An Investigation of the Potential Anti-diabetic (insulinomimetic) Activity of Anti-oxidant Compounds Derived from *Sargassum heterophyllum*

A Thesis Submitted in Fulfilment of the Requirements for the degree of

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By

Mutenta Nsokolo Nyambe

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In dedication to my late mother, Christine Nsokolo

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Table of Contents

List of Figures

List of Schemes

List of Tables

List of Abbreviations

µM	Micromolar
µmol	Micromoles
Ag_2O	Silver oxide
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
$CHCl_3$	Trichloromethane/ chloroform
d	Doublet
dd	Doublet doublet
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
EC_{50}	50% Effective Concentration
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
Fr	Fraction
GDM	Gestational diabetes mellitus
HOPE	Heart Outcomes Prevention Evaluation Study
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IC_{50}	50% Inhibitory Concentration
IDDM	Insulin dependent diabetes mellitus

IDF	International Diabetes Federation
J	Spin-spin Coupling Constant
m	Multiplet
MeOH	Methanol
MHz	Megahertz
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
Mult	Multiplicity
NDK	Noordhoek
NIDDM	Non-Insulin dependent diabetes mellitus
NMR	Nuclear Magnetic Resonance spectroscopy
ºC	Degrees Celsius
PPARγ	Peroxisome proliferator-activated receptor gamma
ppm	Parts per million
PTP1B	Protein tyrosine phosphatase 1B
q	Quartet
s	singlet
SHQA	Sargahydroquinoic acid
SQA	Sargaquinoic acid
STZ	Streptozotocin
TZD	Thiazolidinediones
WHO	World Health Organization
δ	Chemical shift (ppm)

Abstract

In Africa, non-communicable diseases such as diabetes mellitus have been generally neglected. This problem has worsened over the years owing to continuous threats from infectious diseases such as HIV/AIDS, tuberculosis and malaria. Despite this, statistics have shown that by 2030, the African region will have the highest proportional increase in diabetes prevalence. Over 80% of all diabetic deaths occur in developing countries probably not only due to poor equity of access to medication but also due to limited efficacy and side effects associated with the commonly available anti-diabetic agents. Therefore, this creates the desperate need for the development of new anti-diabetic agents that are more efficacious and can be sourced from within the continent.

With oxidative stress as a suggested mechanism underlying the cause of diabetes mellitus and diabetic complications, the discovery of natural anti-oxidants that prevent free radical mediated damage is important for developing new treatment strategies. Marine algae have been identified as good sources for natural anti-oxidants. Unfortunately, very few studies have embarked on the discovery of marine-derived anti-oxidant compounds with potential anti-diabetic activity.

In this project, we investigated the potential anti-oxidant activity of the South African endemic algae *Stypopodium multipartitum, Dictyopterus ligulata, Cystophora fibriosa, Bifurcariopsis capensis, Sargassum* sp. and *Sargassum heterophyllum*. From these studies, *Sargassum heterophyllum* yielded prenylated compounds, the main compound being sargahydroquinoic acid (**3.6**) and the carotenoid metabolite fucoxanthin (**3.8**), which are in part responsible for the radical scavenging activity of the crude extract. Sargahydroquinoic acid (**3.6**) and fucoxanthin (**3.8**) also exhibited significant anti-inflammatory activity.

Sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**) were then semi-synthesized from sargahydroquinoic acid (**3.6**) and their *in-vitro* cytotoxicity profiles evaluated using Chang Liver, HT-29, Caco-2 and 3T3-L1 cell lines prior to anti-diabetic testing. From the semi-synthetic derivatives, sargachromenoic acid (**3.9**) exhibited the most potent anti-oxidant activity (IC_{50} = 6.99 µg/mL). After the evaluation of anti-diabetic activity using 3T3-L1 preadipocyte differentiation, sarganaphthoquinoic acid (**3.10**) showed the most potent insulinomimetic activity at 1.19 µM by inducing a PPARγ response similar to that of rosiglitazone at 1 µM.

Chapter 1

Literature Review

1.1 General Introduction to Diabetes Mellitus

Diabetes mellitus (DM) is a heterogeneous disease which is mainly comprised of poor insulin secretion and lack of insulin sensitivity (Puavilai et al. 1999). Regardless of aetiological cause, diabetes is associated with hyperglycaemia (American Diabetes Association 2009), which is defined as a sustained elevation of glucose in blood plasma. In 1979, The National Diabetes Data Group (NDDG) and the World Health Organisation (WHO) classified diabetes mellitus as Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM) (Alberti and Zimmet 1998). However, due to complications in epidemiologic evaluation and clinical management as well as the discovery of other types of diabetes with different pathophysiologies, a new classification was implemented comprising of four major classifications which are: Diabetes mellitus type I (formally called IDDM), diabetes mellitus type II (formally called NIDDM), Gestational Diabetes Mellitus (GDM) and diabetes secondary to other conditions such as endocrinopathies, drug/chemical-induced, infections and genetic syndromes (Mayfield 1998). The global diabetes morbidity sums up to 346 million people, with a 90% contribution from type II diabetics (WHO 2012). More than 80 % of diabetes associated deaths occur in low to middle income countries with the mortality rate estimated to double between 2005 and 2030 (WHO 2012). These findings therefore disprove the common belief that diabetes is a disease for the 'rich man'.

1.1.1 History of Diabetes

The term 'diabetes' is derived from a greek word '*diabainein*' (διαβητησ), which means '*to pass through*' or '*to siphon*' (Adeghate 2001). The naming of diabetes was due to the classical symptoms of excessive thirst (polydipsia) and frequent urination (polyuria) associated with the disease. An ancient Egyptian medical manuscript discovered by German Egyptologist Georg Ebers (1550 B.C.) known as The *Ebers Papyrus* was the first documentation to mention the polyuric state of the disease which was later clearly described as diabetes mellitus type I by Aretaeus of Cappadocia in 2nd Century A.D (Tattersall 2010). Between the 5th and 6th Century A.D., two Hindu physicians, Charak and Shushrut, were the first to recognize the sweetness of diabetic urine which they referred to as '*Madhumeha*'

meaning '*passing large volumes of sweet urine*' (Ramachandran and Snehalatha 2009). They achieved this diagnosis by tasting the urine or noticing the congregation of ants around it, with the latter being the commonest African traditional way of diagnosing diabetes today (Tattersall 2010). In 1776, a physician to the Liverpool infirmary, Matthew Dobson, was the first to describe hyperglycaemia by showing that the sweetness of diabetic urine was as a result of glucose which preceded and accompanied the glucose in blood (Lock and Bonventre 2008). He further evaporated the urine of his 33 year old patient, Peter Dickonson, to a 'white cake' with a smell and taste that was indistinguishable from sugar (Tattersall 2010). An Edinburgh-trained surgeon John Rollo became the first to coin the term '*mellitus*' to diabetes from the Latin word meaning '*honey*'(Skljarevski 2007).

1.1.2 Global Distribution

Diabetes is a disease that affects all ages and races. The disease has created a significant heath problem and is an economical burden to many people (Narayan et al. 2006). Diabetes has now been considered as a major health threat in the 21st Century (Zimmet 2001). In a report compiled by the International Diabetes Federation (IDF), it has been realised that since the current diabetes morbidity (366 million) is expected to exceed half a billion by the year 2030, the epidemic trajectory of diabetes has become similar to that of climate change and hence, concerted efforts are desperately required to curb the disease (IDF 2012).

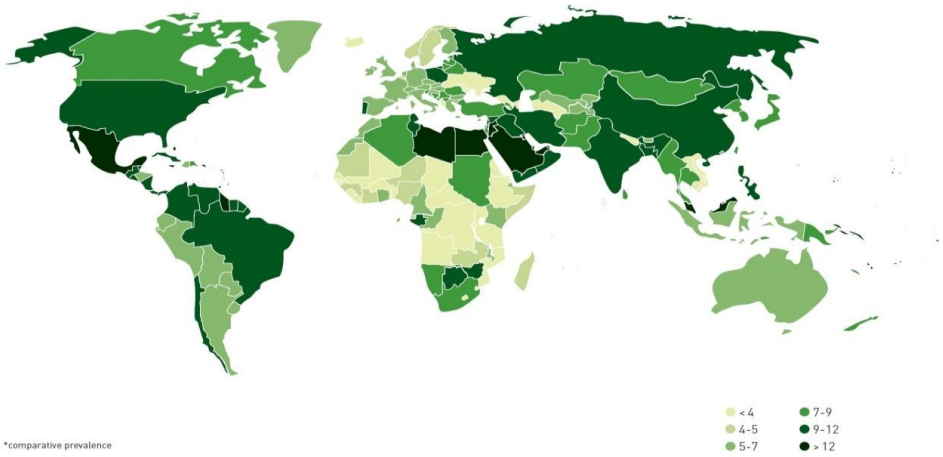


Figure 1.1 Comparative prevalence (%) of diabetes (20-79 years) (International Diabetes Federation 2011)

According to Wild et al., these figures underestimate future diabetes`` prevalence since demographic studies show an increase in the 'diabetic epidemic' even if the levels of obesity remain constant (Wild et al. 2004). It is pertinent to note that even though the prevalence of diabetes in Africa is comparatively less than those from other IDF regions (Figure 1.2), statistics estimate that the African region will experience a 98.1 % proportional increase in diabetes cases by 2030 (Shaw et al. 2010).

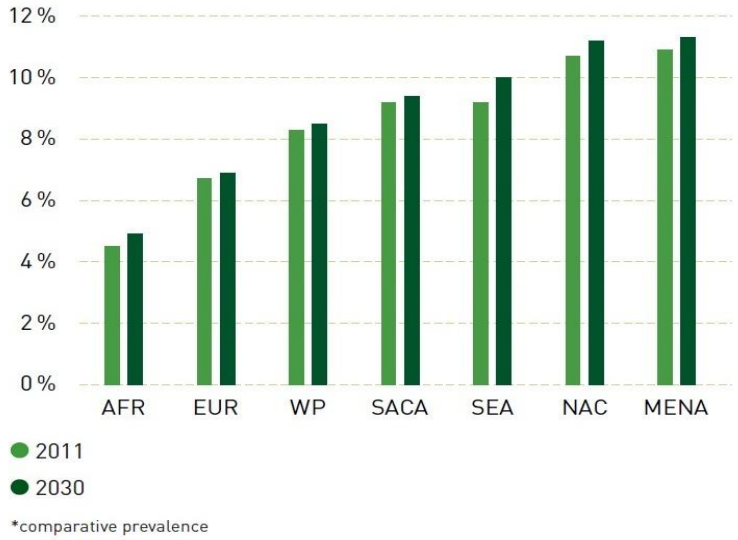


Figure 1.2 Comparative prevalence (%) of diabetes (20-79 years) by IDF region (International Diabetes Federation 2011)[1]

[1] Seven IDF regions were approved by the General Council in 1982 based on the regional structure of the World Health Organisation (WHO) for the strengthening of diabetes association's worldwide. These are; Africa (AFR), Europe (EUR), Middle East and North Africa (MENA), North America and Caribbean (NAC), South and Central America (SACA), South-East Asia (SEA) and Western Pacific (WP).

1.2 Insulin

Discovered in 1922 by Sir Frederick Grant Banting and Dr. Charles Best, Insulin is a crucial regulator of metabolic physiology (Myers and White 1996). Pessin and Saltiel described insulin as "the most potent anabolic hormone known which is also essential for appropriate tissue development, growth and maintenance of whole-body glucose homeostasis" (Pessin and Saltiel 2000). Diabetes mellitus is a disease caused by either lack of insulin (diabetes mellitus type I) or the inability to adequately compensate for a reduced insulin response (diabetes mellitus type II) (Myers and White 1996).

1.2.1 Biosynthesis of insulin

The islets of Langerhans are described as an endocrine organ occupying about 1% of the pancreas characterized by the presence of a central core made up of pancreatic β-cells (Weir and Bonner-Weir 1990) which are unique cells that not only produce but also control the release of insulin to ensure that blood glucose levels are maintained (Lipson et al. 2006). Specialization of pancreatic β-cells for the production and storage of insulin is evidenced by the fact that insulin comprises approximately 10% (10 pg/cell) of the total β-cell protein (Jones and Persaud 2010). Insulin consists of two polypeptide chains (A and B) which are linked by disulphide bonds. It is synthesized as a single polypeptide chain called preproinsulin (Chan et al. 1976;Harper et al. 1981). The gene that codes for preproinsulin is 1355 base pairs in length with a coding region consisting of three exons: the first encoding the signal peptide at the N-terminus of preproinsulin, the second encoding the B chain and part of the C (connecting) peptide, and the third encoding the rest of the C peptide and the A chain (Jones and Persaud 2010). Rapid, mainly co-translational, cleavage of preproinsulin to proinsulin takes place in the rough endoplasmic reticulum. The proinsulin is then transported into the Golgi apparatus to be stored in granular form (Steiner et al. 1985). Through the sequential action of two endopeptidases (prohormone convertases 2 and 3) and carboxypeptidase H, the C peptide chain is removed to liberate two cleavage dipeptides which finally yields insulin (Hutton 1994). Insulin is concurrently stored with C peptide in secretory granules to be released in equimolar quantities by regulated exocytosis normally with >95% of the secretory product as insulin (with C peptide) and <5% as proinsulin (Jones and Persaud 2010).

1.2.2 The insulin receptor

Since the discovery of insulin the most fundamental problem to diabetes research has been that of understanding how insulin works at the cellular level (Sun et al. 1991). The insulin receptor (IR) is a member of a subfamily of receptor tyrosine kinases. Other receptors that belong to this subfamily are the insulin-like growth factor (IGF)-I receptor and the insulin receptor-related receptor (IRR). These receptors are tetrameric proteins characterized by the presence of four glycoprotein subunits (two α and two β) with allosteric enzyme properties (Saltiel and Kahn 2001). The subunits are synthesized on a single mRNA, proteolytically separated and then bound to each other by disulfide bonds to form the insulin receptor with a molecular weight of approximately 340,000 (Ganong 2003). When insulin binds to the α subunit of the insulin receptor, the receptor undergoes conformational changes which trigger the activation of intrinsic tyrosine kinase activity in the β subunit (Liu et al. 2010). This results in the phosphorylation of insulin receptor substrate proteins (IRS) such as IRS-1 (the prototype for this type of molecule) IRS-2, IRS-3, Gab-1 and $p62^{dok}$ functioning as insulin receptor-specific docking proteins to engage multiple downstream signaling molecules (White 1998). The phosphorylated IRS activates two distinct insulin signaling pathways: protein kinase B/glycogen synthase kinase 3β (Akt/GSK3β)[2] and ras-extracellular signal-related kinases (ras-ERK)[3] (Liu et al. 2010). Phosphorylated IRS-1 activates phosphoinositol 3 kinase (PI3K), which subsequently activates Akt2 and GSK and thereby inhibits GSK mediated phosphorylation of glycogen synthesis. This results in the regulation of glycogen and lipid synthesis as well as stimulation of glucose uptake (Figure 1.3) (Liu et al. 2010).

[2] Akt, also known as protein kinase B, is a serine/threonine protein kinase with multiple cellular functions including glucose metabolism, apoptosis and cell migration. Of the 3 genes encoding the Akt family in humans (Akt1, Akt2 and Akt3), Akt2 is important in insulin signalling for the induction of glucose transport. GSK-3 is a serine/threonine protein kinase that mediates the addition of phosphates onto serine and threonine amino acid residues. It is encoded by two genes; GSK-3α and GSK-3β which play a role in type II diabetes mellitus.

[3] Ras is a prototype member of the Ras superfamily of small GTPase proteins. Activated Ras plays a role in cell growth, differentiation and survival. Ras is activated by guanine nucleotide exchange factors encoded by a set of genes called the Son of Sevenless (SOS). ERK, also known as MAPK1 and MAPK3, are serine/threonine kinases (Bivona and Philips 2003).

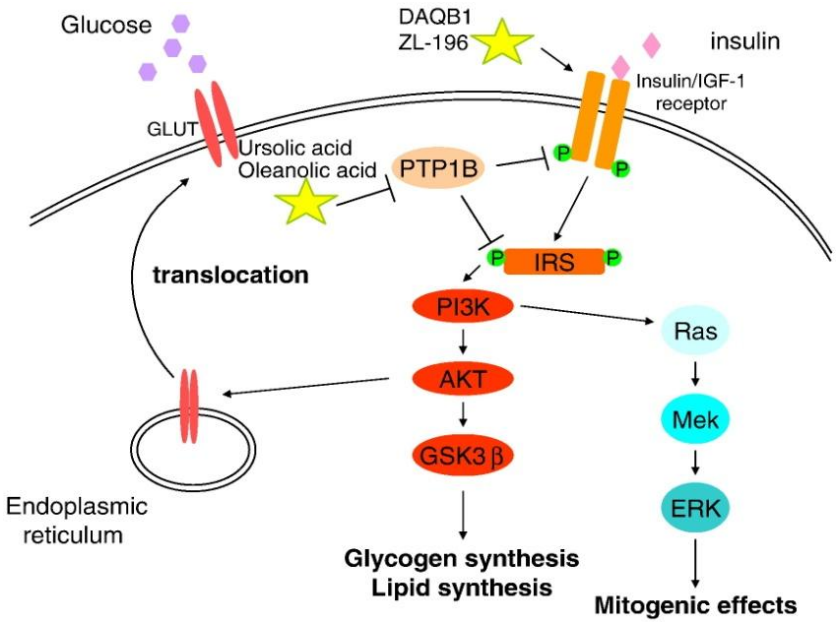


Figure 1.3 The insulin signalling pathway (Liu et al. 2010)

1.3 Pathophysiology of diabetes mellitus type II

Although the etiological cause of diabetes is still undefined, viral infection, autoimmune disease and environmental factors have been implicated (Maritim et al. 2003). The pathogenesis of diabetes mellitus type II is very complicated and usually involves defective β-pancreatic cells and diminished insulin sensitivity. This causes increased rates of hepatic and renal glucose output as well as decreased glucose clearance from the circulation (Kahn 2001). The presence of hyperglycaemia in type II diabetes is normally accompanied by β-cell dysfunction which manifests in several ways such as hypo-insulinaemia, alterations in insulin secretion, an anomaly in the conversion of proinsulin to insulin and decreased release of amylin (Kahn 2001). Even though the presence of defective β-cells is evident in all hyperglycemic patients, the onset of this abnormality and the responsible factors which contribute to this change are still debatable subjects (Kahn 2001). While some suggested that insulin resistance is the primary defect and that β-cell dysfunction comes later due to insulin resistance associated secretory demand (DeFronzo and Ferrannini 1991), data from The United Kingdom Prospective Diabetes Study (UKPDS) suggests that the onset of β-cell dysfunction occurs prior to the development of hyperglycemia and may commence many years before the diagnosis of diabetes (Kahn 2003).

1.3.1 Oxidative stress

Oxidative stress has been defined as "the excessive formation and/ or the insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS)" (Johansen et al. 2005). These ROS and RNS can be divided into free radical and non radical species. Examples of biologically relevant ROS free radical species are superoxide ($\bullet O_2^-$), hydroxyl ($\bullet OH$), peroxyl ($\bullet RO_2$) and hydroperoxyl ($\bullet HRO_2^-$). Examples of ROS non radical species are hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Evans et al. 2002). Examples of RNS free radical species are nitric oxide ($\bullet NO$) and nitrogen dioxide ($\bullet NO_2^-$). Examples of RNS non radical species are peroxynitrite ($ONOO^-$) and nitrous oxide (HNO_2) (Evans et al. 2002). Due to their cardinal role in the pathophysiology of diabetes, superoxide, nitric oxide and peroxynitrite have been the focus of several studies (Johansen et al. 2005).

1.3.1.1 Natural defense system against oxidative stress

Molecular oxygen (O_2) is a terminal electron acceptor in aerobic life which is regarded as a bi-radical because of its possession of two unpaired electrons of parallel spin (Blake et al. 1987). Through enzymatic, non-enzymatic and mitochondrial pathways (Figure 1.4), molecular oxygen undergoes univalent reduction to form superoxide ($\bullet O_2^-$) (Johansen et al. 2005). For instance, when phagocytes are exposed to a certain stimuli their rate of oxygen uptake increases and results in an increased production of superoxide and hydrogen peroxide often referred to as the "respiratory burst" (Babior 1984). From *in vitro* observations, the mitochondrion may be one of the major intracellular sources of reactive oxygen species with ubisemiquinone and ubiquinol as the main sources of mitochondria generated superoxide (Blake et al. 1987). Because of its electronic configuration, superoxide can act as a reducing agent, an oxidizing agent and a nucleophile. Therefore, to prevent cellular auto-oxidative damage as a consequence of free radicals generated during normal metabolism, superoxide is spontaneously dismutated to hydrogen peroxide through catalytic breakdown by the enzymes manganese superoxide dismutase (MnSOD) and copper superoxide dismutase (CuSOD) depending on whether the superoxide is mitochondrial or cytosolic respectively (Figure 1.4) (Blake et al. 1987).

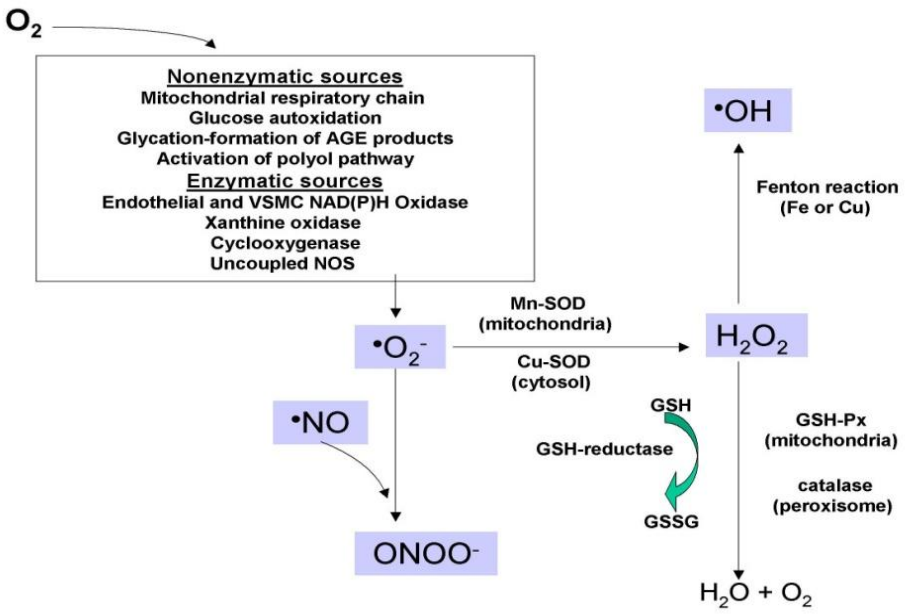


Figure 1.4 Generation of free radicals and the natural anti-oxidant defense mechanism (Johansen et al. 2005).

Hydrogen peroxide is then detoxified by either glutathione peroxidase (GSH-Px) in the mitochondria or catalase (CAT) in the peroxisomes to form water and oxygen. The enzyme glutathione reductase (GSH-reductase) generates glutathione (GSH) from glutathione disulfide (GSSG) (Johansen et al. 2005). Glutathione is both a radical scavenger and a co-substrate for glutathione peroxidase by acting as a hydrogen donor in the elimination of water and oxygen from hydrogen peroxide (Figure 1.4) (Johansen et al. 2005). Excessive formation of free radicals exhausts this defense mechanism causing $\bullet O_2^-$ to react with endothelial •NO leading to the formation of highly reactive peroxynitrite ($ONOO^-$). Similarly, transition metals catalyze the conversion of excess H_2O_2 into •OH radicals through the Fenton reaction (Evans et al. 2002).

1.3.1.2 The role of oxidative stress in diabetes

We have already established that the pathogenesis of diabetes mellitus type II mostly requires defects in both β-pancreatic cells and insulin sensitivity (Kahn 2001). Although the levels of intrinsic anti-oxidant enzymes (MnSOD, CuSOD, GSH-Px and CAT) in β-pancreatic cells are reasonable, the total anti-oxidant enzyme activity may be low thereby making them more susceptible to oxidative damage (Oberley 1988). Studies involving the induction of diabetes in animals using alloxan and streptozotocin (STZ) has increased understanding of the role played by oxidative damage in the cause of diabetes (Wolff 1993). Alloxan (2,4,5,6-

tetraoxohexahydropyrimidine) is converted to dialuric acid, which then undergoes auto-oxidization to yield H_2O_2, O_2, $\bullet O_2^-$ and •OH free radicals (Oberley 1988). The cytotoxic effects of alloxan on β-pancreatic cells is attributed to the efficiency with which the drug is absorbed by the cells, the extent to which the drug interacts with intracellular reducing agents to produce oxidants and decreased amounts of glutathione peroxidase in the islets (Wolff 1993). Despite the mechanism of action of STZ being unknown, inhibition of STZ-induced diabetes by desferrioxamine indicates that STZ toxicity may be a result of free radical reactions catalyzed by transition-metals. According to Wolf (1993), the aetiological cause of diabetes mellitus may at least be a product of oxidative stress resulting from transition metal catalysis and thus, individual variations in the levels of transition metals might determine susceptibility to diabetic complications. During diabetes or insulin resistance, failure of insulin-stimulated glucose uptake by fat and muscle causes a sustained elevation of glucose concentrations in blood plasma. Consequently, this ensuing hyperglycaemia causes an increase in the glucose uptake of insulin-independent tissues leading to the enhancement of oxidant production and impairment of anti-oxidant defenses by multiple interacting pathways (King and Loeken 2004). In the hyperglycaemic state, glucose undergoes monosaccharide auto-oxidation by enolizing to form enediol products which, in the presence of transitional metals produce enediol radical anions (Figure 1.5). These enediol radical anions react with molecular oxygen under physiological conditions to give off dicarbonyl products, superoxide radicals and hydrogen peroxide which, in the presence of transitional metals, further produces hydroxyl radicals. These generated free radicals ultimately result in oxidative stress. Studies have shown that oxidative stress due to hyperglycaemia plays a crucial role in the pathogenesis of diabetic complications (Evans et al. 2002).

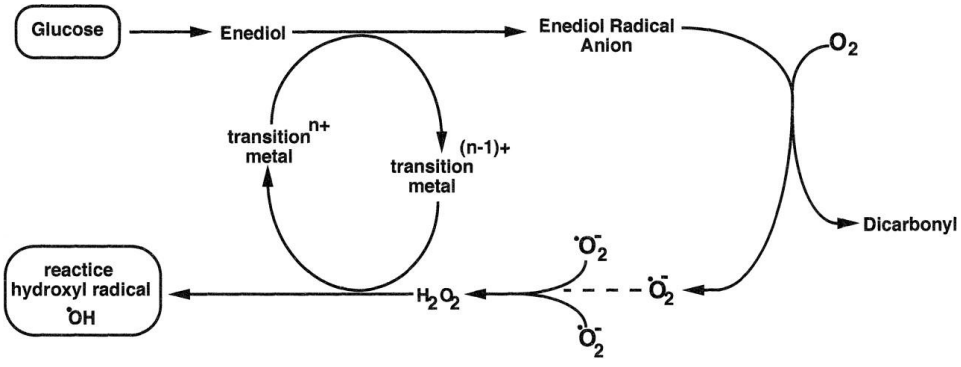


Figure 1.5 Metal catalyzed auto-oxidation of glucose (Bierhaus et al. 1998)

1.4 Presentation of diabetes mellitus

The main feature of patients with diabetes mellitus (DM) type II is the presence of sustained elevation of serum glucose levels while that of DM type I patients is the occurrence of metabolic decompensations such as diabetic ketoacidosis (Pinhas-Hamiel and Zeitler 2007). Increased thirst (polydipsia) and increased urinary volume (polyuria), frequency of low immunity associated infections and about 25% of girls with type II DM have a vaginal monilial infection as their chief complaint at presentation which is exceedingly rare in DM type I but a typical complaint among adult females with DM type II as an indicator of long standing glycosuria. The remaining DM type II patients are subjected to infectious diseases mainly of the bones and the pharynx, excessive weight gain, painful urination and urinary incontinence (Pinhas-Hamiel and Zeitler 2007).

1.5 Diabetic complications

Diabetic complications can be classified into short term and long term effects. The short term effects of diabetes include keto-acidosis, recurrent infections and weight loss. The long term effects of diabetes are generally classified into micro-vascular and macro-vascular complications (Nazimek-Siewniak et al. 2002). The micro-vascular complications include retinopathy, nephropathy and neuropathy (Donaghue et al. 2009) while the macro-vascular complications include cardiovascular diseases such as heart failure, coronary heart disease, stroke, peripheral vascular disease and myocardial infarction (Rosenson et al. 2011). Some of the outcomes of micro-vascular complications include visual disturbances and loss of sight, renal and cardiovascular complications, abnormal sensations, myasthenia and peripheral nervous system disorders (Donaghue et al. 2009). Cardiovascular complications are the leading cause of morbidity and mortality in diabetic patients (Adeghate 2004). About 50% of all diabetic patients die of cardiovascular disease (WHO 2012) with 75% of all deaths caused by myocardial infarction and stroke (Ban et al. 2009). Compared with non-diabetics, diabetic patients have a several-fold increased risk of developing cardiovascular disease comprised of a 10 times higher risk and a 3 times higher risk for type I and type II diabetics respectively (Ban et al. 2009).

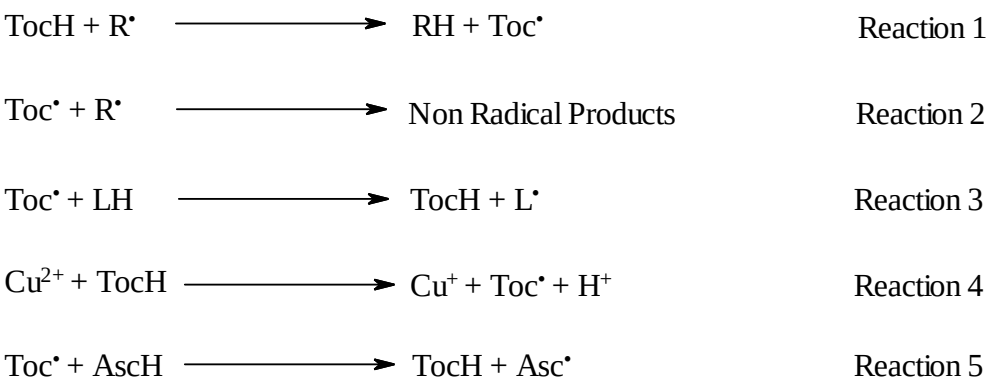
1.5.1 Molecular mechanisms for the cause of diabetic complications

The role of hyperglycaemia in the development of diabetic complications is mainly through the induction of oxidative stress arising from hyperglycaemia associated production and release of ROS and RNS which ultimately leads to abnormal gene expression, faulty signal transduction and apoptosis (via activation of p53 and the cytochrome c-activated caspase 3 pathway) (Adeghate 2004). The four major hypotheses that explain how hyperglycaemia causes diabetic complications are: increased conversion of glucose to sorbitol which ultimately results in the failure to regenerate the reduced form of glutathione, reactions between the products of glucose auto-oxidation and amino acid groups to form advanced glycation end-products (AGE), polyacylglycerol (PAG) mediated activation of protein kinases and alterations in genes and protein function due to hexosamine pathway activation (Brownlee 2001). It was discovered that all these different pathogenic mechanisms reflect a single hyperglycaemia-induced unifying hypothesis which is the overproduction of super oxide ($\bullet O_2^-$) by the mitochondrial electron transport chain (Brownlee 2005). Diabetic complications are a result of lipid peroxidation, protein nitration and DNA damage caused by peroxynitrite ($ONOO^-$), a highly reactive free radical product formed when overproduced $\bullet O_2^-$ reacts with •NO (Johansen et al. 2005) at a rate constant of 6.7×10^9 $M^{-1}s^{-1}$ (Aruoma 1998).

1.5.2 Evaluation of the potential benefits of anti-oxidants

Having explained the involvement of hyperglycaemia-induced oxidative stress in the development of diabetic complications, the theoretical implication is that hypoglycaemic agents with additional anti-oxidant activity should render a long term advantage in avoiding diabetic complications over those which are only focused on the meticulous control of hyperglycaemia. However, the use of anti-oxidants in the management of type II diabetes has been met with huge challenges. For instance, the use of the anti-oxidant α-tocopherol (Vitamin E) as an adjunct to conventional anti-diabetic therapy in clinical trials for the prevention of cardiovascular events has produced conflicting results. The most significant results came from the HOPE and HOPE TOO trials in which supplementation with Vitamin E in patients with vascular disease or diabetes increased the risk of developing heart failure (RR= 1.13) (Lonn et al. 2005). Under high oxidative stress conditions, the main mechanism for the anti-oxidant activity of α-tocopherol involves the direct inactivation of one free radical ($R^\bullet$) by one molecule of α-tocopherol (TocH) (Scheme1.1, Reaction 1) followed by the

scavenging of a second radical by the formed α-tocopheroxyl radical ($Toc^{\bullet}$) (Scheme 4.1, Reaction 2) ultimately resulting in the neutralization of two free radicals with one molecule of α-tocopherol (Kontush et al. 1996).



