The Isolation, Quantification and Synthetic Modification of Antiplasmodial Natural Products from *Sargassum heterophyllum*

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Master of Science (Pharmacy)
Rhodes University

By

Tatenda Carol Munedzimwe

February 2012

For my parents

Salatiel and Catherine Munedzimwe - you are my inspiration

Acknowledgements

Firstly, I would like to thank my supervisor Dr. Denzil Beukes. Thank-you for your advice and guidance throughout this project. Thank-you for believing in me and for showing me that I could complete this project.

Several people have contributed to the success of this project, amongst them I would like to thank the following:

- Professor Robyn Van Zyl for her help with antiplasmodial assays
- Dr Adrienne Edkins for her help with cytotoxicity assays
- Drs. Edith Antunes and Pierre Kempgens for their assistance with NMR experiments
- From the Rhodes University Faculty of Pharmacy technical staff : Mr and Mrs Morley, Ms Prudence Mzangwa, Mr Collin Nontyi and Mr Tich Samkange for technical assistance
- Mrs Yoland Irwin, Ms Catherine Luyt, Drs. Roman Tandlich, Leonie Goosen and Bhupesh Samant for their helpful moral support and advice
- Rhodes University Faculty of Pharmacy staff and post-graduate students for their support
- Rhodes University, the Beit Trust, the Andrew Mellon foundation and the Medical Research Council for funding this project
- Ms Linda Braaten for help with proof reading this thesis
- My G3 Lab mates: Anthonia, Temitope, Maynard, Michael, Mutenta, Denzel and Jameel. Thank you for your assistance and motivation during the long days and nights of lab work
- My great friends: Patricia, Eldinah, Theodore and Amma. Amma – thank-you for believing in me and for helping me find various information when I needed it. Patricia and Eldinah – thank-you for your companionship in the past years, it provided a much needed break from my academic work. Theodore – thank-you for supporting me through the most challenging parts of my project and for allowing me to temporarily replace you with writing up this thesis.

To my dad, thank-you for encouraging me through the hard times and for allowing me never to consider giving up - your strength and courage inspire me. To my mom, thank-you for laughing with me through the hard times and for always cheering me on. Your selfless love

and positive attitude inspire me. To my little brother Tapiwa - I hope you get to look your best at graduation. Thank-you for believing in me and for having faith that I would finish this project. To my sisters: Ngonidzashe and Fadzai, thank-you for your support. Ngoni – thank-you for encouraging me to do this and for allowing me to bounce ideas off you. Fadzi – thank- you for always being available to help me throughout this project and for always being there to brighten up my days.

Finally, to Him who is able to do, immeasurably more than I could ask or imagine, according to His power that is at work within me. Without You and all the special people in my life, this work would not have been possible. Thank - You God.

Table of contents

Acknowledgements.....i
Table of Contents.....iii
List of Figures.....vii
List of Schemes.....x
List of Tables.....x
List of Abbreviations.....xii
Abstract.....xiv
Chapter 1- Literature Review.....1
1.1Malaria.....1
1.1.1 Control of Malaria.....1
1.1.1.1 Commonly used antimalarial agents.....2
1.1.2 The discovery and development of important antimalarial agents 6
1.1.3 Natural products in the treatment of malaria 7
1.2 The Marine environment 8
1.2.1 Antiplasmodial agents from the Marine environment 11
1.3 Research objectives.....14
References.....15
Chapter 2-Extraction and Isolation of Tetraprenylated Toluquinones and Fucoxanthin from *Sargassum heterophyllum*.....20
2.1 Introduction.....20
2.1.1 *Sargassum* species.....20
2.2 Results and Discussion.....23
2.2.1 Extraction and isolation of compounds.....23
2.2.2 Characterization of isolated compounds.....25
2.3 Experimental.....35
2.3.1 General Experimental.....35
2.3.2 Plant Material.....35
2.3.3 Extraction and isolation 35
2.3.4 Compounds.....36
References.....37

