 µg/mL of protein.

This is the first study reporting on the presence of fucoidan in *E. maxima*, a species endemic to South Africa. The L-fucose content in hot water extracts was similar to the content reported for water extracts from *Ecklonia cava*, a species from the Northern Hemisphere (Lee *et al.*, 2012). Extraction with CaCl<sub>2</sub> yielded samples with the lowest levels of contaminants such as protein and uronic acid. Low uronic acid content in extracts suggests low contamination with alginate. L-fucose content, and therefore fucoidan yield, was however also very low. Extraction of fucoidan with CaCl<sub>2</sub> is therefore not recommended for *E. maxima*. This method has however been successfully used to extract copious amounts of fucoidan, relatively free of contaminants, from other brown algal species such as *Undaria pinnatifida* (Mak *et al.*, 2013).

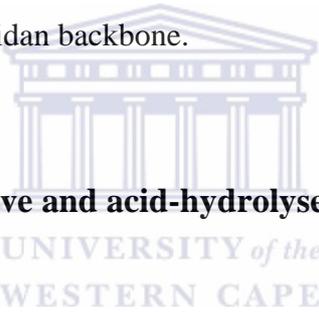
In *L. pallida* extracts, the hot water technique yielded highest L-fucose content and lowest uronic acid levels (Table 3.1). The L-fucose content was however significantly lower compared to *E. maxima* and *S. rugosum*. Fucoidan may be present at lower abundance in cell walls of this species. Lowest sulphate content in acid hydrolysed samples may be attributed to lower fucoidan yield. This is the first study reporting on the presence of fucoidan in *L. pallida*, a species endemic to South Africa.

The *S. rugosum* L-fucose yield, by hot water extraction, was more than five times higher than that of *E. maxima* and *L. pallida*. However, *E. maxima* fucoidan, as seen in acid hydrolysed samples, contained the most sulphate. The fucose-to-sulphate ratio for *E. maxima* was approximately 1:5, the highest reported fucose-to-sulphate ratio to date. The ratios for *S. rugosum* and *L. pallida* were approximately 1:1 and 1:2, respectively (Table 3.1). Fucoidan's bioactivity has been correlated to the sulphation pattern and the sulphate-to-fucose ratio (Morya *et al.*, 2012). Cho *et al.* (2010) conducted a study on the effects of sulphation levels in low and high molecular weight fucoidan compounds on *in vitro* anti-cancer activity. Fucoidan has been partially hydrolysed under mild acid conditions to yield low molecular weight fucoidan and fractionated by membrane ultrafiltration. High (>30 kDa) and low (5-30 kDa) molecular weight fucoidan were oversulphated. The sulphate content increased from 36 to 57% and 32 to 41% for >30 kDa and 5-30 kDa fucoidan, respectively. Cho *et al.* (2010) found that their oversulphated fucoidan displayed greater (15-30%) anti-cancer activity.

Harden *et al.* (2009) extracted fucoidan from sporophytes of *S. rugosum* harvested from New Zealand coastline. Two techniques tested acid (HCl and H<sub>2</sub>SO<sub>4</sub>) and the third relied on CaCl<sub>2</sub>.

All methods yielded sulphated fucans with anti-viral activity against HSV-1 and HSV-2. Assessing the bioactivity of *S. rugosum* from this study and comparing it to bioactivity profiles from fucoidan across the world could aid in elucidating if biogeography is a determining factor in the chemical composition of fucoidan polysaccharides (Mak *et al.*, 2014). Wozniak *et al.* (2015) extracted fucoidan from sporophytes of *S. rugosum* from New Zealand using three different extraction techniques. HCl extraction recovered 34.6% fucose compared to 32.5% with H<sub>2</sub>SO<sub>4</sub> and 35.5% with CaCl<sub>2</sub>. Uronic acid recovery was noted as 1%, 2% and 0%, respectively. They have shown that anti-viral activity was directly correlated with the degree of sulphation.

Fucoidan from *S. rugosum* was selected for further characterization because of its appealing chemical profile including high yields and purity. The hot water method was preferred as fucoidan's native structure and chemical composition were preserved (Kawamoto *et al.*, 2006). Acid extraction and associated acidolysis may result in unwanted degradation of functional groups attached to fucoidan backbone.



### 3.4.2) Sulphate content in native and acid-hydrolysed fucoidan

Fucoidan hydrolysis can be achieved by the following methodologies: enzymatic digestion; acetic-, hydrochloric- or sulphuric acid treatment; gamma-irradiation; or by microwave- and ultrasound-assisted extraction (Ale *et al.*, 2011; Rodriguez-Jasso *et al.*, 2011; Hahn *et al.*, 2012). Acid hydrolysis is considered the most reliable method to degrade fucoidan into mono- and oligomeric units (Lim *et al.*, 2015). Fucoidan was extracted from brown seaweed in its native high molecular weight form by using the hot water method. Extracts were analysed for changes in sulphate content following acid hydrolysis. Higher sulphate content was measured in hydrolysed samples compared to non-hydrolysed (native) fucoidan (Figure 3.14). Dodgson and Price (1962) have shown that the ester sulphate content of polysaccharides can be estimated by measuring SO<sub>4</sub><sup>2-</sup> liberated by acid hydrolysis. Sulphate content measured in native fucoidan polysaccharides prior to acid hydrolysis was however significant (Figure 3.14). Sulphate groups are attached to the backbone of the fucoidan polymer, but may be accessible for analysis. The liberation of L-fucose by acid hydrolysis allows for better assessment of more readily accessible inorganic sulphate. Since the degree of sulphation of fucoidan is critical to the compound's bioactivity, reliable measurement is

crucial. Our results show that acid hydrolysis is required for quantitative analysis of fucoidan sulphate content. Conversely, Xue *et al.* (2001) showed that a water extract from brown seaweed *Laminaria japonica* treated to mild H<sub>2</sub>SO<sub>4</sub> hydrolysis resulted in liberation of L-fucose while the ester sulphate content barely increased.

### **3.4.3) Fucoidan compositional analysis by gas chromatography mass spectroscopy**

Compositional analysis of fucoidan compounds was performed by GC-MS. GC-MS is used to identify and quantify organic compounds in a volatile (i.e. gaseous) state or mixture (Kind and Fiehn, 2010). The affinity of a compound (based on its physiochemical properties) to the stationary phase (i.e. the solid support with a particular coating) enables the separation of consortium of samples into their constituent parts. GC-MS has a longstanding history of successful compositional analysis of small to large biological molecules (Koek *et al.*, 2011a). These molecules include antibiotics, carbohydrates, polysaccharides, proteins and peptides (Koek *et al.*, 2011b). The general GC-MS workflow involves injecting a liquid sample into a hot inlet which converts the sample into a gaseous volatile phase (Banerjee and Mazumdar, 2012). Thereafter, an inert gas transports the volatile compounds through a capillary column (usually of coated glass). This coated column, also known as the stationary phase, passes the sample through the column to a detector over a certain period, referred to as the retention time. Based on the compounds affinity to the stationary phase, the retention time is used to identify the analyte of interest compared to a reference standard. Structural modification of anionic polysaccharides, such as fucoidan, is commonly altered to aid in characterization of these compounds by analytical chemical techniques (Jiao *et al.*, 2011). For example, fucoidan compounds are commonly oversulphated, desulphated, acetylated, or benzylated, in an attempt to develop more effective derivatives to be analysed by mass spectroscopy (Nishino and Nagumo, 1992; Soeda *et al.*, 1993; Soeda *et al.*, 1994; Teruya *et al.*, 2007; Wang *et al.*, 2009).

*S. rugosum* crude fucoidan was isolated by hot water to preserve its native structure and analysed by GC-MS. The polysaccharide consisted mainly of L-fucose (79.4-97.7%) and galactose (0-18.9%) with trace amounts of xylose, mannose, and glucose (Sample A-C, Table

3.2). Algal fucoidan consisting of mainly L-fucose, galactose, and xylose is considered of good quality as these monosaccharides are building-blocks of the fucoidan structure (Ale *et al.*, 2011). The low abundance of trace monosaccharides suggests that the *S. rugosum* fucoidan extract was highly pure. Trace monosaccharides may constitute the building blocks of other algal polysaccharides that are easily co-extracted. Conversely, fucoidan structure is highly heterogeneous and monosaccharides such as galactose, glucose, and mannose may be legitimate substitutions on the fucoidan compound (Ale *et al.*, 2013). Heterogeneity adds to the difficulty in correctly assessing the molecular structure of fucoidan compounds using analytical techniques. GC-MS data supports the results obtained with colorimetric assays, suggesting that fucoidan was successfully extracted from *S. rugosum* and that the extracts were relatively free of contaminants. Wozniak *et al.* (2015) sampled *S. rugosum* from the New Zealand coastline and reported a similar neutral monosaccharide composition by GC-MS. Fucoidan extracts consisted mainly of L-fucose (86-88%) and trace amounts of rhamnose, arabinose, xylose, mannose galactose and glucose (0.2-8%). Inter-species variation in fucoidan composition, i.e. between the same species in different geographic locations, is governed by intrinsic factors such as maturity, age and reproductive cycle (Skriptsova *et al.*, 2012; Maina *et al.*, 2014; Wozniak *et al.*, 2015).

*E. maxima* crude fucoidan analysed by GC-MS consisted mainly of glucose (60.4%) and L-fucose (26.4%), with low abundance of fructose (5.3%), galactose (3.3%), mannose (3.8%) and xylose (1.0%) (Sample I, Table 3.2). High glucose content could be an indication of alginate co-extraction. Glucose and mannose are building-blocks of uronic acids, a major constituent of alginate, abundantly found in the cell walls of brown macroalgae. GC-MS data may explain comparatively low L-fucose content of *E. maxima* extracts as determined by the colorimetric assays (Table 3.2). *E. maxima* hot water fucoidan extracts appeared to be contaminated by alginate as indicated by high uronic acid and glucose content. A similar composition has been presented by Cao *et al.* (2014) for sulphated fucans extracted from *E. cava*, a Northern Hemispheric brown seaweed. Fucoidan has been extracted with 0.01N HCl and consisted mainly of L-fucose (36.1%) and glucose (26.2%), while galactose (15.7%), mannose (11.8%), and small amounts (1-7%) of rhamnose, arabinose and xylose were also present. Conversely, Lee *et al.* (2012) presented data of hot water extracted *E. cava* crude fucoidan with mainly L-fucose (45.2%), galactose (18.2%) and xylose (14.4%). Only small amounts (0.2-10%) of glucose, rhamnose, arabinose and mannose were present. Distinct fucoidan structures can be isolated by different extraction methods (Ale *et al.*, 2013).

Slight modifications in parameters or a condition in the isolation process enhances the variability of chemical composition and subsequent analysis.

*L. pallida* crude fucoidan analysed by GC-MS consisted mainly of L-fucose (73.7%) with a fair amount of galactose (11.9%) and trace amounts of glucose (2.7%), mannose (4.7%), rhamnose (1.24%) and xylose (5.8%) (Sample H, Table 3.2). GC-MS data supports results obtained with colorimetric assays, suggesting that fucoidan extracts were relatively free of contaminants. Xue *et al.* (2001) extracted fucoidan from a Northern Hemispheric kelp *Laminaria japonica* with hot water. Compositional analysis by GC-MS has shown that the presence of L-fucose (52.8%) and galactose (27.4%) are dominant with small amounts (1-6%) of mannose, glucose, rhamnose, xylose and arabinose. Conversely, Xue *et al.* (2004) have reported that a hot water extract from the *L. japonica* consisted mainly out of galactose (57.4%), mannose (15%), L-fucose (12%) and small amounts (2-5%) of glucose, rhamnose, xylose and arabinose were also present. *L. pallida* has a much higher L-fucose ratio than that of its Northern Hemispheric neighbour, *L. japonica*.

Commercially procured crude fucoidan from *F. vesiculosus* (Sigma-Aldrich) (Sample G) was used as a positive control for compositional analysis by GC-MS. *F. vesiculosus* fucoidan consisted mainly of L-fucose (86.2%) with low abundance of galactose (5.7%), mannose (1.4%), rhamnose (2.3%) and xylose (4.5%) (Sample G, Table 3.2). This polysaccharide was extracted with a method using an acid of which the details are not fully disclosed by the supplier. Mak *et al.* (2013) have reported a similar profile for *F. vesiculosus* fucoidan (Sigma-Aldrich), mainly L-fucose (87%) with low abundance of galactose, glucose, mannose and xylose (1-6 %). Results obtained with *F. vesiculosus* control are a confirmation that compositional analyses of brown seaweed extracts reported in this study was correct. L-fucose ratio of fucoidan extracts presented in this study was highest in *S. rugosum* (79-98%) and *F. vesiculosus* (86.2%).

Intra-variations (within the same species in the same location) of fucoidan content has been reported before and it has been inferred that maturity of the alga, the age of the alga, reproductive state, biogeography and seasonality are determining factors (Lee *et al.*, 2006; Skriptsova *et al.*, 2012). Inter-variations (between different/same species in different locations) of fucoidan have also been shown to be a contributing factor to fucoidan composition. The Northern and Southern Hemispheres have different environmental factors such as nutrient content and cycling, light exposure, tidal conditions, and other climate

conditions. To conclude, many variables may affect the heterogeneity of fucoidan compounds but biogeography and extraction methodology are the primary contributors.



### 3.5) Conclusion

Fucoidan was extracted from three species of South African brown seaweed, *Ecklonia maxima*, *Laminaria pallida* and *Splachnidium rugosum*. Three different methods were used to extract fucoidan in an effort to determine the influence of various extraction parameters on the heterogeneity of fucoidan. The hot water extraction method yielded highest L-fucose content, while the calcium chloride salt extraction method yielded fucoidan with lowest uronic acid contamination. Extracts obtained with hydrochloric acid extraction yielded highest uronic acid contamination and sulphate content. High sulphate content may be explained by the liberation of sulphate from fucoidan polymers as a result of acid hydrolysis. All extracts were considered free of protein (<0.01%).

Fucoidan is typically subjected to acid hydrolysis before sulphate assessment. The barium sulphate turbidimetric reaction was used to analyse liberated sulphate. This study has shown that it is possible to comparatively analyse sulphate content of fucoidan extracts without hydrolysis; however, quantitative analysis of sulphate content relies on acid hydrolysis. A drawback of the acid method includes higher alginate co-extracts (as assessed by uronic acid content), whilst fucoidan yield was very low with the salt method. Instead of using one extraction method, a better approach might be to extract fucoidan with hot water to retain its structure in its most native form, apply mild acid hydrolysis to yield lower molecular weight compounds with potentially higher bioactivity and use a salt step to bind and remove cationic contaminants such as alginate, thereby increasing purity. The extraction method for fucoidan and other fucose-containing-sulphated-polysaccharides is critical in maintaining structural integrity and therefore bioactivity. However, the significance of this concept is often overlooked. *Splachnidium rugosum* tissue yielded more than five times more fucoidan than *Ecklonia maxima* and *Laminaria pallida*. Monosaccharide analysis furthermore revealed highest L-fucose ratio. There were only minor compositional differences between fucoidan extracted from the same species. Inter-species variation between *Splachnidium rugosum* from South Africa and New Zealand was more prominent and may be explained by differences in e.g. climate, tidal, and geochemical conditions. The fucose-to-sulphate ratio of fucoidan from *Ecklonia maxima* (1:5) is the highest reported to date. The degree of sulphation is the primary contributor to fucoidan's bioactive properties in mammalian systems. While fucoidan from *Splachnidium rugosum* generated in this study was fractionated and purified (Chapter 4), and

assessed for anti-oxidant and anti-cancer properties (Chapter 5), future studies should also include bioactivity assays with *Ecklonia maxima* extracts.