Scheme 1.1 Reaction schemes for the anti-oxidant and pro-oxidant activity of α-tocopherol

The disadvantage to this anti-oxidant mechanism of α-tocopherol is that it is limited to the scavenging of already formed free radicals and hence may be regarded as a "symptomatic" rather than a causal approach in managing oxidative stress (Ceriello 2003). Several explanations have been given to account for the unpredictability of α-tocopherol in clinical trials despite favourable results *in vitro*. One such explanation is the potential of α-tocopherol to become a pro-oxidant in mild oxidative conditions (Celik et al. 2010). In mild oxidative conditions, if no free radical interacts with $Toc^{\bullet}$, it then directly oxidizes LDL PUFA moieties (LH) giving rise to the PUFA radical ($L^{\bullet}$) (Scheme 1.1, Reaction 3). Also when present in low amounts, Cu^{2+} directly interacts with TocH to form $Toc^{\bullet}$ (Scheme 1.1, Reaction 4). Hence, it appears that the fate of $Toc^{\bullet}$ plays a cardinal role in determining the anti-oxidant/ pro-oxidant activity ratio of α-tocopherol. In mild oxidative conditions, the use of α-tocopherol co-anti-oxidants such as ascorbate (AscH) to recycle $Toc^{\bullet}$ back to TocH restores the anti-oxidant activity of α-tocopherol (Scheme 1.1, Reaction 5). However, under high oxidative stress conditions, α-tocopherol possesses anti-oxidant effects regardless of the concentration of AscH. Therefore, the anti-oxidant activity of α-tocopherol and other compounds bearing the same anti-oxidant mechanism as α-tocopherol is dependent on both the extent of oxidative stress as well as the concentration of co-anti-oxidants (Kontush et al. 1996). Another explanation accounting for the unpredictability of α-tocopherol in clinical trials includes the existence of genetic polymorphisms in the expression of intrinsic anti-oxidant enzymes (Celik et al. 2010).

Currently, the only conventional anti-diabetic agents known to have anti-oxidant properties are the thiazolidinediones (TZD's), a class of drugs which act as PPARγ receptor agonists. Research has proven that the anti-oxidant activity of TZD's is dependent on the activation of the PPARγ receptor (Chung et al. 2011) with the exception of rosiglitazone which, in addition to its PPARγ-dependent anti-oxidant activity, also exhibits a PPARγ-independent inhibition of glucose-induced ROS generation in QZG hepatocytes (Polvani Simone et al. 2012). In the research done by Chung et al., inhibition of PPARγ with a specific PPARγ antagonist GW-9662 significantly reduced the anti-oxidant activity of TZD's (Chung et al. 2011). The ligand-activated PPARγ receptor is thought to regulate the expression of various anti-oxidant and pro-oxidant genes such as MnSOD (a direct target of the PPARγ), Glutathione peroxidase 3 (GPx3), the mitochondrial uncoupling protein 2 (UCP2) and eNOS in response to oxidative stress (Polvani Simone et al. 2012). Therefore, unlike conventional anti-oxidants such as α-tocopherol which have a 'causal' approach in managing oxidative stress, the anti-oxidant mechanism of PPARγ agonists has a 'preventive' approach. There is already much evidence from several studies warranting the ability of PPARγ agonists to prevent cardiovascular complications. PPARγ agonists also prevent atherogenesis and inflammation (Balakumar et al. 2007). This almost implies that PPARγ agonists are a potentially ideal class of anti-diabetic agents superior to the concurrent administration of hypoglycaemic agents with conventional anti-oxidants in the prevention of diabetic cardiovascular complications. However, current TZD's are associated with weight gain, fluid accumulation, pulmonary oedema and macular oedema which ultimately results in congestive heart failure (Shearer and Billin 2007). Since PPARα agonists have been used to treat dyslipidemia via their effects on lowering free triglyceride concentrations in plasma, PPARγ side effects can be avoided by activating both PPARα and PPARγ at the same time resulting in increased metabolism of lipids and sensitivity of insulin (Kim et al. 2008). Because of this, several attempts have been made to develop PPARα/γ dual agonists.

1.5.3 Diabetes as an inflammatory process

An abundance of evidence has emerged to demonstrate a close link between metabolism and immunity (Wellen and Hotamisligil 2005). Recently, much interest has been developed in the concept that prolonged low-grade inflammation and an activated innate immune system have a part to play in the cause of type II diabetes (Fernández-Real and Pickup 2008). The effects of hyperglycaemia on white blood cells such as polymorphonuclear leukocytes (PMN) contributes to the infections and prolonged inflammatory state associated with diabetes (Hand et al. 2007). Therefore, new methods of managing diabetes may be discovered by exploring inflammatory pathways and their inhibitory mechanisms (Fernández-Real and Pickup 2008).

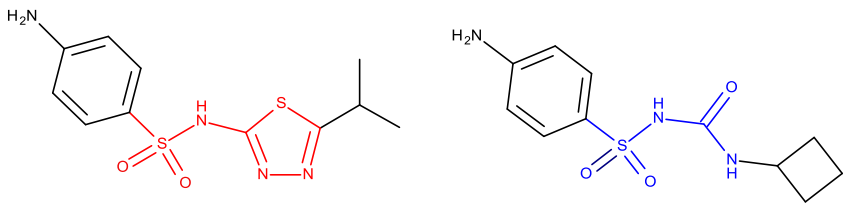
1.6 Anti-diabetic therapeutic agents

Current anti-diabetic treatment aims at managing hyperglycaemia and attenuating the development of diabetic complications (Moller 2001). The current anti-diabetic agents are: Sulphonylureas and other insulin secretagogues such as meglitinides and GLP-1 analogues (which increase insulin secretion); dipeptidyl peptidase-4 (DPP-4) inhibitors (which increase incretin release causing glucagon inhibition and increased insulin secretion); metformin (which decreases glucose output from the liver); peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists (thiazolidinediones, which increase the sensitivity of insulin); α-glucosidase inhibitors (which prevent intestinal glucose uptake); and insulin (which decreases glucose output and increases cellular glucose usage) (Moller 2001).

1.6.1 Structural classification of current anti-diabetic drugs

Oral hypoglycaemic drugs are structurally classified as follows;

1.6.1.1 Sulphonylureas



IPTD Carbutamide

Figure 1.6 Structures of IPTD and Carbutamide (Zavod and Krystenansky 2008)

The sulphonylureas were discovered in the 1950's out of serendipity (Drews 2000). In the 1940's, 2-(*p*-aminobenzenesulfonamido)-5-isopropyl-thiadiazole (IPTD), a compound that was then used for the management of typhoid fever killed several patients by inducing acute and prolonged hypoglycaemia. However, IPTD never became an oral hypoglycaemic due to the discovery of a more effective drug called carbutamide. Carbutamide became the first marketed sulphonyurea (Drews 2000).

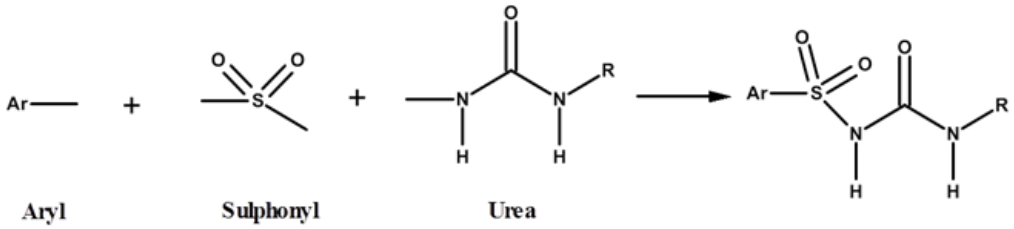


Figure 1.7 The general structure of sulphonylureas

The mechanism of action of sulphonylureas involves the stimulation of β-cell insulin secretion through the activation of ATP-sensitive potassium channels which are comprised of hetero-octameric complexes of two subunits: a sulphonylurea receptor (SUR1) and an inwardly rectifying potassium channel (Zavod and Krystenansky 2008).

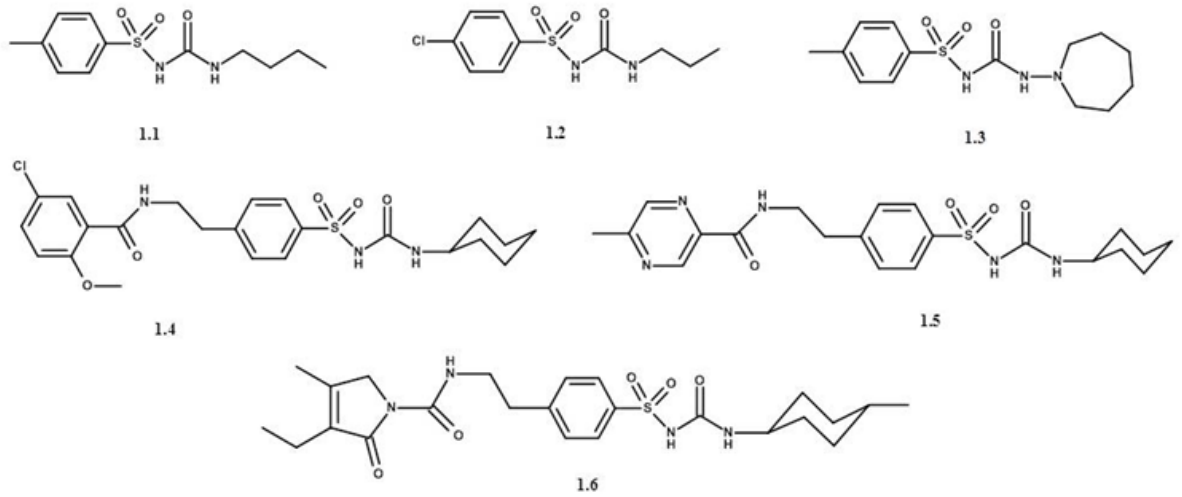


Figure 1.8 Selected structures of first, second and third generation sulphonylureas

Sulphonylureas have been classified as first, second and third generation. Tolbutamide **(1.1)** is a first generation sulphonylurea and the least potent oral hypoglycaemic agent due to its low SUR1 binding affinity. Due to its short half-life, tolbutamide is the most ideal sulphonylurea in senile diabetics and those experiencing moderate to severe kidney disease. Chlorpropamide **(1.2)**, also a first generation sulphonylurea, is more potent and has a more prolonged action (mean elimination t½ 33 h). This is due to its slow metabolism in *vivo* owing to the *p*-Cl substituent which protects the *p*-position from metabolic oxidation. Tolazemide **(1.3)** is a first generation sulphonylurea with a more potent activity than Tolbutamide but a shorter duration of action than Chlorpropamide. Glibenclamide (Glyburide) **(1.4)** and Glipizide **(1.5)** are high potency second generation sulphonylureas with more complex Ar- moieties that facilitate receptor binding and influences their drug excretion profiles. Glimepiride **(1.6)** is a third generation sulphonylurea that was structurally derived from second-generation sulfonylureas by the exchange of an amide moiety with a heterocyclic group so that the drug could bind onto a different region of β-cell receptors and thereby enhancing its potency and increasing its duration of action.

1.6.1.2 Meglitinides

The meglitinides are a relatively new class of insulin secretagogues with repaglinide **(1.7)**, the first drug to be classified under this group, approved for clinical use in 1998 (Nolte and Karam 2007). The mechanism of action of meglitinides involves increase in β-cell insulin secretion resulting from the regulation of potassium efflux through potassium channels. Meglitinides act similar to sulphonylureas because they interact with two binding sites that are common to sulphonylureas and one additional binding site (Nolte and Karam 2007). The structure of repaglinide **(1.7)** is characterized by the presence of the non-sulfonylurea moiety found in glibenclamide **(1.4)** and a salicylic acid derivative (Nattrass and Bailey 1999). Salicylates and sulfonylureas both reduce hyperglycaemia through different mechanisms. A derivative of D-phenylalanine, nateglinide **(1.8)** is related to repaglinide. Repaglinide can be used as monotherapy in combination with biguanides. Due to the lack of sulfur in its structure, repaglinide is idea for use in type II diabetics with sulfur or sulphonylurea allergies. Apart from its special role in isolated postprandial hyperglycaemia, nateglinide offers the lowest risk of hypoglycaemia of all secretagogues and is safe in patients with reduced renal function (Nolte and Karam 2007).

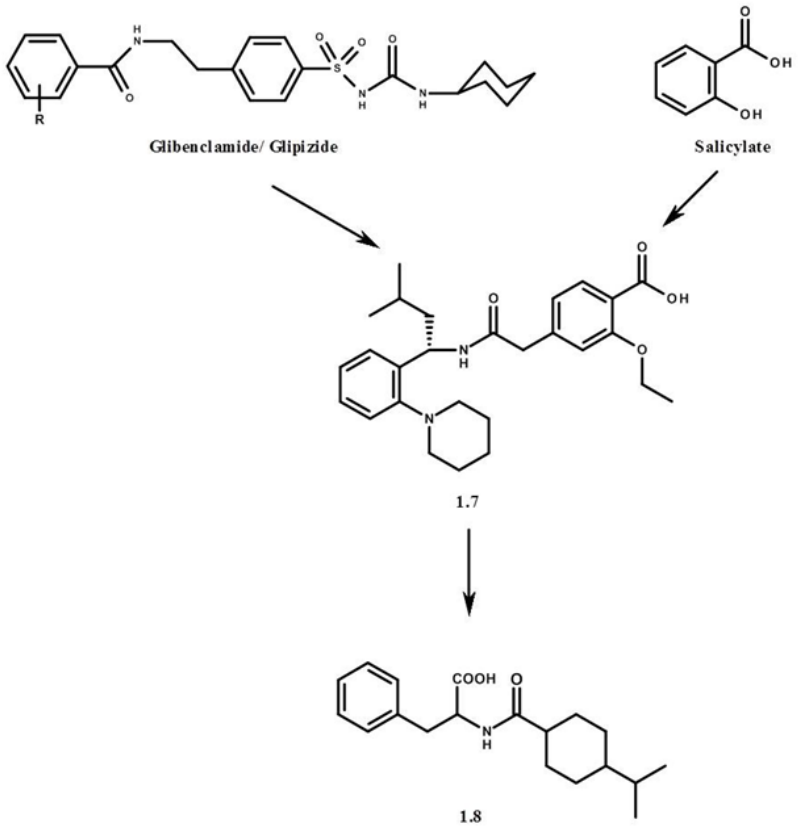


Figure 1.9 Structure derivatization of meglitinides

1.6.1.3 Biguanides

Galegine (isoamylenegaunidine) was discovered from the plant *Galega officinalis* (Goats rue of French lilac) as the main metabolite responsible for the death of grazing animals (Zavod and Krystenansky 2008). In 1918, guanidine was found to possess hypoglycaemic effects but was too toxic for therapeutic use. The biguanides phenformin (**1.9**, phenyethlbiguanide; withdrawn due to lactic acidosis), buformin (**1.10**) and metformin (**1.11**, dimethylguanide) are all guanidine derivatives introduced in the 1950's as oral hypoglycaemics for the management of type II diabetes (Bailey 1992). The mechanism of action of biguanides involves the inhibition of hepatic glucagon dependent glucose output by decreasing the levels of cyclic AMP and the activity of protein kinase A (PKA) (Miller et al. 2013).

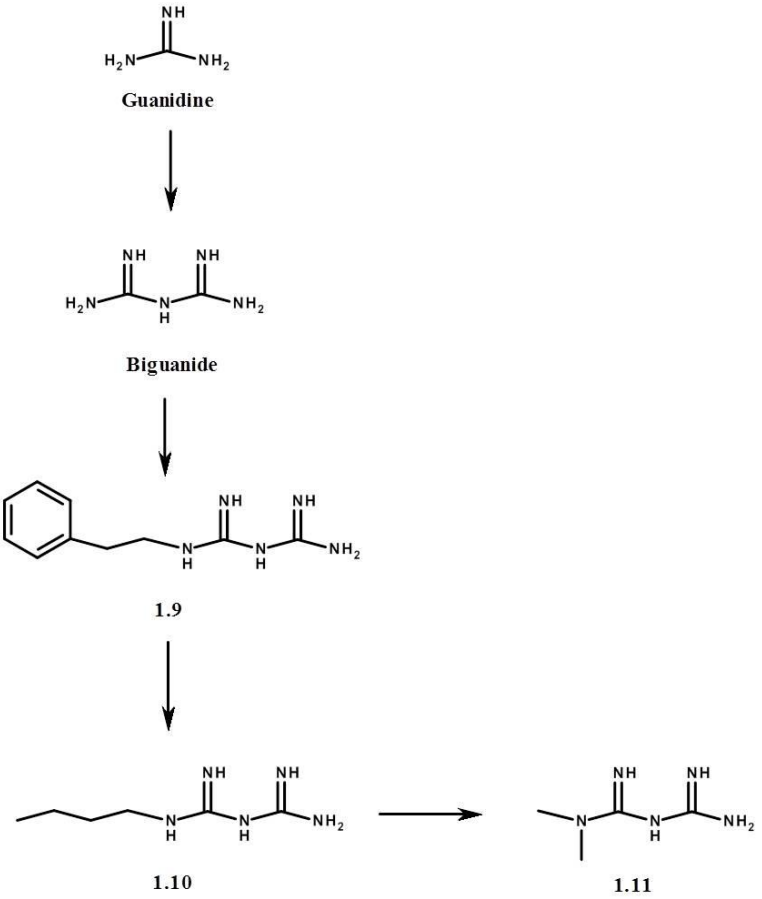


Figure 1.10 Structures of biguanides

1.6.1.4 Gliptins (piperazine and cyanopyrrolidine derivatives)

The gliptins are selective DPP-4 inhibitors that act by increasing the levels of incretin, a hormone with the key function of reducing prandial glucose excursions by increasing glucose-induced insulin secretion from the pancreatic β-cells. The main drugs in this group are: the piperazine derivative, sitagliptin (**1.12**); the cyanopyrrolidine derivative, vildagliptin (**1.13**) and the more recently introduced saxagliptin (Bailey and Krentz 2010).

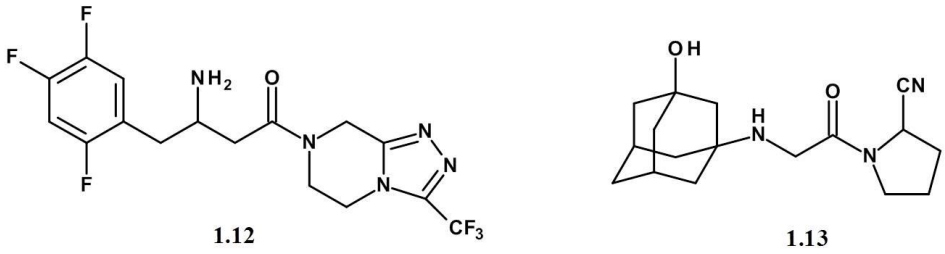


Figure 1.11 Structures of sitagliptin (**1.12**) and vildagliptin (**1.13**)

1.6.1.5 Thiazolidinediones (TZDs)

The anti-diabetic activity of a TZD called ciglitazone was first reported in the early 1980's. This was followed by the identification of the peroxisome proliferator-activated receptor (PPAR) family in the 1990's and the discovery of TZDs as PPAR-γ agonists (Bailey and Krentz 2010). The expression of PPAR-γ receptors is mainly in adipose tissue though they are also expressed in muscles and the liver but to a lesser extent. Ligand activation of PPAR-γ receptors results in the formation of heterodimeric complexes with retinoid X receptors which then bind to nucleotide sequences (AGGTCAXAGGTCA) referred to as peroxisome proliferator response elements (PPRE) that are located in the promoter regions of PPAR-responsive genes (Bailey and Krentz 2010). This causes an alteration in the transcriptional activity of mainly the genes that are involved in lipid and carbohydrate metabolism. Stimulation of PPAR-γ by TZDs promotes the differentiation of preadipocytes into mature adipocytes which are highly sensitive to insulin (Bailey and Krentz 2010). TZDs include troglitazone (**1.14**, removed from the market due to hepatotoxicity), rosiglitazone (**1.15**), pioglitazone (**1.16**) and darglitazone (**1.17**).

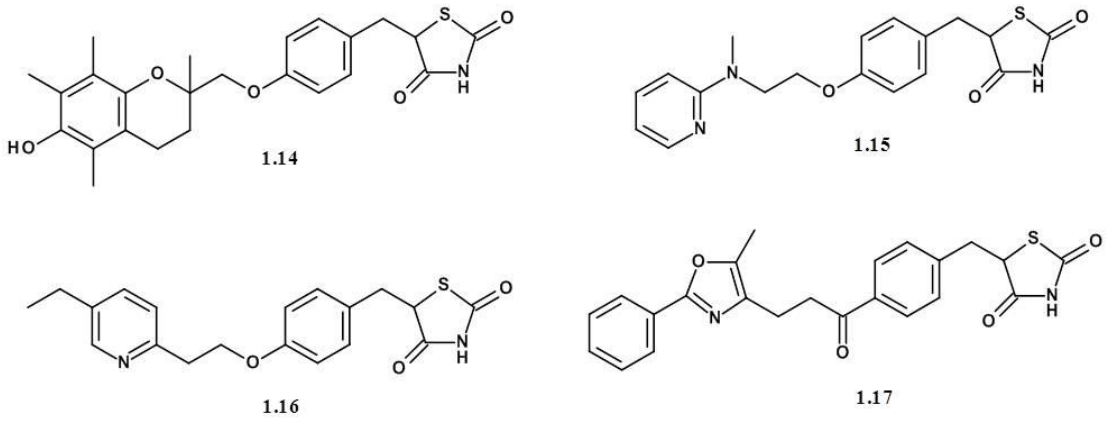


Figure 1.12 Selected structures of thiazolidinediones (TZDs)

1.6.1.6 α-Glucosidase inhibitors (oligosaccharides and polyols)

This group of anti-diabetic drugs act by inhibiting α-glucosidase, an enzyme responsible for the final step of carbohydrate digestion. This inhibition subsequently delays the absorption of sugars. Arcabose (**1.18**), a pseudo-oligosaccharide, was the first α-glucosidase inhibitor to be introduced in the 1990's followed by two agents; a simple aminosugar derivative called miglitol (**1.19**) and a simple amine substituted cyclohexane polyol called voglibose (**1.20**). The major limitation of α-glucosidase inhibitors is their tendency to cause gastro-intestinal side effects resulting from the fermentation of un-absorbed sugars in the gastro-intestinal tract (Bailey and Krentz 2010).

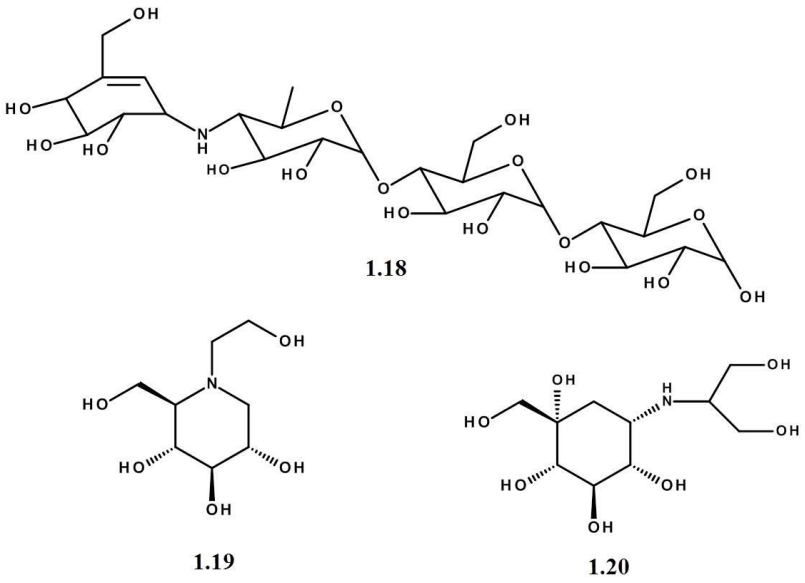


Figure 1.13 Structures of α-glucosidase inhibitors

1.6.2 The search for new anti-diabetic lead compounds from natural sources

Although research outcomes from randomized controlled trials indicate that the reduction of hyperglycemia also reduces the risk of diabetic micro-vascular complications, none of the currently available conventional drugs which aim at reducing hyperglycemia have convincingly demonstrated that they can significantly alter the progressive loss of pancreatic insulin secretion (Baynes 2006). Also, most of the current anti-diabetic drugs are now known to have limited efficacy and tolerability, significant mechanism-based side effects and the tendency to cause weight gain. Therefore, newer therapies with physiological response dependent mechanisms that do not cause weight gain are desperately needed (Moller 2001).

One of the advantages of natural products over synthetic compounds is that they inherently contain a larger scale of structural diversity and hence, have become the major resource for the development of biologically active agents and the discovery of lead compounds (Liu et al. 2010). Current anti-diabetic agents were developed without proper knowledge of the relevant molecular targets or understanding of the disease pathogenesis. However, the understanding of biochemical pathways related to the development of diabetes has since increased (Moller 2001). Mechanistic categories for new therapeutic approaches include those that aim at; reducting hepatic glucose production, increasing glucose-stimulated insulin secretion, molecular targeting of insulin signalling pathways and improving insulin action or secretion (Moller 2001).

1.6.2.1 Terrestrial plant-derived potential anti-diabetic compounds

In combination, traditional treatments for diabetes mellitus and investigative studies on potential anti-diabetic (hypoglycaemic) agents have utilized over 1200 species of plants (Marles and Farnsworth 1995). Deoxyelephantopin (**1.21**, ESD), a sesquiterpene lactone isolated from the plant *Elephantopus carolinianus*, is a selective partial agonist for PPAR-γ which adopts a distinct PPAR-γ binding mode to that of rosiglitazone as determined by molecular modeling (Zou et al. 2008). Ursolic acid **(1.22)**, a pentacyclic triterpenoid isolated from the fruits of a Chinese medicinal plant *Cornus officinalis*, and its synthetic derivative compound UA0713 **(1.23)** are both protein tyrosine phosphatase 1B (PTP1B) inhibitors (Zhang et al. 2006). Resveratrol (RSV), a natural polyphenolic compound isolated from the skin of *Vitis vinifera* (red grapes), is an allosteric SIRT1 activator that mimics the effects of calorie restriction, reduces blood glucose and insulin levels and ameliorates insulin resistance

(Lagouge et al. 2006). Based on RSV, SIRT1 activators 1000-fold more potent than RSV were identified (Liu et al. 2010).

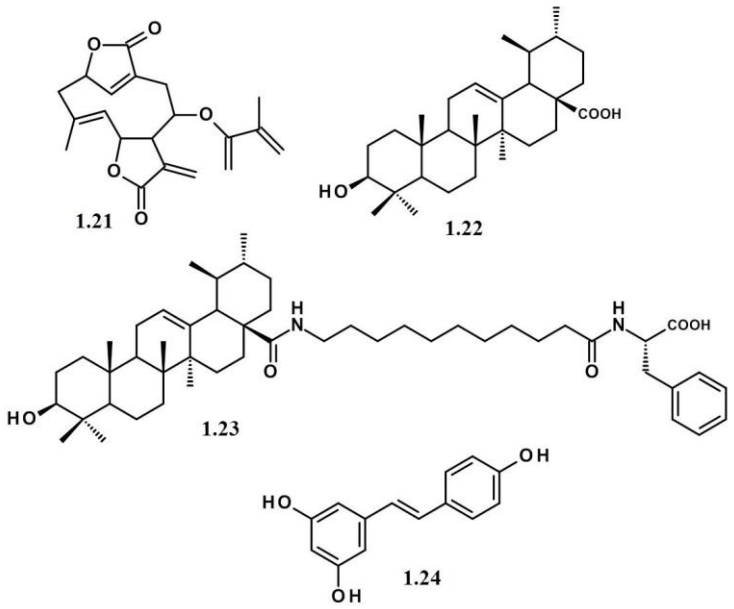


Figure 1.14 Selected structures of plant-derived potential anti-diabetic compounds

1.6.2.2 Marine algae-derived potential anti-diabetic compounds

According to El Gamal (2010), "marine organisms are potentially prolific sources of highly bio-active secondary metabolites that might represent useful leads in the development of lead pharmaceutical agents". Several novel compounds possessing interesting biological activity have been isolated from marine organisms (El Gamal 2010). Such compounds include 1,2-bis(2,3,6-tribromo-4,5-dihydroxyphenyl)-ethane (**1.25**, IC_{50} = 3.5 μmol/L), a PTP1B inhibitor isolated from the brown algae *Symphyocladia latiuscula* (Liu et al. 2011). Hydroxyisoavrainvilleol (**1.26**), another PTP1B inhibitor, has been isolated from the tropical green algae *Avrainvillea nigricans* as well as the red algae *Polysiphonia urceolata* (El Gamal 2010). Several Bromophenols of algal origin have shown α-glucosidase inhibitory activity. Such compounds include bis(2,3-dibromo-4,5-dihydroxybenzyl) ether (**1.27**), purified from the red alga *Polyopes lancifolia* with activity against *Saccharomyces cerevisiae* and *Bacillus stearothermophilus* *α*-glucosidases (IC_{50} values of 0.098 μM and 0.120 μM respectively) (Kim et al. 2010). Octaphlorethol A (**1.28**), a phenolic compound sourced from the brown alga *Ishige foliacea*, has helped to identify PI3K/Akt activation and the AMPK signalling pathway as a new therapeutic target in the management of type II diabetes by its ability to increase glucose uptake via the GLUT4 receptor (Lee et al. 2012).

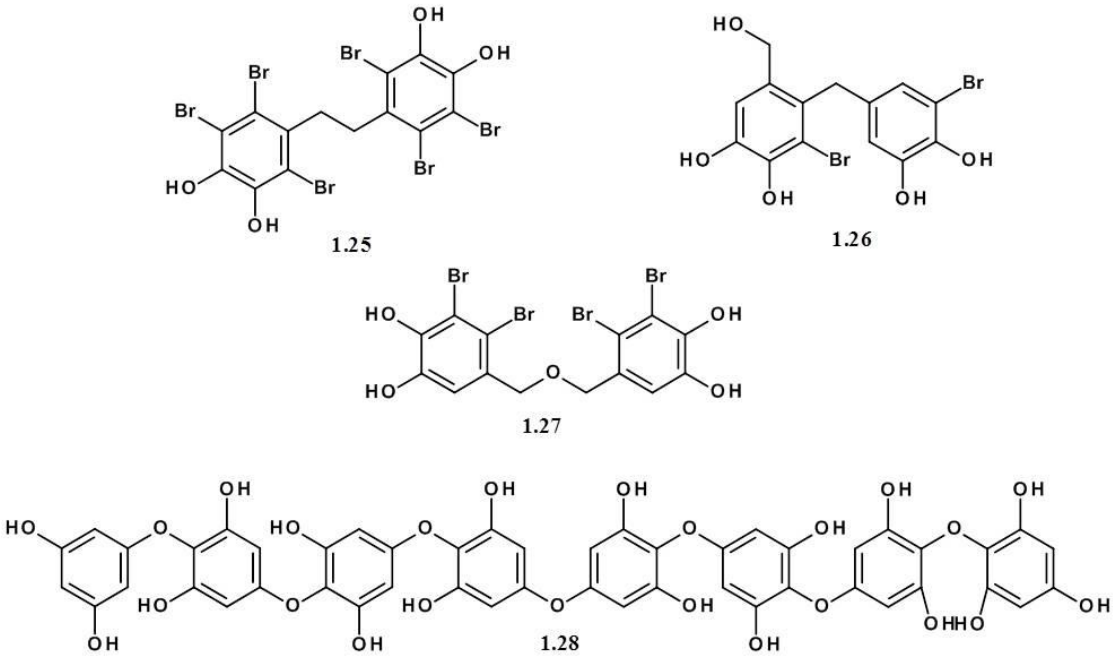


Figure 1.15 Selected structures of marine-derived potential anti-diabetic compounds

1.7 Research objectives

The need for newer compounds in the management of diabetes and the prevention of its complications cannot be over-emphasized. We have theoretically established that anti-oxidant compounds that reduce the accumulation of toxic metabolites of glucose such as free radicals may prevent diabetic complications. Such compounds offer an advantage because the management of diabetes would then also concentrate on the prevention of long term free radical accumulation instead of only focusing on the control of glycaemic levels. Therefore, any treatment approach that combines hypoglycaemic effects with prevention of free radical generation is far much better than one that only focuses on hypoglycaemic effects in preventing diabetic complications.