Chapter 3-Geographical and seasonal Variation of *S. heterophyllum* and *S. elegans*.....40
3.1 Introduction.....40
3.1.1 Variation of Secondary metabolites- a brief overview.....40
3.1.2 Taxonomy of the *Sargassum* Genus.....41
3.1.2.1 Classification in the *Sargassum* Genus.....41
3.2 Results and Discussion.....46
3.2.1 Evaluation of extraction solvents.....46
3.2.2 Investigation of metabolite profile changes between collection site and extraction at the laboratory.....48
3.2.3 Geographical and seasonal variation.....48
3.2.3.1 Intra- and Inter-site variation.....49
3.2.3.2 Seasonal Variation.....54
3.3 Experimental.....57
3.3.1 General Experimental.....57
3.3.2 Plant Material.....57
3.3.3 Optimization of extraction solvents.....57
3.3.4 Intra-site and Inter-site differences and Geographical variation.....58
3.3.5 ^{1}H NMR analysis.....58
References.....59
Chapter 4- A Comparison of four different techniques for the extraction of metabolites from *S. heterophyllum* using qH NMR.....61
4.1 Introduction.....61
4.1.1 Overview of commonly used extraction techniques.....61
4.1.1.1 Extraction by maceration.....62
4.1.1.2 Soxhlet extraction.....62
4.1.1.3 Ultra Sound Assisted Extraction (USAE).....63
4.1.1.4 Microwave Assisted Extraction (MAE).....63
4.1.2 The selection of extraction solvents.....64
4.1.3 Other factors affecting extraction yields.....66
4.2 Quantification of metabolites from natural sources.....66
4.3 NMR as a method of quantification.....67
4.3.1 Applications of qNMR.....68
4.3.2 Quantitative ^{1}H NMR.....69

4.3.2.1 Principles of NMR quantification.....69
4.3.3 Important aspects of q^1H NMR analysis.....70
4.3.3.1 Quantitative experimental conditions.....70
4.3.3.2 Post acquisition data processing.....71
4.3.4 Reference compounds for quantification.....72
4.3.5 Quantitative analysis.....73
4.4 Results and discussion.....75
4.4.1 Effect of drying method on the metabolite profile of *S. heterophyllum*.....75
4.4.2 Development of a quantitative NMR method for the analysis of crude extracts of *S. heterophyllum*.....77
4.4.2.1 Selection of protons of interest.....77
4.4.2.2 Selection of an internal standard for quantification.....79
4.4.2.3 Quantification of metabolites in *S. heterophyllum* 80
4.4.3 Assessment of various extraction techniques.....83
4.4.3.1 Ultrasound Assisted Extraction.....83
4.4.3.2 Microwave Assisted Extraction.....84
4.4.3.3 Maceration and Soxhlet Extractions.....86
4.5 Experimental 90
4.5.1 Solvents and reference compounds.....90
4.5.2 Plant material.....90
4.5.3 Extraction of plant material.....90
4.5.3.1 Qualitative assessment of algae preparation prior to extraction.....90
4.5.3.2 Quantitative investigation of extraction methods.....91
4.5.4 NMR Analysis.....92
4.5.4.1 Sample Preparation.....92
4.5.4.2 NMR apparatus and Parameters.....92
4.5.3.3 Post acquisition processing.....93
References 94
Chapter 5- Structure Activity Relationships of Antiplasmodial and Cytotoxic Sargahydroquinoic acid Derivatives…….....99
5.1 Introduction.....99
5.1.1 The development of ideal antiplasmodial agents.....99
5.1.2 Quinone bearing compounds as antiplasmodial agents.....101
5.2 Results and Discussion.....103