### 3.6) Literature cited

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# Chapter 4. Fractionation and purification of fucoidan

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## Abstract

Fucoidan is a heavily sulphated anionic polysaccharide, found mainly in seaweeds, that exhibits numerous bioactive properties including anti-cancer activity. Bioactivity has been correlated with sulphate-to-fucose ratio and lower molecular weight forms. Crude hot water and acid extracts from *Splachnidium rugosum* tissue were fractionated and purified by anionic ion exchange chromatography. In water extracts, ion exchange chromatography resulted in close to 90% decrease in L-fucose, sulphate and uronic acid, while protein levels increased by 57%. Similar results were reported for acid extracts; however protein content did not change significantly. Purified hot water extracts contained  $25.235 \pm 1.981$   $\mu\text{g/mL}$  L-fucose,  $11.512 \pm 2.352$   $\mu\text{g/mL}$  sulphate,  $3.897 \pm 1.621$   $\mu\text{g/mL}$  uronic acid and  $82.117 \pm 7.186$   $\mu\text{g/mL}$  protein. Comparatively, L-fucose yield in purified acid extracts were almost half and sulphate content was ~4-fold lower. Uronic acid content was higher (~1.7-fold), while protein content was not significantly different. These results show that method of extraction may affect the composition of fucoidan post-purification. Hot water extraction is recommended due to higher fucoidan yield, as reflected by L-fucose content, and higher sulphate-to-fucose ratio. High protein content after ion exchange chromatography was however of concern. Since mucilage in *Splachnidium rugosum* thallus had no detectable protein present, fucoidan was precipitated from mucilage with ethanol. Fucoidan yield of mucilage was >15-fold higher than in purified hot water extracts with a sulphate-to-fucose ratio of ~1:1. The average molecular weight of native fucoidan in mucilage was estimated to be 2367 kDa. The polysaccharide was hydrolysed by gamma-irradiation levels of 10-50 kGy to fractions ranging between 60 and 15.5 kDa. It is recommended that the dosage of gamma-irradiation should not exceed 50 kGy, considering the drastic drop in molecular weight at higher levels.

## 4.1) Introduction

Fucoidan is a heavily sulphated anionic polysaccharide with an L-fucose backbone (Ale *et al.*, 2013). Extracts from seaweed are of highly heterogeneous structure with different branching patterns and a variable molecular weight (Fitton *et al.*, 2015). Structure and size are impacted by species, origin, extraction method and downstream processing (Ale *et al.*, 2013). Fucoidan can be classified into three groups based on size (Senthilkumar *et al.*, 2013). HMWF is >10,000 kDa, MMWF ranges between 10-10,000 kDa, and LMWF is usually <10 kDa. Fucoidan is often fractionated by acid hydrolysis, irradiation, an enzyme cocktail, or by microwave- and ultrasound-assisted extraction (Ale *et al.*, 2011; Rodriguez-Jasso *et al.*, 2011; Hahn *et al.*, 2012). Numerous studies have reported that the bioactivity of fucoidan in mammalian systems is both compound size- and dosage-dependent (Yumi *et al.*, 2009; Cho *et al.*, 2011; Lee *et al.*, 2012; Morya *et al.*, 2012; Kimura *et al.*, 2013; Zhang *et al.*, 2013; Kwak, 2014). Recent studies have reported that LMWF possesses different bioactive characteristics to that of HMWF (Choi and Kim, 2013). HMWF has been shown to have a low affinity *in vitro* as a bioactive compound, possibly due to low solubility (attributed to its large size) which hinders its use as a drug. It has been shown that LMWF is an effective anti-oxidant, anti-coagulant and a cellular proliferator of healthy cells. Conventionally, LMWF is obtained by acid hydrolysis of the polymer or by enzymatic degradation (Lee *et al.*, 2012; Ale *et al.*, 2013; Pielesz, 2014). The disadvantages of acidolysis include uneven size distribution and unwanted removal of sulphate groups, while enzymatic degradation requires a complex metabolic process. No universal fucoidan hydrolysing enzyme (i.e. fucoidanase or fucanase) has been identified (Wu *et al.*, 2011).

Gamma ( $\gamma$ )-irradiation is a commonly used security measure to kill food borne pathogens and remove contaminants from food products processed for long term storage (Choi *et al.*, 2014). Gamma-irradiation has been shown to effectively reduce the size of fucoidan (Choi and Kim, 2013; Lim *et al.*, 2015), resulting in increased anti-oxidant and cytotoxic bioactivities in mammalian systems (Lee *et al.*, 2009; Sung *et al.*, 2009; Abad *et al.*, 2013). Increasing radiation dosages, measured in kGy, has been shown to cleave high molecular weight (HMW) polysaccharides into low molecular weight (LMW) monosaccharide or oligosaccharide sugars (Byun *et al.*, 2008; Choi *et al.*, 2009a). The main advantage of  $\gamma$ -irradiation is that sugars are fractionated at glycosidic bonds, and not at random bonds and

functional groups which may alter the structural integrity of the compound (Choi *et al.*, 2009b, Sung *et al.*, 2009).

Ion exchange chromatography (IEC) separates compounds based on net surface charge. Interaction with a charged chromatography medium depends on a molecule's overall charge, charge density and surface charge distribution. In the IEC process, ions or polar molecules are separated based on their affinity for the ion exchanger, i.e. the column. Analyte molecules are retained on the column due to a physical phenomenon known as columbic interactions. The surface of the stationary phase (i.e. the fixed medium that makes up the column) contains the functional groups (R-X) that interact with analyte ions of opposite charge. An IEC medium comprises a matrix of spherical particles substituted with ionic groups that are negatively (cationic) or positively (anionic) charged. As fucoidan is a heavily sulphated polysaccharide its overall charge is negative. As IEC separates compounds based on charge, fucoidan, an anionic polysaccharide, would bind to a positively charged ligand (CTAB) attached to the column medium (Celite<sup>®</sup>).

Literature has shown that by refining fucoidan into a more purified compound, the bioactivity of fucoidan could be increased, especially the potency of the compound as an *in vitro* drug candidate (Gerwick and Moore, 2012). Conversely, crude algal extracts may possess synergistic activities in biological systems (Michalak and Chojnacka, 2015). This study aimed to purify fucoidan, assess the bioactivity of low molecular weight, crude, and purified fucoidan extracted from brown macroalgal species of South Africa by exploiting the physicochemical properties of the polysaccharide.

#### **4.1.1) Aims and objectives of this chapter**

The aim of this Chapter was to develop an effective purification and fractionation strategy for fucoidan compounds extracted from *S. rugosum*. This aim was further broken down into two objectives:

- (1.) Development of an effective fucoidan fractionation method which has minimal effects on the structural integrity of fucoidan. Commonly used fractionation techniques,  $\gamma$  -irradiation and anionic exchange chromatography, were evaluated.

- (2.) Development of a novel purification protocol for fucoidan compounds extracted from *S. rugosum*.



## 4.2) Methods and material

### 4.2.1) Fucoidan samples and compositional analysis

Crude fucoidan was extracted from *S. rugosum* whole algal tissue (thallus fingers) by using hot water or acid extraction. Methods were described in Section 3.2.2. The clear viscous gel or “mucilage” was separated from tissue by cutting off the tip of thallus fingers and squeezing out the gel (Figure 4.1). Mucilage was stored at  $-80^{\circ}\text{C}$  for analysis by  $\gamma$ -irradiation. For L-fucose analysis, mucilage and empty thallus tissue were dried, followed by a hot water extraction and ethanol precipitation (Section 3.2.2 and 4.2.3 respectively).

Colorimetric analysis of L-fucose, sulphate and uronic acid content was determined with the cysteine sulphuric acid, barium salt and carbazole sulphuric acid methods, respectively (Section 3.2.3). Protein content was analysed by the Bradford assay (Section 3.2.3).



**Fig. 4.1.** *Splachnidium rugosum* mucilage isolated from algal tissue. **A.** Wild *S. rugosum* sporophyte photographed at Grotto Bay, Western Cape, South Africa ( $33.5014^{\circ}$  S,  $18.3192^{\circ}$  E). **B.** Mucilage squeezed out of *S. rugosum* thallus (left), empty thallus (centre), and whole alga tissue (right).

## 4.2.2) Anion exchange/silica column chromatography

### 4.2.2.1) Creating and packing the column

Glass wool (Sigma-Aldrich) was inserted into the bottom of a 50 mL syringe (DuraSyringe<sup>®</sup>) to act as a plug for the column slurry. The syringe was mounted vertically to a retort stand (Figure 4.2). A fucoidan-CTAB complex was formed by adding 20 mL of 10 mg/mL crude *S. rugosum* fucoidan extracts (Section 3.2.2), prepared in 25mM sodium acetate (NaOAc: Sigma-Aldrich), to 20 mL of 10% (w/v) CTAB. The solution was vortexed for 30 min and precipitated at RT for 30 min. The fucoidan-CTAB complex was mixed with ~40-50 mL of a 30% (w/v) Celite<sup>®</sup> (Sigma-Aldrich) solution (1:2), forming a fucoidan-CTAB-Celite slurry. The slurry was left to settle for 1 hour at RT, the supernatant was removed (~20-30 mL), and the slurry was packed into the syringe column.



**Fig. 4.2. Fucoidan purification columns.** Crude fucoidan extracts from *S. rugosum* algal tissue ( $n=3$ ) were purified by IEC. The syringe column (stationary phase) was packed with a fucoidan-CTAB-Celite slurry (solid adsorbent). A range of mobile phases were passed over the column to elute fractions containing different concentrations of fucoidan-salts. Gravity and external pressure (i.e. a peristaltic pump) were applied.

#### **4.2.2.2) Washing and eluting fucoïdan salts off the column**

Once the column had settled and dried the following wash and eluent buffers were passed through the column to elute fucoïdan-CTAB salts: 2x column vol 0.05% CTAB (w/v) prepared in 0.05M NaOAc, 3x column vol 30% (w/v) EtOH prepared in 0.05M NaOAc, 3x column vol 60% (w/v) EtOH in 0.05M NaOAc, and 3x column vol 100 % (w/v) EtOH in 0.05M NaOAc. Eluted fractions were captured and assessed colorimetrically for L-fucose content by a modified cysteine sulphuric acid method developed by Dische and Shettles (1948) (Section 3.2.3.).

#### **4.2.2.3) Dialysis of fucoïdan salts**

Fractions containing highest concentrations of L-fucose were pooled and dialysed against two buffers. Firstly, the fucoïdan-CTAB alcoholic fractions (60-90 mL) were added to 230 mL Slide-A-Lyzer™ Dialysis Flasks 3.5K MWCO (Thermoscientific) and dialysed against 1-2 L of 0.2M NaCl buffer (10-20x vol dialysis buffer) overnight at 4°C. Dialysis displaced CTAB and created a fucoïdan-Na<sup>+</sup> complex. The complex was dialysed against 1-2 L of UV-treated autoclaved ddH<sub>2</sub>O buffer (10-20x vol dialysis buffer) overnight at 4°C. Na<sup>+</sup> was displaced with H<sub>2</sub>O, thereby creating a homogenous fucoïdan solution in water. The fucoïdan solution was lyophilised at -50°C in a freeze-dryer (Labconco FreeZone<sup>1</sup> Benchtop Freeze-dryer) for 3-5 days until a dry white powder was formed.

#### **4.2.3) Gamma-irradiated fucoïdan mucilage**

##### **4.2.3.1) Gamma-irradiation**

*S. rugosum* mucilage was transferred into 50 mL glass Schott bottles and subjected to  $\gamma$ -irradiation at High Processing Energy Cape (Pty) (Ltd) (HEPRO), Montague Gardens, Western Cape Province, South Africa (-33.871105, 18.525371). Mucilage was  $\gamma$ -irradiated at ambient temperature at 10, 30, 50, and 80 kGy with a cobalt 60 irradiator. Dosimetry was

conducted with a B3 DoseStix® dosimeter and measured with a Genesys G20 spectrophotometer. Non-irradiated mucilage (0 kGy) was used as the untreated control. Samples were maintained at 4°C after irradiation. This experiment was conducted as an initial trial experiment ( $n=1$ ).

#### 4.2.3.2) Ethanol precipitation

Fifty mL samples of *S. rugosum* untreated and  $\gamma$ -irradiated mucilage were precipitated overnight at 4°C in 5x vol of 96% (v/v) technical ethanol (Sigma-Aldrich). Alcoholic mucilage solutions were pelleted at 7,000 x g at 4 °C for 10 min. Supernatant was discarded and pellets were dried at 40°C for 24-48 hours. Dried pellets were macerated in a mortar and pestle to a fine powder.

#### 4.2.4) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry



Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry (MS) of irradiated fucoidan was run performed using an UltrafleXtreme MALDI ToF/ToF system (Bruker Daltonics, Bremen, Germany) with instrument control through Flex control 3.4. Samples were physically spotted onto an MTP 384 Ground steel MALDI target. Spectra were acquired in a linear negative mode with an ion source 1 of 20 kV, ion source 2 of 18.88 kV and with a lens of 7 kV. A number of 250 laser shots were summed up at a scan range of  $m/z = 500-2200$ . Spectra were also acquired in positive reflector mode between a scan range of  $m/z$  500-2000 Da.

Matrices used were  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA). A combinatorial approach was conducted whereby various matrix:sample ratios were prepared since there is no set standard for fucoidan preparation for analysis on MALDI-TOF MS. Therefore, optimization was conducted with matrix type, sample to matrix ratio, ionization sources, and both linear and positive modes of ionization.

#### 4.2.5) Statistical analysis

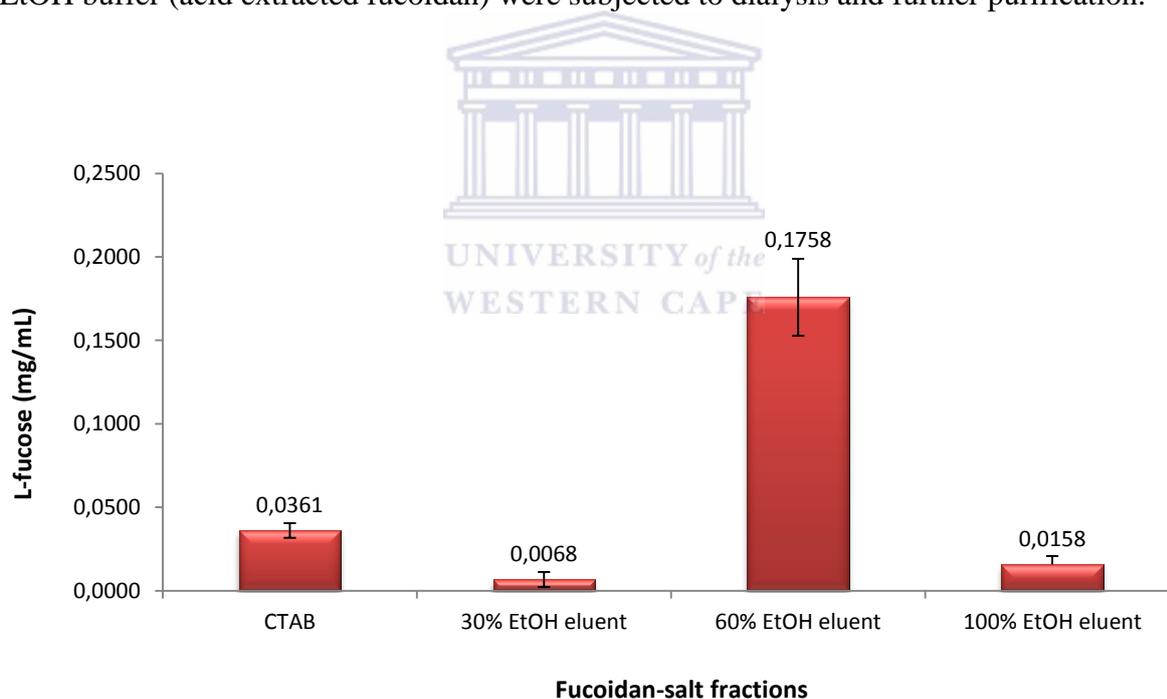
All results presented (excluding  $\gamma$ -irradiation) were from three independent replicate experiments and each seaweed sample came from three biological sources. For each biological repeat, three technical repeats were performed. Standard error was calculated using Microsoft<sup>®</sup> Office Excel 2010 Version 14.0.7153.500. Significant differences between chemical composition of crude fucoidan extracts and of purified compounds were assessed by *Student t*-test. The assessment was determined using Microsoft<sup>®</sup> Office Excel 2010 Version 14.0.7153.500 and GraphPad Prism 6 Version 6.01.