The aim of this research project is to discover anti-oxidant compounds from marine algae with potential anti-diabetic activity. An ideal anti-diabetic compound in this case would be one that, in addition to hypoglycaemic activity, would in low concentrations relative to the substrate to be oxidized, exhibit anti-oxidant activity by being able to delay, retard or prevent either auto-oxidation or free radical-mediated oxidation. This type of compound will hopefully be achieved by the following:

(i) Performing a literature review of marine algae reported to have anti-oxidant activity
(ii) Screening of the selected marine algae
(iii) Bioactivity guided isolation of anti-oxidant/anti-diabetic natural products
(iv) Structural characterization of the natural products and synthetic analogues
(v) *In-vitro* anti-oxidant/anti-diabetic evaluation of the natural products and synthetic analogues

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Chapter 2

In-vitro screening of selected South African marine algae

2.1 Introduction

Current paradigms in the development of new drug entities focus on obtaining a high molecular diversity that maintains reasonable drug-like properties (Koehn and Carter 2005). In this approach to drug discovery, natural products show a higher promise over synthetic and combinatorial compounds because they are chemically diverse and biochemically specific (Koehn and Carter 2005). The exploration of natural products that possess anti-oxidant activity and prevent oxidative damage is important for the discovery and development of new treatment strategies against obesity-related diseases such as diabetes mellitus (Lee et al. 2011). Similar to other photosynthesizing plants, exposure of marine algae to intense light and high oxygen concentrations results in the generation of free radicals and other strong oxidizing agents (Nahas et al. 2007). The fact that marine algae remain stable during storage with no signs of oxidative damage in their structural components suggests the presence of protective anti-oxidant systems (Nahas et al. 2007). Through the protection of cells from oxidative damage, marine-derived anti-oxidants have shown their importance as bioactive compounds against oxidative stress related diseases (Kelman Dovi et al. 2012).

2.1.1 Selection of South Africa endemic marine algae for screening

In order to achieve the aim of discovering marine derived potential anti-diabetic compounds with preliminary anti-oxidant activity, we performed a literature search for marine algae from specific algal genera that have been documented to possess anti-oxidant activity. We achieved this by investigating species of algal genera from which anti-oxidant compounds have been previously isolated. Such anti-oxidant compounds include the phenolics taondiol **(2.1)**, stypodiol **(2.2)**, stypoldione **(2.3)** and atomaric acid **(2.4)** (Figure 2.2) isolated from the Aegean brown sea weed *Taonia atomaria* (Nahas et al. 2007). These compounds were also previously isolated from the alga *Stypopodium zonale* collected in Spain (Wessels et al. 1999). However, the South African *S. zonale* has not yet been studied. Since species of the genus *Stypopodium* are not easily morphologically distinguished from those of *Taonia*, this suggests a possible botanical misidentification of the Canarian *T. atomaria* with the likelihood that *T. atomaria* from the Canary Islands is in reality *S. zonale* (Soares et al. 2003).

The genus *Dictyopteris* is known to produce sulphated polysaccharides with superoxide radical scavenging activity such as those from *Dictyopteris delicatula* (Costa et al. 2010). These sulphated polysaccharides include several fucoidans isolated from the brown algae *Dictyopteris polypodioides* (Sokolova et al. 2011).

Cystophora and *Cystoseira*, the two major genera of the *Cystoseiraceae* family, are known for their production of relatively complex meroditerpenoids. *Cystophora* produces both isoprenoid and non-isoprenoid secondary metabolites with various functionalised carbon skeletons characterized by linear and cyclic C_{18} terpenoid metabolites (Reddy and Urban 2008). The Australian brown algae *Cystophora moniliformis* produces farnesylacetone derivatives **(2.5)** and **(2.6)** (Figure 2.2) (Reddy and Urban 2008), also isolated from the honey bee entomopathogenic fungus *Ascosphaera apis*, with reported anti-oxidant activity (Gallardo et al. 2008). There are three main classifications to the *Cystoseira* genus designated as categories A-C. These categories are mainly based on the type of metabolites produced. The metabolites produced from category A do not contain diterpenes while those from category B possess linear diterpenes. The metabolites produced from category C are either linear, cyclic or rearranged meroditerpenoids (Reddy and Urban 2008). Triprenyltoluquinols such as **(2.7)** (occurring either as an E′ or *Z*′ isomer on the position indicated by the wavy bond in Figure 2.2) and tetraprenyltoluquinols such as **(2.8)** (Figure 2.2) both isolated from *Cystoseira crinite* exhibit DPPH and TBARS radical scavenging anti-oxidant activity (Reddy 2009). Six cyclised tetraprenyltoluquinols and five stereoisomers were previously isolated from the South African species *Cystophora fibrosa* with the same amentol skeleton as the compounds isolated from the species *C. crinite* (Laird and van Altena 2006).

From the *Cystoceiraceae* family are found the genera *Bifurcaria* and *Bifurcariopsis*. The main species arising from *Bifurcaria* are: *B. bifurcata*, found on Atlantic coasts; *B. brassicaeformis*, found on western and Indian Ocean coasts of South Africa; and *B. galapagensis*. A rich array of acyclic diterpenes have been previously isolated from *B. bifurcata* (Daoudi et al. 2001) and crude extracts of the algae have shown marked DPPH radical scavenging activity (Zubia et al. 2009). The genus *Bifurcariopsis* is monotypic and the generitype *B. capensis* is known only from South Africa (Daoudi et al. 2001). However, the key metabolites responsible for the anti-oxidant activity of *B. bifurcata* have not yet been identified.

Several species of the genus *Sargassum* have shown radical scavenging effects (Budhiyanti et al. 2012). A chromene mojabanchromanol **(2.9)** isolated from *Sargassum siliquastrum* possesses remarkable anti-oxidant activity (Cho et al. 2008). The tetraprenyltoluquinols thunbergol A **(2.10)** and thunbergol B **(2.11)** (Figure 2.2) isolated from *S. thunbergii* both exhibit significant radical scavenging activity and inhibition of morpholinosydnonimine (Linsidomine or SIN-1) generated peroxynitrite (Seo et al. 2006). Similar prenylated toluquinols have been isolated from the South African genus *S. heterophyllum* (Afolayan et al. 2008).

During the selection of algae for screening studies, we were constrained by lack of access. Hence, we focused on those algae that were accessible and could be collected in reasonable quantities, many of which were endemic to South Africa and had not been extensively studied. The algae that were selected are: *Stypopodium zonale* (**a**), *Dictyopteris ligulata* (**b**), *Cystophora fibrosa* (**c**), *Bifurcariopsis capensis* (**d**), *Sargassum sp*. (**e**) and *Sargassum heterophyllum* (**f**) (Figure 2.1).

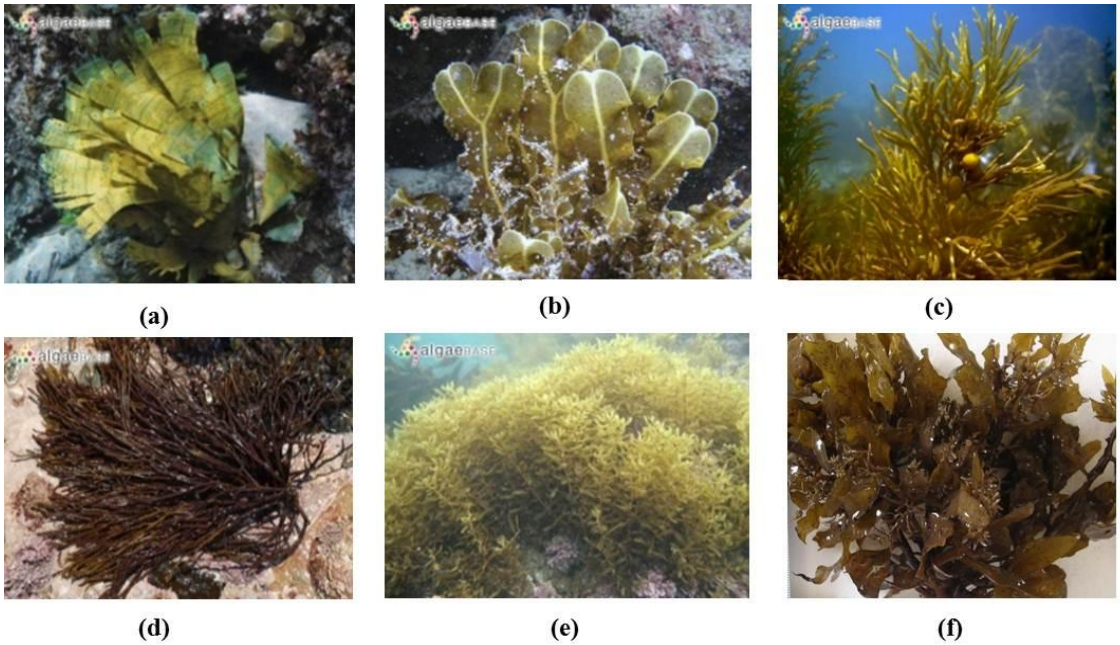


Figure 2.1 Images of *S. zonale* **(a)**, *D. ligulata* **(b)**, *C. fibrosa* **(c)**, *B. capensis* **(d)**, *Sargassum* sp. **(e)** and *S. heterophyllum* **(f)** (Guiry and Guiry 2012)

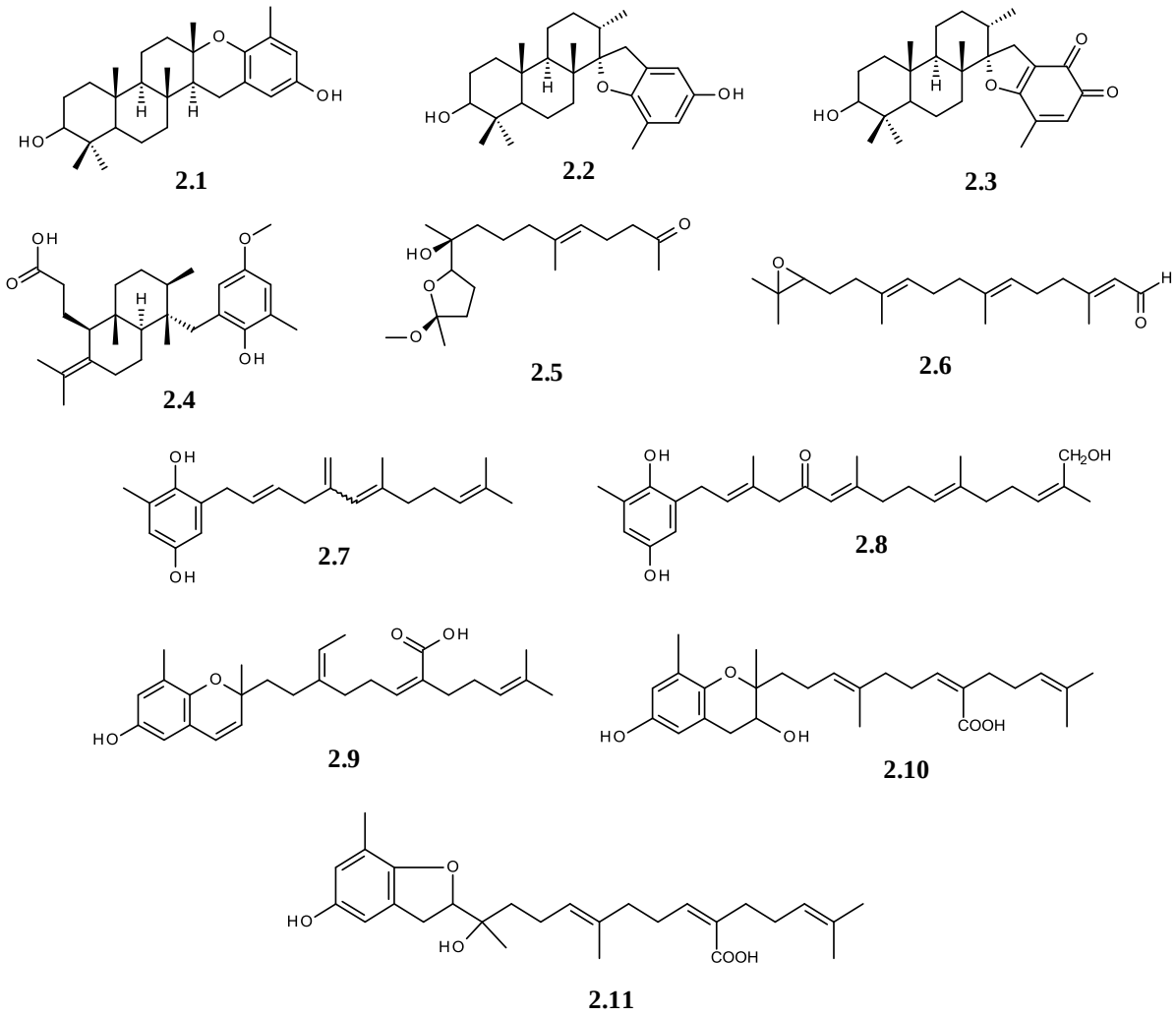


Figure 2.2 A selection of anti-oxidant compounds isolated from various algal species

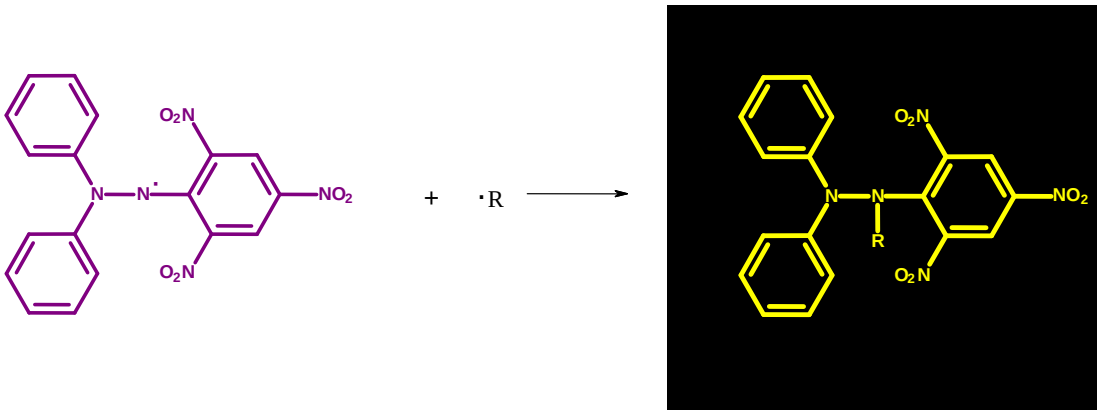
2.2 Anti-oxidant/radical scavenging screening of crude algal extracts and fractions

In our search for potential anti-oxidant/radical scavenging compounds, it is of great importance to emphasize that radical scavenging activity does not fully guarantee anti-oxidant activity. According to Tirzitis and Bartosz (2010), "antiradical activity is characterised by the ability of a compound or extract to react with and thereby neutralize free radicals while anti-oxidant activity represents the ability to inhibit the process of oxidation" (Tirzitis and Bartosz 2010). The general methods employed in the determination of anti-oxidant/radical scavenging activity include the 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), the 2,2-diphenyl-1-picrylhydrazyl (DPPH), the ferric reducing antioxidant power (FRAP) and the oxygen radical absorption capacity (ORAC) assays (Thaipong et al. 2006). In this study, we employed the DPPH assay because of its cost effectiveness and high reproducibility. The FRAP assay is equally as reproducible as the

DPPH assay but we could not use this method because our test samples were insoluble in FRAP reagent. Besides the ORAC assay being an expensive alternative due to the involvement of expensive equipment, the ABTS and ORAC assays are relatively less reproducible than the FRAP and DPPH assays (Thaipong et al. 2006).

2.2.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay

The DPPH assay has been described as a simple, rapid and convenient method independent of sample polarity for the screening of radical scavenging activity (Marxen et al. 2007). The purple coloured diphenylpicrylhydrazyl **(2.12)** free radical is stable in methanolic solution. In the presence of a potent anti-oxidant/radical scavenging compound or extract, purple coloured diphenylpicrylhydrazyl radical is spontaneously converted to yellow coloured diphenylpicrylhydrazine **(2.13)** with an absorbance maximum centred at about 520 nm (Marxen et al. 2007). This assay is based on the principle that accepting a hydrogen atom from an anti-oxidant agent results in the reduction of the hydrazyl radical to form the hydrazine stable compound with a concomitant decrease in absorbance at 513 nm. It is important to emphasise that this mechanism of hydrogen atom transfer is different from that of biological radical scavenging which occurs through electron transfer.



2.12 **2.13**

Figure 2.3 Anti-oxidant mediated conversion of diphenylpicrylhydrazyl **(2.12)** (purple) to diphenylpicrylhydrazine **(2.3)** (yellow)

·R= Hydrogen radical donated from anti-oxidant compound/extract

2.3 Anti-inflammatory assay

Macrophages represent a highly heterogeneous group of hematopoietic cells present in almost all tissues including adipose. When microphages are triggered by stimuli, their response can be categorized into two distinct and mutually exclusive activation programs known as classical and alternative. Classical activation causes a highly inflammatory phenotype and mainly occurs in response to bacterial stimuli such as lipopolysaccharide (LPS) and interferon gamma (IFN-γ). The classically activated macrophages then produce a myriad of pro-inflammatory signals which can alter the functionality of its surrounding cells. In addition, these activated cells produce various highly reactive oxidants including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS). Therefore, the production of NO and consequent nitrate accumulation is conveniently used as a marker for classically activated macrophages.

Adipose tissue contains both adipocytes and resident macrophages which can produce cytokines. Pro-inflammatory cytokines derived from adipose tissue contribute to the pathogenesis of insulin resistance via changes in the phosphorylation status of IRS1 in the insulin signalling cascade. Numerous studies have demonstrated increased accumulation of macrophages in adipose tissue of obesity models of metabolic syndrome. Hence, the inhibition of an inflammatory response represents a potential therapeutic target to treat obesity related type II diabetes.

2.4 *In-vitro* cytotoxicity assays

Cell based *in-vitro* cytotoxicity assays are generally designed for studying individual compounds of known structure and concentration. It is important to keep in mind that cell based cytotoxicity analysis of botanical or natural extracts are difficult to interpret in terms of predicting toxicity *in vivo*, because these extracts contain mixtures of compounds and their concentrations will differ depending on which compounds reach the liver or other organs after oral administration.

Cytotoxicity assays are used as the first phase in anti-diabetic screening to evaluate the suitability of the test samples for *in vitro* cell based assays. This includes establishing parameters such as solubility in culture medium and general cellular toxicity profiling. For samples with poor aqueous solubility it is difficult to establish accurate dose response curves as higher doses do not produce the expected higher response. Similarly there is a high

likelihood that basal toxicity of the samples can disguise the anti-diabetic potential of any active constituents within the extract. Together, these factors define the practical limits within which the anti-diabetic data can be interpreted.

2.4.1 The Chang Liver cell line

The Chang liver cell line originated from a normal liver obtained from a Chinese boy (Chang Shih-man 1954) and was later transformed *in-vitro.* Controversy exists in literature regarding the current authenticity of the Chang Liver cell line as it is believed to have been contaminated by HeLa cells prior to its deposition in cell banks such as the American Type Culture Collection (ATCC) (Masters 2002). The presence of HeLa markers (DNA sequences) and the absence of PCR amplifiable Y chromosome DNA sequences are considered as evidence that these cells now represent a derivative of HeLa cells. Despite these findings, there are a number of publications which suggest that these cells possess characteristics associated with hepatocyte function (Ludueña et al. 1977). Therefore, the use of this cell line as a model for normal human liver should be interpreted with caution as we have no definitive proof as to the authenticity of our Chang liver cell line. On the other hand, even if they do not represent hepatocytes, Chang Liver cells provide an acceptable model to explore general cellular cytotoxicity as many of the known drug induced cytotoxic mechanisms such as those involving mitochondrial function, calcium levels, cellular death pathways, free radical accumulation, enzymatic activity, cellular transportation and generation of harmful metabolites are common to most cells.

2.4.2 The HT-29 and Caco-2 cell lines

The HT-29 and Caco-2 are human colon carcinoma cell lines established by Jorgen Fogh in 1964 and 1974 respectively (Rousset 1986). These cells have gained increased interest due to their ability to express differentiation features characteristic of mature intestinal cells such as enterocytes or mucus cells. Because of these characteristics, HT-29 and Caco-2 cell lines have been used in various fields of investigation related to intestinal cell differentiation, function and malignant transformation (Rousset 1986). In this study, our main rationale for the use of these cell lines is not based on the aforementioned characteristics but rather to explore the cytotoxicity profiles of our extracts and fractions against both normal and malignant cells. However, outside the scope of this research, selective cytotoxicity of any of our test samples towards a particular malignant cell line is indicative of the need for its further research in that particular pathology.

2.4.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

A modification of the original MTT assay (Mosmann 1983) was used to evaluate the cytotoxicity of the extracts. MTT (**2.14**) is a yellow tetrazolium salt which is converted by dehydrogenase enzymes in viable cells to an insoluble purple compound called formazan resulting from the cleavage of the tetrazolium ring. The amount of purple formazan that is extracted from the cells is proportional to the number of viable cells. Since the assay quantifies the number of viable cells at the end of the treatment period, it is not possible to distinguish between treatments that have a cytotoxic (cell killing) or cytostatic (cell growth inhibiting) effects. It has been shown that plant extracts with strong anti-oxidant activity can also convert MTT to the purple formazan (Muraina et al. 2009) thereby masking cytotoxic effects. To avoid this, the cells are incubated with the extract for 48 hours after which the extract is removed from the cells before the addition of MTT.

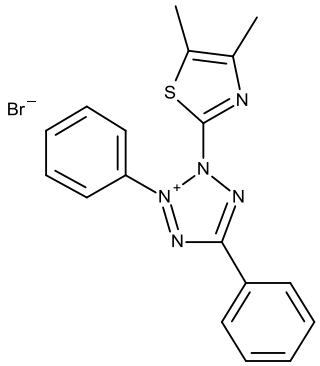


Figure 2.4 The structure of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, **2.14**)

2.5 Chapter aims

In Chapter one, we described the diabetic state as a low-grade inflammatory process and explained the significant role that free radical mediated oxidative stress plays in the pathophysiology of the disease. We also discussed the potential role of anti-oxidants in the management of type II diabetes mellitus. This chapter aims at identifying and screening various marine algae for anti-oxidant and anti-inflammatory activity and further evaluating their potential *in-vitro* cytotoxicity.

2.6 Results and Discussion

2.6.1 Collection and extraction of South African algae

Stypopodium zonale (**DH110218-1**), *Dictyopteris ligulata* (**DH110218-2**), *Cystophora fibrosa* (**DH110218-3**), *Bifurcariopsis capensis* (**DH110218-4**) and *Sargassum* sp. were collected from De Hoop near Cape Town while *Sargassum heterophyllum* (**NDK101124**) was collected from Noordhoek near Port Elizabeth.

A standard procedure was implemented for the extraction of the algae. The algal biomasses were extracted with MeOH followed by DCM/MeOH (2:1). The extracts of each alga were then combined and sufficient deionised water was added to allow separation of the aqueous MeOH and CH_2Cl_2 phases. The desired crude extracts for *S. zonale* (**MN-11-8a**, 362.7 mg), *D. ligulata* (**MN-11-8b**, 467.1 mg), *C. fibrosa* (**MN-11-8c**, 778.6mg), *B. capensis* (**MN-11-8d**, 1331 g), *Sargassum* sp. (**MN-11-8e**, 69.1 mg) and *S. heterophyllum* (**MN-11-62**, 1240 mg) were obtained after concentration of the CH_2Cl_2 phase under rotary evaporation.

2.6.2 DPPH Anti-oxidant/radical scavenging activity of algal extracts

The DPPH anti-oxidant/radical scavenging activity of the crude extracts varied from undetectable to that nearly equivalent to ascorbic acid. From the results, extracts from *S. heterophyllum* (**MN-11-62**, IC_{50} = 20.93 µg/mL) and *B. capensis* (**MN-11-8d**, IC_{50} = 27.10 µg/mL) showed the most potent DPPH radical scavenging activity. *S. heterophyllum* (**MN-11-62**) exhibited a higher DPPH radical scavenging activity than that of ascorbic acid (IC_{50} = 24.07 µg/mL) while *C. fibrosa* (**MN-11-8e**) did not show any activity at all the test concentrations. The anti-oxidant activity of the *Sargassum* genera has been reported in literature. Examples of *Sargassum* species that have shown potent anti-oxidant activity include extracts from *S. hystrix*, (Budhiyanti et al. 2012), *S. boveanum* (Zahra et al. 2007) and *S. spagiophyllum* (Suresh et al. 2012). However, this account is the first to report the anti-oxidant activity of extracts from *B. capensis*.

Table 2.1 IC_{50} values for the DPPH radical scavenging activity of *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**) crude extracts

Crude Extract	**IC_{50} (µg/mL)**
Ascorbic acid	24.07
8a	48.95
8b	254.7
8c	72.61
8d	27.10
8e	0.00
62	20.93

2.6.3 Potential Anti-inflammatory activity of algal extracts

Stimulation of RAW267.4 cells with bacterial LPS (1 µg/mL) resulted in a greater than 200 fold increase in the production of nitrate, indicating classical activation to the inflammatory phenotype. Aminoguanidine, an inhibitor of both iNOS activity and its expression strongly inhibited nitrate production. *D. ligulata* (**8b**) showed pro-inflammatory activity by increasing nitrate production in the absence of LPS (Figure 2.5). Pro-inflammatory activity will normally risk an exacerbated inflammatory response. With the exception of *D. ligulata* (**8b**), there was no significant pro-inflammatory activity observed in the rest of the algal extracts. Significant inhibition of nitrate production was evident in crude extracts of *C. fibrosa* (**8c**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**), with the latter having significant inhibitory activity at both test concentrations (12.5 and 25 µg/mL). These effects also appear to be independent of toxicity as revealed through corresponding maintenance of cell viability as assayed using MTT. Anti-inflammatory activity by inhibition of nitrate production in murine RAW 264.7 macrophages has been reported in extracts from the *Sargassum* specie, *S. thunbergii* (Yang et al. 2010). The anti-inflammatory activity of the *Cystophora* specie, *C. torulosa*, has also been previously documented (Baker 1984). To our knowledge, there has not been any previous report documenting the potential pro-inflammatory activity of *D. ligulata* (**8b**).

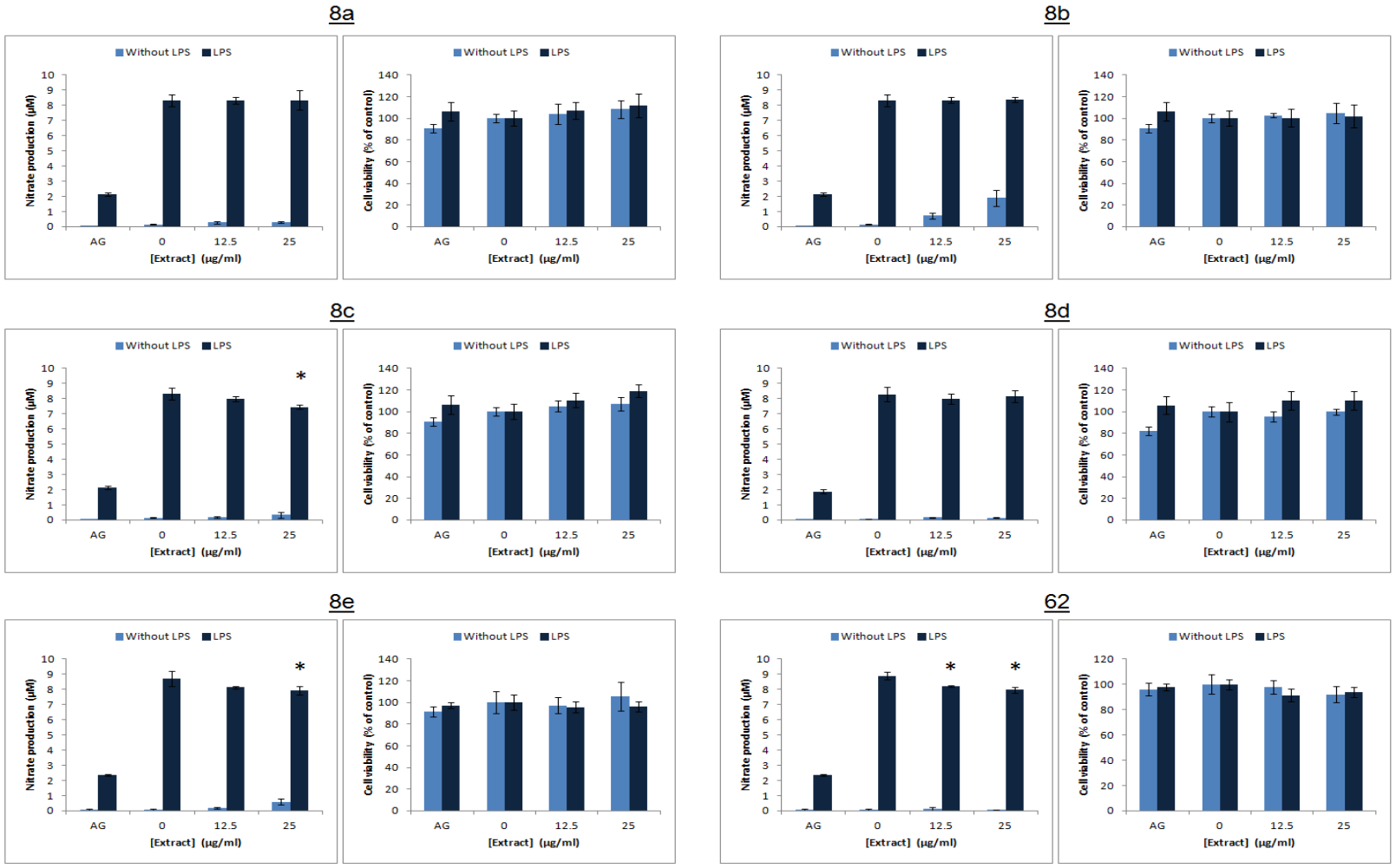


Figure 2.5 Nitrate production and cell viability in murine macrophages (RAW264.7) treated with *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**) crude extracts.

Significant ($p<0.05$) reductions in the levels of nitrate are indicated as (*)

2.6.4 Cytotoxicity profiles of the crude algal extracts

There are two main reasons for the assessment of the toxicity of a compound/extract prior to *in-vitro* anti-diabetic testing. Firstly, toxic effects tend to override the anti-diabetic endpoints thereby creating difficulty in the accurate assessment of anti-diabetic properties. Secondly, when attempting to establish an anti-diabetic dose response, severe toxicity at high doses gives rise to a negative trend. Samples which display EC_{50} values below 100 µg/mL in Chang liver cells may not to be suitable for cell based assays without the consideration of lowering the test concentrations. Although it is possible to do so, it is also important to bear in mind that the sensitivity of the assay methods may become limiting. The individual EC_{50} values for the crude extracts are presented in Table 2.2.

Table 2.2 EC_{50} values for *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**) crude extracts against Chang liver, HT-29 and Caco-2 cells as obtained using the MTT assay

Sample code	**EC_{50} Chang Liver (µg/mL)**	**EC_{50} HT-29 (µg/mL)**	**EC_{50} Caco-2 (µg/mL)**
α-tocopherol	>500	ND	ND
8a	61.67	92.47	73.12
8b	150.7	19.55	27.08
8c	21.79	36.6	31.73
8d	> 500	>500	>500
8e	139.9	>500	>500
62	15.31	71.5	63.8

ND = not determined

From the results and as expected, the positive control α-tocopherol displayed no cytotoxicity (EC_{50} > 500 µg/mL) towards Chang liver cells (Table 2.1). With EC_{50} values > 500 µg/mL for all cell lines, *B. capensis* (**8d**) did not show any cytotoxicity. *Sargassum* sp. (**8e**) had no cytotoxicity towards HT-29 and Caco-2 cells (EC_{50} > 500 µg/mL) but had selective cytotoxicity towards Chang liver cells (EC_{50} = 139.9 µg/mL). *D. ligulata* (**8b**) was generally cytotoxic towards all cell lines but with relatively less cytotoxicity towards Chang liver cells (EC_{50} = 150.7 µg/mL). The cytotoxic activity of polysaccharides isolated from *Dictyopteris* species such as *D. polypodioides* has been previously reported (Sokolova et al. 2011). *C.*

fibrosa (**8c**) was relatively more cytotoxic than the rest of the algae with EC_{50} values of 21.79, 36.6 and 31.73 µg/mL against Chang liver, HT-29 and Caco-2 cell lines respectively. The cytotoxicity of algae from the *Cystophora* genus has been reported in literature. Such examples include the antitumoural activity of the tetraprenyl toluquinols usneoidol Z and Usneoidol E isolated from *Cystophora usneoides* (Urones et al. 1992). *S. heterophyllum* (**62**) was also generally cytotoxic to all cell lines with selective cytotoxicity towards Chang liver cells (EC_{50} = 15.31 µg/mL). The cytotoxicity of *S. zonale* (**8a**) had a similar profile towards all cell lines with no signs of selectivity.