5.2.1 Chemical Modification of Sargahydroquinoic acid….....103
Oxidation of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)**.....103
Methylation and acetylation of sargahydroquinoic acid **(1.38)**.....108
Attempted conversion of **1.38** to sargachromenol **(2.10)**.....114
Formation of sargaquinol **(5.5)** and sargachromendiol **(5.6)** 114
Conversion of sargaquinol **(5.5)** to *Z*-sargaquinal **(5.7)**.....116
Conversion of sargahydroquinoic acid **(1.38)** to sargalactone **(5.8)**.....119
5.2.2 Bioactivity of sargahydroquinoic derivatives and naturally occurring prenylated toluquinones …….....122
Antiplasmodial assay.....122
Cytotoxicity assay.....122
5.3 Experimental.....126
5.3.1 General Experimental 126
5.3.2 Plant material.....126
5.3.3 Extraction and isolation of sargahydroquinoic acid.....126
5.3.4 The Chemical Modification of Sargahydroquinoic acid **(1.38)**…….....127
5.3.4.1 Oxidation of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)**.....127
5.3.4.2 Methylation and acetylation of sargahydroquinoic acid.....129
5.3.4.3 Acetylation of sargahydroquinoic acid.....130
5.3.4.4 Attempted synthesis of sargachrommenol **(2.10)** 131
5.3.4.5 Reduction of sargahydroquinoic acid to sargaquinol **(5.5)** …….131
5.3.4.6 Conversion of sargaquinol **(5.5)** to *Z*-sargaquinal **(5.7)**.....133
5.3.4.7 Conversion of sargahydroquinoic acid **(1.38)** to sargalactone **(5.8)**.....133
5.3.5 Antiplasmodial Assays.....134
5.3.6 Cytotoxicity Assay.....134
References.....135
Conclusion……..139
Supplementary Data……..……See attached CD

List of Figures

Figure 1.1 Map showing countries or areas in which the transmission of malaria occurs 2

Figure 1.2 *Plasmodium* lifecycle showing the phases targeted by specific antimalarial agents…….....3

Figure 1.3 Some of the currently used antimalarial agents.....5

Figure 1.4 Marine derived Bioactive compounds from natural products that have either landed in clinical trials or provided lead compounds for new drugs.....10

Figure 2.1 Selected structural classes of metabolites isolated from *Sargassum* species.....21

Figure 2.2 Prenylated metabolites isolated from *Sargassum* species.....22

Figure 2.3 ^{1}H NMR spectrum of compound **1.38** (400 MHz, $CDCl_3$).....26

Figure 2.4 ^{1}H NMR spectrum of compound **1.39** (400 MHz, $CDCl_3$).....26

Figure 2.5 ^{13}C NMR spectrum of compound **1.39** (400 MHz, $CDCl_3$) with the insert showing an expansion of the region between δ 188.6 and δ 187.4 ppm.....27

Figure 2.6 ^{1}H NMR spectrum of compound **1.40** (400 MHz, $CDCl_3$).....30

Figure 2.7 ^{1}H NMR spectrum of compound **2.10** (400 MHz, $CDCl_3$).....31

Figure 2.8 ^{1}H NMR spectrum of **1.41** (400 MHz, $CDCl_3$).....32

Figure 3.1a Figure 3.1a Photograph of *S. elegans*.....43

Figure 3.1b Diagram of *S. elegans* and *S. heterophyllum* 43

Figure 3.2 Map of South Africa's coastline.....44

Figure 3.3 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* (PA100331) obtained from extraction with different solvents at the site of collection and with CH_2Cl_2- MeOH (2:1) at the laboratory……..……..47

Figure 3.4 a ^{1} H NMR (400 MHz, $CDCl_3$) spectrum of crude *S. heterophyllum* extracts (PA100727-11a and 12a) collected from different pools at Port Alfred in July.....50

Figure 3.4 b ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts (PA100924-9c) collected from different pools at Port Alfred in September.....50

Figure 3.4 c ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts (PA100924-10c & 11c) collected from different pools at Port Alfred in September.....51