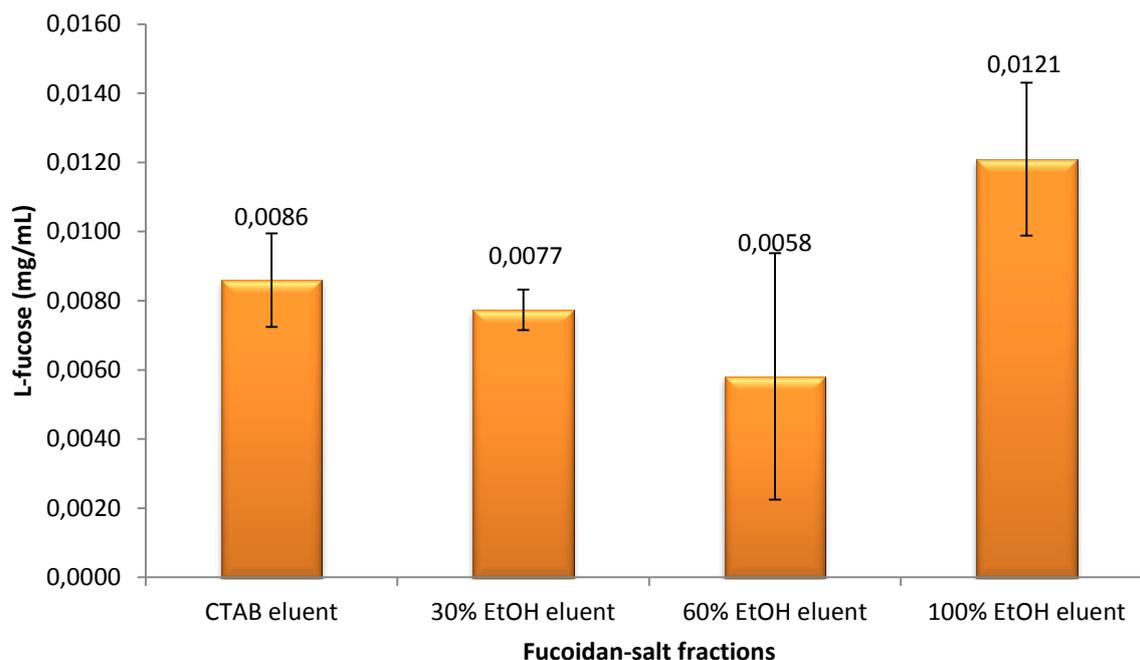
## 4.3) Results

### 4.3.1) Assessing ion exchange eluate fractions for fucoidan content

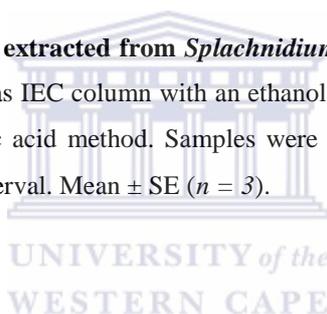
Crude hot water and acid extracts from *S. rugosum* tissue were loaded separately onto columns for purification by IEC. Fractions eluted by IEC were assessed for L-fucose content. L-fucose containing fractions were pooled to ensure maximum recovery of FCSPs. The hot water extraction method yielded FCSPs that were most efficiently eluted with 60% (w/v) EtOH in 0.05M NaOAc buffer ( $p < 0.05$ ), yielding an L-fucose content of  $0.176 \pm 0.023$  mg/mL (Figure 4.3). With the acid extraction method, no significant differences ( $p > 0.05$ ) in elution efficiency were recorded in an EtOH concentration range of 30-100% (Figure 4.4). Eluates obtained with the 60% EtOH buffer (hot water extracted fucoidan) and the 100% EtOH buffer (acid extracted fucoidan) were subjected to dialysis and further purification.



**Fig. 4.3. L-fucose content of fucoidan extracted from *Splachnidium rugosum* tissue with a hot water method.** CTAB:fucoidan salts were eluted from an IEC column using an ethanol concentration gradient and L-fucose content was determined by a cysteine sulphuric acid method. Samples were assessed for significant differences by a Student *t*-test with a 95% confidence interval. Mean  $\pm$  SE ( $n = 3$ ).



**Fig. 4.4. L-fucose content of fucoïdan extracted from *Splachnidium rugosum* tissue with an acid method.** CTAB:fucoïdan salts were eluted from as IEC column with an ethanol concentration gradient. L-fucose content was determined by a cysteine sulphuric acid method. Samples were assessed for significant differences by a Student *t*-test with a 95% confidence interval. Mean  $\pm$  SE ( $n = 3$ ).

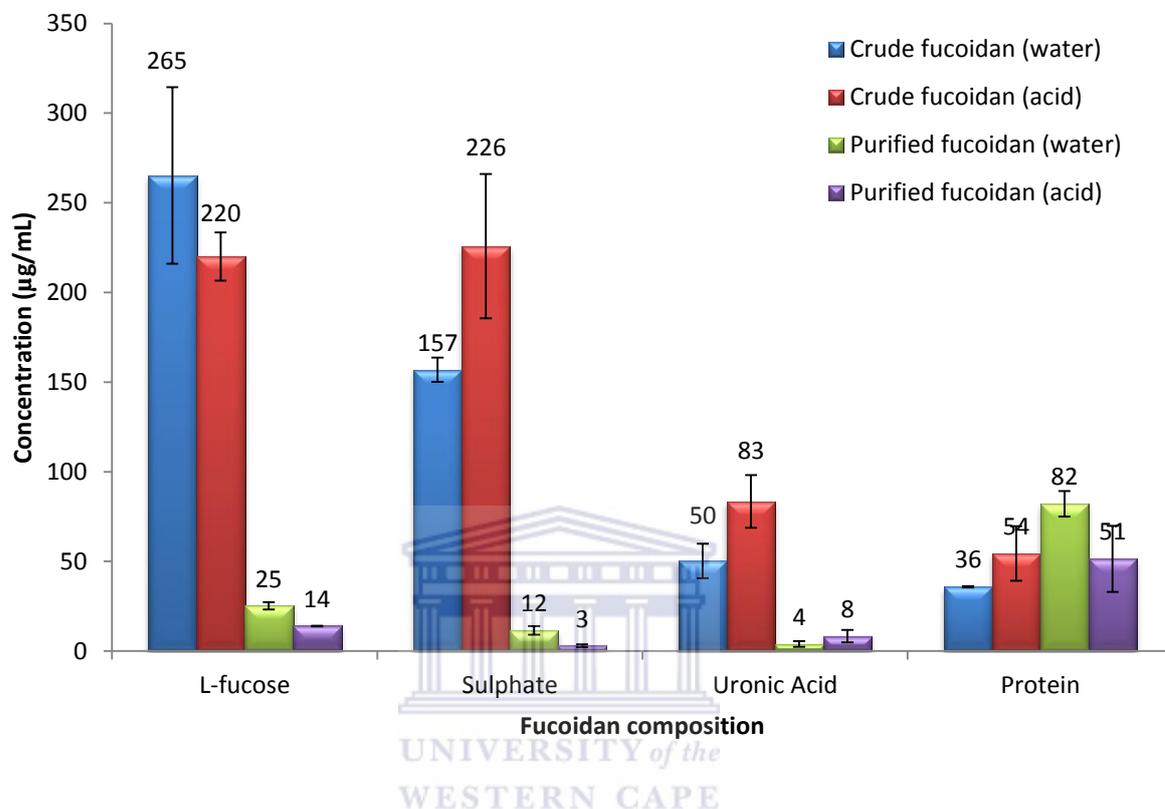


#### 4.3.2) Compositional analyses of crude fucoïdan extracts before and after ion exchange chromatography

Crude hot water and acid extracts from *S. rugosum* tissue were subjected to IEC. Chemical composition was compared before and after IEC (Figure 4.5). Yield differences are presented in Table 4.1 as percentage change. For crude water extracts, IEC resulted in more than 90% decrease in L-fucose, sulphate and uronic acid, while protein content increased by 57% ( $p < 0.05$ ). Similar results were reported for acid extracts; however the protein content did not change significantly ( $p > 0.05$ ).

Fucoïdan extracted by the hot water method contained  $25.235 \pm 1.981 \mu\text{g/mL}$  L-fucose and  $11.512 \pm 2.352 \mu\text{g/mL}$  sulphate. Acid extraction yielded significantly ( $p < 0.05$ ) less L-fucose and sulphate,  $13.976 \pm 0.164 \mu\text{g/mL}$  and  $2.963 \pm 0.787 \mu\text{g/mL}$ , respectively. Hot water fucoïdan extracts contained  $3.897 \pm 1.621 \mu\text{g/mL}$  of uronic acid compared to  $8.308 \pm 3.470 \mu\text{g/mL}$  in acid extracts ( $p < 0.05$ ). Water and acid extracts contained  $82.117 \pm 7.186 \mu\text{g/mL}$

and  $51.450 \pm 18.449 \mu\text{g/mL}$  protein, respectively. The difference in protein content obtained with different extraction methods was not statistically significant ( $p > 0.05$ ).



**Fig. 4.5. Chemical profile of *Splachnidium rugosum* fucoïdan extracts.** Crude water and acid extracts were purified by IEC and all samples analysed for L-fucose, sulphate, uronic acid, and protein content. Samples were assessed for significant differences by a Student *t*-test with a 95% confidence interval. Mean  $\pm$  SE ( $n = 3$ ).

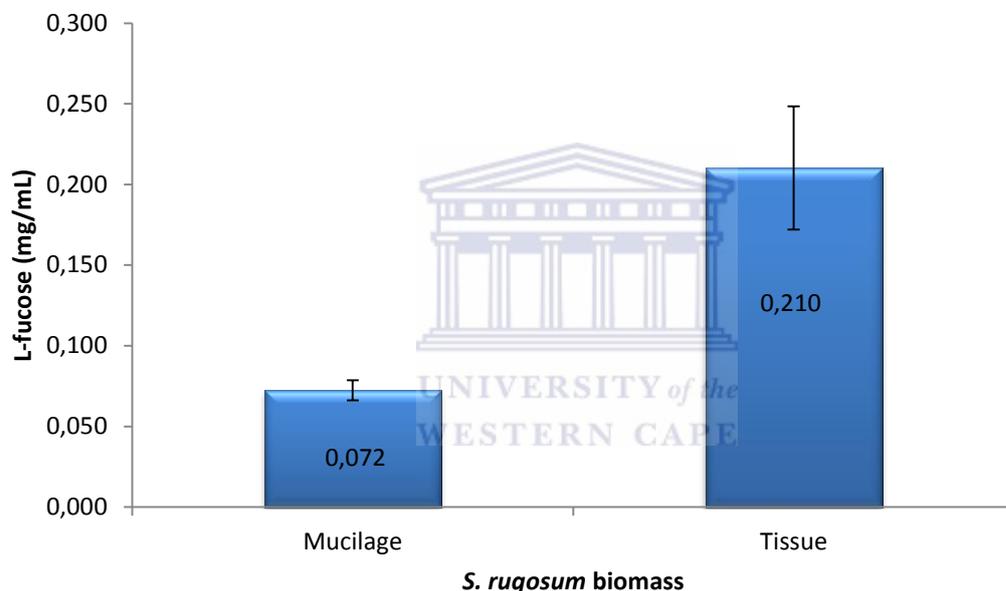
**Table 4.1. Yield of fucoïdan constituents gained (+) or lost (-) after ion exchange chromatography.**

Species	Fucoïdan sample	L-fucose %	Sulphate %	Uronic acid %	Protein %
<i>S. rugosum</i>	Purified water extract	-89.4%	-86.9%	-87.5%	+43.9%
<i>S. rugosum</i>	Purified acid extract	-88.6%	-98.7%	-90.4%	-5.6%

### 4.3.3) Gamma-irradiated fucoidan

#### 4.3.3.1) *Splachnidium rugosum* fucoidan content in mucilage and cellular tissue

*S. rugosum* mucilage was removed and dried alongside the empty thallus tissue, followed by a hot water extraction and ethanol precipitation (Section 3.2.2). The mucilage contained  $0.072 \pm 0.0063$   $\mu\text{g}/\text{mL}$  L-fucose (17% yield), while the tissue contained  $0.210 \pm 0.0382$   $\mu\text{g}/\text{mL}$  (49.6% yield) (Figure 4.6).

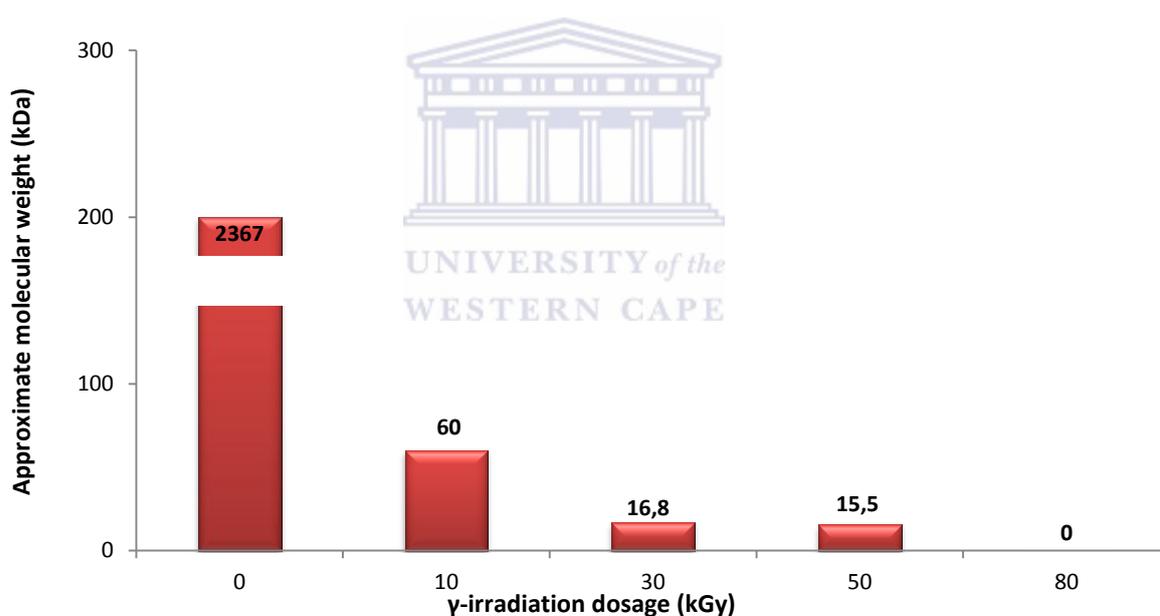


**Fig. 4.6.** L-fucose content of fucoidan in *Splachnidium rugosum* mucilage and cellular tissue. *S. rugosum* algal tissue was divided into mucilage and cellular tissue. Fucoidan was extracted from dried samples with the hot water method, precipitated by ethanol, and assessed for L-fucose content. Mean  $\pm$  SE ( $n = 3$ ).