In this chapter, we managed to identify and screen various marine algae for anti-oxidant and anti-inflammatory activity and further evaluated their potential *in-vitro* cytotoxicity. From the identified algae, we determined that extracts from *S. heterophyllum* (**MN-11-62**, IC_{50} = 20.93 µg/mL) and *B. capensis* (**MN-11-8d**, IC_{50} = 27.10 µg/mL) had the most potent DPPH radical scavenging activity. We also established that among the crude extracts that inhibited nitrate production in murine macrophages, *S. heterophyllum* (**62**) possessed the highest anti-inflammatory activity. From the screened algae, crude extracts of *B. capensis* and *Sargassum* sp. had the least cytotoxicity towards the tested cell lines. By exhibiting the most potent anti-oxidant/radical scavenging and anti-inflammatory activity, *S. heterophyllum* crude extract showed the highest promise for bio-active compounds relevant to diabetes mellitus.

2.7 Experimental

2.7.1 General experimental

All solvents used were analytical grade without need for further purification from commercial sources. Extraction solvents and culture mediums were sourced from Sigma Aldrich® and Hyclone® respectively. The EC_{50} values of each extract were calculated from a minimum 5-point dose response curve using GraphPad Prism 4 software package.

2.7.2 Algal Material

Stypopodium zonale (**DH110218-1**), *Dictyopteris ligulata* (**DH110218-2**), *Cystophora fibrosa* (**DH110218-3**), *Bifurcariopsis capensis* (**DH110218-4**) and *Sargassum* sp. were collected from De Hoop near Cape Town while *Sargassum heterophyllum* (**NDK101124**) was collected from Noordhoek near Port Elizabeth. The algae were transported to the laboratory in ice, frozen and stored at – 4 ^{o}C until the time of extraction. Sample authentication was done by anatomical comparison with voucher specimens at the Division of Pharmaceutical Chemistry, Rhodes University, Grahamstown, South Africa.

2.7.3 Extraction of marine algae

After defrosting under running water, the *Stypopodium zonale* (**DH110218-1**) biomass was soaked in MeOH for 30 min after which the MeOH was decanted and the retained algae heated at 40 ^{o}C for 30 min in CH_2Cl_2/MeOH (2:1). MeOH and CH_2Cl_2/MeOH mixtures were pooled and sufficient deionised water added to allow phase separation of the CH_2Cl_2 and the MeOH/H_2O phases. The CH_2Cl_2 phase was then collected separately and dried *in vacuo* to yield the desired crude extract (**MN-11-8a**, 362.7 mg).

Dictyopteris ligulata (**DH110218-2**), *Cystophora fibrosa* (**DH110218-3**) and *Bifurcariopsis capensis* (**DH110218-4**) biomasses were blended in MeOH. CH_2Cl_2 was further added and the CH_2Cl_2/MeOH (2:1) mixture heated at 40 ^{o}C. MeOH and CH_2Cl_2/MeOH mixtures were pooled and sufficient deionised water added to allow phase separation of the CH_2Cl_2 and the MeOH/H_2O phases. The CH_2Cl_2 phase was then collected separately and dried *in vacuo* to yield the desired crude extracts **MN-11-42a** (*Dictyopteris ligulata*), **MN-11-41a** (*Cystophora fibrosa*) and **MN-11-43a** (*Bifurcariopsis capensis*).

The *Sargasssum heterophyllum* (**NDK101124**) biomass was soaked in MeOH for 1 h after which the MeOH was decanted and the retained algae heated at 40 ^{o}C for 30 mins in CH_2Cl_2/MeOH 2:1. The MeOH and CH_2Cl_2/MeOH mixtures were then pooled and sufficient deionized water added to allow phase separation of the CH_2Cl_2 and the MeOH/H_2O phases. The CH_2Cl_2 phase was then collected separately and dried *in vacuo* to yield the desired crude extract (**MN-11-62**, 12.4g).

2.7.4 Sample preparation for *in-vitro* assays

The dried extracts were reconstituted in DMSO to 100 μg/μL and then diluted with complete medium to the desired test concentrations. The final DMSO content in the assays was kept below 1%, a concentration with minimal effect on cell viability.

2.7.5 The DPPH Anti-oxidant assay

The test samples were diluted in EtOH/H_2O (1:1) from 10 mg/100 μL stocks prepared in DMSO. 5 μL of each sample was pipetted into individual wells of a 96-well plate and then a 120 μL of Tris-HCl buffer (50 mM, pH7.4) was added followed by 120 μL of freshly prepared DPPH solution (0.1 mM in ethanol). The plate was incubated for 20 min at room temperature and the absorbance read at 513 nm. The percentage of DPPH scavenging was calculated as [(A- B/A) × 100], where A represents absorbance without test samples and B represents absorbance in the presence of test samples. Ascorbic acid was used as a positive control (EC_{50} = 24.07 μg/mL).

2.7.6 Anti-inflammatory assay

The murine peritoneal macrophage cells (RAW267.4) were cultured in DMEM containing 10% FCS from LONZA®. Cells were seeded into 96 well plates at a density of 8 X 10^4 cells/well and allowed to attach overnight. The cells were then treated with 1 μg/mL LPS (SIGMA®) and two concentrations of test sample (12.5 and 25 μg/mL) for 18 h. To measure nitrate levels, 50 μL of the spent culture medium was removed and added to an equal volume of Griess reagent (SIGMA®). The absorbance was measured at 540 nm using a microplate reader and the nitrate concentrations were calculated by comparison with the absorbance to sodium nitrate standard solutions. Aminogaunidine (SIGMA®) was used as positive control to demonstrate the inhibition of nitrate production. Cell viability was simultaneously measured using the standard MTT assay.

2.7.7 *In-vitro* Cytotoxicity assays

The Chang liver cells were seeded into 96-well culture plates (TTP) at 10 000 cells/well in EMEM supplemented with 10% fetal bovine serum (FBS) and left for 24 hrs. Algal extracts were added and the cells incubated for a further 48 hrs after which the medium was replaced with 200 µL of MTT (Sigma®) (0.5 mg/mL in EMEM). After a further 2 hrs of incubation at 37 °C, the MTT was removed and the purple formazan product dissolved in 200 µL of DMSO.

The HT-29 and Caco-2 cells were seeded into 96-well culture plates (TTP) at 5 000 cells/well in DMEM supplemented with 10% fetal bovine serum (FBS) and left for 24 hours. Algal extracts were added and the cells incubated for a further 48 hrs after which the medium was replaced with 200 µL MTT (Sigma®) (0.5 mg/mL in DMEM). After 3 hrs of incubation at 37 °C, the MTT was removed and the purple formazan product dissolved in 200 µL DMSO.

The absorbance was measured at 560 nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). All incubation steps were carried out in a 37 °C humidified incubator with 5% CO_2. EC_{50} values were calculated from a minimum 5-point dose response curves using GraphPad Prism 4 software package.

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Chapter 3

Isolation and synthetic modification of prenylated toluhydroquinone and toluquinone metabolites from *Sargassum heterophyllum*

3.1 Introduction

Plant derived natural products form the basis of sophisticated traditional medicine systems that have been in existence for thousands of years (Cragg and Newman 2005). In some parts of the world, natural remedies are still the only treatment available. Conventional drugs are either extracted from natural sources or synthesized using natural compounds as templates or precursors (Pomponi 2001). With the discovery of over 21,855 marine-derived compounds possessing novel mechanisms of action (Blunt et al. 2013), the unexplored marine environment harbours the vastest biological and chemical diversity (Lui 2012). Marine macroalgae, or 'seaweeds' as they are most generally called, have been used as crude drugs for vitamin supplementation, in the treatment of iodine deficiency states, in the management of pregnancy associated anaemia, for the alleviation of various intestinal disorders and as hypocholesterolaemic and hypoglycaemic agents with the latter property claimed for the seaweeds *Cystoseira barbata*, *Sargassum confusum* and *Junia rubens* (Blunden 2002). The potential of marine natural products to compete with synthetic compounds as future pharmaceuticals and their contribution to the cure of human disease will depend on their increased isolation from natural sources, the development of new technologies and efficient collaborations between multi-disciplinary scientists (Lui 2012).

3.1.1 Marine pharmacognosy of the *Sargassum* C. Agardh genus

Sargassum C. Agardh is one of the marine macroalgal genera from the class Phaeophyceae (brown algae). This genus is present in much of the tropics and has been described as large, economically important and ecologically dominant (Marimuthu et al. 2012). The Phaeophyceae has five recognized subgenera: *Phyllotrichia* (Areschoung) J. Agardh, *Schizophycus* J. Agardh, *Bactrophycus* J. Agardh, *Arthrophycus* J. Agardh and *Sargassum* (Stiger et al. 2000). *Sargassum* C. Agardh is the largest genus within the Phaeophyceae and includes more than 400 described species that are globally distributed in tropical and subtropical regions (Stiger et al. 2000). Phytochemistry studies have only been reported on an estimate of 62 species of the *Sargassum* genus (Reddy 2009).

The east coast of South Africa is characterised by a high wave-energy environment and one of its most common genera of intertidal algae is *S. crassifolium*, *S. elegans*, *S. heterophyllum*, *S. lendigerum* and *S. obovatum* (Critchley et al. 1991). According to Stiger et al., "the taxonomy of the *Sargassum* genera has been regarded as notoriously difficult due to morphological plasticity" (Stiger et al. 2000). *S. heterophyllum* possesses a large discoid holdfast for attachment which gives rise to a main axis. The main axis gives rise to primary laterals which float due to the presence of vesicles filled with air and partitioned into receptacle producing laterals. The receptacles produced by these laterals possess gametangia inside their male and female conceptacles. The presence of both male and female conceptacles makes *S. heterophyllum* receptacles to be androgenous (Critchley et al. 1991).

3.1.2 Previously isolated compounds from *Sargassum* species

Sargassum species are known to produce secondary metabolites of structural classes such as plastoquinones, chromanols, chromenes, steroids and glycerides (Reddy 2009). From these structural classes, much interest has been focused on the isolation of prenylated compounds due to their potential to possess a wide variety of biological activities. These hydroquinone and quinone moieties are derived from shikimic acid while their prenylated side chains may be derived from either the mevalonate (MVA) or the non-mevalonate (MEP) biosynthetic pathway (Eisenreich et al. 2004).

Examples of biologically active prenylated compounds derived from the *Sargassum* genera (Figure 3.1) include anticholinesterase plastoquinones such as sargaquinoic acid **(3.1)** isolated from *S. sagamianum* (Choi et al. 2007), the endothelin inhibitor nahocol A **(3.2)** (a biogenetic precursor of ubiquitous prenyl-hydroquinones or benzoquinones) isolated from *S. autumnale* (Tsuchiya et al. 1998), the bone resorption inhibitory activity and antiviral activity (against human cytomegalovirus) of the meroditerpenoids 14′,15′-dihydroxysargahydroquinone **(3.3)** and 11′,12′-dihydro-11′,12′-dihydroxysargaol **(3.4)** isolated from *S. micracanthum* (Komai et al. 2006) and the potential PPAR-γ transactivation activity of plastoquinones such as meroterphenol C **(3.5)** isolated from Korean *S. yezoense* (Kim et al. 2011). Research has also shown that most of these prenylated compounds possess anti-oxidant properties with the hydroquinones being more potent than their corresponding quinones.

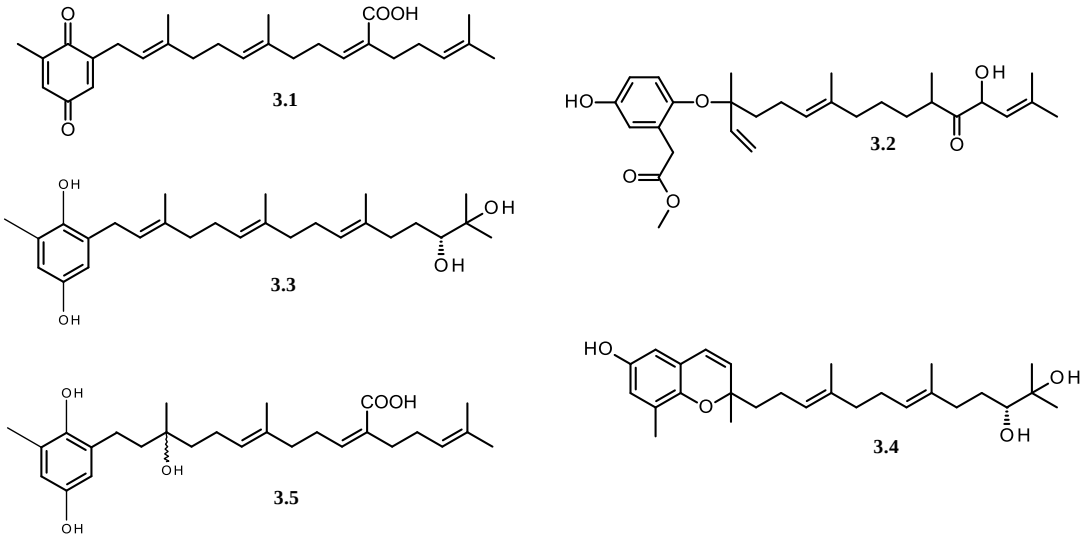


Figure 3.1 Prenylated compounds isolated from the *Sargassum* genera possessing various biological activities.

3.1.3 Bioactive compounds isolated from *S. heterophyllum*

Prenylated toluquinols isolated from *S. heterophyllum* have been reported to have antiplasmodial activity. Afolayan et al. (2008) screened 22 marine algae, of which the *S. heterophyllum* extract showed the most promising antiplasmodial activity (Afolayan et al. 2008). The tetraprenyl toluquinol sargaquinal (**3.7**) and the carotenoid *all-tans*-fucoxanthin (**3.8**) both showed good antiplasmodial activity towards a chloroquine sensitive *P. falciparum* D10 strain. The *S. heterophyllum* metabolites sargahydroquinoic acid (**3.6**) and sargaquinoic acid (**3.1**) have both been reported as dual agonists for PPARα/γ (Kim et al. 2008). Sargachromenoic acid (**3.9**), a metabolite from *S. heterophyllum* (Munedzimwe 2012), has been reported to potentiate nerve growth factor (NGF) mediated neurogenesis and is therefore, a potential neuroprotective agent for the prevention of progressive neuronal death characteristic of neurodegenerative diseases (Tsang et al. 2005).

3.1.4 Chapter aims

In Chapter 2, after screening several algae, we discovered that crude extracts obtained from *S. heterophyllum* exhibited the most potent anti-oxidant and anti-inflammatory activity. In this Chapter, we describe the isolation and characterization of pure natural products from *S. heterophyllum* as well as the preparation of a series of synthetic derivatives for further pharmacological studies.

3.2 Results and Discussion

3.2.1 Extraction and isolation of metabolites from *Sargassum heterophyllum*

Specimens of *S. heterophyllum* (**NDK101124**) were collected on the 24th of November, 2010 from Noordhoek, Port Elizabeth on the southeast coast of South Africa. The alga was first extracted with MeOH followed by CH_2Cl_2/MeOH (2:1). Extracts were combined and sufficient deionised water was added to allow separation of the aqueous MeOH and CH_2Cl_2 phases. A dark green oil (**MN-11-62**) was obtained after concentration of the CH_2Cl_2 phase under rotary evaporation. The ^{1}H NMR spectrum of the crude extract (Figure 3.2) exhibited two singlets at δ 6.46 and 6.48, and a doublet at δ 3.28. These signals are characteristic of prenylated hydroquinones/quinones previously isolated from this alga (Afolayan et al. 2008) and are likely to be responsible for its anti-oxidant activity.

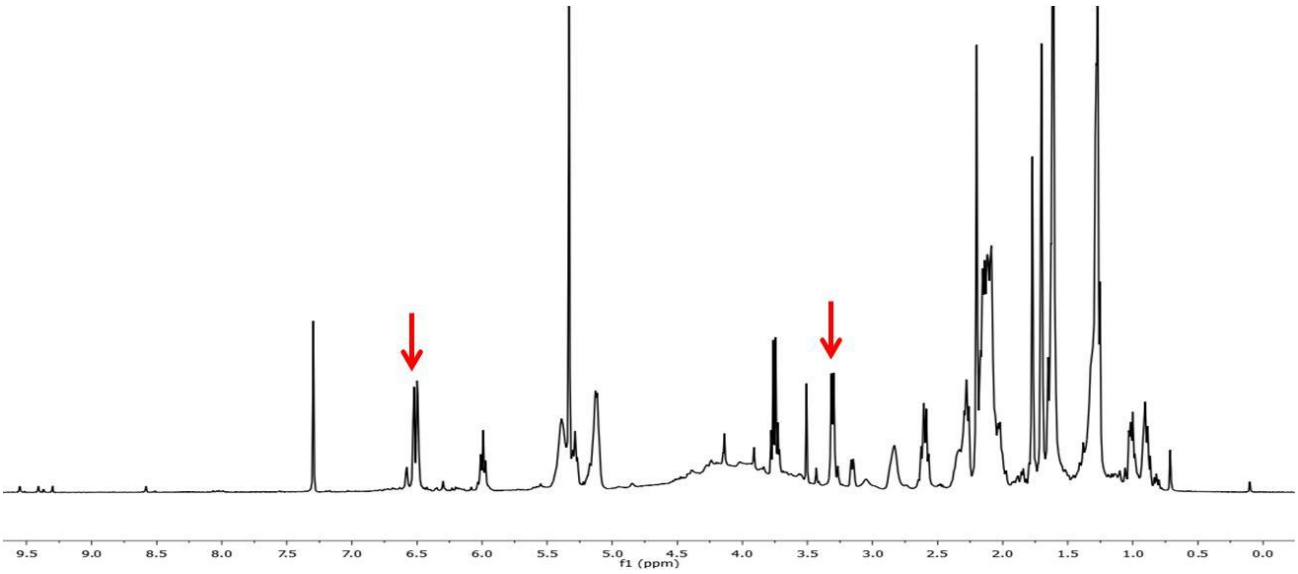
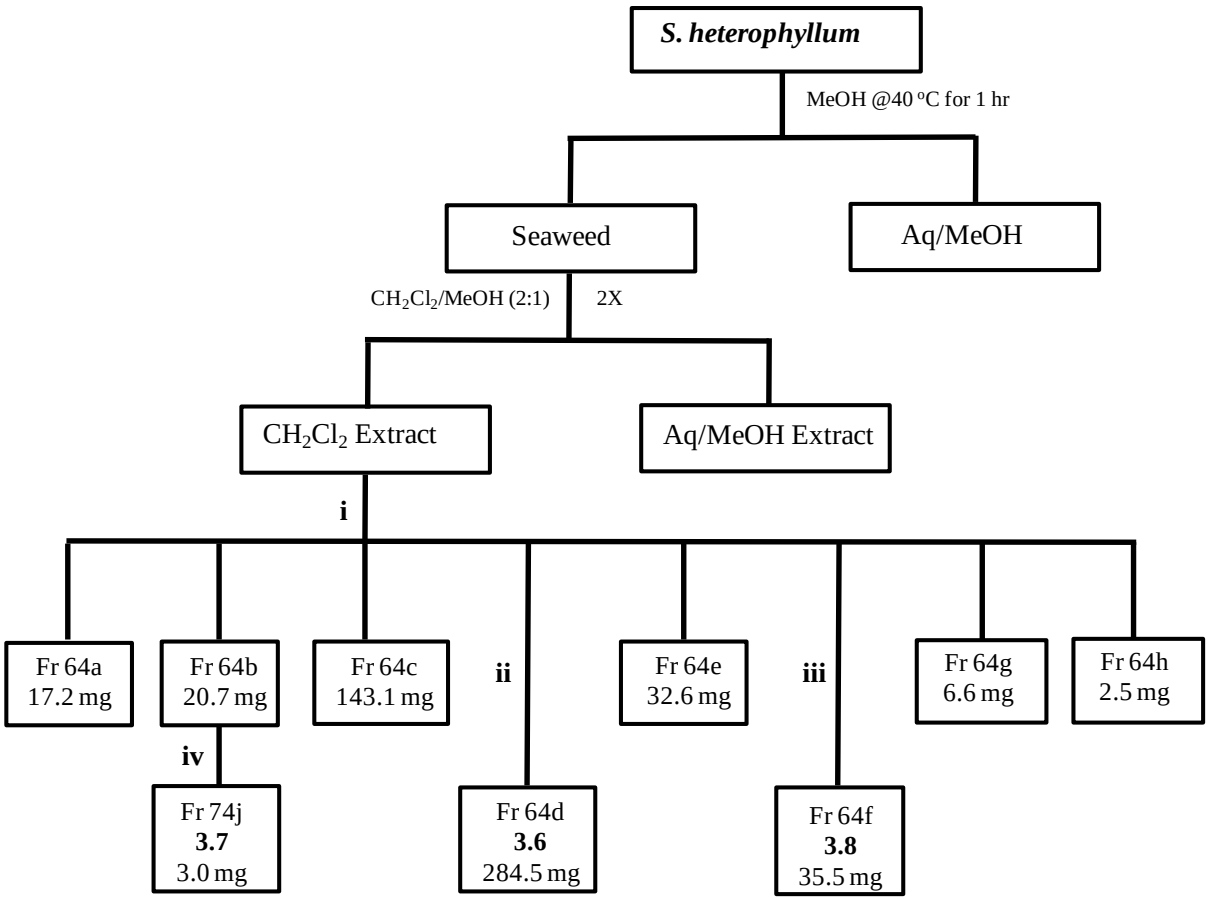


Figure 3.2 ^{1}H NMR spectrum ($CDCl_3$, 400MHz) of *S. heterophyllum* crude extract

The red arrows indicate signals characteristic of prenylated quinones/hydroquinones

Fractionation of the crude extract by silica gel column chromatography using a step-gradient of increasing polarity (hexane-EtOAc-MeOH) yielded nine crude fractions (**64a-i**). Further purification, by either silica column chromatography or HPLC, was guided by DPPH free radical scavenging activity and ^{1}H NMR spectroscopy (Scheme 3.1, Figure 3.3) to yield compounds **3.6**, **3.7** and **3.8**.



Scheme 3.1 Isolation of compounds **3.6**, **3.7**, and **3.8** from *S. heterophyllum*

Conditions: (i) Silica gel column chromatography of **MN-11-62**, mobile phase hexane-EtOAc. **(ii)** Silica gel column fraction, hexane-EtOAc (7:3). **(iii)** Silica gel column fraction, hexane-EtOAc (4:6). **(iv)** Normal Phase HPLC fraction, hexane-EtOAc (9:1)

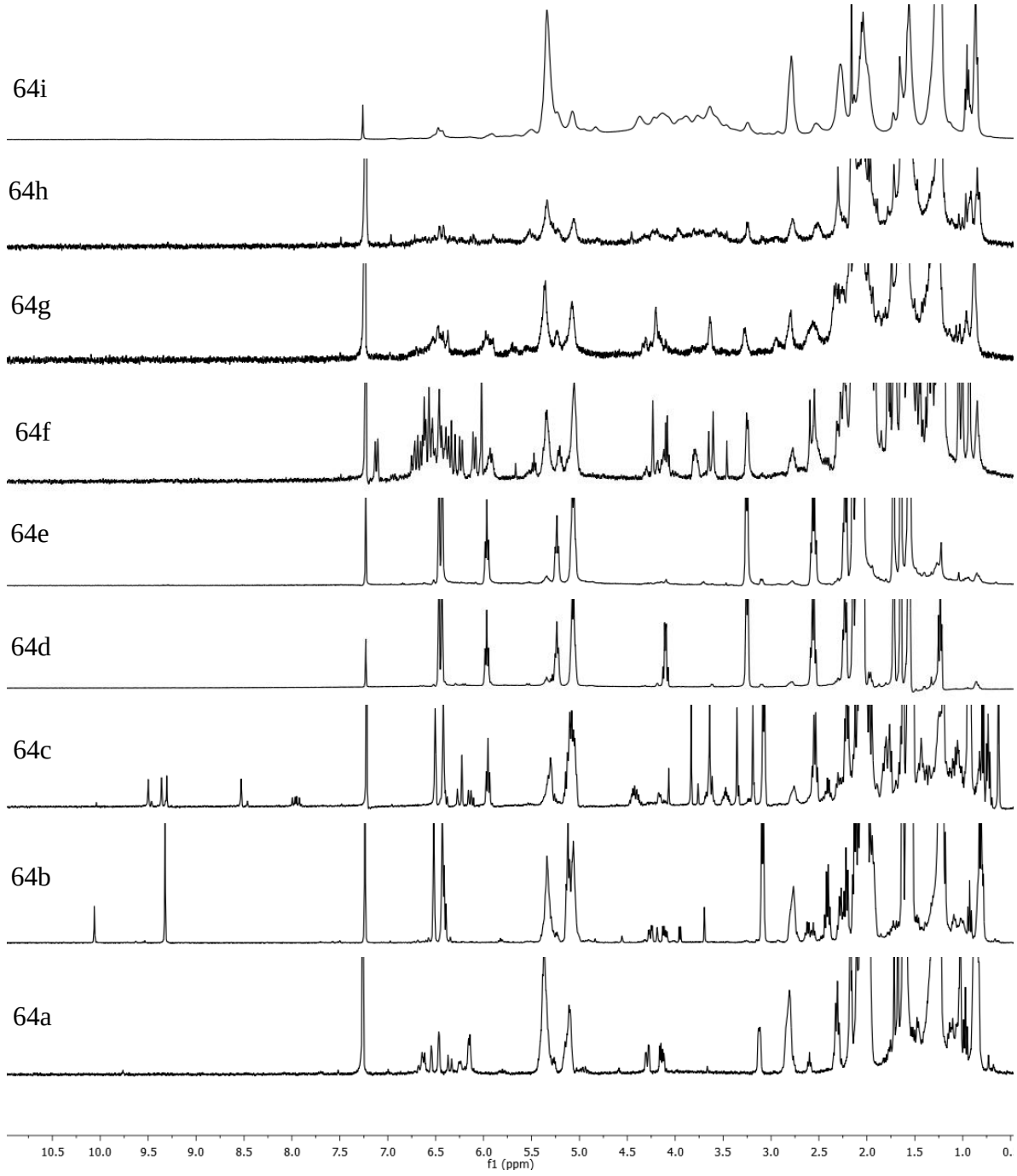


Figure 3.3 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of *S. heterophyllum* silica gel column fractions **64a-i**

3.2.2 Structural characterization of isolated compounds

3.2.2.1 Characterization of compound 3.6

Compound **3.6** was the major compound isolated from the crude extract. It's ^{1}H NMR spectrum showed key signals in the aromatic region at δ 6.46 and 6.50 and a methylene doublet at δ 3.29. The ^{13}C NMR spectrum of compound **3.6** showed 27 carbon resonances, including a carbonyl signal at δ 172.7 characteristic of a carboxylic acid functional group. Comparison of ^{1}H and ^{13}C NMR spectroscopic data with that found in literature for metabolites previously isolated from *S. heterophyllum* confirmed that compound **3.6** was sargahydroquinoic acid (SHQA) (Munedzimwe 2012). This compound has been previously isolated from several *Sargassum* species including *S. heterophyllum* (Afolayan et al. 2008), *S. yezoense* (Kim et al. 2011), and *S. thunbergii* (Seo et al. 2006). The trace impurity at δ 2.17 is residual acetone.

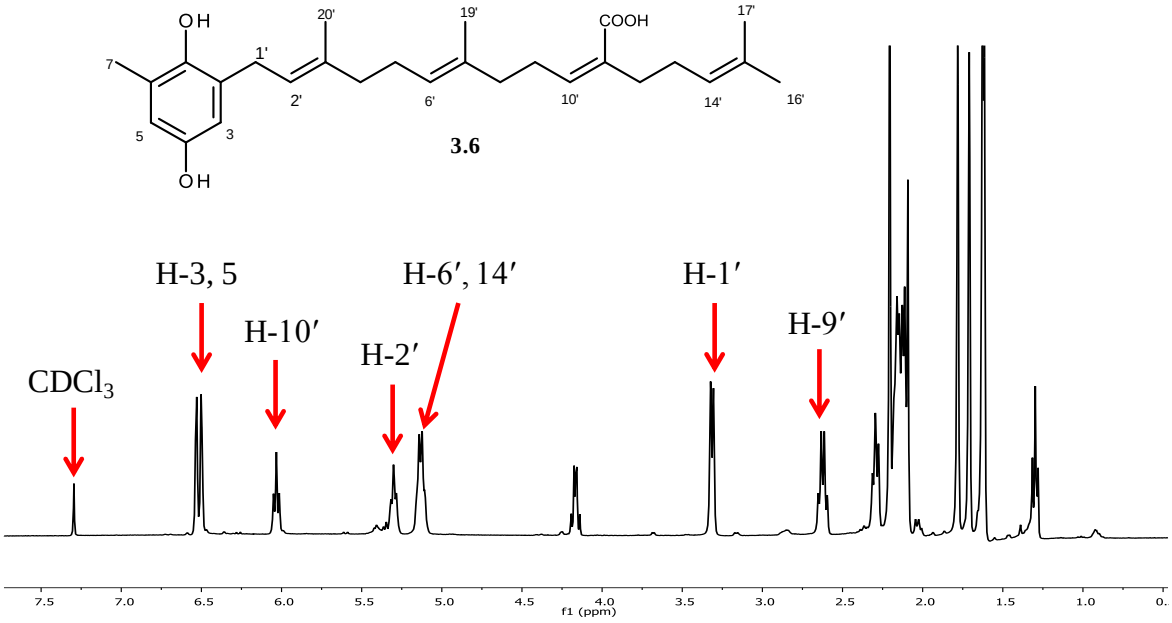


Figure 3.4 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.6**

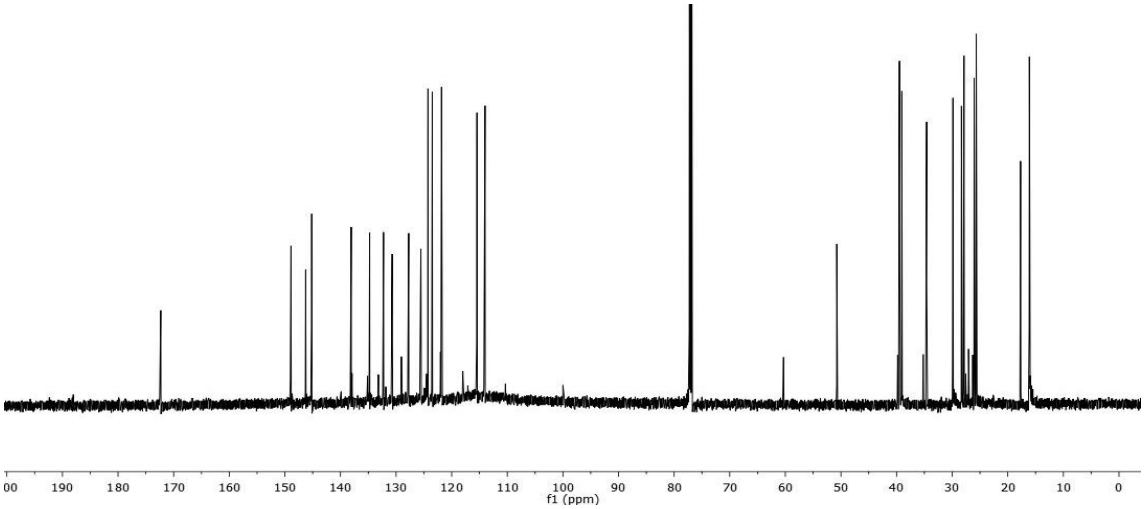


Figure 3.5 ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of compound **3.6**

3.2.2.2 Characterization of compound 3.7

Compound **3.7** was isolated from fraction **64b** (Figure 3.3) and its ^{1}H NMR spectrum (Figure 3.6) showed a sharp aldehyde singlet at δ 9.34. The latter showed an HSQC correlation to a carbon signal at δ 195.2 which, combined with the loss of the carboxylic acid carbon signal at δ 172.7 (in **3.6**) confirmed the presence of an aldehyde moiety. Furthermore, the ^{13}C NMR spectrum of compound **3.7** (Figure 3.6) also showed the presence of a benzoquinone carbonyl at δ 188.3. Comparison of ^{1}H NMR and ^{13}C NMR spectroscopic data with those found in literature helped us to identify compound **3.7** as 2′*E*,6′*E*,10′*E*- sargaquinal and not 2′*E*,6′*E*,10′*Z*- sargaquinal.