Figure 3.5 a ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts (NDK100328-4 &2) collected from different pools at Noordhoek in March 51
Figure 3.5 b ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts (NDK100328-1 & 5) collected from different pools at Noordhoek in March.....52
Figure 3.5 c ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts (NDK100727-4b) collected from different pools at Noordhoek in July.....52
Figure 3.6 a ^{1} H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts (KOS100329-5) collected from different pools at Kenton on Sea in March.....53
Figure 3.7 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts collected from different sites in March (PA- Port Alfred, NDK- Noordhoek, KOS- Kenton on Sea)…….....54
Figure 3.8 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts collected from Port Alfred in July and September….....55
Figure 3.9 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* and *S. elegans* extracts collected from Noordhoek in March.....55
Figure 4.1 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of crude extracts obtained from *S. heterophyllum* dried by different methods.....76
Figure 4.2 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of sargahydroquinoic acid **(1.38)**, sargaquinoic acid **(1.39)** and sargachromenol **(2.10)** showing protons selected for integration in qNMR.....78
Figure 4.3 ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of crude *S. heterophyllum* with internal standard (DMF- N,N-dimethylformamide, SCHR- sargachromenol **(2.10)**, SHQA- sargahydroquinoic acid **(1.38)** and SQA- sargaquinoic acid **(1.39)**).....79
Figure 4.4 HSQC spectrum of crude *S. heterophyllum* spiked with DMF (600 MHz, $CDCl_3$).....80
Figure 4.5 Amount of sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) extracted using USAE…….....83
Figure 4.6 Amount of sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) using MAE.....84
Figure 4.7 Amount of sargahydroquinoic acid (SHQA), sargachromenol (SCHR)

and sargaquinoic acid (SQA) extracted by maceration and soxhlet extraction…….....86

Figure 5.1 Proposed changes to the hydroquinone and carboxylic moiety of sargahydroquinoic acid moieties in order to assess their effect on antiplasmodial and cytotoxic activity **(1.38)** ….....102

Figure 5.2 ^{1}H NMR spectra of sargahydroquinoic acid **(1.38)** and sargaquinoic acid **(1.39)** (400 MHz, $CDCl_3$) 104

Figure 5.3 ^{1}H NMR spectrum of the crude product obtained from the Ag_2O catalysed oxidation of sargahydroquinoic acid (400 MHz,$CDCl_3$).....105

Figure 5.4 ^{1}H NMR spectrum of compound **5.1** (400 MHz, $CDCl_3$) with insert showing an expansion of the region between δ 8.1 and δ 7.4 ppm.....105

Figure 5.5 Key HMBC correlations for compound **5.1**.....106

Figure 5.6 Proposed mechanism for the conversion of **1.38** to **5.1**.....108

Figure 5.7 ^{1}H NMR spectra of compounds **5.2** and **5.3** (400 MHz, $CDCl_3$) 109

Figure 5.8 ^{13}C NMR spectrum of compound **5.3** (100 MHz, $CDCl_3$) with inserts showing expansions of regions between δ 169.0 and δ 168.0 ppm and δ 53 and δ48 ppm.....110

Figure 5.9 ^{13}C NMR spectra of compound **5.4** (100 MHz, $CDCl_3$) with inserts showing expansions of regions between δ 171.0 and δ 168.0 ppm and δ 23 and δ 19 ppm…….....113

Figure 5.10 DEPT135 NMR spectrum of compound **5.4** (100 MHz, $CDCl_3$) with the insert showing an expansion of the region between δ 23 and δ 19 ppm….....113

Figure 5.11 The proposed mechanism for the conversion of sargahydroquinoic acid **(1.38)** to sargachromenol **(2.10)**.....114

Figure 5.12 ^{1}H-NMR spectra of sargaquinol (**5.5)** and sargachromendiol **(5.6)** (400 MHz, $CDCl_3$) 115

Figure 5.13 ^{1}H-NMR spectra of the natural (**1.40)** and derived **(5.7)** sargaquinal (400 MHz, $CDCl_3$) 117

Figure 5.14 ^{1}H NMR spectrum of compound **5.8** (600 MHz, $CDCl_3$) with the insert showing an expansion f the region between δ 7.1 and δ 6.6 ppm 120

List of Schemes

Scheme 2.1 Isolation of compounds **1.38**, **1.39**, **1.40**, **1.41** and **2.10** from *S. heterophyllum*.....24

Scheme 2.2 Reaction scheme showing the conversion of sargahydroquinoic acid **(1.38)** to sargachromenol **(2.10)**.....34

List of Tables

Table 2.1 ^{1}H and ^{13}C NMR (400 and 100 MHz) data for compounds **1.38**, **1.39**, **1.40** and **2.10** recorded in $CDCl_3$.....28