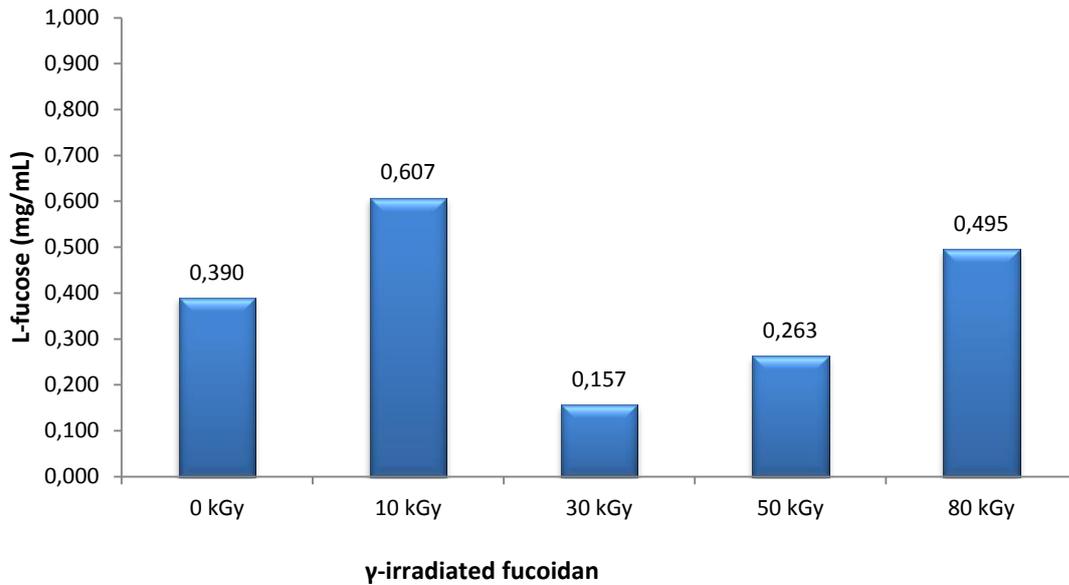
#### 4.3.3.2) Assessment of $\gamma$ -irradiated fucoidan

Mucilage was removed from *S. rugosum* thalli and fucoidan was precipitated by ethanol. Fucoidan were exposed to  $\gamma$ -irradiation, and concentrated again by ethanol precipitation. Increasing dosages of  $\gamma$ -irradiation (kGy) fractionated fucoidan into LMW compounds ranging from 60 to 15.5 kDa (Figure 4.7). Native fucoidan extracted from the untreated

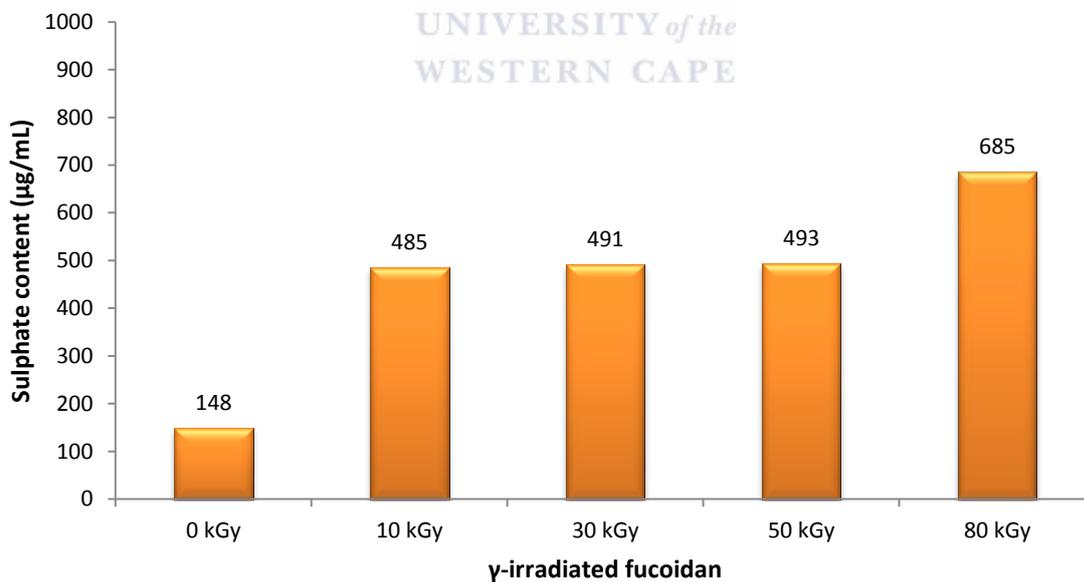
control (0 kGy) was estimated at 2367 kDa. At 10 kGy of  $\gamma$ -irradiation, L-fucose content increased 1.5-fold, from 0.390 to 0.607 mg/mL (Figure 4.8). At a dosage of 30 kGy, L-fucose concentration dropped drastically to 0.157 mg/mL, followed by a steady increase at higher dosages reaching 0.495 mg/mL at 80 kGy. The sulphate content of native fucoidan (148  $\mu$ g/mL) steadily rose with an increase in radiation (Figure 4.9). The highest sulphate content (685  $\mu$ g/mL) was measured at 80 kGy. Mucilage was free of protein (results not presented). Uronic acid content remained constant at  $\sim$ 55  $\mu$ g/mL up to 50 kGy, but dropped to 19  $\mu$ g/mL at 80 kGy (Figure 4.10). All data are comparatively presented in Figure 4.11 to depict effects of different doses of  $\gamma$ -irradiation on yield and purity.



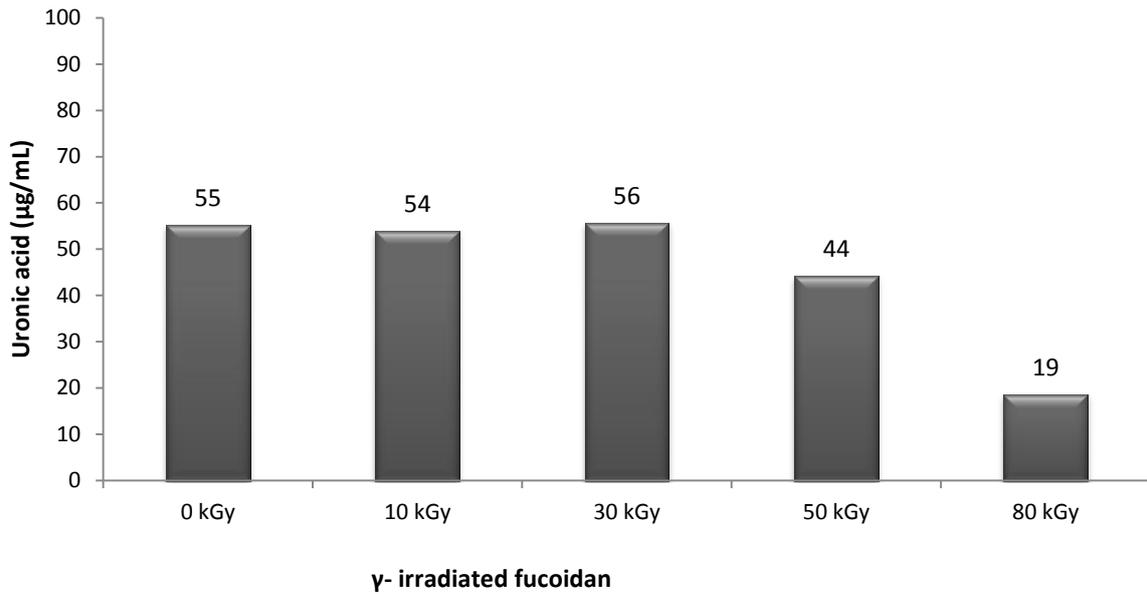
**Fig. 4.7. Molecular weight of gamma-irradiated fucoidan.** Fucoidan was precipitated from *S. rugosum* mucilage with ethanol and exposed to increasing dosages of  $\gamma$ -irradiation (kGy). Molecular weight was determined by HPLC.



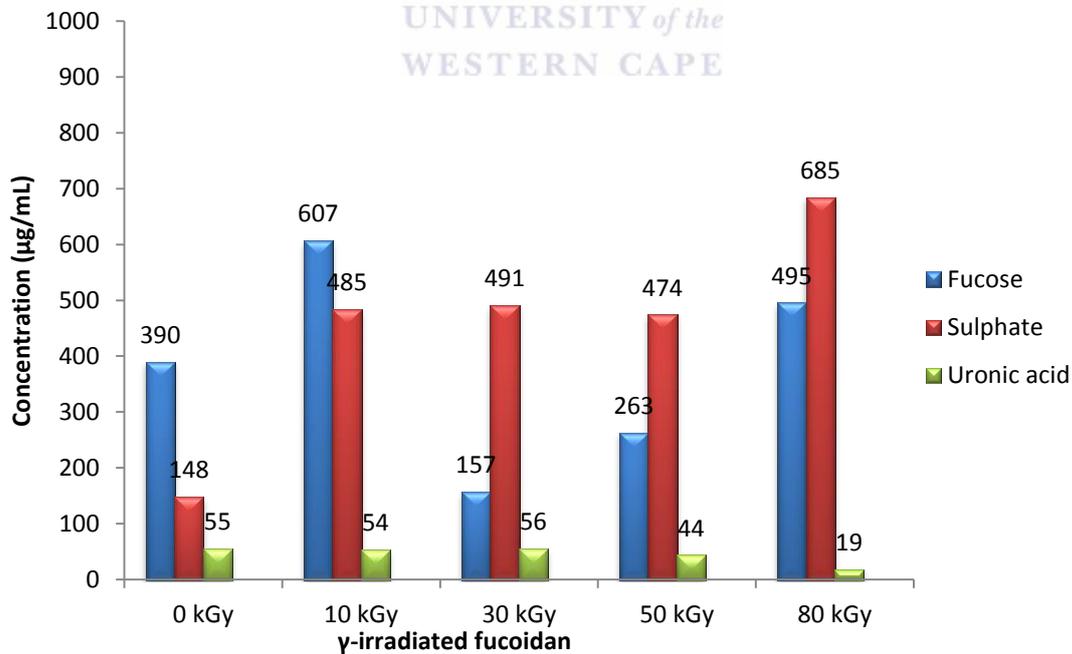
**Fig. 4.8. L-fucose content of gamma-irradiated and non-irradiated fucoidan.** Fucoidan was precipitated from *S. rugosum* mucilage with ethanol. Fucoidan was exposed to increasing dosages of  $\gamma$ -irradiation (kGy) to break down the polysaccharide into oligosaccharide units. L-fucose content was determined by a cysteine sulphuric acid method.



**Fig. 4.9. Sulphate content of gamma-irradiated fucoidan.** Fucoidan was precipitated from *S. rugosum* mucilage with ethanol. Fucoidan was exposed to increasing dosages of  $\gamma$ -irradiation (kGy) to break down the polysaccharide into oligosaccharide units. Sulphate content was determined by turbidimetric barium salt method.



**Fig. 4.10. Uronic acid content of gamma-irradiated fucoidan.** Fucoidan was precipitated from *S. rugosum* mucilage with ethanol. Fucoidan was exposed to increasing dosages of  $\gamma$ -irradiation (kGy) to break down the polysaccharide into oligosaccharide units. Uronic acid content was determined by a sulphuric acid carbazole method.



**Fig. 4.11. Comparative profile of *Splachnidium rugosum* gamma-irradiated fucoidan.** Fucoidan was precipitated from *S. rugosum* mucilage with ethanol. Fucoidan was exposed to increasing dosages of  $\gamma$ -irradiation (kGy) to break down the polysaccharide into oligosaccharide units. L-fucose, sulphate, uronic acid contents were comparatively assessed. No protein was detected.

#### **4.3.3.3) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of gamma-irradiated fucoidan**

Gamma-irradiated fucoidan was subjected to MALDI-TOF MS to further elucidate molecular structure. Several matrices and sample mixtures were assessed in both linear and positive modes of ionization. Spectra produced by MALD-TOF did not present clear definitive peaks, and ions of interest could not be confidently identified under current parameters and conditions.

### **4.4) Discussion**

Chapter 3 assessed the suitability of various fucoidan extraction methods from a range of brown seaweed species of South Africa. Crude *S. rugosum* extracts yielded 5-7 times more L-fucose, irrespective of method of extraction. This chapter presents the fractionation and purification of crude water and acid extracts from *S. rugosum* tissue, as well as from *S. rugosum* mucilage. This chapter also assessed the chemical profiles presented by all purified and fractionated fucoidan extracts.

#### **4.4.1) Assessing ion exchange eluates for fucoidan content**

Fucoidan is a heavily sulphated water-soluble polysaccharide with a net negative charge. Its anionic nature allows for purification by IEC. In this study, a differential precipitation process was utilised which is commonly used to purify pathogenic bacterial lipopolysaccharides for vaccine development. Positively charged CTAB was added to negatively charged fucoidan, thereby forming a CTAB:fucoidan complex. This complex (or polysaccharide salt) is insoluble in water but soluble in ethanol (EtOH). Celite<sup>®</sup> (0.02-0.1 mm pore size), which acts as filter trap, was added and mixed into the solution thereby capturing and retaining most insoluble impurities. The Celite:CTAB:fucoidan slurry was packed into a column and allowed to settle. The CTAB:fucoidan complex was eluted by washing the column with a concentration gradient of ethanolic buffers. The eluates (containing the

CTAB:fucoïdan) were dialysed against sodium chloride (NaCl) to remove the CTAB (CTAB<sup>+</sup> was displaced with Na<sup>+</sup>), thereby forming a Na:fucoïdan complex. The Na:fucoïdan salt is insoluble in alcoholic solution and hence precipitated. Following centrifugation, the supernatant was discarded and the pellet that contained water-soluble fucoïdan was dissolved in water. The Na:fucoïdan salt was dialysed against ddH<sub>2</sub>O to displace Na<sup>+</sup> and lyophilised.