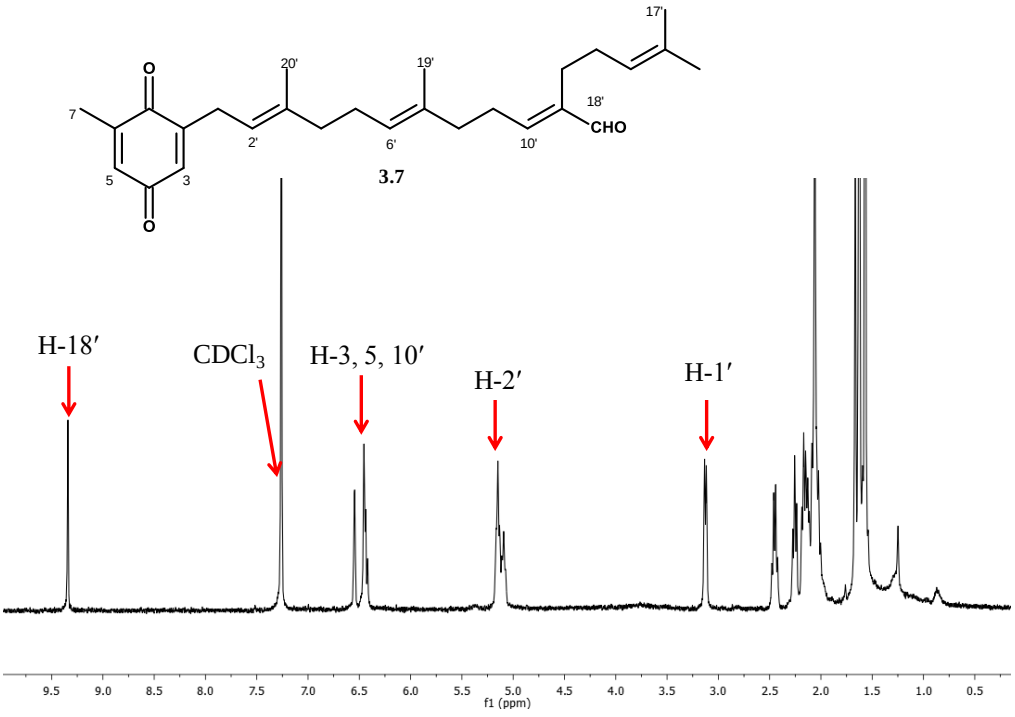


Figure 3.6 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.7**

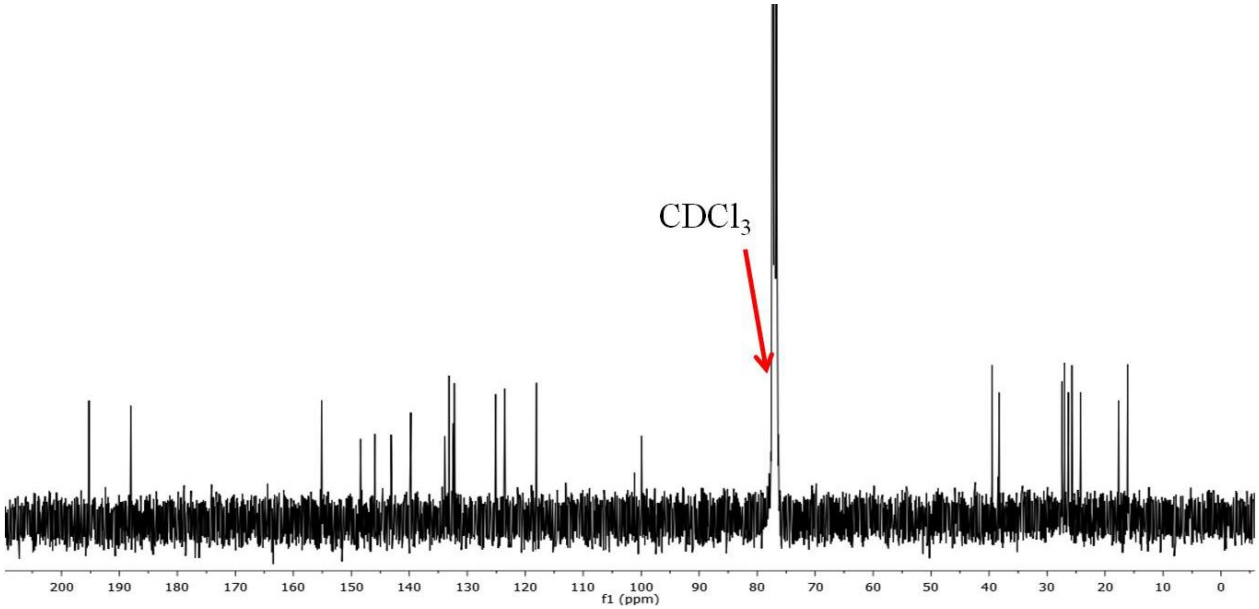


Figure 3.7 ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of compound **3.7**

Compound **3.7** has been previously isolated from several *Sargassum* species including *S. serratifolium* (Kusumi et al. 1979) and *S. heterophyllum* (Afolayan et al. 2008) whereas its isomer has been successfully synthesized from sargaquinoic acid (compound **3.1**) (Kusumi et al. 1979).

Table 3.1 ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data for compounds **3.6** and **3.7**

	3.6		**3.7**	
Carbon No.	δ_C	δ_H **mult, J_{Hz}**	δ_C	δ_H **mult, J_{Hz}**
1	146.2		188.3	
2	125.5		146.2	
3	115.4	6.5, br s	132.4	6.55, br s
4	148.9		187.9	
5	114.1	6.46, br s	132.4	6.44, m
6	127.7		148.4	
7	16.1	2.17, s	16.0	2.06, m
1′	29.9	3.29, d, 6.90	27.5	3.12, d, 6.8
2′	121.8	5.27, t,	118.1	5.15, m
3′	138		139.7	
4′	39.4	2.08, m	39.5	2.06, m
5′	26.0	2.11, m	26.3	2.09, m
6′	124.2	5.10, d, 6.70	125.1	5.11, m
7′	134.7		133.9	
8′	39.0	2.07, m	38.3	2.15, m
9′	28.3	2.58, q, 7.5	27.0	2.44, q, 7.6
10′	145.1	6.00, t, 7.2	155.1	6.42, m
11′	130.7		143.1	
12′	34.6	2.26, t, 7.6	27.5	2.26, t, 8.0
13′	27.8	2.11, m	25.7	2.15, m
14′	123.5	5.09, d, 7.2	123.5	5.15, m
15′	132.2		133.1	
16′	25.6	1.68, s	24.2	1.66, s
17′	17.7	1.58, s	17.7	1.56, br s
18′	172.7		195.2	9.34, s
19′	16.0	1.59, br s	16.0	1.63, s
20′	16.1	1.75, s	16.1	1.62, s

3.2.2.3 Characterization of compound 3.8

The ^{1}H and ^{13}C NMR spectra of compound **3.8** (Figures 3.8, 3.9 and Table 3.2) showed no resemblance to those of compounds **3.6** and **3.7** with a total of 42 resonances in the ^{13}C NMR spectrum mostly observed in the olefinic region (δ 100 and 150) and several overlapping peaks on the ^{1}H NMR spectrum (Figure 3.8). Upon isolation, compound **3.8** had a strong red-orange colour that suggested the presence of conjugated double bonds characteristic of carotenoid compounds as an attribute of conjugated pi systems that are able to absorb light of lower energy levels and reflect the complimentary colours. Comparison of the ^{1}H and ^{13}C NMR spectroscopic data with known carotenoids isolated from *S. heterophyllum* (Afolayan et al. 2008) allowed for the identification of compound **3.8** as fucoxanthin.

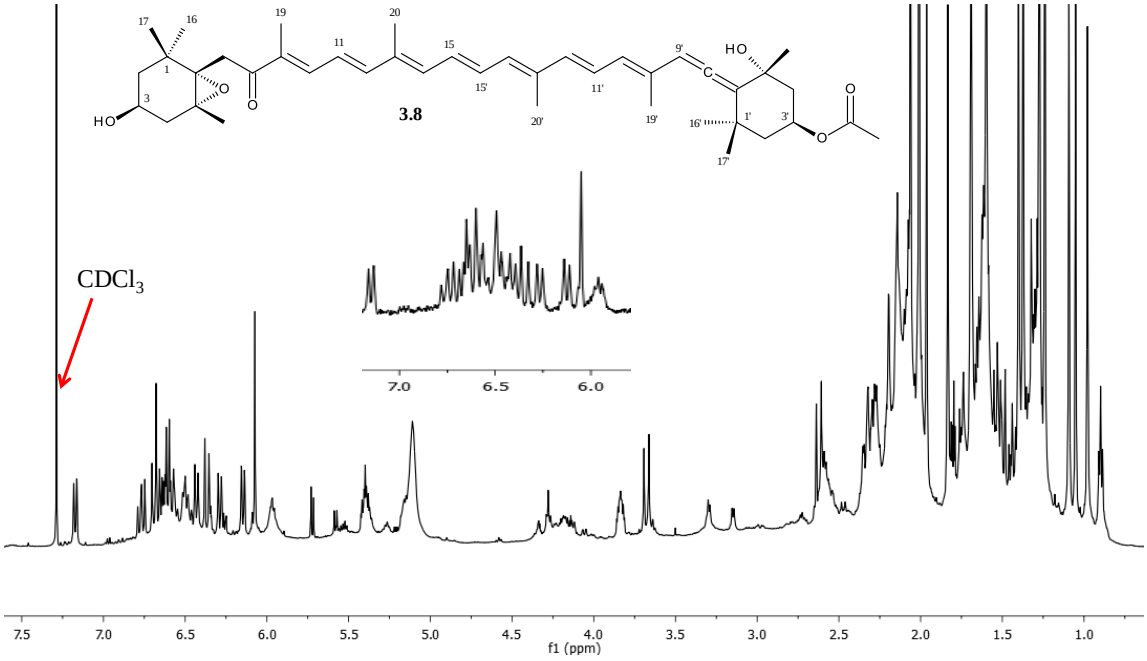


Figure 3.8 ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **3.8**

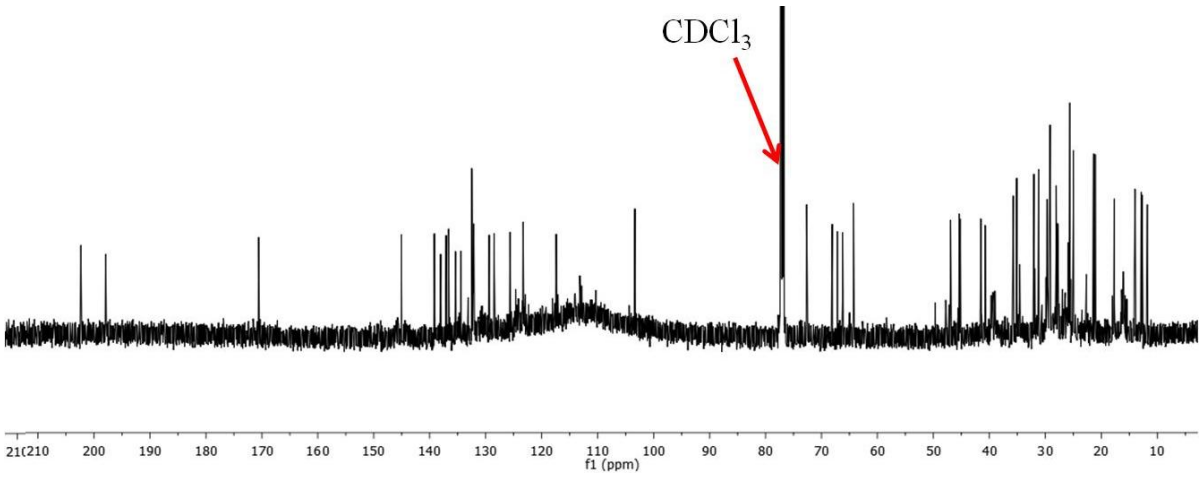


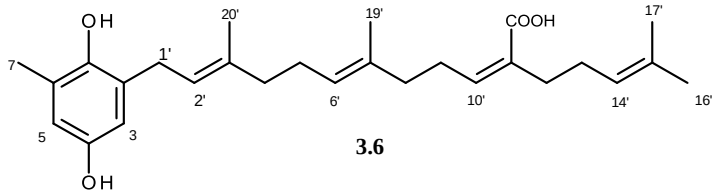
Figure 3.9 ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of compound **3.8**

Table 3.2 ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data for compound **3.8**

Carbon No.	**δ_C**	**δ_H mult, J_{Hz}**	**Carbon No.**	**δ_C**	**δ_H mult, J_{Hz}**
1	35.72		11	123.4	6.54
1′	35.12		11′	125.65	6.58
2	47.0		12	145.1	6.66
2′	45.40	1.48	12′	137.1	6.33
3	64.3	3.82	13	135.4	
3′	8.1	5.37	13′	138.04	
4	41.50	1.74	14	136.6	6.39
4′	45.20	1.51	14′	132.13	6.25
5	66.23		15	129.37	6.63
5′	72.66		15′	132.48	6.75
6	67.16		16	25.00	1.03
6′	117.4		16′	21.37	1.81
7	40.74	2.58, 3.641	17	28.10	0.96
7′	202.35	3.63	17′	32.04	1.07
8	197.9		18	21.10	1.22
8′	103.34	6.05	18′	31.20	1.35
9	134.43		19	11.80	1.94
9′	132.5		19′	13.97	1.81
10	139.2	7.13	20	12.71	1.99
10′	128.5	6.11	20′	12.87	1.99

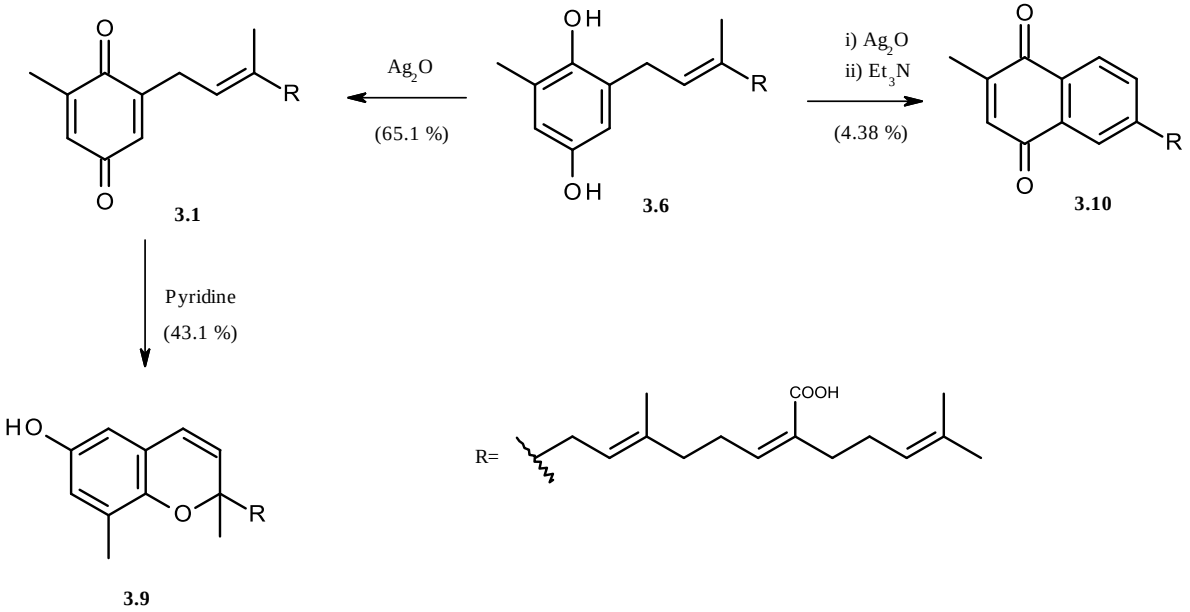
3.2.3 Synthesis of sargahydroquinoic acid derivatives

Although sargaquinoic acid (SQA, **3.1**) and sargachromenoic acid (**3.9**) have been isolated from *S. heterophyllum* in minute quantities (Munedzimwe 2012), we decided to synthesize these compounds from sargahydroquinoic acid (SHQA, **3.6**), which could be isolated in gram quantities from the alga.



3.6

Sargaquinoic acid (**3.1**) has been previously isolated from *S. heterophyllum* (Afolayan et al. 2008) and has been synthesized by the oxidation of SHQA using MnO_2 and Ag_2O (Munedzimwe 2012). Thus, the oxidation of SHQA (**3.6**) with Ag_2O gave SQA (**3.1**) in 65.1 % yield (Scheme 3.2). Not surprisingly, the major differences in the ^{1}H NMR spectra of the two compounds were due to the signals of H-3, H-5 and H-1′. These protons resonate at δ 6.50, 6.46 and 3.29, respectively, in SHQA (Figure 3.10 and Table 3.1) and at δ 6.56, 6.47 and 3.12, respectively, in SQA (Figure 3.10 and Table 3.3). The ^{13}C NMR spectrum of SQA clearly shows carbon signals at δ 187.9 and 188.0 due to the presence of the quinone moiety.



3.1 **3.6** **3.10** **3.9**

Scheme 3.2 Synthesis of sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**)

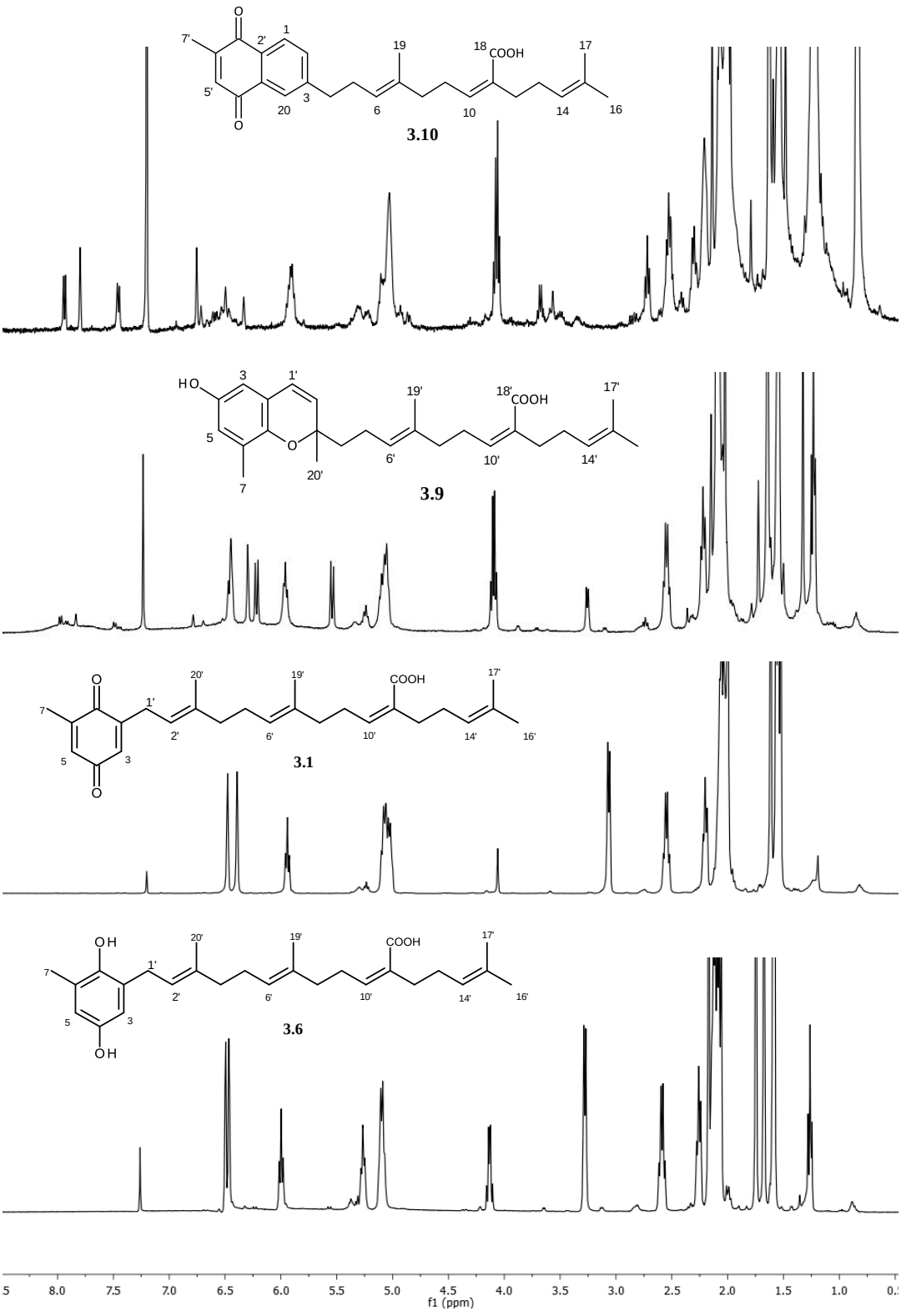
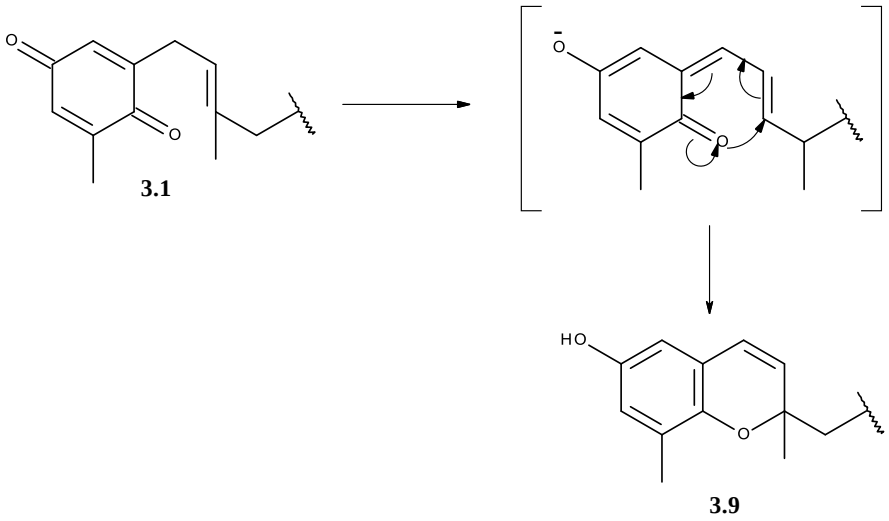


Figure 3.10 ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of compounds **3.1**, **3.6**, **3.9** and **3.10**

Sargachromenoic acid (**3.9**) has been reported from *S. fallax* (Reddy and Urban 2009) as well as from *S. serratifolium* (Kusumi et al. 1979). The synthetic transformation of hydroquinones into quinones with subsequent conversion to chromenols has been reported (Kusumi et al. 1979;Pérez-Castorena et al. 2002). It has also been suggested that this conversion occurs from hydroquinones directly to chromenols by cyclization between the hydroxyl of the hydroquinone and the C-3′ of the polyprenylated side chain (Scheepers 2006). In our hands, the treatment of SQA with pyridine at room temperature gave sargachromenoic acid (**3.9**) in almost quantitative yields. A tautomerism/electrocyclization mechanism has been proposed for the formation of chromenes from quinones (Scheme 3.3).

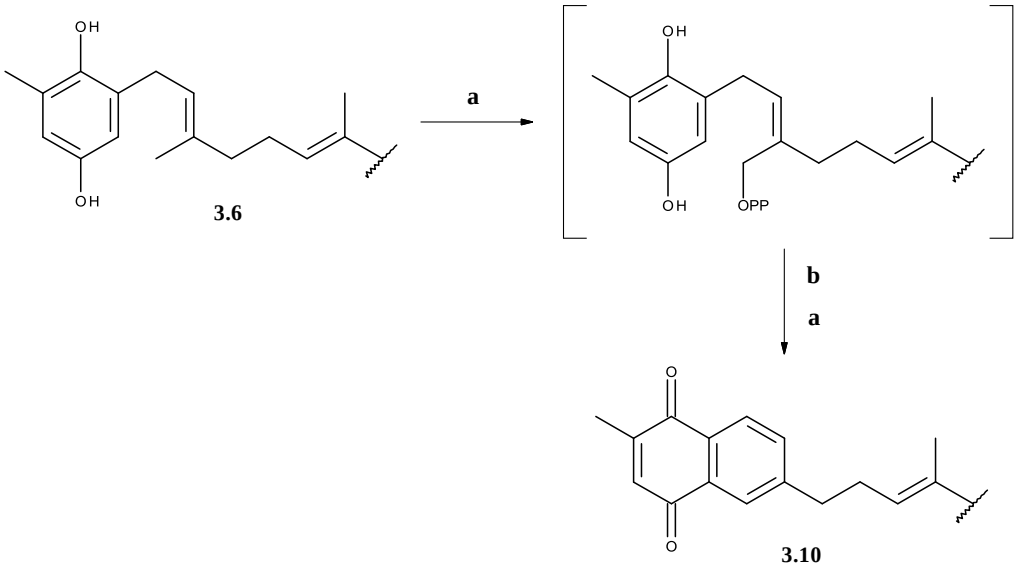


Scheme 3.3 Proposed mechanism for the synthetic conversion of sargaquinoic acid (**3.1**) to sargachromenoic acid (**3.9**) (Lumb et al. 2008)

The most diagnostic feature of the ^{1}H NMR spectrum of compound **3.9** (Figure 3.10) was the two mutually coupled olefinic protons at δ 6.26 and 5.58. In addition, signals due to the protons H-3 and H-5 had shifted from δ 6.53 and 6.45 in SQA to 6.32 and 6.47 in **3.9**. Comparison of ^{1}H and ^{13}C NMR spectroscopic data with that found in literature (Munedzimwe 2012) confirmed the identification of compound **3.9** as sargachromenoic acid.

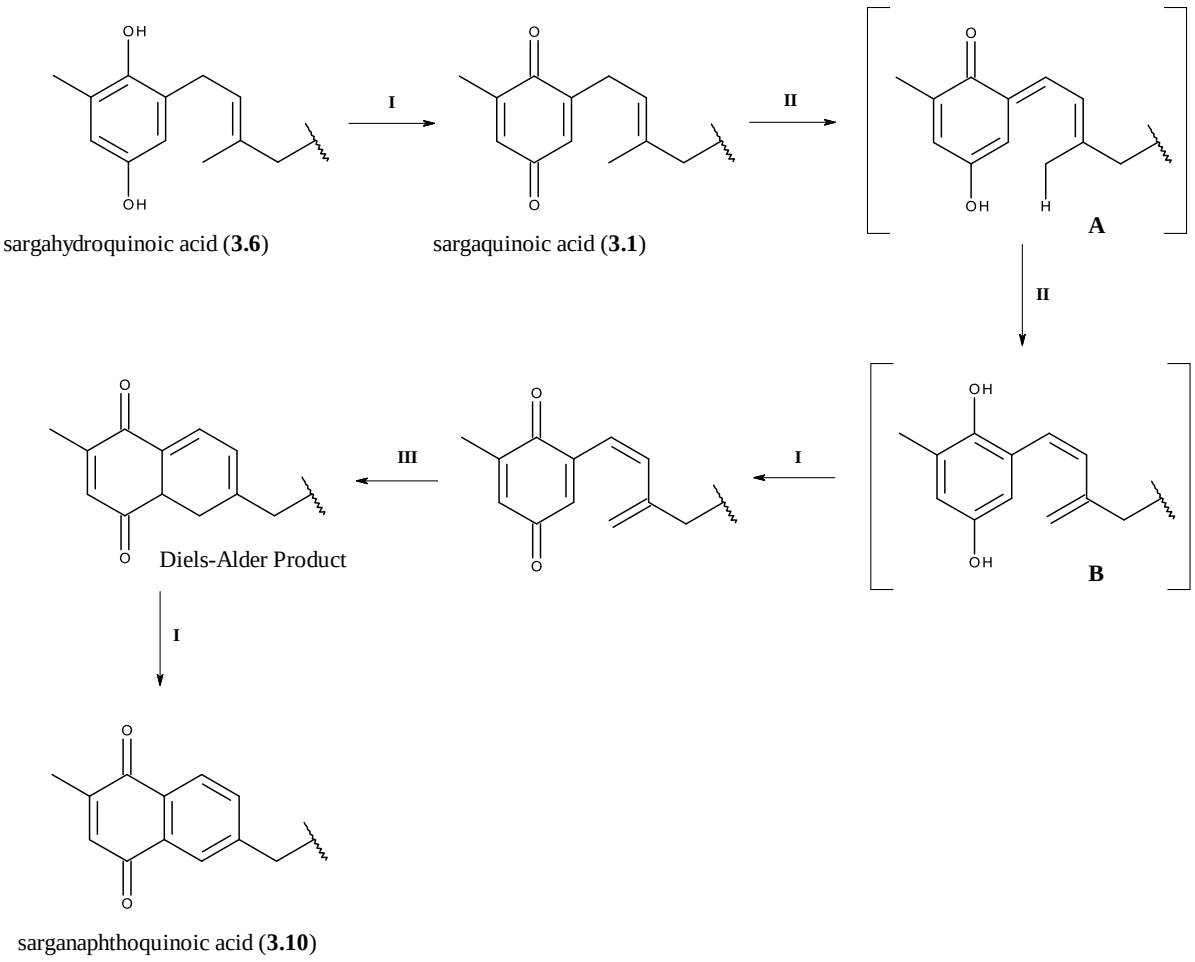
Munedzimwe (2012) previously reported the isolation of compound **3.10** as a minor side product during the Ag_2O catalyzed oxidation of SHQA to SQA (Munedzimwe 2012). A related compound, chabrolonaphthoquinone A, was previously isolated from the soft coral *Nephthea chabrolii* differing by the geometry of the $\Delta^{10\text{-}11}$ double bonds and the positioning of the methyl substituent at C-5′ rather than C-6′ (Sheu et al. 2004). Scheme 3.4 shows a proposed biosynthetic pathway by Sheu et al. in which oxidation of SHQA at C-20 forms an

intermediate which is subsequently alkylated and oxidized to form compound **3.10**. The proposed mechanism for the synthesis of compound **3.10** from SQA includes the oxidation of SHQA to SQA followed by tautomerism to intermediate A which, after oxidation to the quinone is able to undergo a Diels-Alder reaction to intermediate B which after further oxidation gives the naphthoquinoic acid (Scheme 3.5). Considering the proposed mechanism, we reasoned that addition of a base subsequent to the oxidation of SHQA to SQA should facilitate the formation of **3.10**. The reaction of SHQA with six equivalents of Ag_2O for 24 h followed by three drops of triethylamine and stirring at room temperature for a further 24 h gave **3.10** in 43.1 % yield after purification.



Scheme 3.4 Proposed biosynthetic pathway for sarganaphthoquinoic acid (**4.2**) from sargahydroquinoic acid (**3.6**). (**a**) Oxidation, (**b**) Friedel-Craft's alkylation (Sheu et al. 2004)

From the ^{1}H NMR spectrum of compound **3.10** (Figure 3.11), the upfield signals at δ 7.52, 7.86 and 8.00 are typical of the naphthoquinone system. In addition, one of the aromatic singlets shifted downfield from δ 6.46 in SHQA to 6.81. Comparison of ^{1}H and ^{13}C NMR spectroscopic data with that found in literature (Munedzimwe 2012) confirmed the identification of compound **3.10** as sarganaphthoquinoic acid.



Scheme 3.5 Proposed mechanism for the synthetic conversion of sargahydroquinoic acid (**3.6**) to sarganaphthoquinoic acid (**3.10**)

(**I**) Oxidation. (**II**) Tautomerism. (**III**) Diels-Alder reaction.