Table 2.2 ^{1}H and ^{13}C NMR (400 and 100 MHz, $CDCl_3$) data for compound **1.41**.....33

Table 3.1 Summary of collection sites, collected species and dates of collection.....49

Table 4.1 Physical properties of extraction solvents used in the current study.....65

Table 4.2 Solvents used to extract specific metabolites from *Sargassum* sp.....65

Table 4.3 Examples of natural product based studies in which QNMR was utilized as the method of analysis.....69

Table 4.4 T1 values of selected protons.....81

Table 4.5 Range of chemical shifts used in the integration of selected NMR signals.....81

Table 4.6 Integration limits used to select the most suitable integration limits for DMF.....81

Table 4.7 Signal to noise ratios (S/Ns) calculated from peaks of each metabolite.....82

Table 4.8 Masses (mg /g of dry biomass) of crude extract, sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) extracted by each extraction technique.....88-89

Table 4.9 Acquisition parameters for the NMR analysis of all samples 92

Table 5.1 List of oxidising agents investigated for the conversion of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)**.....104

Table 5.2 NMR spectroscopic data for compound **5.1** (600 and 100 MHz, $CDCl_3$).....107

Table 5.3 Comparison of ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data for compounds **5.2** and **5.3** with 2D NMR data for the more stable **5.3**.....111

Table 5.4 Comparison of NMR spectral data for the natural (**1.40**) and derived aldehydes **(5.7)** ($CDCl_3$) in addition to 2D NMR data for **5.7**.....118
Table 5.5 NMR spectroscopic data for compound **5.8** 121
Table 5.6 Bioassay results for compounds **1.38 - 1.40** and **5.1 -5.8**.....123
Table 5.7 Antiplasmodial activity of compounds **1.38 - 1.40** and **5.1 - 5.8.1 - 5.11**.....124

List of Abbreviations

$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
COSY	^1H-^1H Homonuclear Correlation Spectroscopy
°C	Degrees Celsius
d	Doublet
dd	Doublet of doublet
DCM	Dichloromethane
DEPT	Distortionless Enhancement of Polarisation Transfer
DMF	N,N-dimethyl formamide
DOXP	1-deoxy-D-xylulose-5-phosphate
EIMS	Electron Impact Mass Spectrometry
EtOAc	Ethyl Acetate
EtOH	Ethanol
V	Volt
Fr	Fraction
GC	Gas Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
HSQC	Heteronuclear Single Bond Correlation
Hz	Hertz
IC_{50}	Inhibitory Concentration 50
IR	Infra Red Spectroscopy
J	Spin-Spin coupling constant (Hz)
KOS	Kenton on Sea
m	Multiplet
MS	Mass Spectroscopy
m/z	Mass to charge ratio
MeOH	Methanol
MHz	Mega hertz
mult	Multiplicity
NDK	Noordhoek

NMR	Nuclear Magnetic Resonance Spectroscopy
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PA	Port Alfred
ppm	Parts per million
q	Quartet
qNMR	Quantitative NMR
s	Singlet
SCHR	Sargachromenol
SHQA	Sargahydroquinoic acid
SQA	Sargaquinoic acid

Abstract

Malaria is one of the most deadly parasitic diseases known to man. Although the number of malaria cases reported each year is decreasing, this disease continues to pose health and economic problems mainly in developing countries. Significant progress has been made in the fight against this disease. This includes the discovery and development of potent antimalarial agents. However, the development of resistance to most of these potent antimalarials has made the development of new antiplasmodial agents of paramount importance.

Several promising antiplasmodial agents have been found from the marine environment. Amongst these are the tetraprenylated toluquinols from the brown alga: *Sargassum heterophyllum*. These metabolites have been reported to exhibit a range of antiplasmodial activity; however, the mechanisms by which these compounds bring about their antiplasmodial activity and the pharmacophoric groups responsible for such activity are unknown.