Fractions eluted by IEC containing L-fucose were pooled to ensure maximum recovery of FCSPs. Eluent buffers prepared in different concentrations of alcoholic solvents have been shown to elute polysaccharides of different molecular weight and chemical composition (Gardell, 1957; Bouveng *et al.*, 1963; Zhan *et al.*, 2011; Han *et al.*, 2014). The hot water extraction method yielded FCSPs that were most efficiently eluted with 60% (w/v) EtOH in 0.05M NaOAc buffer ( $p < 0.05$ ), yielding an L-fucose content of  $0.176 \pm 0.023$  mg/mL (Figure 4.3). Similarly, Zvyagintseva *et al.* (1999) reported that a concentration gradient of ethanolic buffers could elute various types of water-soluble polysaccharides from brown seaweeds. However, with the acid extraction method, no significant difference in elution efficiency was recorded at different concentrations of ethanol (Figure 4.4). Eluates obtained with the 60% EtOH buffer (hot water extracted fucoïdan) and the 100% EtOH buffer (acid extracted fucoïdan) were subjected to dialysis and further purification.

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#### 4.4.2) Assessment of fucoïdan purity

Crude hot water and acid extracts from *S. rugosum* tissue were fractionated and purified by anionic IEC. IEC resulted in close to 90% decrease in L-fucose and sulphate content of both extracts (Table 4.1). This decrease is not unexpected, as the sulphate groups are attached to the L-fucose backbone of the fucoïdan polysaccharide. Higher sulphate content in crude acid extract (Figure 4.5) is explained by liberation of sulphate by acid hydrolysis during extraction. Once purified, sulphate content was however ~4-fold lower than in water extracts, while L-fucose content was about 2-fold less. Liberated sulphate was likely removed by the IEC column. Since bioactivity of fucoïdan correlates positively with sulphate content, or more specifically, to sulphate-to-fucose ratio (Jiao *et al.*, 2011), extraction with hot water is recommended. Chapter 5 will delve into the efficacy of the crude versus purified fucoïdan compounds as potential anti-oxidants and anti-cancer agents.

The extraction method employed may alter fucoidan's chemical composition on an ionic level, which may explain the drastic drop in fucoidan yield, as reflected by L-fucose content, after purification by IEC. This could be attributed to the negative charge of the sulphate groups attached to the L-fucose backbone which is the primary contributor to the compound's overall negative charge (Ponce *et al.*, 2003; Mak *et al.*, 2013). A similar phenomenon has been reported by Ale *et al.* (2012) with fucoidan extracts from *Sargassum* species. The sulphate-to-fucose ratio of fucoidan molecules after acid extraction was dependent on concentration of acid, time of extraction, and concentration of ethanol during precipitation. Mabeau *et al.* (1990) showed that fucans extracted with acid from brown seaweeds *Pelvetia canaliculata*, *Fucus vesiculosus*, *Sargassum muticum* and *Laminaria digitata*, and precipitated by EtOH, could be solubilized by Triton X-100 (a surfactant similar to CTAB). Once purified by anion IEC, these fucans displayed a different ionic composition to its crude form. Lee *et al.* (2012) reported that purification of a hot water extract from *Ecklonia cava* by anion IEC yielded FCSPs with varying ionic compositions. Our study confirmed that the method of extraction can affect the composition of fucoidan post-purification.

Significantly higher uronic acid content in crude and purified acid extracts may be explained by co-extraction of alginate. Uronic acid is the primary component of alginate, a cell wall polysaccharide commonly found in brown seaweeds (Lee and Mooney, 2012). Considering that uronic acid often contaminates fucoidan as a co-extract during the isolation process (Ale *et al.*, 2013), its content may be a good indication of fucoidan purity. Conversely, uronic acid is not always a contaminant but may be attached to the fucoidan compound as discrete short branches (Ale *et al.*, 2013). Uronic acid content of crude extracts (water and acid) decreased by close to 90% after IEC (Table 4.1), suggesting that uronic acid is not an integral part of *S. rugosum* fucoidan structure. Saboural *et al.* (2014) included a calcium acetate step in their purification protocol, as the calcium ions bind to uronic acid which can be precipitated out at a later stage. However, in the present study the addition of calcium chloride resulted in only a 10% decrease in uronic acid content of *S. rugosum* fucoidan (data not shown).

While IEC resulted in ~90% decrease in fucoidan yield, no protein was lost, raising questions as to the efficacy of the purification method. Zvyagintseva *et al.* (2003) also reported high protein content of water-soluble polysaccharides extracted from brown seaweeds after purification. The authors hypothesised that purified FCSPs may be a glycoconjugate for proteins and therefore has a high binding affinity for protein. They also reported on high protein content of water-soluble polysaccharides from brown macroalgal *Laminaria* and

*Fucus* species separated chromatographically based on hydrophobic affinities. Proteins were removed from a *Fucus evanescens* fucoidan fraction, containing the largest amount of protein (12%), with the Sevag method (1934). It entailed chloroform and *n*-butanol treatment, followed by polysaccharide precipitation with 80% aqueous EtOH and an acetone wash step. As a result, the molecular weight decreased from 200-500 kDa to 20-30 kDa and a UV spectra peak at 285 nm disappeared. The authors concluded that fucoidan may have formed a complex with protein. High protein content in IEC purified *S. rugosum* extracts may therefore support the notion that fucoidan can exist as glycoconjugates to proteins.

#### **4.4.3) Fucoidan content of *Splachnidium rugosum* mucilage**

Gamma-irradiation is common practice for sterilization and decontamination in the food sector (Farkas, 1998; Howard, 2010). Gamma rays released by radiation are of a high ionizing nature and are therefore hazardous, disrupting genetic and cellular mechanisms of bacteria and pests. Gamma-irradiation has been utilised as a safe means to hydrolyse algal polysaccharides into LMW fractions without disrupting structural or chemical integrity (Choi *et al.*, 2013). This was accomplished by exposing algal material to high-energy photons emitted from an isotope source (e.g. Cobalt 60), thereby disrupting electrons in the product by ionization. This form of ionization can disrupt glycosidic bonds that hold polysaccharides together and degrade them into LMW oligosaccharides (Abad *et al.*, 2013). This study investigated the use of  $\gamma$ -irradiation to fractionate fucoidan. From a range of endemic brown algal species screened, *S. rugosum* was selected as a model organism for this trial experiment due to highest fucoidan content, irrespective of extraction method utilised (Chapter 3, Table 3.2). Fucoidan forms part of the intracellular mucilage of numerous brown seaweed species, and has been shown to be present in the extracellular mucilage of *L. digitata* fronds and on the fruiting tips of *Ascophyllum nodosum* (Black, 1954). Thalli of *S. rugosum* are uniquely filled with a clear sticky viscous gel. Considering the difficulties in extracting pure fucoidan from cellular matter, we assessed the presence and purity of fucoidan in clear thalli mucilage. *S. rugosum* mucilage was removed and dried alongside the rest of the algal tissue, followed by a hot water extraction and ethanol precipitation. The mucilage contained L-fucose (0.072  $\mu\text{g}/\text{mL}$ ), albeit in lower L-fucose concentration than fucoidan extracted from cellular tissue of empty thallus (0.210  $\mu\text{g}/\text{mL}$ ) (Figure 4.6). The mucilage was however free of

cellular debris and protein (results not shown), yielding fucoidan in a comparatively pure form as assessed by chemical profiling. This is the first account of *S. rugosum* fucoidan extraction and assessment from different anatomical parts of the alga.

#### 4.4.3.1) Assessment of gamma-irradiated fucoidan

Mucilage was removed from *S. rugosum* thalli and fucoidan was precipitated with ethanol. Native fucoidan in unexposed (0 kGy) mucilage was estimated at 2367 kDa (Figure 4.7). Gamma-irradiation (10, 30 and 50 kGy) fractionated fucoidan into LMW compounds of 60.0, 16.8 and 15.5 kDa, respectively (Figure 4.7). Cleavage of glycosidic bonds in the polymer was successfully achieved with increasing dosage of irradiation. In a study by Choi *et al.* (2009a), *F. vesiculosus* fucoidan was similarly hydrolysed by  $\gamma$ -irradiation from 217 kDa to 37, 15, and 10 kDa at 10, 30, and 50 kGy, respectively. Other polysaccharides from brown seaweeds such as  $\beta$ -glucan, laminaran and alginate have also been hydrolysed into low molecular weight by  $\gamma$ -irradiation (Nagasawa *et al.*, 2000; Byun *et al.*, 2008; Choi *et al.*, 2013). The dosage of  $\gamma$ -irradiation is not recommended to exceed 50 kGy, considering the drastic drop in molecular weight at higher levels (Figure 4.7). Factors such as molecular weight and chemical composition are determining variables in fucoidans bioactivities. Choi *et al.* (2009a) have shown that an increase in dosages of  $\gamma$ -irradiation and associated decrease in molecular weight was directly correlated with an increase of carbonyl groups and double bonds in fucoidan, as well as bioactivity.

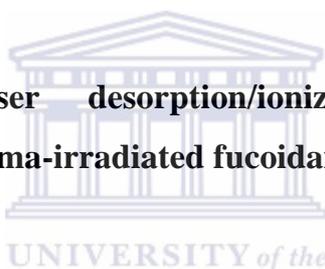
At 10 kGy of  $\gamma$ -irradiation, L-fucose content increased ~1.5-fold, from 0.390 to 0.607 mg/mL (Figure 4.8). Colorimetric determination of L-fucose content included an acid hydrolysis step to liberate L-fucose from the fucoidan polysaccharide. An increase in L-fucose content upon irradiation may be explained by an increase in L-fucose liberation and other monosaccharide sugars upon fractionation. Interestingly, at a dosage of 30 kGy, liberated L-fucose concentration dropped drastically to 0.157 mg/mL, followed by a steady increase at higher dosages reaching 0.495 mg/mL at 80 kGy.

Sulphate content was assessed by a turbidimetric barium salt assay, as described in Chapter 3 (Section 3.2.3), which does not include an acid hydrolysis step. Data presented are therefore an indication of sulphate liberated by fractionation. Irradiation increased sulphate content

>3-fold and concentration remained constant up to 50 kGy (Figure 4.9). At 80 kGy, sulphate content was increased ~4.6-fold compared to unexposed control. Fractionation by irradiation may expose and liberate sulphate groups attached to the backbone of the fucoidan polymer. Results furthermore confirmed high sulphate content of *S. rugosum* fucoidan as determined in Chapter 3. Sulphate content has been shown to be a key determinant in fucoidan's bioactivities, especially in its anti-viral activity against encapsulated viruses (Fitton, 2011). Further research needs to be conducted *in vitro* to test if the sulphation level of fucoidan from *S. rugosum* plays a pivotal role in its bioactivity.

Uronic acid was determined colorimetrically by a sulphuric acid carbazole assay described in Chapter 2 (Section 3.2.3). Uronic acid content did not appear to be affected by irradiation below 80 kGy (Figure 4.10). All samples were free of protein as determined by Bradford assay.

#### **4.4.4) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of gamma-irradiated fucoidan**



MALDI-TOF is a soft ionization technique - along with electrospray ionization (ESI) - that is extensively and effectively utilized in analytical chemistry for the analysis of fragile biological macromolecules such as DNA, protein, polysaccharides and glycoconjugates (Sato *et al.*, 2007), as conventionally MS methodologies tend to fragment fragile macromolecules (Schiller *et al.*, 2004). MALDI and ESI allow for less harsh retrieval of ions from large biomolecules in a gaseous phase (Gross and Strupat, 1998).

No standardised protocol for MALDI-TOF MS analysis of fucoidan samples has been published due to the heterogeneity of fucoidan compounds. Optimisation with regards to sample concentration, matrix type, matrix to sample ratio, and ionization source selection were necessary. The following MS steps (reviewed by Mann and Talbo, 1996; Karas and Krüger, 2003) were followed: (i) fucoidan samples were mixed with appropriate matrix and applied to a metal MALDI plate ("spotting"); (ii) the spotted sample-matrix mixture was irradiated by a pulsed laser, ablation was initiated (i.e. removal of matrix material on a surface by an erosive process, e.g. vaporization) followed by sample desorption; (iii) analyte

molecules were ionized by protonation or deprotonation in the hot plume of the ablated glass; and (iv) accelerated ions were analysed by MS.

Matrices used for this analysis were  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA). Various matrix:sample ratios were analyzed since there is no set standard for fucoidan analysis by MALDI-TOF MS. Limited literature is available on MALDI-TOF MS analysis of fucoidan extracted from seaweed species. Therefore, optimization was conducted with matrix type, sample to matrix ratio, ionization sources, and both linear and positive modes of ionization.

The choice and usage of matrix materials in MALDI-TOF MS improves the desorption and ionization processes, thereby allowing for the assessment of non-volatile thermally accessible molecules (Sze *et al.*, 1998). The precise mechanism of matrix enhancement is not fully understood, but it has been reported that matrices such as 2,5-dihydroxybenzoic acid (DHB) and CHCA yield respectable reproducibility and sensitivity for the analysis of biomolecules. Generally, sample preparation includes a dilution of a small quantity of analyte (approximately 1-10 pmol) within a bigger molar excess of matrix solution, followed by drying the resultant mixture to produce a solid deposit on the metal matrix plate. The analyte-matrix mixture is deposited into the mass spectrometer and analysed by laser pulse of large intensity. The laser desorption of solid analyte-matrix deposits presents several disadvantages including low shot-to-shot reproducibility, short sample longevity, and an excessive dependence on sample preparation methods. The problem of shot-to-shot reproducibility is generally caused by laser-based modifications of the surface structure of the analyte-matrix deposit, and the heterogeneity of the solid deposit. The latter variable is also attributed to the variation in mass spectra obtained from different regions of a sample. Over the last few years numerous novel sample preparation methodologies have been developed to enhance the homogeneity of the analyte-matrix deposit. These methods include vacuum drying, co-crystallization of analyte and matrix molecules by the addition of a “seed” layer, and precoating a matrix substrate for deposition of analytes, and rapid evaporation of matrix followed by an application of the analyte solution in a matrix insoluble solvent.

Spectra were achieved but identification of the peaks could not be defined due to the complex nature of FCSPs, as no database has been established to verify peaks (data not shown). Anastyyuk *et al.* (2009) reported the successful analysis of fucoidan from the brown seaweed *F. evanescens* by MALDI-TOF and tandem ESI-MS. However, their samples were

depolymerized by solvolysis, not  $\gamma$ -irradiation, and a DHB matrix was employed. To date, there are only a few matrices reported that can detect polysaccharides with molecular weights larger than 3000 Da by MALDI MS (Hsu *et al.*, 2007). Hsu *et al.* (2007) reported that 2,4,6-trihydroxyacetophenone (THAP) is a good matrix for MALDI-TOF MS analysis of polysaccharides with a broad mass range. They reported that large polysaccharides, dextrans, glycoproteins and polysialic acids can be successfully detected by MALDI-TOF MS using a THAP matrix.



## 4.5) Conclusion

Crude fucoidan extracted from *Splachnidium rugosum* whole thalli tissue, with hot water or acid, was purified and fractionated by anionic ion exchange chromatography. This is the first study incorporating a novel hexadecyltrimethylammoniumbromide/Celite<sup>®</sup> strategy for fucoidan purification. Low fucoidan yield and high protein content after purification was of concern but may be improved with further optimisation. Conversely, purified fucose-containing-sulphated-polysaccharides may have a high binding affinity for protein as a large amount of protein was purified with fucoidan. Previous studies have shown fucoidan can be a glycoconjugate for proteins. Mucilage from *Splachnidium rugosum* thallus was found to be free of protein and cellular debris. Fucoidan was precipitated from mucilage with ethanol at relatively high yield and purity, and had an average molecular weight of 2367 kDa. The polysaccharide was hydrolysed by gamma-irradiation to fractions ranging between 60 and 15.5 kDa. Further research needs to be conducted to determine whether fractionated and purified fucose-containing-sulphated-polysaccharides from *Splachnidium rugosum* have an increased bioactivity over that of its crude and high molecular weight counterparts. The establishment of a database for fucose-containing-sulphated-polysaccharides or similar sulphated sugars would aid in the detection and elucidation of fucoidan compound's structural architecture by matrix assisted laser desorption/ionization time-of-flight mass spectroscopy.