Table 3.3 ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data for compounds **3.1** and **3.9**

	3.1		**3.9**	
Carbon No.	**δ_C**	**δ_H mult, J_{Hz}**	**δ_C**	**δ_H mult, J_{Hz}**
1	188.0		145.8	
2	145.9		121.1	
3	133.1	6.53, s	110.4	6.32, d, 2.5
4	187.9		148.5	
5	132.2	6.45, s	117.2	6.47, d, 2.4
6	148.5		126.0	
7	177.7	2.08, m	15.7	2.13, s
1′	27.5	3.12, d, 7.3	122.9	6.26, s
2′	117.9	5.11, dt	130.7	5.58, d, 9.8
3′	139.8		77.7	
4′	39.6	2.08, m	40.8	1.67, s
5′	26.3	2.10, m	22.8	2.05, m
6′	124.5	5.11, m	124.9	5.12, dt, 19, 6.4
7′	134.6		134.1	
8′	39.0	2.10, m	39.3	2.06, m
9′	28.2	2.58, q, 7.5	28.1	2.59, q, 7.3
10′	145.4	6.00, t, 7.0	144.5	5.98, t, 7.3
11′	130.6		130.4	
12′	34.5	2.25, t, 7.7	34.9	2.25, t, 7.4
13′	27.9	2.10, m	28.1	2.13, s
14′	123.4	5.11, m	123.3	5.12, dt, 19, 6.4
15′	132.2		132.3	
16′	25.6	1.66, s	25.7	1.67, s
17′	16.1	1.59, s	17.9	1.57, s
18′	172.7		172.2	
19′	16.0	1.60, s	15.6	1.57, s
20′	16.1	1.61, s	25.9	1.68, s

In this Chapter, we managed to isolate and characterize the pure natural products sargahydroquinoic acid (**3.6**), *E*-sargaquinal (**3.7**) and fucoxanthin (**3.8**) from *S. heterophyllum*. From sargahydroquinoic acid (**3.6**), we then prepared the synthetic derivatives sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**) for further pharmacological studies.

3.3 Experimental

3.3.1 General experimental procedures

All extractions and chromatography utilised HPLC grade solvents supplied by Lichrosolv® (Merck, Germany). NMR experiments were obtained on a Bruker Avance 400 MHz NMR spectrometer using standard pulse sequences. All HPLC solvents were filtered through a 0.45 μm filter before use. Normal phase HPLC was performed using a Spectra-Physics IsoChrom pump, Whatman® Partisil 10 (9.5 mm × 500 mm) semi-preparative column and a Waters 410 differential refractometer attached to a 100 mV full scale Rikadenki chart recorder.

3.3.2 Algal Material

The algal specimen of *S. heterophyllum* (**NDK101124**) was collected from Noordhoek, near Port Elizabeth, on the southeast coast of South Africa on the 24th of November 2010. The algal specimens were transported to the laboratory on ice where it was immediately frozen and stored until the time of extraction. For purposes of identification and authentication the algal material was morphologically compared with previous voucher specimens of *S. heterophyllum*. A voucher specimen is kept at the Division of Pharmaceutical Chemistry, Rhodes University, Grahamstown, South Africa.

3.3.3 Extraction and Isolation of metabolites

Frozen algal material (**NDK101124**) was allowed to defrost under running distilled water. Defrosted algae were then soaked in MeOH for 1 h after which the MeOH was decanted and the retained algae heated at 40 ^{o}C for 30 min in CH_2Cl_2/MeOH (2:1, 150 mL x3). MeOH and CH_2Cl_2/MeOH (2:1) mixtures were pooled and sufficient water added to allow for the separation of the CH_2Cl_2 and the MeOH/H_2O phases. CH_2Cl_2 phase was then collected and dried *in vacuo* to yield the desired crude extract (**MN-11-62**, 12.4g). A portion of the crude extract (0.95 g) was applied to a silica gel column (10 g) and the column eluted using a series of solvents (50 mL each) of increasing polarity. This yielded the following fractions: **64a** (hexane-EtOAc, 10:0, 17.2 mg), **64b** (hexane-EtOAc, 9:1, 20.7 mg), **64c** (hexane-EtOAc, 8:2, 143.1 mg), **64d** (hexane-EtOAc, 7:3, compound **3.6**, 284.5 mg), **64e** (hexane-EtOAc, 6:4, 32.6 mg), **64f** (hexane-EtOAc, 4:6, compound **3.8**, 35.5 mg), **64g** (hexane-EtOAc, 2:8, 6.6 mg), **64h** (EtOAc, 2.5 mg) and **64i** (MeOH-EtOAc, 1:1, 207.7 mg). Fractions **64d** and **64f** contained pure sargahydroquinoic acid (**3.6**, 284.5 mg, 30% extracted yield) and fucoxanthin

(**3.8**, 35.5 mg, 3.74% extracted yield) respectively. Normal phase HPLC of fraction **64b** (20.7 mg) yielded compound **3.7** (3.0 mg, 15.4% dry weight).

Sargahydroquinoic acid (3.6)

Dark-green gum; ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 3.1; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 3.1.

E-Sargaquinal (3.7)

Orange-yellow oil; ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 3.1; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 3.1.

Fucoxanthin (3.8)

Red-orange oil; ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 3.2; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 3.2.

3.3.4 Semi-synthesis of derivatives

3.3.4.1 Oxidation of sargahydroquinonic acid (3.6) to sargaquinoic acid (3.1)

To a solution of sargahydroquinoic acid (165.8 mg) in $CHCl_3$ (8 mL) and MeOH (7 mL) was added Ag_2O (100 mg) and the resulting mixture was stirred at room temperature for 24 h. The mixture was filtered through a column of activated charcoal and passed through a plug of silica gel (hexane-EtOAc, 4:6, 10 mL) to yield sargaquinoic acid (**3.1**, 108 mg, 65.1 % dry weight) as a yellow compound after concentration under reduced pressure. All spectroscopic data for **3.1** are consistent with those previously reported (Afolayan et al. 2008;Munedzimwe 2012).

Sargaquinoic acid (3.1)

Yellow oil (65.1%); ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 3.3; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 3.3.

3.3.4.2 Conversion of sargaquinoic acid (3.1) to sargachromenoic acid (3.9)

To sargaquinoic acid (56.1 mg) was added pyridine (3 mL) and stirred at room temperature for 15 h under nitrogen gas. The extract was solubilised in EtOAc (10 mL), acidified with 5M HCl and extracted 3 times, each time collecting the EtOAc layer. The EtOAc extract was then filtered through Na_2S to yield sargachromenoic acid (**3.9**, 24.2 mg, 43.1 % dry weight) after concentration under reduced pressure. All spectroscopic data for **3.9** are consistent with those previously reported (Munedzimwe 2012).

Sargachromenoic acid (3.9)

Orange-yellow oil (43.1 %); ^{1}H NMR data ($CDCl_3$, 400 MHz) see Table 3.3; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 3.3.

3.3.4.3 Conversion of sargahydroquinoic (3.6) acid to sarganaphthoquinone (3.10)

To a solution of sargahydroquinoic acid (111.9 mg) in EtOH (15 mL) was added six equivalents of Ag_2O (326 mg) and stirred at room temperature for 24 h. To the reaction mixture was then added 3 drops of triethylamine (TEA) and further stirred for another 24 h. The resulting crude extract was filtered through a 5 g silica column (hexane-EtOAc, 7:3, 2 mL) to yield sarganaphthoquinoic acid (**3.10**, 4.9 mg, 4.38% dry weight) after concentration

under reduced pressure. All spectroscopic data for **3.10** are consistent with those previously reported (Munedzimwe 2012).

Sarganaphthoquinoic acid (3.10)

Orange-yellow oil (4.38%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J*=7.9 Hz), 7.86 (s), 7.52 (dd, *J*=7.9 Hz), 6.81 (s), 5.97 (t, *J*=7.3 Hz), 5.16 (t, *J*=7.0 Hz), 2.78 (t, *J*=7.6 Hz), 2.59-2.55 (m), 2.36-2.32 (m), 2.25 (m), 2.18 (s), 2.13-2.12 (m), 2.05-2.02 (m), 1.67 (s), 1.58 (s).

^{13}C NMR (100 MHz, $CDCl_3$) δ 185.44, 185.41, 171.9, 149, 148.2, 145, 136, 136, 136, 133.8, 132.3, 132.2, 130.6, 130.2, 126.7, 125.9, 123.4, 123.2, 39.01, 36.17, 29.1, 28.19, 28.19, 25.63, 17.65, 16.44, 15.94.

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Chapter 4

In-vitro anti-diabetic (insulinomimetic) activity of sargahydroquinoic acid semi-synthetic derivatives

4.1 Introduction

In Chapter 3, we identified the main metabolite obtained from the dichloromethane crude extract of *S. heterophyllum* as sargahydroquinoic acid (SHQA, **3.6**) from which sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**) were synthesized. Other compounds isolated from the dichloromethane crude extract of *S. heterophyllum* included *E*-sargaquinal (**3.7)** and fucoxanthin (**3.8**). However, the vulnerability of aldehyde compounds such as **3.7** to nucleophilic attack at the carbonyl carbon and hence, their high reactivity in biological systems gave reason to avoid the inclusion of this compound in our *in-vitro* anti-diabetic studies. With much research of this nature already done on fucoxanthin (**3.8**), this chapter only seeks to investigate the potential anti-diabetic activity of SHQA and its semi-synthetic derivatives (compounds **3.1**, **3.9** and **3.10**).

4.1.1 Mechanistic selection of an *in-vitro* anti-diabetic model

The potential anti-diabetic activity of a compound or extract may be assessed clinically using human subjects, *in-vivo* using animal models or *in-vitro* using a variety of test experiments. While each level of assessment has merits and demerits, the role of *in-vitro* models as an initial screening tool is significant in the determination of the anti-diabetic activity of test compounds or extracts (Soumyanath and Srijayanta 2006). By having a controlled system within which experimental variables of a test compound can be measured, *in-vitro* models enable the study of biological mechanisms of action as a scientific advantage over *in-vivo* models in addition to ethical and cost advantages (Sceats 2010). Several existing *in-vitro* anti-diabetic models are employed either for random screening or for the determination of the mechanisms of action of compounds and extracts. Since diabetes is a heterogeneous group of disorders characterized by a variety of pathogenic abnormalities, it offers numerous therapeutic targets that can be exploited in the quest of finding new treatment options. The four anti-diabetic mechanisms of action or therapeutic targets mainly tested for are; inhibition of carbohydrate digestive enzymes, the impairment of glucose uptake from the small intestinal lumen, stimulation of β-pancreatic insulin release, insulinomimetic or insulin-

sensitizing activity at insulin target tissues (e.g. liver, skeletal muscle and adipocytes) and antagonism of glucagon activity (Soumyanath and Srijayanta 2006).

Currently used *in-vitro* anti-diabetic assays incorporate most of these therapeutic targets. However, it is important to note that these *in-vitro* assays do not represent a comprehensive anti-diabetic screening system and that compounds or extracts that show lack of anti-diabetic activity on one particular therapeutic target may show positive results on another target. Therefore, with these limitations associated with *in-vitro* assays, there is no guarantee to ascertain anti-diabetic activity in *in-vivo* models post *in-vitro* studies. Hence, results obtained from *in-vitro* studies should be considered as an initial screening process for the identification of compounds or extracts with specific anti-diabetic mechanisms which then need to be followed by bioassay guided fractionation in the case of extracts and *in-vivo* anti-diabetic models. In addition to direct anti-diabetic therapeutic targets, other secondary anti-diabetic properties such as anti-oxidant and anti-inflammatory activity as well as cytotoxicity profiles need to be considered.

4.1.1.1 Adipocytes as an insulin target tissue

Once thought only as a storage organ for triacylglycerol, the development and biochemistry of adipose tissue has substantially gained interest and intrigued research for decades. The adipocyte has been described as "a dynamic cell that plays a fundamental role in energy balance and overall body homeostasis" (Bernlohr et al. 2002). In humans, the adipose organ consists of brown adipose tissue (BAT) and white adipose tissue (WAT) (Farmer 2008). Of the two, WAT is the most predominant and functions as an energy store in the form of triglyceride-containing intracellular droplets. The WAT also secretes a wide range of hormones and 'adipocytokines' which affect the functioning of various tissues such as the brain, muscles, and the liver (Gesta et al. 2007). However, since these 'adipocytokines' are mostly neither 'cytokines' nor 'cytokine-like', suggestions have been made for the adoption of the term 'adipokine' to describe a protein that is secreted from and synthesized by adipocytes (Trayhurn and Wood 2004). The adipokines are normally divided into pro-inflammatory and anti-inflammatory. The monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are examples of pro-inflammatory adipokines while adiponectin is an example of anti-inflammatory adipokine (Trayhurn and Wood 2004). The BAT are mainly specialized for thermogenesis, which is heat generated via the accelerated oxidation of triacylglycerides within brown adipocytes facilitated by large

numbers of mitochondria. The BAT also exclusively expresses uncoupling protein-1 (UCP-1), a proton transporter that uncouples electron transport from ATP production and allows the energy to be dissipated as heat (Farmer 2008;Ricquier and Bouillaud 2000). The induced up-regulation of mitochondrial activity and acquired BAT-like properties such as the expression of UCP-1 in WAT (Toh et al. 2008) has formed the basis for using adipocytes as an anti-obesity therapeutic target. The carotenoid fucoxanthin has been reported to have anti-obesity effects through this particular mechanism of action (Maeda et al. 2005).

The formation of adipocytes (adipogenesis) is a differentiation process governed by transcriptional cascades involving a regulated set of gene expression events. The peroxisome proliferator activated receptor γ (PPARγ) has been termed by Rosen and MacDougald as "the 'master regulator' of adipogenesis" (Rosen and MacDougald 2006). The PPARγ is sufficient to differentiate fibroblasts into mature adipocytes without which no other factor has yet been realised (Rosen and MacDougald 2006). The PPARγ is therefore not only crucial for adipogenesis but is also a requirement for the maintenance of the differentiated state (Rosen and MacDougald 2006). Hence, a compound or extract that stimulates the differentiation of preadipocytes into mature adipocytes is most likely a PPARγ agonist. The PPARγ is predominately expressed in adipose tissue with lower levels of expression in other tissues such as cardiac, renal and hepatic tissues (Shearer and Billin 2007). The association of PPARγ activation and consequent adipogenesis with an increase in tissue insulin sensitivity provided the basis for the development of TZD's as a class of anti-diabetic drugs (see Chapter 1, Section 1.6.1.5).

4.1.1.2 Sargahydroquinoic acid and sargaquinoic acid as potential PPARα/γ agonists

PPARγ agonists (TZD's) are a potentially ideal class of anti-diabetic agents in the prevention of diabetic cardiovascular complications. However, current TZD's are associated with weight gain, fluid accumulation, pulmonary oedema and macular oedema which ultimately results in congestive heart failure (Shearer and Billin 2007). Since PPARα agonists have been used to treat dyslipidemia via their effects on lowering free triglyceride concentrations in plasma, PPARγ side effects may be circumvented by the combined activation of PPARα/γ which should result in a synergistic enhancement of lipid metabolism and insulin sensitivity (Kim et al. 2008). Because of this, several attempts have been made to develop PPARα/γ dual agonists.

According to a study performed by Kim et al., Sargahydroquinoic acid (**3.6**) and its quinone congener sargaquinoic acid (**3.1**) both isolated from *S. yezoense* were reported to be dual agonists for PPARα/γ by increasing receptor transcriptional activity (Kim et al. 2008). The development of several PPARα/γ agonists including muraglitazar, tesaglitazar and ragaglitazar has been discontinued due to adverse effects such as oedema, heart failure, renal dysfunction and carcinogenesis (Shearer and Billin 2007). This is because a balance in the binding affinity of PPARα/γ dual agonists towards both receptor subtypes is required for optimal biological activity such that supra therapeutic activation of either PPARα or PPARγ results in adverse effects. Therefore, there is need for the development of new PPARα/γ selective agonists with balanced activity. In the study performed by Kim et al., the PPARα activation of SHQA was higher (4.3-fold) than that on PPARγ (2.1-fold) while SQA had a more balanced activation on PPARα and PPARγ (2.6-fold and 3.0-fold respectively) (Kim et al. 2008). Considering that the potential anti-diabetic mechanism of action of SHQA (**3.6**) and possibly its derivatives may be via the PPARγ which is predominantly expressed in adipose tissue, we decided to use adipocytes as the therapeutic target tissue through the 3T3-L1 preadipocyte differentiation assay.

4.1.1.3 The 3T3-L1 preadipocyte differentiation

The adipocytes are highly sensitive to insulin and hence, act as a target site for the study of insulinomimetic or insulin sensitizing activity. Insulin in adipocytes causes glucose uptake (controlled by the GLUT4 glucose transporter), lipogenesis and inhibition of triglyceride hydrolysis. The differentiation of preadipocytes into primary adipocytes, glucose uptake, lipogenesis and inhibition of lipolysis in differentiated cells are all insulinomimetic effects (Soumyanath and Srijayanta 2006). The 3T3-L1 is a preadipocyte cell line that was originally developed by clonal expansion from murine Swiss 3T3 cells (Zebisch et al. 2012). The ability of the 3T3-L1 preadipocytes to differentiate from fibroblasts to adipocytes makes them suitable as an anti-diabetic model for the observation of insulinomimetic effects. For the transformation of 3T3-L1 fibroblasts into mature adipocytes, the cells are normally treated post-confluence with either a combination of recombinant insulin (1 μg/mL), dexamethasone (DEX, 0.25 μM), and 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM) or a PPARγ agonist such as rosiglitazone (Zebisch et al. 2012).

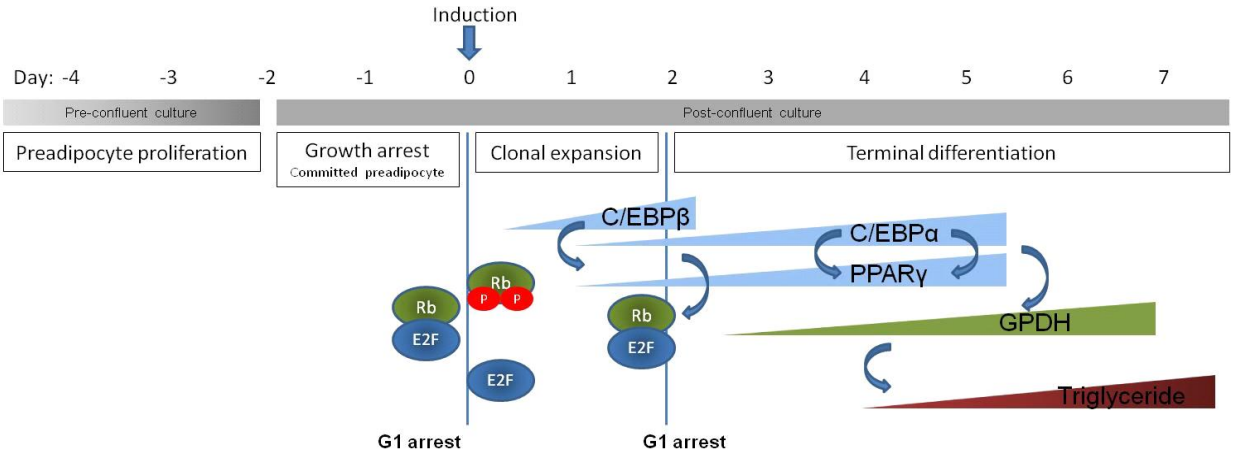

Figure 4.1 Schematic overview of the sequential steps involved in the 3T3-L1 differentiation process

In-vitro cultured proliferating 3T3-L1 preadipocytes cells contain high levels of retinoblastoma protein (Rb) in phosphorylated early gene 2 factor (E2F) -nonbinding form which is required for the transcription of the S phase genes (Figure 4.1). As cells progress towards contact inhibition and consequent growth arrest, the level of phosphorylated Rb decreases resulting in enhanced pRb-E2F complex formation and growth arrest at G1 phase (Johnson 2005). Only once preadipocytes become growth arrested can they be induced to differentiate. Activated transcription factor CCAAT/enhancer binding protein β (C/EBPβ) and activated transcription factor CCAAT/enhancer binding protein δ (C/EBPδ) are the first transcription factors to be induced after the exposure of preadipocytes to differentiating agents with C/EBPβ primarily responsive due to DEX (Ntambi and Young-Cheul 2000). Upon induction, the levels of C/EBPβ rapidly increase and growth arrested preadipocytes undergo mitotic clonal expansion involving two cycles of cell division. C/EBPβ in turn regulates the expression of the two major adipogenesis transcription factors, C/EBPα and PPARγ (Clarke et al. 1997), which together coordinate the dephosphorylation of Rb with subsequent growth arrest and the cessation of mitotic clonal expansion. Activated transcription factor CCAAT/enhancer binding protein α (C/EBPα) and PPARγ also promote the sustained expression of numerous adipocyte specific genes including GPDH which is required for the formation of triglyceride droplets (the main phenotypic feature of mature adipocytes). Both C/EBPα and PPARγ, the latter in combination with the obligate heterodimeric partner, the retinoid X receptor alpha (RXRα), have been shown to bind

regulatory elements within the promoter of the cytosolic glycerol-3-phosphate dehydrogenase (cytGPDH) gene.

4.1.2 Chapter aims

In Chapter 3, we managed to isolate and characterize pure natural products from crude fractions of *S. heterophyllum* and prepared a series of synthetic derivatives. In this chapter, we explore the potential anti-oxidant/radical scavenging activity, anti-inflammatory activity and cytotoxicity of *S. heterophyllum* fractions and pure natural products. Finally, we investigate and rationalize the potential anti-diabetic activity of SHQA and synthetic derivatives.

4.2 Results and Discussion

4.2.1 DPPH anti-oxidant/radical scavenging Assays

S. heterophyllum crude fractions were evaluated for DPPH radical scavenging activity using ascorbic acid as standard. Fractions **64c** (mainly SQA, IC_{50} = 19.48 µg/mL), **64d** (IC_{50} = 4.01 µg/mL) and **64e** (IC_{50} = 3.32 µg/mL) exhibited strong DPPH radical scavenging activity more potent than ascorbic acid (IC_{50} = 24.07 µg/mL) (Table 4.1). There should be background fucoxanthin absorbance in fraction **64f** which would interfere with the DPPH quantification and as such, it was not possible to reliably determine its antioxidant activity under these experimental conditions. However, extensive research has already been undertaken to show among many others, the anti-oxidant, anti-inflammatory and anti-cancer activity of fucoxanthin (Peng Juan et al. 2011).

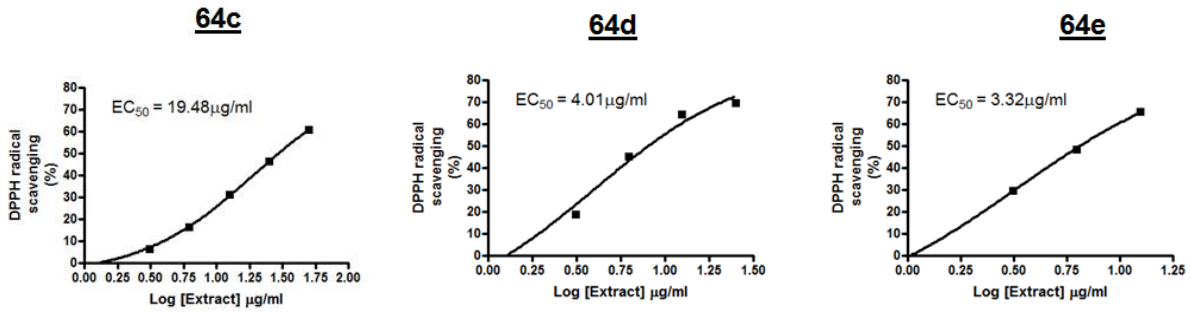

Figure 4.2 Titration curves for DPPH anti-oxidant activity of fractions (**64c**), (**64d**) and (**64c**)

Table 4.1 IC_{50} values for the DPPH radical scavenging activity of *S. heterophyllum* fractions

Crude fraction	**IC_{50} (µg/mL)**
64a	>500
64b	113.9
64c	19.48
64d	4.01
64e	3.32
64g	43.14

The pure compounds SHQA (**3.6**), SQA (**3.1**), sargachromenoic acid (**3.9**), sarganaphthoquinoic acid (**3.10**) and a SQA/chlorophyll fraction (**64c**) were evaluated for DPPH anti-oxidant/radical scavenging activity also using ascorbic acid as standard (Table 4.2). SHQA (**3.6**, IC_{50} = 6.20 µg/mL) and sargachromenoic acid (**3.9**, IC_{50} = 6.99 µg/mL) exhibited strong DPPH radical scavenging activity more potent than ascorbic acid (IC_{50} = 24.07 µg/mL). The DPPH radical scavenging activity of SHQA and sargachromenoic acid has been previously documented (Pérez-Castorena et al. 2002). It is a known fact that the reduced forms of vitamin E and coenzyme Q groups such as hydroquinones, chromanols and chromenols normally function as protective anti-oxidants. However, the ability of such compounds to function in these capacities of electron transfer and antioxidant activity directly depends upon the oxidation potential of the compound, which is also partly dependent upon the nuclear substituents (Moore et al. 1964). Not surprisingly, SHQA showed more potent DPPH radical scavenging activity than SQA (IC_{50} = 95.76 µg/mL) as it is generally known that hydroquinones are more potent radical scavengers than their quinone congeners. However, the SQA/chlorophyll fraction (**64c**) (IC_{50} = 19.48 µg/mL) possessed a more potent DPPH radical scavenging activity than SQA alone. This 'synergism' may have possibly resulted from the additive DPPH radical scavenging activity of chlorophyll which has been previously reported (Endo et al. 1985). Endo et al. proposed that the radical scavenging activity of chlorophyll involves a third order reaction in which electron donation from the π-cation radical of the porphyrin and subsequent 'molecular complexing' results in free radical neutralization.

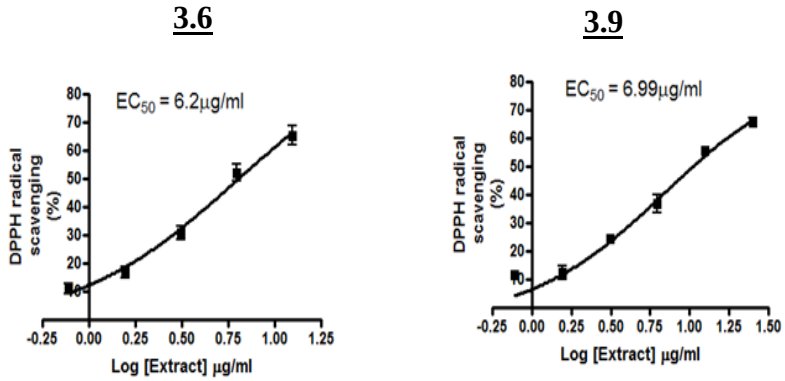

Figure 4.3 Titration curves for DPPH radical scavenging activity of sargahydroquinoic acid (**3.6**) and sargachromenoic acid (**3.9**)

Table 4.2 IC_{50} values for the DPPH radical scavenging activity of sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**)

Compound	**IC_{50} (µg/mL)**
3.6	6.20
3.1	95.76
3.9	6.99
3.10	226.5

These results indicate that *S. heterophyllum* yields prenylated compounds with potent anti-oxidant activity which may be exploited for therapeutic purposes. Since these results are based on *in-vitro* studies, further research is required in order to determine the biological relevance of DPPH radical scavenging capacity in *in-vivo* diabetic models.

4.2.2 Potential anti-inflammatory activity of *S. heterophyllum* fractions

S. heterophyllum fractions were evaluated for anti-inflammatory activity. Fractions **64c** (SQA/chlorophyll mixture), **64d** (SHQA) and **64f** (fucoxanthin) produced a significant decrease in nitrate production at both test concentrations, with fraction **64c** being relatively less potent by only having a significant effect at the highest test concentration (Figure 4.4). The anti-inflammatory activity of fucoxanthin has been previously documented (Peng Juan et al. 2011). SHQA significantly attenuated nitrate production, indicating that this compound may also be considered to possess anti-inflammatory properties. A research on the anti-inflammatory activity of plastoquinone derivatives isolated from natural sources also reported that SHQA significantly reduced TPA-induced mouse ear oedema (Pérez-Castorena et al. 2002). Further research is however required to define the mechanism through which SHQA inhibits NO production and to assess the potential for synergism between fucoxanthin and SHQA. Under the conditions of the anti-inflammatory assay there was no evidence for cytotoxicity toward the RAW cells and thus, it can be assumed that the inhibition of nitrate production was not due to differences in the relative cytotoxicity.

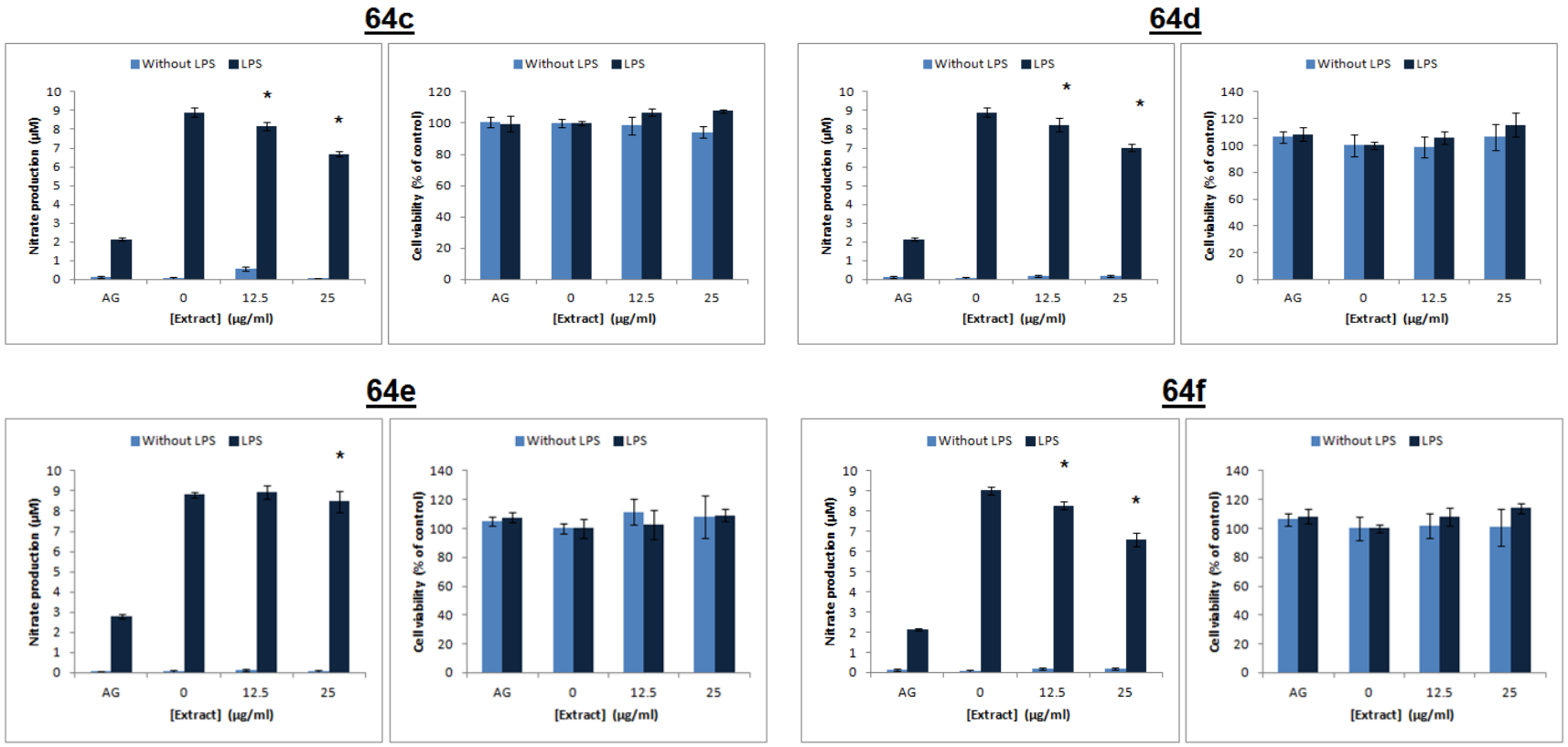

Figure 4.4 Nitrate production in mouse macrophages (RAW264.7) treated with *S. heterophyllum* fractions (**64c**), (**64d**), (**64e**) and (**64f**).[4]

[4] Data represents the mean ± STDev of four replicates from a single experiment. Significant ($p<0.05$) reductions in the levels of nitrate are indicated as (*)

4.2.3 Cytotoxicity assays for crude extracts and pure compounds

Cytotoxicity towards Chang Liver, HT-29 and Caco-2 cell lines was evaluated for *S. heterophyllum* crude extracts. There appeared to be strong cytotoxicity of *S. heterophyllum* fractions on Chang Liver, HT-29 and Caco-2 cells (Table 4.3). This is consistent with studies that have been done to show the selectivity of *Sargassum* extracts towards HT-29 and Caco-2 cell apoptosis (Khanavi Mahnaz et al. 2010). Fucoxanthin (**64f**), the most potent sample tested, was previously reported to inhibit the proliferation of HT-29 and Caco-2 cells through inducing cell cycle arrest in the G_o/G_1phase at low concentrations (25 μM) and apoptosis at higher concentrations (Das et al. 2005). Not surprisingly, the aldehyde mixture, **64b**, was the second most cytotoxic fraction while fraction **64i** was selective towards Chang Liver cells. Taken together these results indicate the presence of multiple cytotoxic compounds in the extracts of *S. heterophyllum* with potential anticancer properties.