Two species of *Sargassum* algae were encountered during the course of this project. From the investigation of the geographical and seasonal variation of metabolites of *S. heterophyllum* and *S. elegans* we established that there were no significant intra and inter site variations amongst metabolite profiles of both species both within and between the sampled seasons. These results enabled us to establish that the collection of both species from three different sites on the eastern coast of South Africa namely; Kenton on Sea, Port Alfred and Noordhoek in autumn, winter or spring would qualitatively yield the same metabolites. A comparison of metabolite profiles of both species also revealed no qualitative differences between metabolites of *S. heterophyllum* and *S. elegans*.

The quantities of selected prenylated metabolites extracted from *S. heterophyllum* using four different extraction techniques was also assessed using qNMR as the method of quantification. This led to the identification of optimal extraction techniques and conditions for the extraction of sargahydroquinoic acid **(1.38)**, sargaquinoic aid **(1.39)** and sargachromenol **(2.10)** from *S. heterophyllum*. From this study, the extraction of algae by

soxhlet extraction using EtOH as the extraction solvent led to the extraction of the highest quantities of sargahydroquinoic acid. The potential of other extraction techniques such as microwave assisted extraction, to yield high quantities of the selected metabolites were also identified.

With gram quantities of sargahydroquinoic acid **(1.38)** in hand, this compound was modified by oxidation, reduction, acetylation, methylation and cyclization reactions to yield nine derivatives. The derivatives and four naturally occurring prenylated toluquinols were assessed for antiplasmodial and cytotoxic activity against the FCR-3 Gambian Chloroquine resistant strain of *P. falciparum* and the MDA-MB-231 breast carcinoma cell line respectively. Comparison of antiplasmodial data for all twelve compounds showed that the hydroquinone moeity of sargahydroquinoic acid **(1.38)** is important for antiplasmodial activity while esterification of the carboxylic acid group in **1.38** resulted in more potent antiplasmodial compounds. Of all twelve compounds, compound **5.2**, the hydroquinone methyl ester of **1.38** was found to be the most potent antiplasmodial compound with an IC_{50} value of 1.94 μM and a selectivity index of 22.68.

Chapter 1

Literature Review

1.1 Malaria

Parasitic diseases such as malaria, leishmaniasis, trypanosomiasis, giardiasis and amebiasis are prevalent throughout the world (Mancini et al., 2009). Malaria is one of the most deadly of these diseases (Mancini et al., 2009; Ravichandran et al., 2007). This disease is caused by four strains of the *Plasmodium* parasite namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Williams and Lemke, 2002; Balint, 2005). Of these parasites, *Plasmodium falciparum* causes the most virulent form of disease in humans (Williams and Lemke, 2002). In 2009, 225 million cases of malaria were reported (World Health Organisation, 2010b). Of these cases, an estimated 781,000 deaths, mostly amongst children in Africa, were also recorded (World Health Organisation, 2010b). From the 106 countries in which malaria is endemic, countries in Sub-Saharan Africa are amongst the most widely affected (World Health Organisation, 2010b; Laurent and Pietra, 2006). Apart from adversely affecting human health and contributing to childhood mortality, malaria also decreases gross domestic productivity in countries in which it is endemic (Laurent and Pietra, 2006; Chima et al., 2003).

1.1.1 Control of Malaria

The control of malaria is dependent on various forms of chemotherapy with a higher emphasis on drug therapy compared to the use of malaria prophylaxis (Olliaro, 2001). Presently, drugs from different classes are used to treat malaria (World Health Organisation, 2010a). The selection these agents is dependent on several factors including patterns of parasite resistance to antimalarial agents in the relevant area (World Health Organisation, 2010a).