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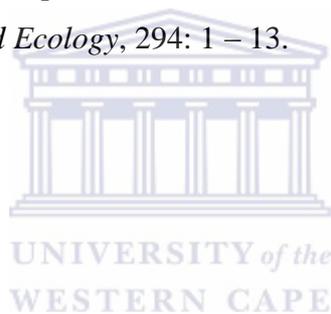
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# Chapter 5. Bioactivity of fucoidan

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## Abstract

Fucoidan is a promising marine-derived agent that holds enormous potential for the pharmaceutical industry because of its organic nature, bioactive properties, and opportunities for sustainable harvesting from seaweeds. Hot water crude fucoidan extracts from *Ecklonia maxima*, *Laminaria pallida*, and *Splachnidium rugosum* were assessed for anti-oxidant activity by measuring the ability to scavenge free radicals and the capacity to reduce copper ions using the 2,2-Diphenyl-1-picrylhydrazyl and Cupric Reducing Antioxidant Capacity assays, respectively. *Ecklonia maxima* crude fucoidan displayed the highest anti-oxidant activity and capacity, having the potential to scavenge reactive oxygen species as well as the capacity to reduce copper to less toxic forms in mammalian systems. *Splachnidium rugosum* showed the weakest anti-oxidant activity and lowest reducing capacity (chelating activity). Crude fucoidan extracts from algae tissue were fractionated and purified by anionic ion exchange chromatography (Chapter 4). Fucoidan was also precipitated from *Splachnidium rugosum* mucilage with ethanol and fractionated by gamma-irradiation. In this study we assessed the anti-cancer activity of crude and purified hot water *Splachnidium rugosum* extracts, as well as non-irradiated (native) and gamma-irradiated fucoidan, and commercially procured fucoidan for anti-cancer activity against MCF-7 breast cancer cells by the WST-1 cell viability assay. *Fucus vesiculosus* fucoidan displayed highest half maximal inhibitory concentration of 0.25 mg/mL. *Splachnidium rugosum* crude and purified fucoidan displayed a half maximal inhibitory concentration of 0.7 mg/mL and 0.029 mg/mL, respectively. Low cytotoxicity of *Splachnidium rugosum* crude and purified fucoidan against non-cancerous breast epithelial cell line, MCF-12A was reflected by half maximal inhibitory concentration values of 2 mg/mL of 0.663 mg/mL, respectively. The cancer specific selectivity of purified *Splachnidium rugosum* fucoidan was therefore much higher as reflected by selectivity indices of 22.86 compared to 2.86. Native and low molecular weight gamma-irradiated fucoidan also showed bioactive properties including anti-cancer activity as seen by the reduction of cell

proliferation *in vitro*, the ability to scavenge free radicals, and the capacity to reduce copper ions.



## 5.1) Introduction

Natural products have been used as therapeutic agents for centuries in folk and traditional medicines (Imhoff *et al.*, 2011; Edwards, 2015). Communities located along coastlines or along neighbouring towns depend on marine derived products for food, medicines, and economic exports. Marine organisms have been shown to contain a number of biologically active compounds, including polysaccharides displaying therapeutic effects in humans and other mammalian systems (Michalak and Chojnacka, 2015). Fucoidan is a marine derived sulphated polysaccharide that possesses numerous bioactivities, including, anti-bacterial (Kandasamy *et al.*, 2015), anti-coagulant (Jiao *et al.*, 2011), anti-inflammatory (Chen *et al.*, 2008), anti-oxidant (Wang *et al.*, 2010), anti-viral (Ponce *et al.*, 2003), anti-protozoal (Torres *et al.*, 2014), anti-cancer (Zhang *et al.*, 2011), anti-angiogenic (Ustyuzhanina *et al.*, 2014) and immunomodulatory activities (Ale *et al.*, 2011). Bioactivity is governed by molecular weight, charge distribution, degree of sulphation, and chemical affinity to other biomolecules such as proteins and viral epitopes (Wozniak *et al.*, 2015). Fucoidan of macroalgal origin is diversely branched compared to that of marine invertebrates (Ale *et al.*, 2013). Algal fucoidan consists of a backbone of  $\alpha$  (1-3)-L-fucopyranose residues or of alternating  $\alpha$  (1-3) and  $\alpha$  (1-4)-linked L-fucopyranosyls, which in either case may be substituted with sulphate or acetate. Side branches contain fucopyranoses or other glycosyls such as uronic acid. To further add to heterogeneity of algal fucoidan, the branching pattern of monomeric units and functional groups appear different in each species of brown algae. The complex chemical and molecular structure of fucoidan has not yet been fully elucidated (Ale *et al.*, 2012). Fucoidan has been reported to have cytotoxic effects against several human cancerous cell lines, reducing the proliferation of tumour growth, angiogenesis, and metastasis (Ustyuzhanina *et al.*, 2014). Anti-cancer activity is displayed in a dosage- and size-dependent manner (Cho *et al.*, 2011). The anti-cancer properties of fucoidan have been examined *in vitro* and *in vivo*, and clinical trials have recently been initiated in human patients (Kimura *et al.*, 2013). Despite its wide range of biological activities, fucoidans mode of action remains to be elucidated.

There are many types of free radicals, but those of most concern in biological systems are derived from oxygen, and are known collectively as ROS. Oxidative phosphorylation is a vital part of the respirative cycle in eukaryotes, whereby nutrients are oxidized into adenosine triphosphate (ATP) for energy (Dean and Allikimets, 1995). ROS are released as a

by-product, such as oxygen ions and peroxides, and are essential in maintaining cellular homeostasis and signalling (Wang *et al.*, 2010). However, when ROS is heavily accumulated due to environmental stress, it can have devastating long term effects on biological macromolecules (Franco *et al.*, 2008). ROS may oxidize cellular proteins and cleave DNA which could cause genetic mutagenesis, cardiovascular disease, cancerous tumour formation and senescence (aging) (Halliwell and Aruoma, 1991; Halliwell and Gutteridge, 2006). This phenomenon is known as oxidative stress. Diets rich in food containing natural containing anti-oxidants are the most common prescribed method to combat oxidative stress (Finkel and Holbrook, 2000; Roberts *et al.*, 2002). Anti-oxidants are molecules that can scavenge and neutralise free radicals which may offer a natural alternative as a cancer therapeutic, opposed to chemically synthesised drugs with harmful side-effects. The food industry incorporates synthetic anti-oxidants in their products to prevent lipid peroxidation caused by ROS (Wang *et al.*, 2010). Some of these anti-oxidants such as butylates hydroxyanisole (BHA), butylatedhydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) may be carcinogenic or toxic to human health (Ito *et al.*, 1989). To overcome health issues, numerous MNPs have been tested for anti-oxidant activity and potential application in food industry or in biological systems (Rupérez *et al.*, 2002).

Biogeography is an important factor in fucoidan chemistry and may therefore influence fucoidan bioactivity. This study reports on the anti-cancer and anti-oxidant activities of fucoidan compounds extracted and purified from South African brown macroalgae.

### **5.1.1) Aim and objectives of this chapter**

The aim of this Chapter was to assess the bioactivity of fucoidan extracted from brown macroalgae of South Africa. The aim was broken down into three objectives:

1. Assessment of anti-cancer activity of crude, purified, and  $\gamma$ -irradiated fucoidan in the breast cancer cell line MCF-7, using cell viability assays.
2. Assessment of cytotoxicity of crude, purified, and  $\gamma$ -irradiated fucoidan in the non-cancerous breast epithelial cell line MCF-12A, using cell viability assays .

3. Assessment of anti-oxidant activity of crude fucoidan by measuring the ability to scavenge free radicals, and anti-oxidant capacity by measuring copper chelating ability.



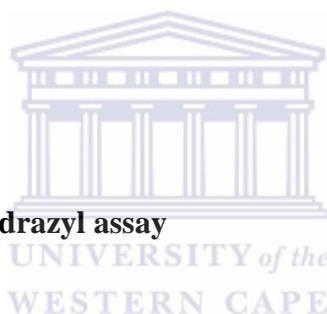
## 5.2) Methods and material

### 5.2.1) Fucoidan samples

Crude hot water fucoidan extracts (Section 3.2.2) from whole algae material of *E. maxima*, *L. pallida*, *S. rugosum*, as well as commercially procured fucoidan (Sigma-Aldrich) (Section 3.2.3) from *F. vesiculosus* were assessed for anti-oxidant activity. FCSPs were assessed for anti-cancer activity and cytotoxicity. These included crude water extracts and fractions purified by IEC (Section 4.2.2), as well as high molecular weight (native) fucoidan precipitated from *S. rugosum* mucilage and 10 kGy  $\gamma$ -irradiated lower molecular weight fractions (Section 4.2.3).

### 5.2.2) Anti-oxidant screening

#### 5.2.2.1) 2,2-Diphenyl-1-picrylhydrazyl assay



Crude hot water fucoidan extracts were tested for anti-oxidant activity by measuring the radical scavenging ability of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radicals. This was achieved by the DPPH assay described by Mensor *et al.* (2001) and Mak *et al.* (2013), with minor modifications. The assay was performed in triplicate. Fucoidan samples were diluted to 0.05-4 mg/mL in ddH<sub>2</sub>O. Fucoidan (100  $\mu$ L) was mixed with 250  $\mu$ L methanolic 0.3mM DPPH (Sigma-Aldrich). In sample blanks, DPPH was substituted for ddH<sub>2</sub>O. Ascorbic acid (Vitamin C: Sigma-Aldrich) was included in assays for comparative analysis. A negative control was prepared that consisted of 100  $\mu$ L methanol (Sigma-Aldrich) and 250  $\mu$ L methanolic DPPH (0.3mM). Reactions were incubated at RT for 30 min, and the absorbance was measured at 518 nm in a 96-well microtitre plate (Greiner) with a microtitre plate reader (Nano spectrophotometer: BMG LABTECH). The following equation was used to calculate antioxidant activity, as described by Mensor *et al.* (2001):

$$\text{Antioxidant activity (\%)} = 100 - \left[ \frac{(Abs_{\text{sample}} - Abs_{\text{sample blank}}) * 100}{(Abs_{\text{negative control}})} \right]$$

### 5.2.2.2) Cupric Reducing Antioxidant Capacity assay

Crude hot water fucoidan extracts were assessed in triplicate for anti-oxidant capacity by Cupric Reducing Antioxidant Capacity (CUPRAC) assay, which measures the reducing power of copper as described by Mak *et al.* (2013) and Apak *et al.* (2008). Fucoidan samples were diluted to 0.5-3 mg/mL in ddH<sub>2</sub>O and 100  $\mu$ L was added to a solution containing 100  $\mu$ L 0.01M copper chloride (CuCl<sub>2</sub>: Sigma-Aldrich), 100  $\mu$ L 1M ammonium acetate (NH<sub>4</sub>Ac: Sigma-Aldrich) (pH 7.0), 100  $\mu$ L 0.075M neocuproine (in EtOH) and 10  $\mu$ L ddH<sub>2</sub>O. In blanks, samples were substituted for ddH<sub>2</sub>O. The reaction was incubated at RT for 10 min, and the absorbance measured at 450 nm in a 96-well microtitre plate (Greiner) with a microtitre plate reader (Nano spectrophotometer: BMG LABTECH, USA). An increase in absorbance is indicative of an increase in copper reducing ability.

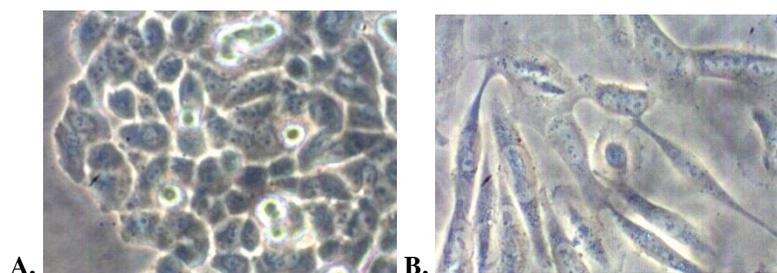
### 5.2.3) Anti-cancer and cytotoxic screening

#### 5.2.3.1) Culturing (seeding) cell lines



Immortalized *Homo sapiens* breast cancer cell line MCF-7 (ATCC<sup>®</sup>) and non-cancerous human breast epithelial cell line MCF-12A (ATCC<sup>®</sup>) were aseptically cultured *in vitro* at the Animal Cell Culture Laboratory of the Department of Biotechnology, University of the Western Cape. All reagents and media were removed from storage and allowed to reach RT. A vial containing 2 mL of cryopreserved MCF-7 cells was removed from a -150°C cryogenic storage freezer (Cryo Freezer Conquerer -150°C, OPERON Co., Ltd.) and partially thawed. Cells were resuspended in 9 mL Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12: GIBCO<sup>®</sup> Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS: Biochrom The Scientific Group) and 100 U/mL penicillin-streptomycin (PENSTREP) (Lonza WhiteSci). Cells were pelleted by centrifugation (2,500 rpm for 3 min) and resuspended in 10 mL DMEM/F12. The suspension was transferred to 25cm<sup>2</sup> flasks (Greiner) and incubated in a humidified atmosphere inside a water-jacketed incubator (Labtech) at 37°C with 5% carbon dioxide (CO<sub>2</sub>). Growth rate of cells were manipulated with 1:5, 2:5, or 3:5 dilution with media. Cells were cultured to 70-80% confluency (Fig 4.1) and inspected visually under a light microscope (Nikon TMS).

MCF-12A cell line was cultured under the same conditions with the following modification: DMEM/F12 (GIBCO® Life Technologies) was supplemented with 10% (v/v) FBS (Biochrom), 100 U/ml PENSTREP (Lonza), 20 ng/mL LONG® human epidermal growth factor (EGF: SAFC Biosciences), 0.01 mg/ml bovine insulin (Roche) and 500 ng/mL hydrocortisone (Sigma-Aldrich).



**Figure 5.1. Confluent culture of cells visualized and captured by light microscope (200X magnification).**  
A. Confluent MCF-7 cells B. Confluent MCF-12A.

### 5.2.3.2) Maintaining cells in culture

All reagents and media were removed from storage and allowed to reach RT. Cells in culture were inspected daily under a light microscope to determine cell confluency. Twenty four hours after seeding a culture, 5 mL of culture medium was replaced with fresh medium. This process was subsequently repeated every 48 hours until a desired confluency was reached.

### 5.2.3.3) Sub-culturing (“splitting”) cell lines

Once cells had reached a desired confluency, used culture medium was discarded and the flask was rinsed with 10 mL 1X phosphate-buffered saline (PBS: Biochrom). Monolayers of attached cells were enzymatically dissociated from the flask by trypsinization. Cells were trypsinized by adding 3 mL 0.25% (v/v) trypsin containing 0.02% (v/v) ethylenediaminetetraacetic acid (trypsin-EDTA) and incubated at 37°C with 5% CO<sub>2</sub> for 2-5 min. Flasks were microscopically examined to ensure complete detachment of cells and reincubated if necessary. Three mL of culture medium was added to the cell suspension to

inactivate trypsin, and the suspension was transferred to a 15 mL centrifuge tube (Greiner). Cells were pelleted by centrifugation (2,500 rpm for 3 min) and resuspended in 10 mL fresh culture medium. Appropriate aliquots of suspension were transferred to 25cm<sup>2</sup> flasks and incubated at 37°C with 5% CO<sub>2</sub>.