Table 4.3 EC_{50} values for *S. heterophyllum* fractions against Chang Liver, HT-29 and Caco-2 cells as obtained using the MTT assay.(nd = not determined)

Fraction/ compound	**Sample purity**	**EC_{50} Chang Liver (μg/mL)**	**EC_{50} HT-29 (μg/mL)**	**EC_{50} Caco-2 (μg/mL)**
α-tocopherol	Pure	> 500	Nd	Nd
64a	Crude fraction	39.55	150.2	126.8
64b	Sargaquinal Isomers	13.59	25.9	14.06
64c	SQA/chlorophyll	52.73	35.06	23.12
64d	Pure SHQA	82.2	114.8	58.25
64e	Crude fraction	266.4	369.5	146.1
64f	Pure fucoxanthin	12.11	19.83	14.82
64g	Crude fraction	32.46	116.5	117.6
64h	Crude fraction	75.05	Nd	Nd
64i	Crude fraction	70.53	> 500	> 500

Cytotoxicity towards 3T3-L1 and Chang Liver cell lines was evaluated for SHQA (**3.6**), SQA (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**). All the test compounds were more selective for 3T3-L1 than Chang Liver cells (Table 4.2) with the most selective being SHQA (SI = 7.9) and SQA (SI = 8.0). Literature has documented the selective cytotoxicity of SQA towards metastatic MDA-MB-231 breast cancer cells (Mare et al. 2012) as well as the human keratinocyte cell line HaCaT via apoptosis (Choi et al. 2007). The selective cytotoxicity of SHQA towards P338 cells has also been reported (Reddy and Urban 2009). As we explained in Chapter 1, one of the main reasons for the assessment of the potential cytotoxicity of a test compound or extract prior to *in-vitro* anti-diabetic testing is to avoid the overriding of anti-diabetic endpoints by toxic effects which make the accurate assessment of anti-diabetic properties very difficult. Considering that a proliferatory phase is required for 3T3-L1 cells to differentiate, cytostatic or cytotoxic compounds often inhibit adipogenesis in this assay. Hence, it is important to ensure that the inhibition of proliferation does not mask the effects of potential PPAR agonists.

Table 4.4 Cytotoxicity IC_{50} values for sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinone (**3.10**) against Chang Liver and 3T3-L1 cell lines

Compound Code	**Chang Liver** IC_{50} (µg/mL)	**3T3-L1** IC_{50} (µg/mL)	**Selectivity index** (Chang/3T3-L1)
3.6	43.16	5.45	7.9
3.1	92.85	11.55	8.0
3.9	53.56	11.4	4.7
3.10	43.50	20.63	2.1

The relatively strong cytotoxicity of these compounds toward proliferating 3T3-L1 cells (Table 4.4 and Figure 4.5) raised the concern that this effect could override any PPAR activity. For that reason, we defined the lowest toxic dose of the test compounds and used that concentration as a starting point for our anti-diabetic assay. Therefore, it was decided to test for 3T3-L1 differentiation at concentrations 10µg/mL and below.

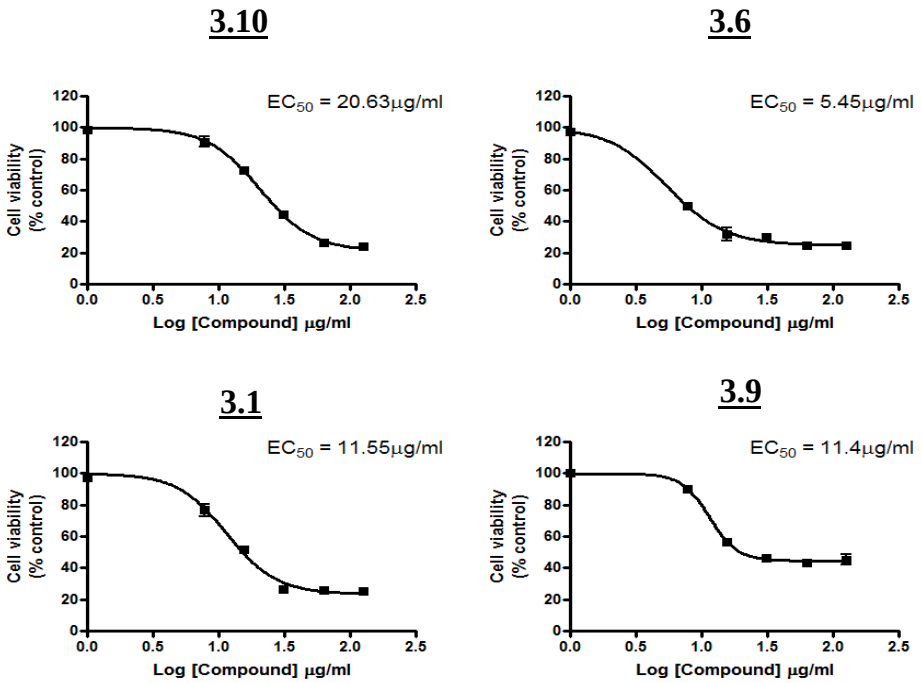

Figure 4.5 Dose dependent inhibition of proliferating 3T3-L1 cell viability by sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**)

4.2.4 3T3-L1 preadipocyte differentiation assay

The potential anti-diabetic (insulinomimetic) activity of sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**) and sarganaphthoquinoic acid (**3.10**) was evaluated using the 3T3-L1 preadipocyte differentiation assay (Figure 4.6 and 4.7). Despite previously raised concerns regarding the cytotoxicity of these test compounds towards 3T3-L1 cells, there was an appreciable increase in the relative accumulation of triglycerides in the adipocytes, indicating positive interaction with the PPARγ. It appears that proliferating and pre-growth arrested cells are more sensitive to the toxic effects of these compounds. In future it would be advisable to simultaneously assess the relative cell density so as to exclude potential toxicity. The most significant response was obtained from sarganaphthoquinoic acid (**3.10**). At test concentrations of 1 µg/mL (0.24 µM) and 5 µg/mL (1.19 µM), sarganaphthoquinoic acid (**3.10**) produced a response similar to that of rosiglitazone at 1 µM. Sargachromenoic acid (**3.9**) also induced a strong PPARγ response but it was only significant at 5 µg/mL (1.14 µM). The PPARγ activity of sargahydroquinoic acid (**3.6**) was weakly positive at 5 µg/mL (1.17 µM) while sargaquinoic acid (**3.1**) did not reveal any potential PPARγ activity at all test concentrations. The absence of any PPARγ activity with SQA conflicts with earlier research done by Kim et al. in which both SHQA and SQA produced very significant PPARγ mediated preadipocyte differentiation (Kim et al. 2008). However, considering that Kim et al. used 10 µM each of SHQA and SQA for the induction of differentiation while we used a maximum of 1.17 µM of SHQA and 1.18 µM of SQA, it is highly possible that increasing the test concentrations would also result in an increased PPARγ activity of these compounds. Even then, this still implies that sarganaphthoquinoic acid (**3.10**) is a more superior PPARγ agonist than SHQA and SQA. To our knowledge, this is the first report on the PPARγ-mediated anti-diabetic activity of sarganaphthoquinoic acid (**3.10**). In an attempt to improve the side effect profiles of current PPARγ agonists, research has explored the replacement of the thiazolidine ring with other 'acidic head groups' which have lesser side effects. Such examples include a study performed by Sundriyal et al. (2008) in which, after replacement of the thiazolidine ring with a 1,4-naphthoquinone moiety, the newly synthesized compounds still retained PPARγ activity comparable to pioglitazone (Sundriyal et al. 2008). This shows that the 1,4-naphthoquinone, sarganaphthoquinoic acid (**3.10**), is a potential PPARγ agonist which may have lesser side effects compared to current thiazolidinediones. Also, 1,4-naphthoquinones are commercially available, less costly and easily derivatized.

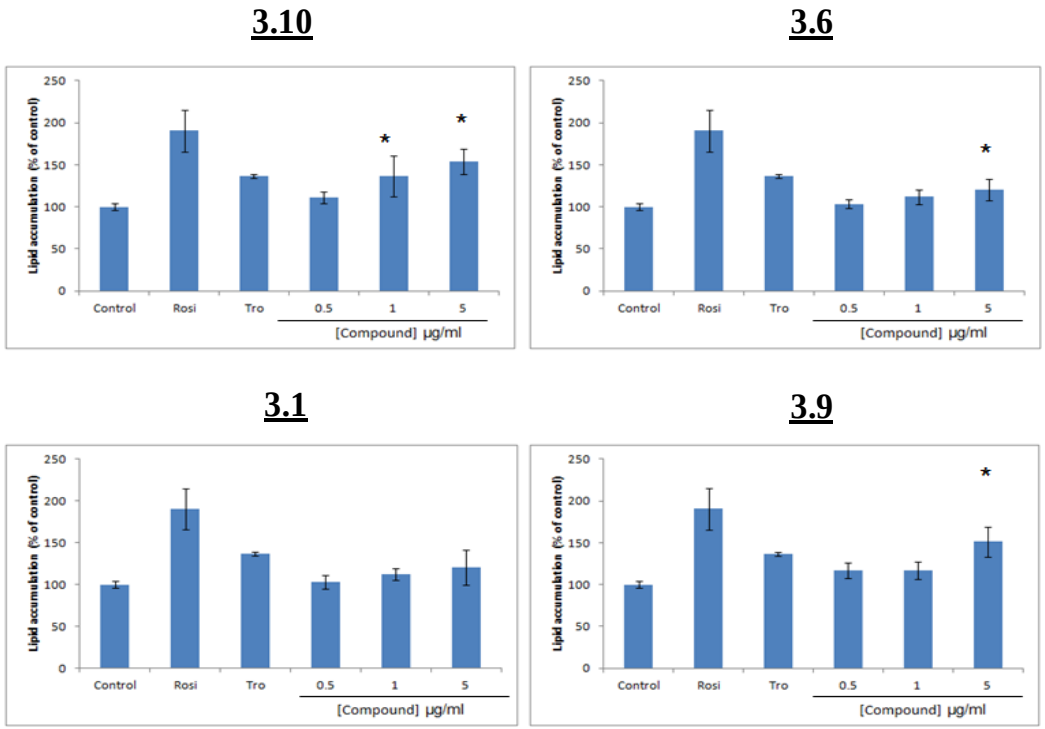

Figure 4.6 Dose dependent lipid accumulation in differentiating 3T3-L1 cells after treatment with sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**), sarganaphthoquinoic acid (**3.10**) and positive controls rosiglitazone and troglitazone[5]

[5] Each data point represents the mean±STDev for triplicate wells of a single experiment while the astericks (*) indicate a significant increase in lipid accumulation.

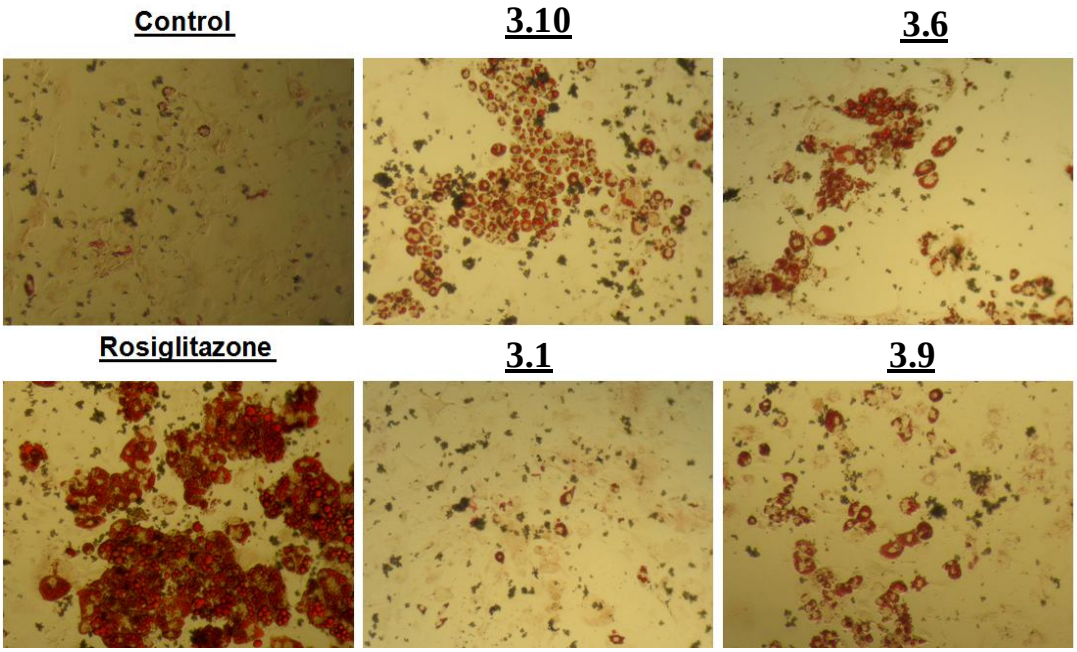

Figure 4.7 Representative images of oil Red O stained 3T3-L1 cells after treatment with sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**), sargachromenoic acid (**3.9**), sarganaphthoquinoic acid (**3.10**) and rosiglitazone

In this chapter, after exploring the potential anti-oxidant/radical scavenging activity and anti-inflammatory activity of *S. heterophyllum* fractions and pure natural products, SHQA (**3.6**, IC_{50} = 6.20 µg/mL) and sargachromenoic acid (**3.9**, IC_{50} = 6.99 µg/mL) exhibited the most potent DPPH radical scavenging activity while the fractions **64d** (SHQA) and **64f** (fucoxanthin) produced the most significant anti-inflammatory activity. After investigating the potential anti-diabetic activity of SHQA and synthetic derivatives, sarganaphthoquinoic acid (**3.10**) had the most significant PPARγ-mediated response similar to that of rosiglitazone. Sargachromenoic acid (**3.9**) also induced a strong response while that of sargahydroquinoic acid (**3.6**) was weakly positive compared to results earlier documented by Kim et al. (2008). It is however important to note that Kim et al. used a different protocol to investigate differentiation. The protocol implemented by Kim et al. used a combination of insulin (INS), 3-isobutyl-1-methylxanthin (IBMX) and dexamethasone (DEX) which is likely to amplify the induction of differentiation due to PPARγ activation through an indirect increase in PPARγ expression and not receptor-ligand binding. From a theoretic point of view, it is not an absolute requirement to include INS/IBMX/DEX as is evident in the positive controls we used which, in my opinion, represents a more direct evaluation of PPARγ activation.

4.3 Experimental

4.3.1 General experimental procedures

The culture mediums were sourced from Sigma Aldrich® (South Africa) and Hyclone® (Thermal Fisher, USA). The IC_{50} values of the test compounds were calculated from a minimum 5-point dose response curve using GraphPad Prism 4 software package.

4.3.2 DPPH radical scavenging assay

Test samples were diluted in ethanol/water (1:1) from 10 mg/100 µL stocks prepared in DMSO. 5 µL of each sample was placed into each well of a 96-well plate followed by the addition of 120 µL of Tris-HCl buffer (50 mM, pH7.4) and 120 µL of freshly prepared DPPH solution (0.1 mM in ethanol). The plate was incubated for 20 minutes at room temperature with the absorbance read at 513nm. The percentage of DPPH radical scavenging was calculated as [(A- B/A) × 100] where A represents the absorbance in the absence of test samples and B represents the absorbance in the presence of test samples. Ascorbic acid was used as a positive control (EC_{50} = 24.07µg/mL).

4.3.3 Anti-inflammatory assays

Anti-inflammatory assays were performed as previously reported in Chapter 2, Section 2.6.6.

4.3.4 Cytotoxicity assays

3T3-L1 cells were seeded into 96-well culture plates (TTP) at 3 000 cells/well in DMEM supplemented with10% fetal bovine serum (FBS) and left for 24 hrs. Test compounds were added and the cells incubated for a further 48 hrs after which the medium was replaced with 200 µL of MTT (0.5 mg/mL in DMEM). Samples were then incubated for 3 hr at 37°C after which MTT was removed and the purple formazan product dissolved in 200 µL DMSO. Absorbance was measured at 560 nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). All incubation steps were carried out in a 37 °C humidified incubator with 5% CO_2. Procedures for the cytotoxicity assays on Chang Liver cells were as previously reported in Chapter 2, Section 2.6.7.

4.3.5 3T3-L1 preadipocyte differentiation assay

Prior to the induction of differentiation, 3T3-L1 cells were routinely maintained in DMEM containing new born calf serum. Cells were seeded at a density of 3000 cells/well into 96-well plates and allowed to reach 100% confluence. Two days post-confluence, the cells were treated for a further two days with DMEM medium now supplemented with FBS (to induce mitotic clonal expansion) and the indicated concentrations of test compounds or the control substances rosiglitazone and troglitazone (1 μM, final concentration). Cells were then cultured for an additional 7 days in normal culture medium (DMEM, 10% FBS with inducers) and the medium replaced every two to three days. Triglyceride accumulation, a marker for adipocyte differentiation, was measured by Oil red-O staining. The Oil Red O stained lipids were extracted in isopropanol and measured at 510 nm. The sample results were then compared to controls using a two-tailed Student's t-test assuming equal variances.

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Chapter 5

Conclusion

Several studies have been done to search for terrestrial plant-derived natural products with potential anti-diabetic activity. However, there are very few studies that have embarked on investigating the potential anti-diabetic activity of marine algae. Through a literature review guided identification of specific marine algal genera and evaluation of their anti-oxidant activity, this research has shown that South African marine algae and specifically *Sargassum heterophyllum* (**62**) is a potentially good source of bioactive anti-oxidant compounds with higher potencies than conventional anti-oxidants. This research also serves as the first publication to document the anti-oxidant/radical scavenging activity of *Bifurcariopsis capensis* (**8d**). However, due to limitations of quantity, further isolation and characterization of the compounds responsible for the anti-oxidant activity of *B. capensis* (**8d**) was not undertaken. Taking into consideration that the DPPH radical scavenging/anti-oxidant assay that was employed uses a hydrogen atom transfer mechanism, further research may be improved by incorporating more biologically relevant anti-oxidant assays that mimic the electron transfer mechanism of biological systems. A good example of such is the cellular anti-oxidant assay (CAA) which also accounts for the uptake, metabolism and location of anti-oxidant compounds within cells.

Despite relatively high *in-vitro* cytotoxicity profiles exhibited by *S. heterophyllum* extracts and crude fractions, the alga portrayed very interesting biological activity. The *S. heterophyllum* crude extract (**62**) as well as its metabolites sargahydroquinoic acid (**3.6**) and fucoxanthin (**3.8**) showed significant anti-inflammatory activity. Therefore, this research proves that *in-vitro* cytotoxicity assays should never be the only screening method to determine the selection of potentially bioactive crude extracts *in-vivo*. The *in-vitro* cytotoxicity of fucoxanthin (**3.8**) when directly applied to cultured cells, as was the case in our study, has been widely reported. It is known that fucoxanthin (**3.8**) undergoes enzymatic deacetylation in the intestinal lumen to form fucoxanthinol which is then further metabolized into amarouciaxanthin A in the liver. Fucoxanthinol and amarouciaxanthin A are the active but non cytotoxic forms of fucoxanthin *in-vivo*.

In the evaluation of anti-diabetic (insulinomimetic) activity, sarganaphthoquinoic acid (**3.10**) showed the strongest PPARγ activity by producing a response similar to that of rosiglitazone

at 1 µM. Sargachromenoic acid (**3.9**) also induced a strong PPARγ response at 5 µg/mL. Sargachromenoic acid (**3.9**) exhibited significant anti-oxidant activity (IC_{50} = 6.99 µg/ml) in addition to PPARγ activity while sarganaphthoquinoic acid (**3.10**) did not show any anti-oxidant activity. In Chapter one, we defined our ideal anti-diabetic agent as one that possesses anti-oxidant activity in addition to hypoglycaemic effects. By that definition, sargachromenoic acid (**3.9**) would appear to have more promise as an anti-diabetic agent than sarganaphthoquinoic acid (**3.10**). Further research may find it beneficial to evaluate the potential anti-inflammatory activity of the semi-synthetic derivatives which was not determined in this study.

Appendix I

DPPH Radical Scavenging Activity

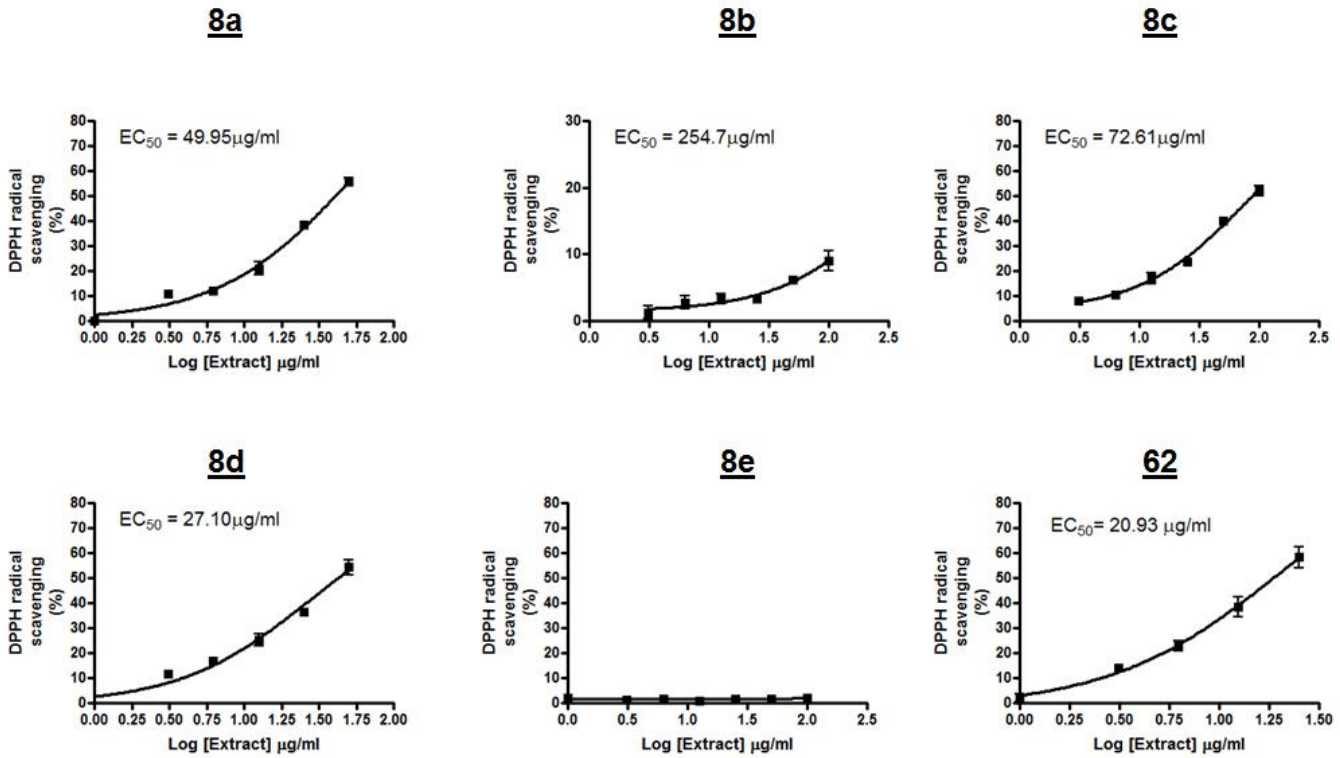

Figure A1.1 Titration curves for DPPH radical scavenging activity of the crude extracts *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**)

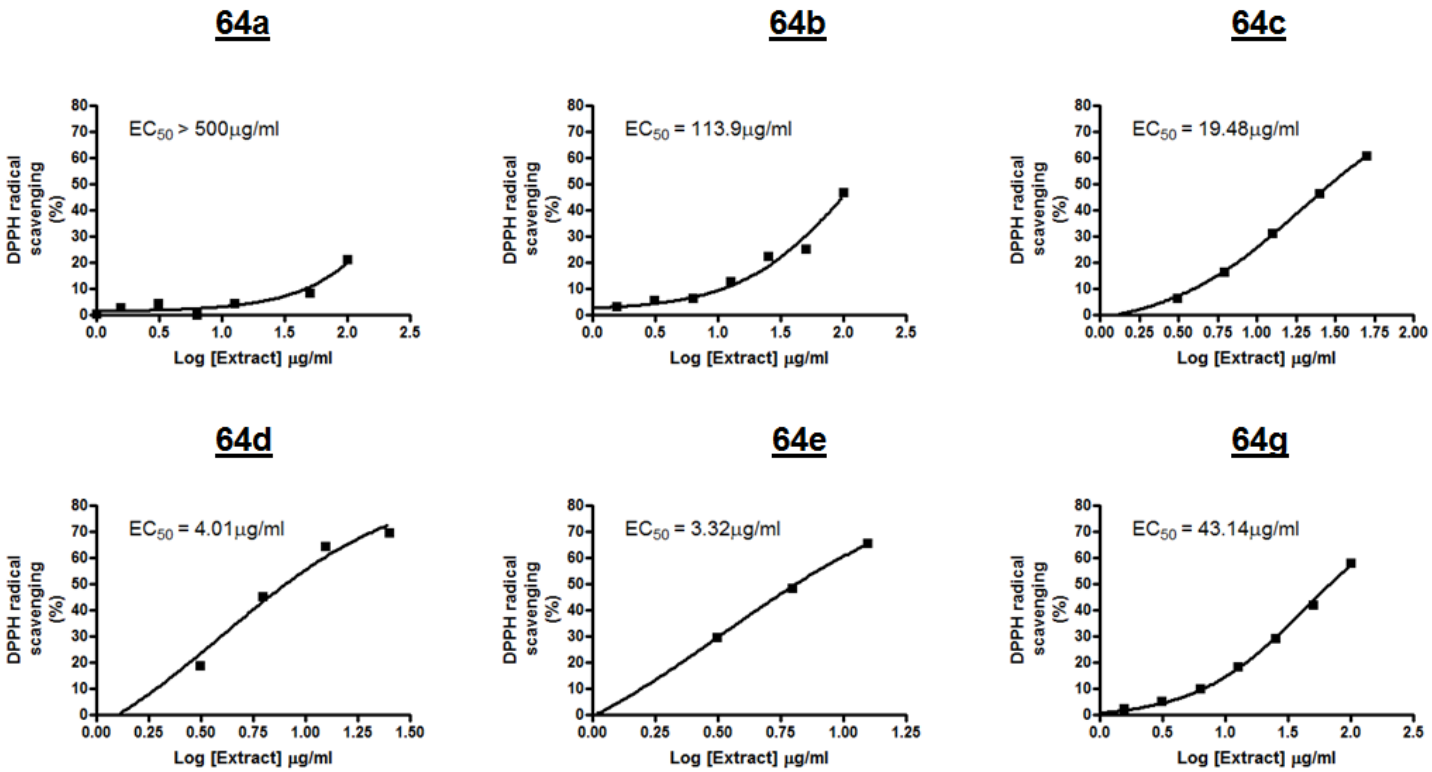

Figure A1.2 Titration curves for the DPPH radical scavenging activity of *Sargassum heterophyllum* crude fractions (**64a**), (**64b**), (**64c**), (**64d**), (**64e**) and (**64g**)

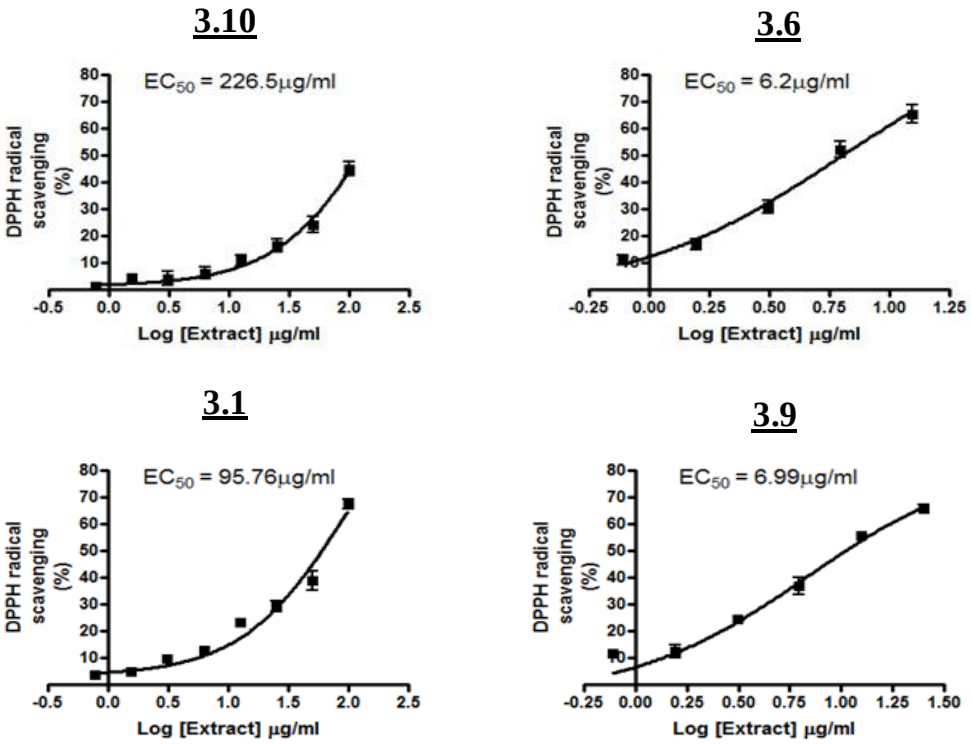

Figure A1.3 Titration curves for DPPH radical scavenging activity of sarganaphthoquinoic acid (**3.10**), sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**) and sargachromenoic acid (**3.9**)

Appendix II

Anti-inflammatory Assays

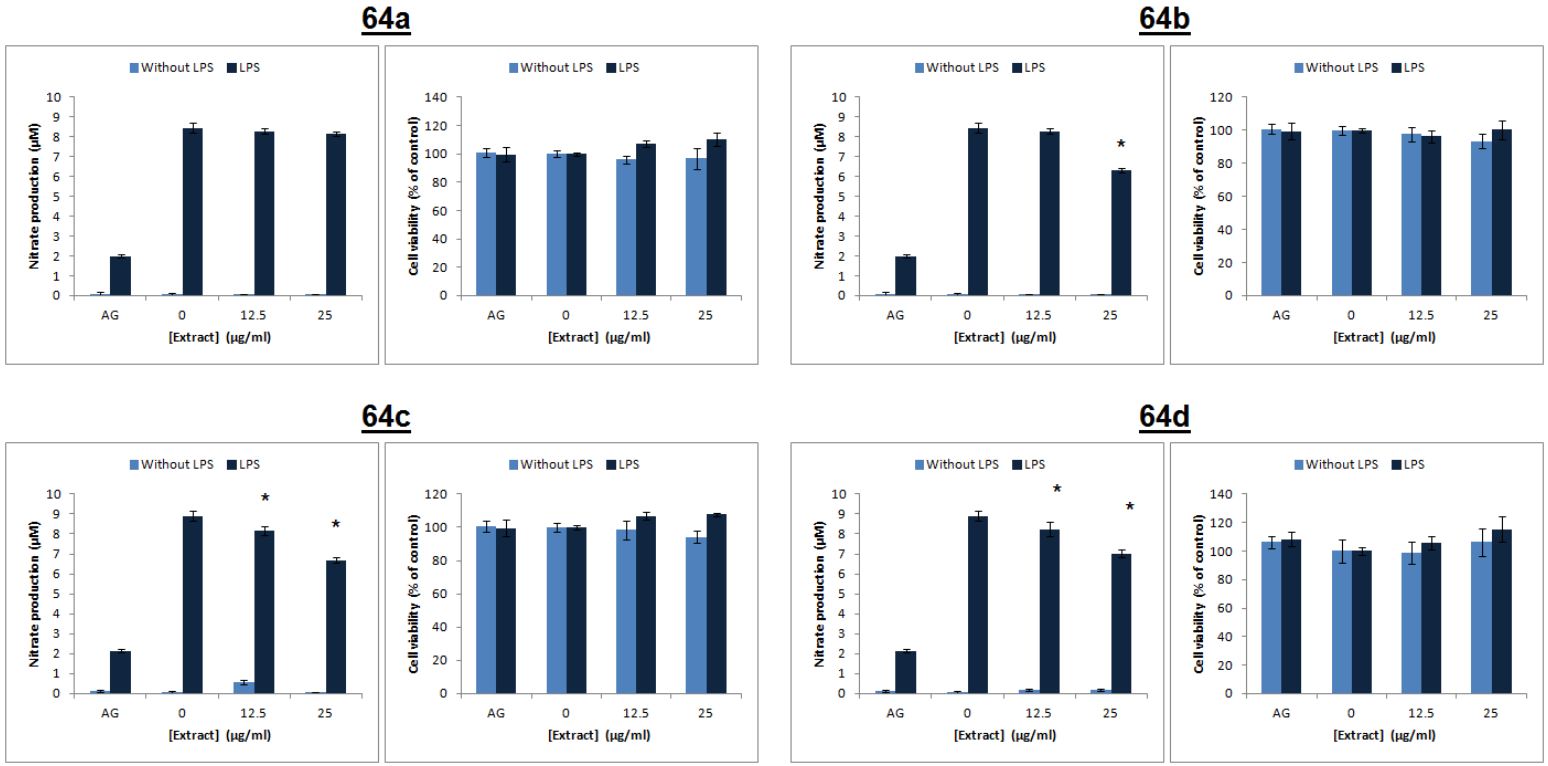

Figure A2.1a Nitrate production in mouse macrophages (RAW264.7) treated with *S. heterophyllum* fractions (**64a**), (**64b**), (**64c**) and (**64d**)