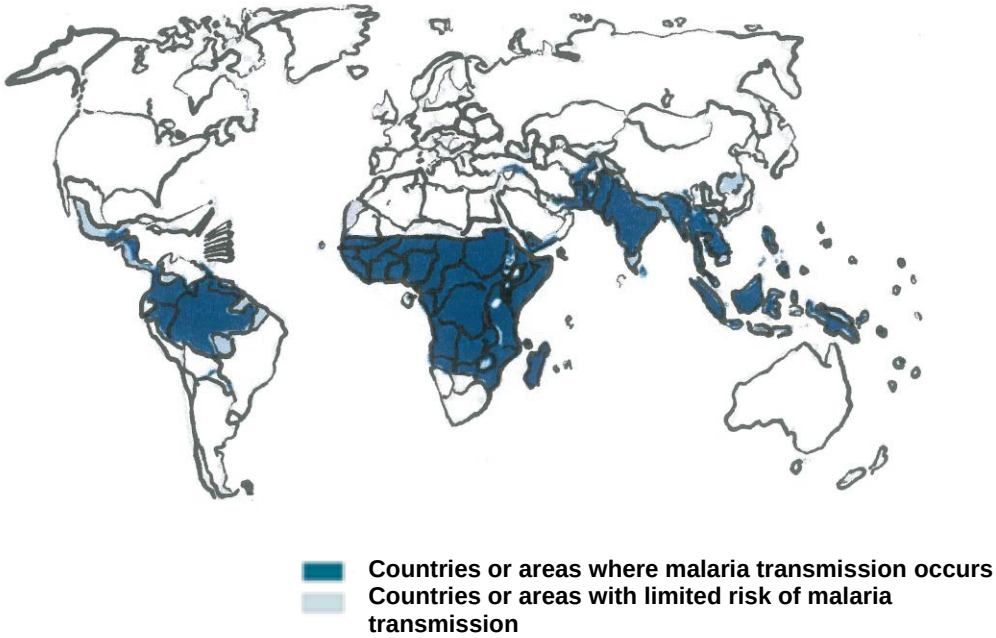


Figure 1.1 Map showing countries or areas in which the transmission of malaria occurs (World Health Organisation, 2011b)

1.1.1.1 Commonly used antimalarial agents

Currently used antimalarial agents target different stages of the *Plasmodium* parasite's life cycle (figure 1.2). The folate antagonists inhibit the synthesis of essential nucleotides (Olliaro, 2001). By mimicking the substrate: para-amino benzoic acid (PABA) the sulphonamide folate antagonists; sulfadoxine **(1.1)**, sulfalene and dapsone inhibit the enzyme dihydropteroate synthase (DHPS) (Olliaro, 2001). Other folate antagonists such as pyrimethamine **(1.2)** and the biguanides: proguanil **(1.3)** and chlorproguanil exert their antiplasmodial activity by competing with natural substrates for the active site of dehydrofolate reductase (DHFR), another enzyme involved in the synthesis of folates in the parasite. In this manner, the folate antagonists are able to target all stages of the asexual cycle as nucleotides are essential for development throughout the parasite's lifecycle (Olliaro, 2001). Atovaquone **(1.4)** is structurally classified as a naphthoquinone antimalarial. The only one in this class of compounds, this agent acts mainly on the mitochondrial functions of the parasite (Olliaro, 2001). Like other antimalarials, several mechanisms by which this agent exerts its activity have been proposed. The most widely accepted hypothesis is that atovaquone selectively inhibits electron transport in the mitochondrial membrane, resulting in

parasite death (Srivastava et al., 1997). Despite the relatively unique mode of action displayed by this agent, its use in monotherapy readily leads to parasite resistance (Olliaro, 2001). Atovaquone is thus used in combination with proguanil, a folate antagonist with which it has a synergistic effect (Olliaro, 2001; Mather et al., 2005).