#### **5.2.3.4) Cryopreservation of cells**

Cells at 70-90% confluency that were not required for immediate usage were preserved at -150°C. Firstly, cells were detached from flasks by trypsinization and pelleted by centrifugation (as described in Section 5.2.3.3). Cell pellets were resuspended in 10 mL fresh culture medium containing 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich), transferred to 1.5 mL cryovials (SPL Life Sciences) and stored in a cryogenic freezer.

### **5.3.3) Assay for cellular proliferation**



#### **5.3.3.1) Cell counting and viability analysis**

All reagents and media were removed from storage and allowed to reach RT. Cells at desired confluency were seeded into a 96-well flat bottom microtitre plate (Greiner). Once attached to the surfaces of the microtitreplate, cells were assayed for cell viability, cell proliferation, and cytotoxicity. Firstly, cells were trypsinized (Section 5.2.2.3) followed by mixing a 10 µL aliquot of cell suspension with 10 µL of trypan blue dye (Invitrogen). Thereafter, 10 µL of the mix was transferred to a Countess Chamber Slide (Invitrogen). Cell count or cell viability was recorded on a Countess™ automated cell counter (Invitrogen). Cells were seeded into a 96-well microtitre plate at densities of 2 000 cells/mL to 50 000 cells/mL (in a final volume of 100 µL/well), and incubated for 24, 48 and 72 hours at 37°C with 5% CO<sub>2</sub>. Optimal conditions were selected for subsequent experiments after analysis of the absorbance spectra (Section 5.3.3).

### **5.3.3.2) Drug compound preparation and administration**

Stock solutions of FCSPs (10 mg/mL) were prepared in UV-treated ddH<sub>2</sub>O or media as diluent. Cells at desired confluency were treated with 100 µL of drug mix (70 µL diluent: 30 µL drug) to final concentrations ranging from 0.03125 to 4 mg/mL per well. To administer the drug dosages, spent media was removed and wells were washed twice with 100 µL of 1X PBS. Thereafter, 100 µL of the drug-dosage-mix was added to the well and the plate was incubated for 24, 48, and 72 hours at 37°C with 5% CO<sub>2</sub>. Optimal conditions were selected for subsequent experiments after analysis of the absorbance spectra (Section 5.3.3).

### **5.3.3.3) Bioactivity assay**

An aliquot of the cell proliferation reagent 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1; Roche) was removed from storage and allowed to thaw in the dark. Ten µL of WST-1 was added to each well of a microtitreplate, which contained controls, blanks, or samples. Plates were incubated at 37°C for 1, 2, and 4 hours. Absorbance were spectrophotometrically analysed against a background control used as a blank with a microtitre plate reader (Nano spectrophotometer: BMG LABTECH) between 420-480 nm. A reference absorbance was measured at 610 nm. Once analysis of the absorbance spectra was completed, optimal conditions were selected for subsequent experiments.

## 5.3) Results

### 5.3.1) Anti-oxidant activity of fucoidan

Hot water crude fucoidan extracts and a commercially procured sample were assessed for anti-oxidant activity. The ability of seaweed extracts to scavenge free radicals was measured by the DPPH assay (Table 5.1).

**Table 5.1. Anti-oxidant activity of seaweeds as defined by ability to scavenge free radicals.**

Family	Seaweed species	Anatomy	<sup>a</sup> EC <sub>50</sub> ± SD (mg/ml)
Fucaceae	<sup>b</sup> <i>Fucus vesiculosus</i>	n/a	4.2 ± 1.4
Splachnidiaceae	<i>Splachnidium rugosum</i>	Whole algae	3.0 ± 0.4
Laminariaceae	<i>Laminaria pallida</i>	Whole algae	2.7 ± 0.9
Lessoniaceae	<i>Ecklonia maxima</i>	Whole algae	2.5 ± 1.5
n/a	<sup>c</sup> Vitamin C	n/a	1.3 ± 0.7

<sup>a</sup>Values obtained from regression lines with a 95% confidence level. EC<sub>50</sub> is defined as the half maximal effective concentration of DPPH. Standard deviation (SD) is calculated from the mean of three independent experiments

<sup>b</sup>Commercially procured crude fucoidan from Sigma-Aldrich

<sup>c</sup>Vitamin C was used as a positive control

\*n/a = not applicable

The CUPRAC assay assessed anti-oxidant capacity by measuring the ability of the test compound to reduce copper ions. *E. maxima* crude fucoidan displayed the highest ( $p < 0.05$ ) reducing ability, almost 5-fold higher than *S. rugosum* (Figure 5.2).

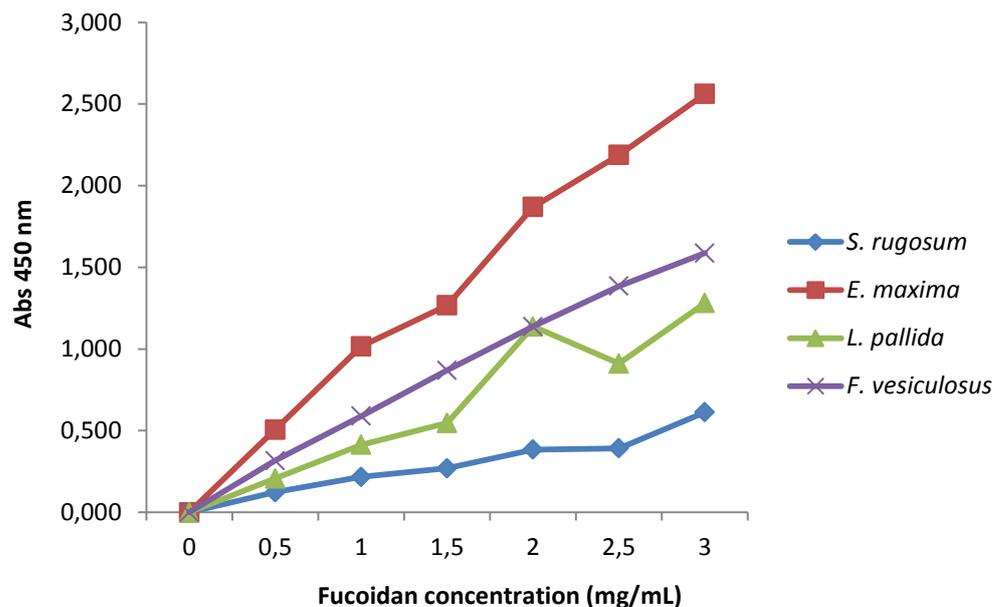


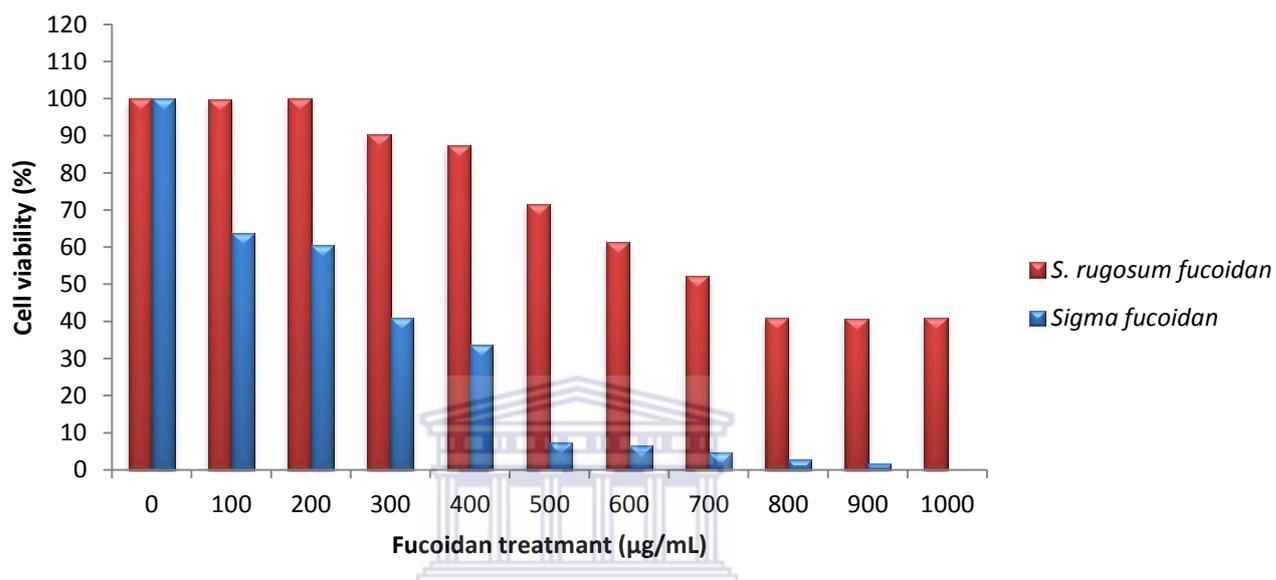
Fig. 5.2. Anti-oxidant capacity of crude fucoïdan extracts as measured by the CUPRAC assay.

### 5.3.2) Anti-cancer and cytotoxicity screening of fucoïdan

FCSPs from *S. rugosum* were assessed for *in vitro* anti-cancer activity and cytotoxicity against MCF-7 and MCF-12A cell lines, respectively. Commercial crude fucoïdan from *F. vesiculosus* was used as a control. Optimal conditions for the WST-1 cell viability assay are presented in Table 5.2. Fucoïdan displayed anti-cancer activity in a dose-dependent manner (Figure 5.3). IC<sub>50</sub> is defined as the fucoïdan concentration that inhibits 50% of cell growth. Compared to crude water extracts, IEC purified *S. rugosum* fucoïdan possessed lowest IC<sub>50</sub> values of 0.029 mg/mL and 0.663 mg/mL against MCF-7 and MCF-12A cells, respectively (Table 5.3). Purified fucoïdan however displayed the highest cancer specific selectivity index of 22.86.

**Table 5.2. Optimal WST-1 assay conditions for cytotoxicity screening of fucoïdan from this study.**

Cell seeding density	Drug exposure time	WST-1 incubation time	Absorbance wavelength
$5 \times 10^4$ cells/mL	24 hours	2 hours	430 nm



**Fig. 5.3. MCF-7 cell viability after 24 hr treatment with *Splachnidium rugosum fucoïdan*.** Commercially available fucoïdan (Sigma-Aldrich) was used as a control. Graph represents cell viability (%) after assessment with WST-1 cell proliferation reagent (Roche).

**Table 5.3. IC<sub>50</sub> values and selectivity index of fucoidan compounds against various cell lines.**

Cell line	Cellular origin	Cell type	Compound origin	Molecular weight (kDa)	<sup>a</sup> IC <sub>50</sub> (mg/ml)	<sup>b</sup> Selectivity index (SI)
<b>Crude tissue extract</b>						
MCF7	Human (breast)	Epithelial (cancerous)	<i>F. vesiculosus</i>	20.5	0.25	nd <sup>c</sup>
<b>Crude tissue extract</b>						
MCF7	Human (breast)	Epithelial (cancerous)	<i>S. rugosum</i>		0.70	
MCF12A	Human (breast)	Epithelial (non-cancerous)	<i>S. rugosum</i>	nd <sup>c</sup>	2.00	2.86
<b>IEC purified tissue extract</b>						
MCF7	Human (breast)	Epithelial (cancerous)	<i>S. rugosum</i>		0.03	22.86
MCF12A	Human (breast)	Epithelial (non-cancerous)	<i>S. rugosum</i>	<3.5	0.66	
<b>Mucilage Precipitate</b>						
MCF7	Human (breast)	Epithelial (cancerous)	<i>S. rugosum</i>	2367	2.00	2.00
MCF12A	Human (breast)	Epithelial (non-cancerous)	<i>S. rugosum</i>		4.00	
<b>γ -Irradiated (10 kGy) mucilage</b>						
MCF7	Human (breast)	Epithelial (cancerous)	<i>S. rugosum</i>	60	4.00	nd

<sup>a</sup>IC<sub>50</sub> is defined as the fucoidan concentration that inhibits 50% of cell growth. Results are the mean of three independent experiments.

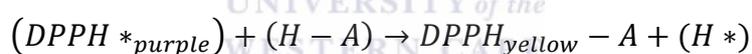
<sup>b</sup>Selectivity indices were calculated by dividing the IC<sub>50</sub> mean of non-cancerous cell line divided by the mean of the cancerous cell line.

<sup>c</sup>not determined

## 5.4) Discussion

### 5.4.1) Anti-oxidant activity of fucoidan by 2,2-Diphenyl-1-picrylhydrazyl assay

Crude and commercial fucoidan samples were tested for antioxidant activity by measuring the radical scavenging ability using the DPPH assay. The principle of the assay is described below. DPPH is a stable free radical that possesses an extra electron that delocalizes the orbit of the entire molecule (Szabo *et al.*, 2007). This delocalization allows DPPH to appear as a dark purple coloured substance in a methanolic solvent. When DPPH is added to a solution containing a hydrogen atom donor (H-A) (such as an anti-oxidant), a non-radical form of DPPH is formed (DPPH-A) (Brand-Williams *et al.*, 1995). That is, the free radicals are scavenged by anti-oxidant, which in turn causes a colour shift from deep purple to pale yellow (Mak *et al.*, 2013). The equation below depicts the colorimetric reaction that occurs when an anti-oxidant scavenges the overly protonated DPPH-H molecule (purple) to the neutralised DPPH molecule (yellow):

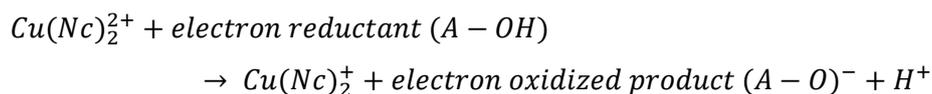


The half maximal effective concentration (EC<sub>50</sub>) is a pharmacological term used to represent the concentration of a bioactive compound that induces a response halfway between the baseline and maximum after a specified exposure time (Sebaugh, 2011), in other words, the concentration where 50% of its maximal effect is observed. Vitamin C is a natural anti-oxidant (Liu *et al.*, 2008) and was used as a positive control in this study. Vitamin C displayed the strongest anti-oxidant activity as indicated by lowest EC<sub>50</sub> of 1.3 ± 0.7 mg/mL (Table 5.1). Ayoola *et al.* (2008) reported an EC<sub>50</sub> of 0.054 mg/mL for Vitamin C. Vitamin C is an efficient free radical scavenger, and therefore an ideal positive control in DPPH assays. The anti-oxidant activity of all fucoidan samples as determined by the DPPH assay were dosage-dependent (Table 5.1). *F. vesiculosus* commercial fucoidan displayed weakest anti-oxidant activity seen by highest EC<sub>50</sub> value of 4.2 ± 1.4 mg/mL (Table 5.1). The commercial sample is sold as a crude acid extract from *F. vesiculosus*. In contrast, all South African seaweed samples were extracted with hot water to retain native polysaccharide

structure, which may explain higher anti-oxidant activity at lower doses. *S. rugosum* crude fucoidan displayed anti-oxidant activity with an EC<sub>50</sub> of 3.0 ± 0.9. There is no published literature on the assessment of *S. rugosum* fucoidan anti-oxidant activity. *L. pallida* crude fucoidan displayed the second strongest anti-oxidant activity with an EC<sub>50</sub> of 2.7 ± 0.9. Activity furthermore appears to be stronger than its Northern Hemispheric neighbour *L. japonica*. Wang *et al.* (2010) have reported EC<sub>50</sub> values were in the range of 3.6-3.7 mg/mL for several LMW fucoidan derivatives extracted from *L. japonica*. *E. maxima* crude fucoidan displayed the strongest anti-oxidant activity as seen by lowest EC<sub>50</sub> of 2.5 ± 1.5. Kuda *et al.* (2007) have extracted water-soluble polysaccharides (including fucoidan and alginate) from *Ecklonia stolonifera* and *Ecklonia kurome*. Water extracts have shown a stronger anti-oxidant scavenging ability than alcoholic extracts from the same species, due to the presence of large concentrations of phlorotannin and phenolic compounds. Maina *et al.* (2014) reported that *E. maxima* possess an abundance of phlorotannins, a natural anti-oxidant found in brown macroalgae. Phlorotannins may be contained within crude fucoidan extracts from *E. maxima*, which could aid in the compound's effectiveness as an anti-oxidant. The ability of an anti-oxidant to neutralise the free radical DPPH has been accredited to several mechanisms including protonation, radical scavenging, chain inhibition, and deterrence of constant hydrogen abstraction (Wang *et al.*, 2009). Strong anti-oxidant activity of fucoidan may be due to the hydrogen-donating ability of fucoidan to form a stable DPPH-H molecule (Wang *et al.*, 2010). The presence of sulphate groups may activate the hydrogen atom of the anomeric carbon. The sulphate content as well as the sulphate-to-fucose ratio may influence anti-oxidant activity of fucoidan.