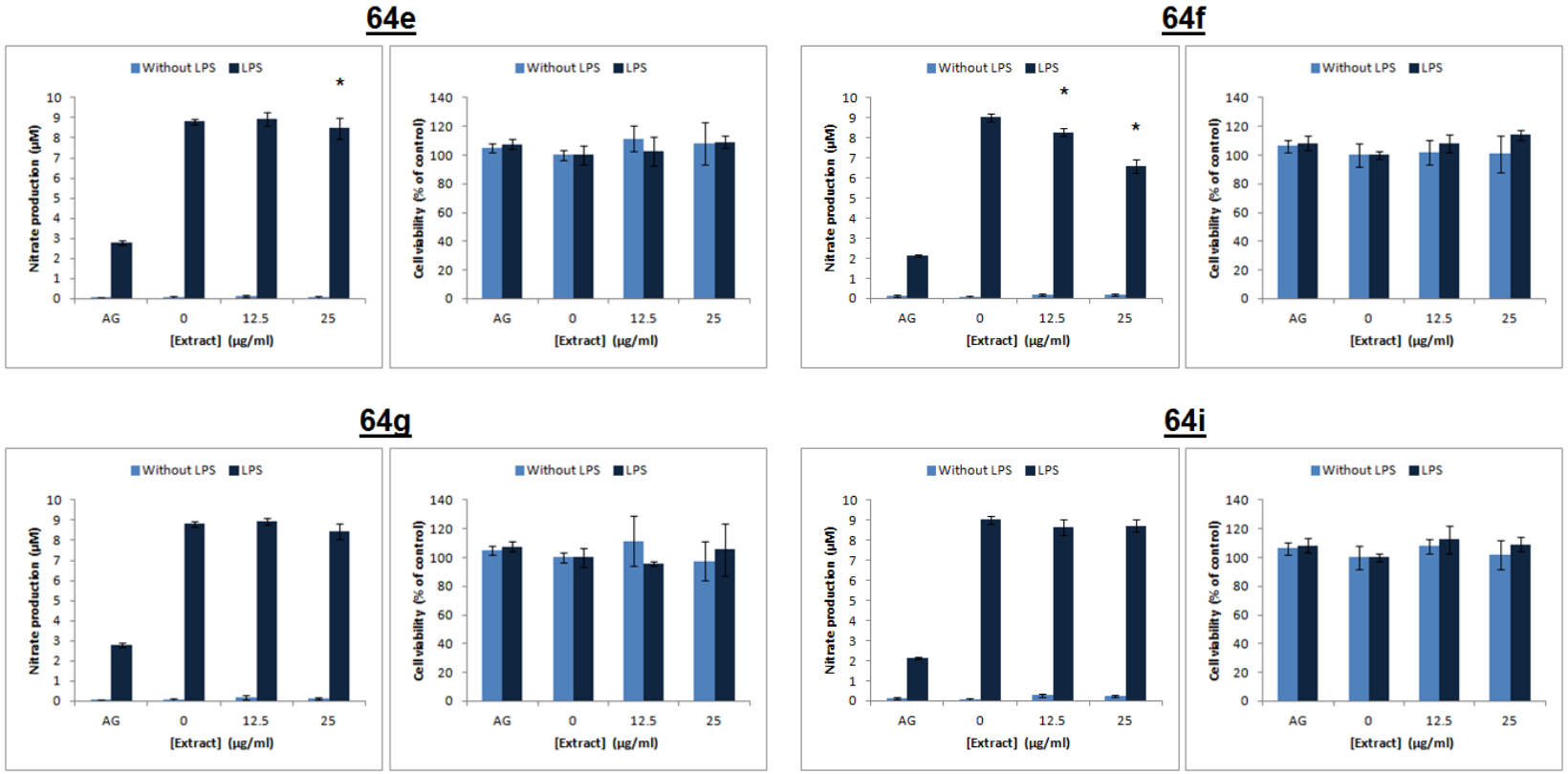

Figure A2.1b Nitrate production in mouse macrophages (RAW264.7) treated with *S. heterophyllum* fractions (**64e**), (**64f**), (**64g**) and (**64i**)

Appendix III

Cytotoxicity Assays

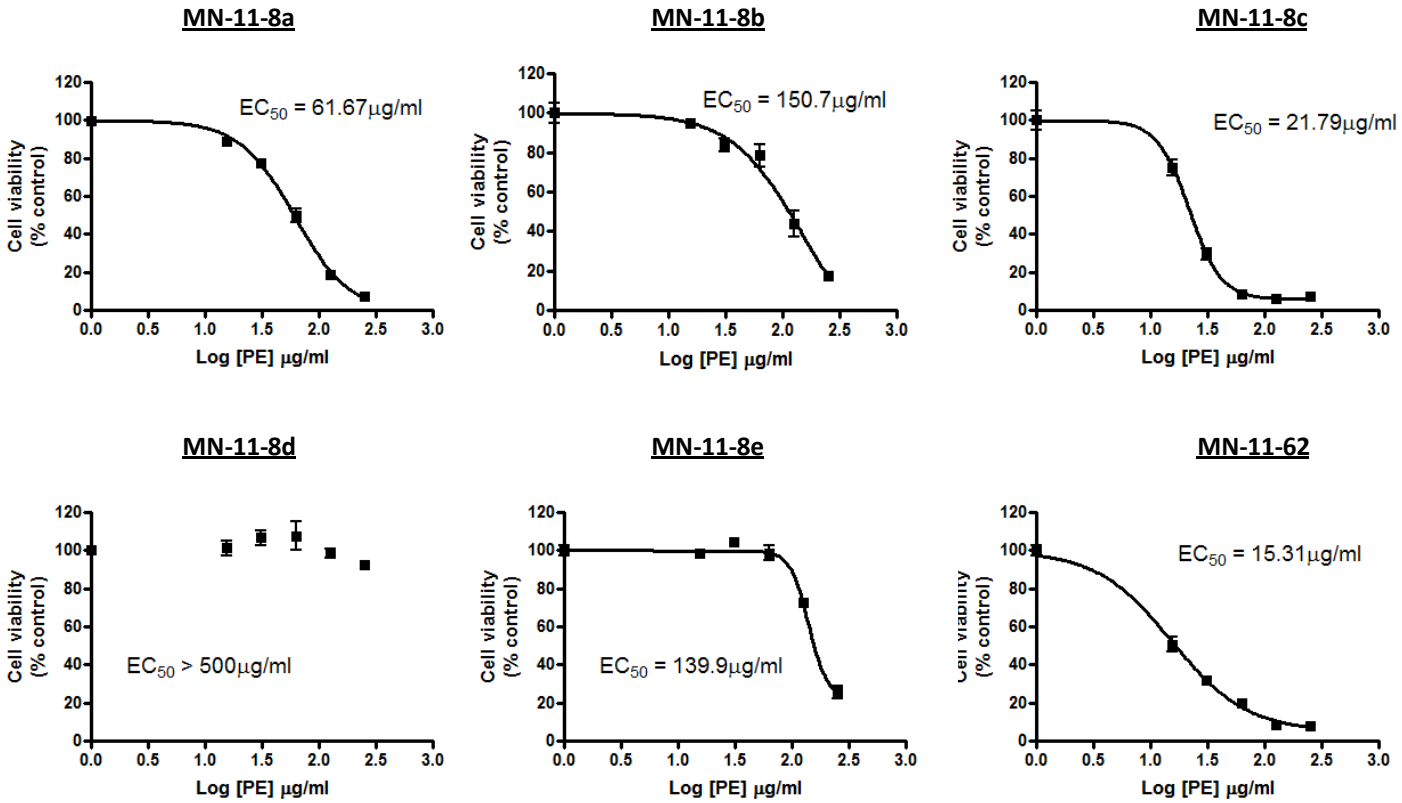

Figure A3.1 Dose dependent inhibition of Chang liver cell viability by *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**) crude extracts

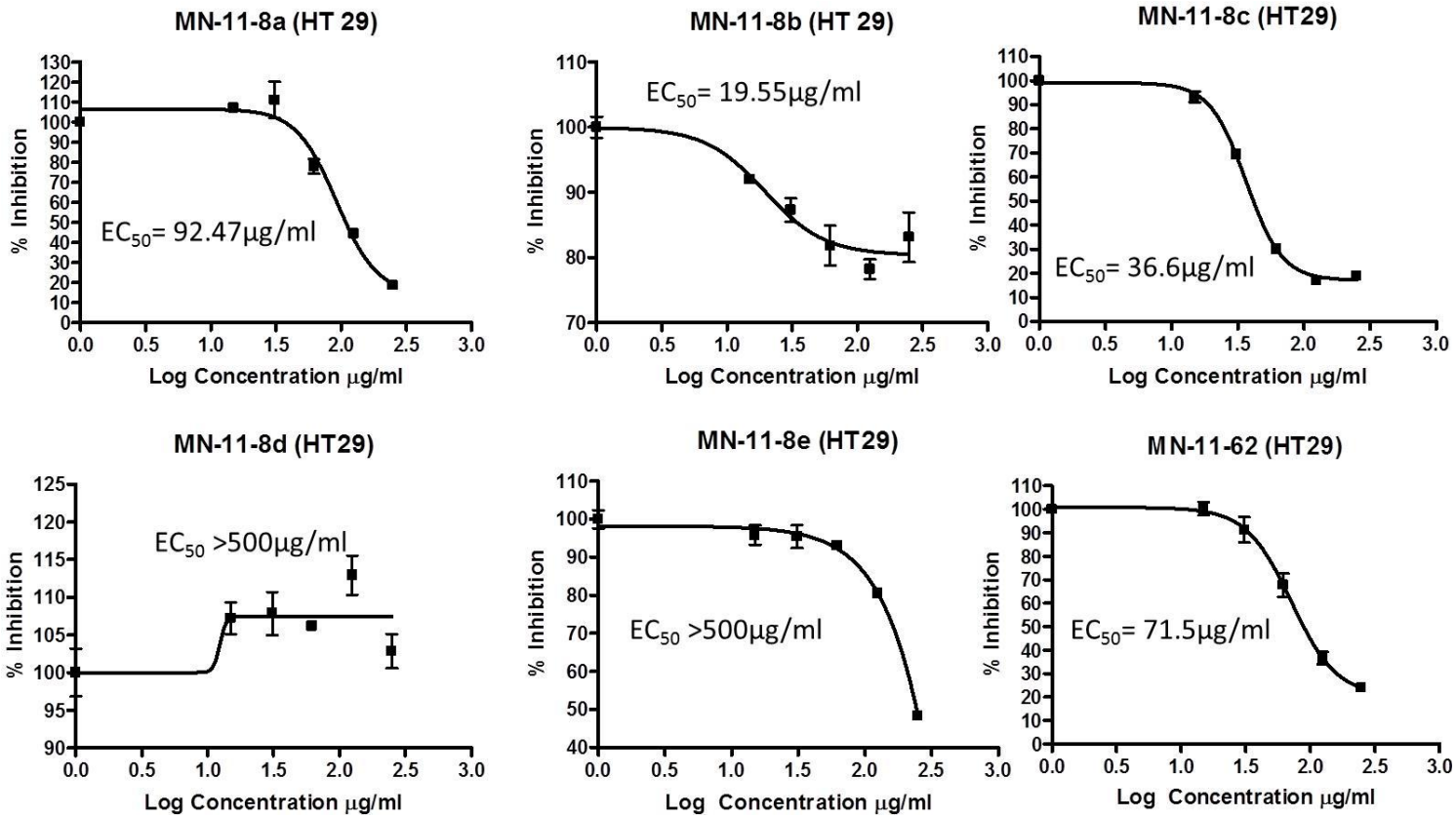

Figure A3.2 Dose dependent inhibition of HT-29 cell viability by *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**) crude extracts

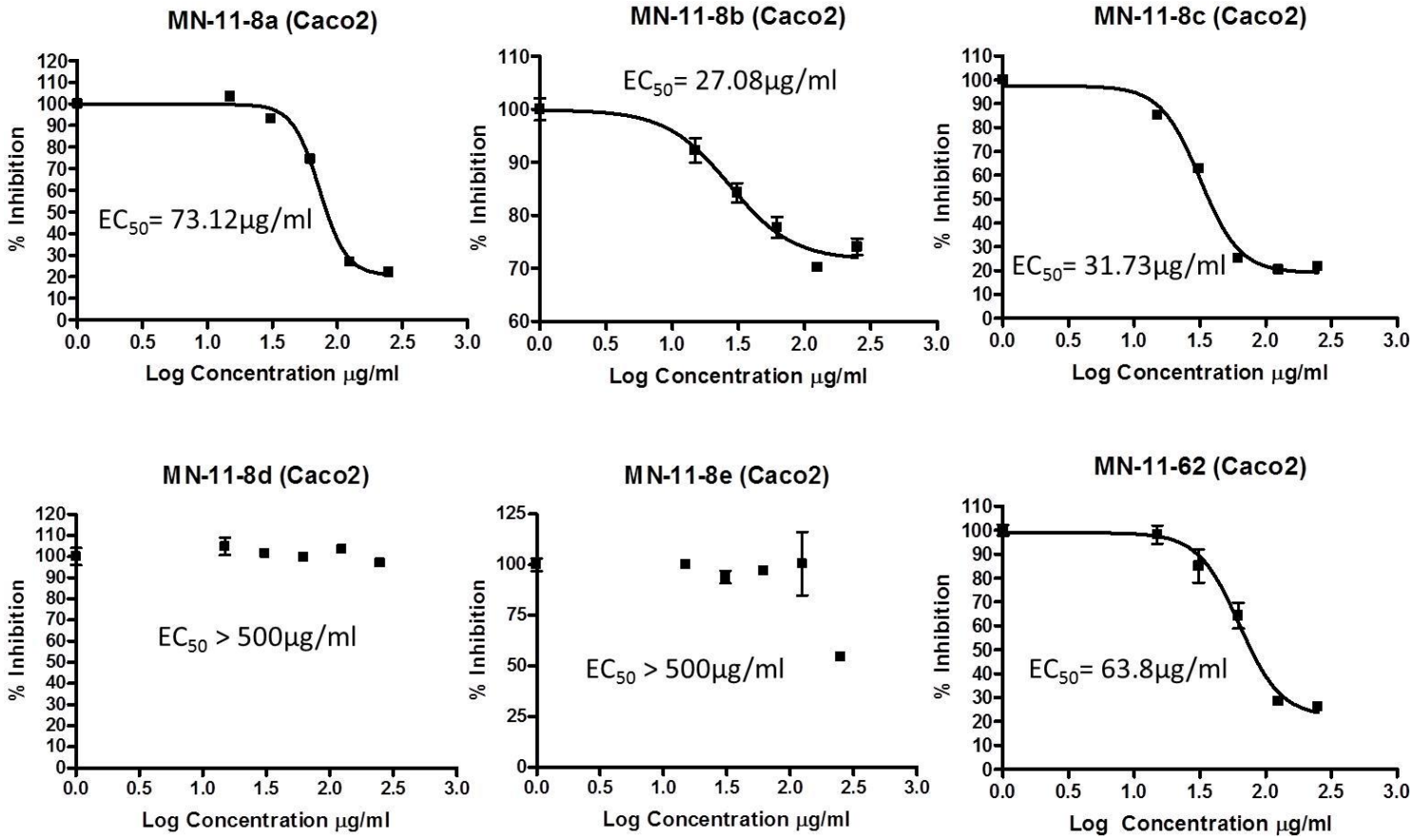

Figure A3.3 Dose dependent inhibition of Caco-2 cell viability by *S. zonale* (**8a**), *D. ligulata* (**8b**), *C. fibrosa* (**8c**), *B. capensis* (**8d**), *Sargassum* sp. (**8e**) and *S. heterophyllum* (**62**) crude extracts

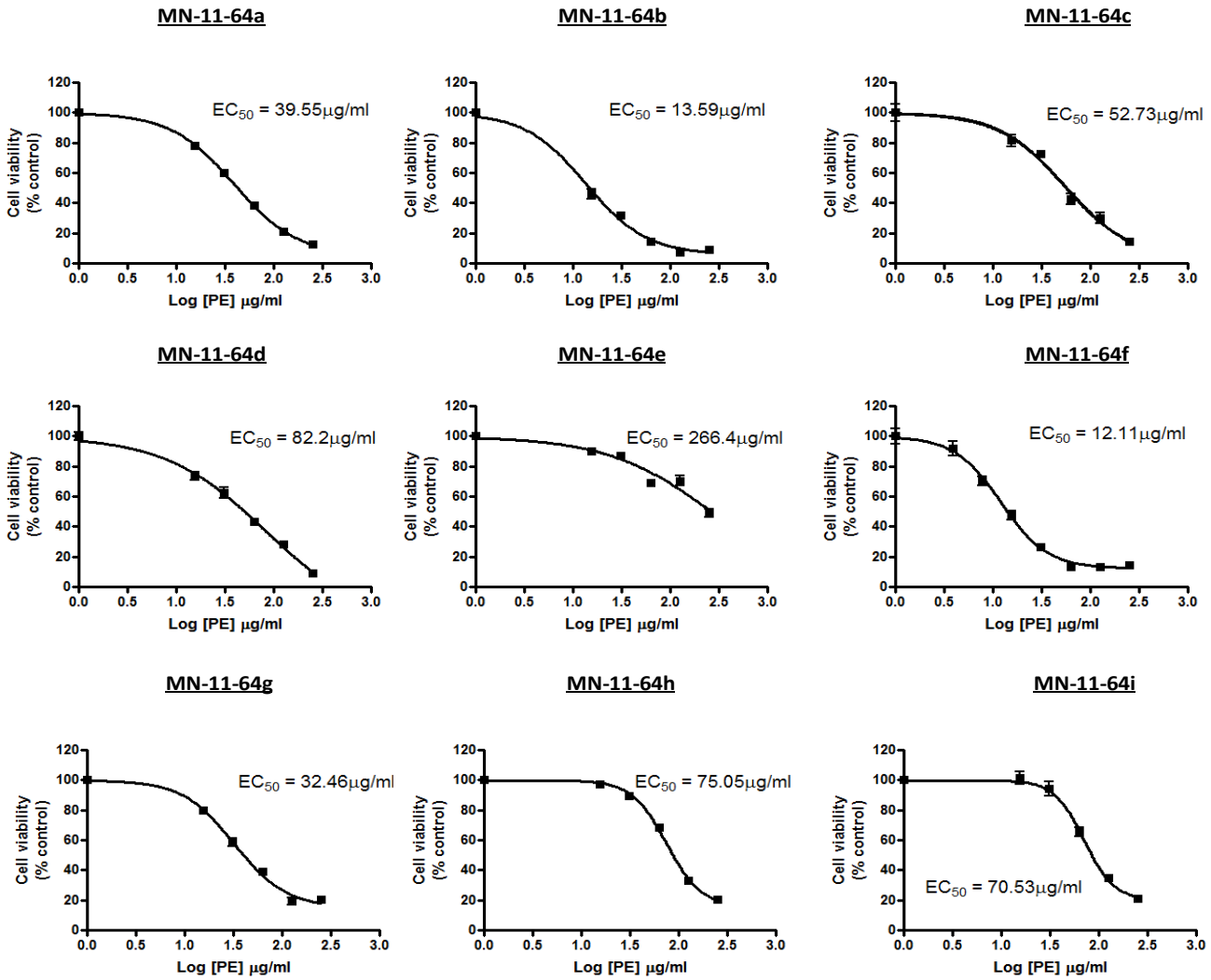

Figure A3.4 Dose dependent inhibition of Chang liver cell viability by *S. heterophyllum* fractions (**64a**), (**64b**), (**64c**), (**64d**), (**64e**), (**64f**), (**64g**), (**64h**) and (**64i**)

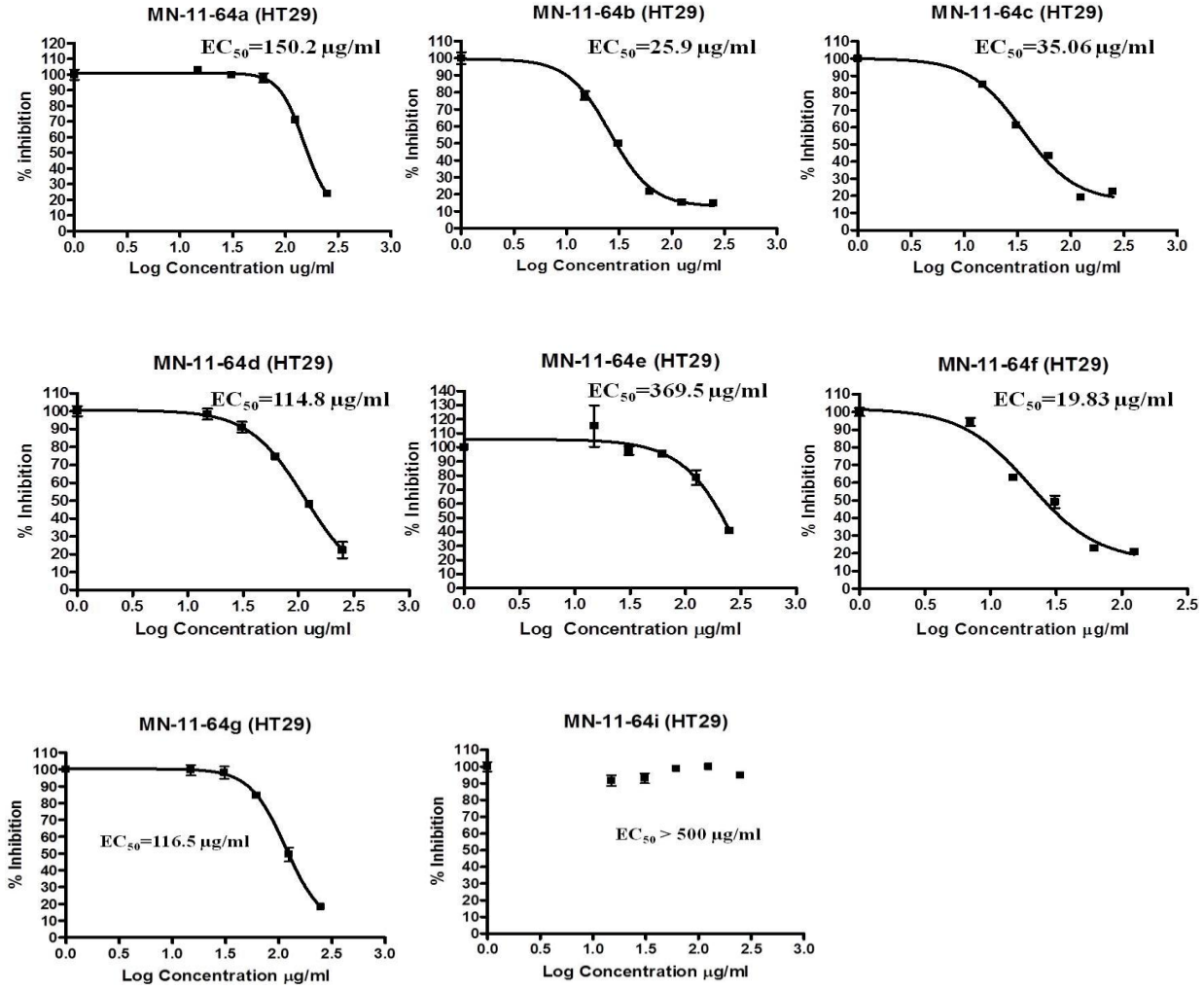

Figure A3.5 Dose dependent inhibition of HT-29 cell viability by *S. heterophyllum* fractions (**64a**), (**64b**), (**64c**), (**64d**), (**64e**), (**64f**), (**64g**), (**64h**) and (**64i**)

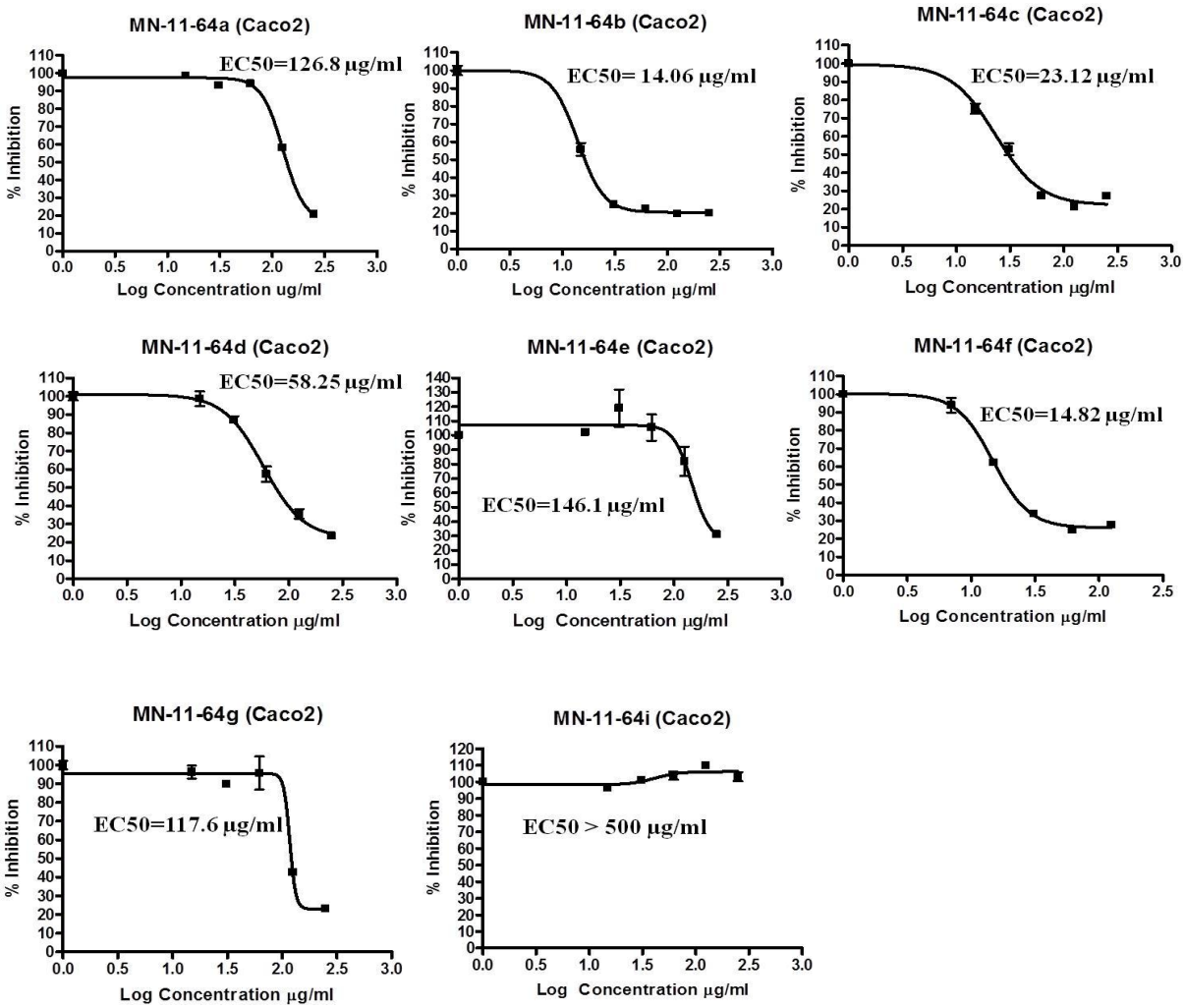

Figure A3.6 Dose dependent inhibition of Caco-2 cell viability by *S. heterophyllum* fractions (**64a**), (**64b**), (**64c**), (**64d**), (**64e**), (**64f**), (**64g**), (**64h**) and (**64i**)

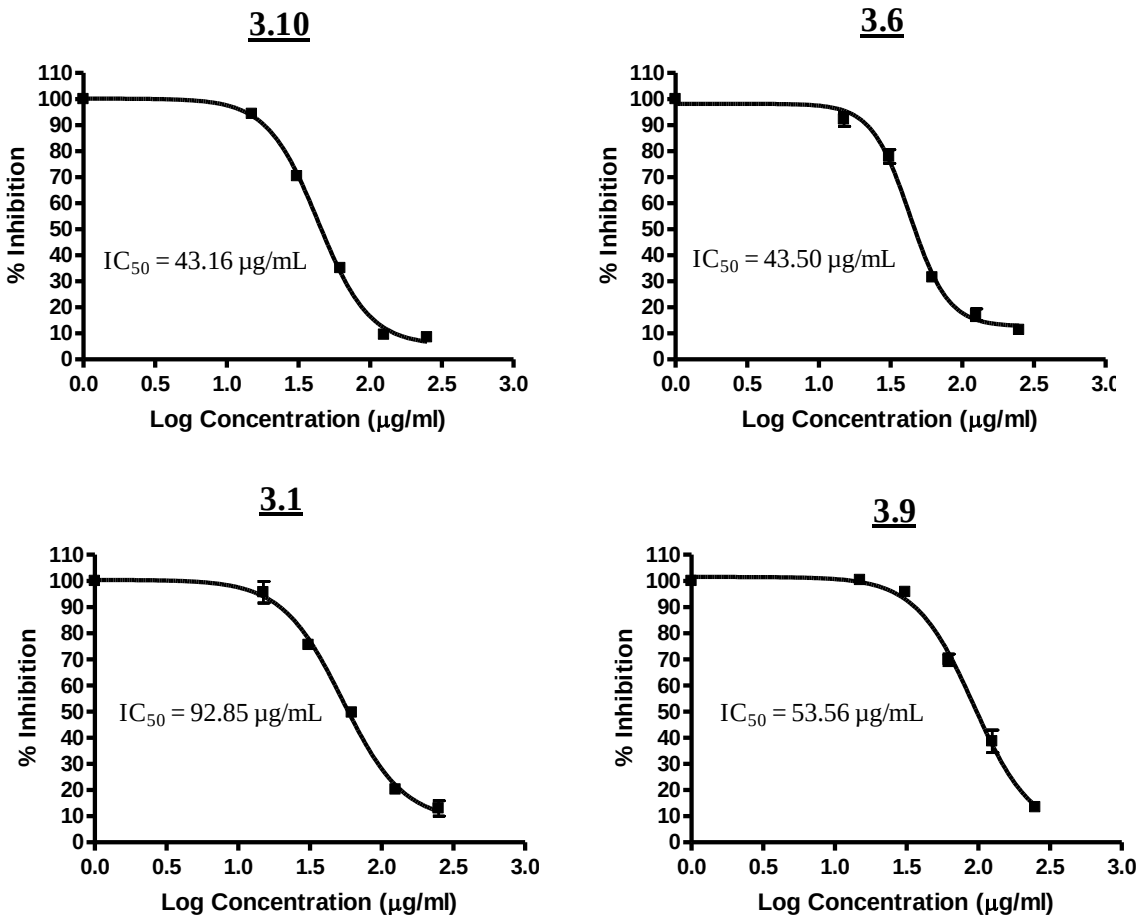

Figure A3.7 Dose dependent inhibition of Chang liver cell viability by sarganaphthoquinoic acid (**3.10**), sargahydroquinoic acid (**3.6**), sargaquinoic acid (**3.1**) and sargachromenoic acid (**3.9**)