#### **5.4.2) Anti-oxidant capacity of fucoidan by Cupric Reducing Antioxidant Capacity assay**

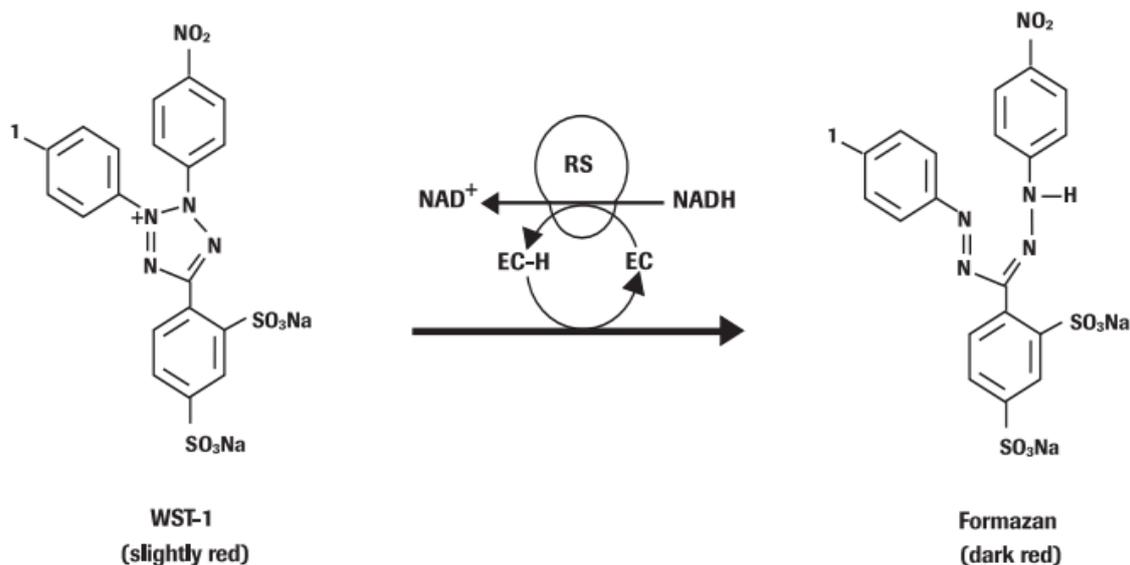
Crude and commercial fucoidan samples were assessed for anti-oxidant capacity with the CUPRAC assay (Apak *et al.*, 2008). The principle of the assay works by reducing Cu(II) to Cu(I) where absorbance is recorded at a maximum wavelength of 450 nm. Cu(II)-Nc acts as the chromogenic oxidising reagent, which reacts with an electron reductant antioxidant (A-OH). This gives rise to the CUPRAC chromophore, copper(I)-neocuproine (Cu(I)-Nc) chelate (Güçlü *et al.*, 2006). The reaction formula is illustrated below:



Crude fucoidan extracts (3 mg/mL) reduced  $\text{Cu}^{2+}$  to  $\text{Cu}^+$  in a dosage-dependent manner (Figure 5.2). A higher absorbance measurement at a wavelength of 450 nm indicates a stronger CUPRAC anti-oxidant capacity. *E. maxima* showed the strongest ability to reduce copper. The anti-oxidant properties of *F. vesiculosus* and *L. pallida* were similar ( $p > 0.05$ ). *S. rugosum* displayed weakest capacity, ~4.3-fold lower than *E. maxima*.

### 5.4.3) Anti-cancer and cytotoxicity screening of fucoidan

FCSPs were assessed for *in vitro* anti-cancer activity and cytotoxicity against MCF-7 and MCF-12A cell lines, respectively. The WST-1 cell viability assay was chosen for anti-cancer and cytotoxic analyses due to its robustness, stability, user-friendliness, sensitivity, accuracy, and proven track record of success (Berridge and Tan, 1998). The principle of this assay is based on the conversion of stable water-soluble tetrazolium salts (WST) into soluble formazan by cellular enzymes, namely mitochondrial dehydrogenases (Figure 5.4). Tetrazolium salts are cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. Dehydrogenases are released extracellularly, and the colorimetric conversion takes place at the cell surface. Living cells produce NAD(P)H by glycolysis, which is essential for the conversion of WST-1 (slightly red) to formazan (dark red). Dead cells will appear light red to yellow (depending on the colour of the culture medium). The formazan dye content is quantitated colorimetrically and correlates to the number of metabolically active cells in the culture medium.



**Fig. 5.4. Schematic illustrating the conversion of WST-1 to formazan.** EC = electron coupling reagent, RS = mitochondrial succinate-reductase system (Taken from Roche protocol: <https://pim-eservices.roche.com/LifeScience/Document/c4c412ae-96ed-e311-98a1-00215a9b0ba8>)

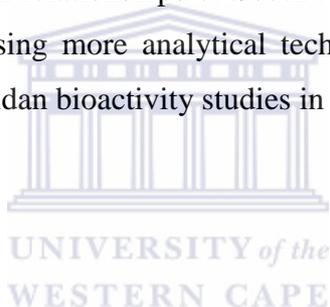
Fucoidan from *S. rugosum* species was selected for bioactivity studies, as the polysaccharide could be extracted at the highest yield and purity compared to extracts from other species (Chapter 3). The anti-cancer activity of *S. rugosum* crude fucoidan was compared with activity of commercially procured crude fucoidan from *F. vesiculosus*. Bioactivity increased with an increase in fucoidan concentration in a dosage-dependent manner (Figure 5.3). *F. vesiculosus* fucoidan displayed higher activity with an  $IC_{50}$  of 0.25 mg/mL. Mak *et al.* (2014) reported a similar  $IC_{50}$  for commercially procured *F. vesiculosus*. *S. rugosum* crude and purified fucoidan displayed an  $IC_{50}$  of 0.7 mg/mL and 0.029 mg/mL, respectively (Table 5.3). Higher efficacy of purified fucoidan compared to crude is likely due to low molecular weight (<3.5 kDa). Factors such as molecular weight and chemical composition are determining variables in fucoidans bioactivities. Choi *et al.* (2009) have shown that an increase in dosages of  $\gamma$ -irradiation and associated decrease in molecular weight was directly correlated with an increase of carbonyl groups and double bonds in fucoidan, as well as bioactivity. Low cytotoxicity of *S. rugosum* crude and purified fucoidan against non-cancerous breast epithelial cell line MCF-12A was reflected by  $IC_{50}$  values of 2 mg/mL and 0.663 mg/mL, respectively. The cancer specific selectivity of purified *S. rugosum* fucoidan was therefore much higher as reflected by an almost 10-fold increase in selectivity index (SI), 22.86 compared to 2.86. Prayong *et al.* (2008) have presented that plant extracts with SI

values greater than 3 for cancer cell lines are considered high selectivity. Low cytotoxicity have of fucoidan has also been reported for non-cancerous cell lines such as HDFb (derma fibroblast), HUVEC (umbilical vein), and HEK-293 (embryonic kidney).

Native fucoidan (2367 kDa) has been precipitated from *S. rugosum* mucilage with EtOH and fractionated into low molecular weight compounds using  $\gamma$ -irradiation (Chapter 4). Exposure to 10, 30 and 50 kGy has yielded fractions of 60.0, 16.8 and 15.5 kDa, respectively. Native and 60 kDa fractions were assayed for activity against MCF-7, with no difference in inhibition profile (results not shown). Native fucoidan and 60 kDa fraction displayed an IC<sub>50</sub> value of 2 mg/mL and 4 mg/mL respectively against MCF-7 breast cancer cells (Table 5.3). Anti-cancer activity in mucilage was furthermore considerably less than IEC purified samples, which may be explained by low molecular weight of purified polysaccharide (<3.5 kDa). Future studies should include comparative analysis of the lower molecular fractions obtained at higher doses of  $\gamma$  -irradiation. Choi and Kim (2013) showed that 10 kGy gamma irradiated fucoidan (38 kDa) from *F. vesiculosus* (Sigma-Aldrich) showed a greater cytotoxicity to cancer cell lines AGS, MCF-7, and HepG-2 (IC<sub>50</sub> values of 2-4 mg/mL), compared to its native counterpart (217 kDa). The authors presented similar chemical and bioactive profiles to that of  $\gamma$  -irradiated fucoidan from *S. rugosum*.

## 5.5) Conclusion

Fucose-containing-sulphated-polysaccharides from brown macroalgae displayed high anti-oxidant activity and may have potential as a natural dietary supplement. The health implications of natural anti-oxidants are significant as these compounds have the ability to quench ROS under oxidative stress environments. This is the first study assessing fucoidan from *S. rugosum* for anti-cancer activity. Fucoidan extracts often consist of carbohydrate polymer mixtures, the composition of which is influenced by the method of extraction. Bioactivity not only depends on purity of the extract, but also on chemical structure of fucoidan polysaccharide, which is governed by species and biogeography. An anti-cancer drug should be effective at low concentrations, and possess little or no cytotoxicity to non-cancerous cells, characteristics displayed by *S. rugosum* purified fucoidan. Further studies aim to elucidate structure-function relationships of South African fucoidan compounds by in depth profiling of compounds using more analytical techniques such as nuclear magnetic resonance, as well as *in vivo* fucoidan bioactivity studies in animal models.



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## Chapter 6. General Conclusion

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Fucoidan was extracted from three species of South African brown seaweed, *Ecklonia maxima*, *Laminaria pallida* and *Splachnidium rugosum*. Three different methods were used to extract fucoidan in an effort to determine the influence of various extraction parameters on the heterogeneity of fucoidan. Extraction methods employed water, acid or salt. The hot water extraction method yielded highest L-fucose content, while the calcium chloride salt method yielded fucoidan with lowest uronic acid contamination. Extracts obtained with hydrochloric acid extraction yielded highest uronic acid contamination and sulphate content. High sulphate content may be explained by the liberation of sulphate from fucoidan polymers as a result of acid hydrolysis. All extracts were considered free of protein. Fucoidan is typically subjected to acid hydrolysis before sulphate assessment. The barium sulphate turbidimetric reaction was used to analyse liberated sulphate. This study has shown that it is possible to comparatively analyse sulphate content of fucoidan extracts without hydrolysis. However, quantitative analysis of sulphate content relies on acid hydrolysis. A drawback of the acid method includes higher alginate co-extracts (as assessed by uronic acid content), whilst fucoidan yield was very low with the salt method. Instead of using one extraction method, a better approach might be to extract fucoidan with hot water to retain its structure in its most native form, apply mild acid hydrolysis to yield lower molecular weight compounds with potentially higher bioactivity, and use a salt step to bind and remove cationic contaminants such as alginate, thereby increasing purity. The extraction method for fucoidan and other fucose-containing-sulphated-polysaccharides is critical in maintaining structural integrity and therefore bioactivity. However, the significance of this concept is often overlooked. *Splachnidium rugosum* tissue yielded >5 times more fucoidan than *Ecklonia maxima* and *Laminaria pallida*. Monosaccharide analysis furthermore revealed highest L-fucose ratio. There were only minor compositional differences between fucoidan extracted from the same species. Inter-species variation between *Splachnidium rugosum* from South Africa and New Zealand was more prominent and may be explained by differences in e.g. climate, tidal, and geochemical conditions. The fucose-to-sulphate ratio of fucoidan from *Ecklonia maxima* (1:5) is the highest reported to date. Degree of sulphation is the primary

contributor to fucoidan's bioactive properties in mammalian systems. While fucoidan from *Splachnidium rugosum* generated in this study was fractionated and purified, and assessed for anti-oxidant and anti-cancer properties (Chapter 4), future studies should also include bioactivity assays with *Ecklonia maxima* extracts.

Crude fucoidan extracted from *Splachnidium rugosum* whole thalli tissue, respectively with hot water and acid, was purified and fractionated by anionic ion exchange chromatography. This is the first study incorporating a novel Celite<sup>®</sup> column for fucoidan purification. Low fucoidan yield and high protein content after purification were of concern but may be improved with further optimisation. On the other hand, purified fucose-containing-sulphated-polysaccharide may have a high binding affinity for protein. Mucilage from *Splachnidium rugosum* thallus proved free of protein and cellular debris. Fucoidan was precipitated from mucilage with ethanol at relatively high yield and purity and an average molecular weight of 2367 kDa. The polysaccharide was hydrolysed by gamma-irradiation to fractions ranging between 60 and 15.5 kDa. Further research needs to be conducted to determine whether fractionated and purified fucose-containing-sulphated-polysaccharide from *Splachnidium rugosum* have an increased bioactivity over that of its crude and high molecular weight counterparts. The establishment of a database for fucose-containing-sulphated-polysaccharide or similar sulphated sugars would aid in the detection and elucidation of fucoidan compound's structural architecture by matrix assisted laser desorption ionization time-of-flight mass spectroscopy.

Fucose-containing-sulphated-polysaccharides from brown macroalgae displayed high anti-oxidant activity and may have potential as a natural dietary supplement. The health implications of natural anti-oxidants are significant as these compounds have the ability to quench reactive oxygen species under oxidative stress environments. This is the first study assessing fucoidan from *Splachnidium rugosum* for anti-cancer activity. Fucoidan extracts often consist of carbohydrate polymer mixtures, the composition of which is influenced by method of extraction. Bioactivity not only depends on purity of extract, but also on chemical structure of fucoidan polysaccharide, which is governed by species and biogeography. An anti-cancer drug should be effective at low concentrations, and possess little or no cytotoxicity to non-cancerous cells, characteristics displayed by *Splachnidium rugosum* purified fucoidan. Further studies aim to elucidate structure-function relationships of South African fucoidan compounds by deeper profiling of compounds using more analytical

techniques such as nuclear magnetic resonance, as well as assessing fucoidan bioactivity in *in vivo* animal models.