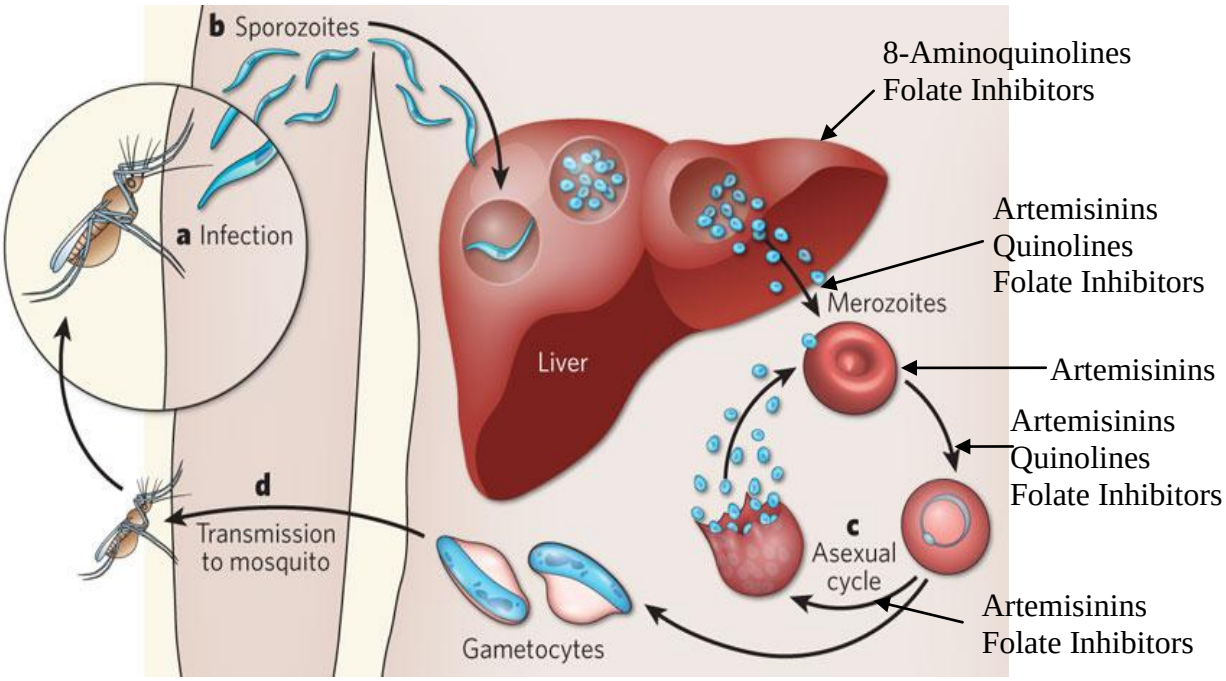


Figure 1.2 *Plasmodium* life cycle showing the phases targeted by specific antimalarial agents (Olliaro, 2001; Michalakis and Renaud, 2009)

The quinoline group of antimalarial compounds contains the most commonly known antimalarial agents. This class of antimalarial agents is made up of the 4-amino-quinolines such as chloroquine **(1.5)**, amodiaquine **(1.6)** and piperaquine **(1.7)**, the amino alcohols: quinidine **(1.8)**, quinine **(1.9)**, mefloquine **(1.10)**, halofantrine **(1.11)** and lumefantrine in addition to primaquine **(1.12)**, an 8-amino-quinoline (Olliaro, 2001). The most widely accepted mode of action for this class of compounds is that they act by targeting the parasite's disposal of haem in the intra erythrocytic asexual stage of the parasite's life cycle (Foote and Cowman, 1994). During the blood stages of its lifecycle, the parasite accumulates haem from the host's red blood cells. The accumulation and exposure of this substance to the parasite's cells is toxic, hence the parasite sequesters haem into a membrane bound food vacuole in which the haem is broken down by various mechanisms (Foote and Cowman,

1994; Olliaro, 2001). The quinolines are weak bases which are neutral at physiological pH and protonated at more acidic pH levels (Foote and Cowman, 1994). These compounds readily enter the food vacuole at physiological pH. Upon entry into the relatively acidic food vacuole, the quinolines become ionised with 4-aminoquinolines such as chloroquine becoming diprotonated (Foote and Cowman, 1994). The quinolines thus lose their ability to cross the membrane and accumulate in the vacuole where they interfere with the breakdown of toxic haem which in turn accumulates, resulting in the lyses of parasite cell components (Olliaro, 2001). Chloroquine in its diprotonated state exerts this effect by inhibiting haem polymerase, an enzyme responsible for the detoxification of haem in the parasite (Foote and Cowman, 1994).

The artemisinins **(1.13-1.17)** also target the detoxification of haem in the parasite lifecycle, killing both the asexual parasite stages in the blood as well as young gametocytes (Olliaro, 2001; White, 2008). It is widely accepted that the artemisinins are activated by the homolytic cleavage of their endoperoxide moiety in the presence of Fe^{2+} in the parasite (O'Neill et al., 2010; Olliaro, 2001). This leads to the formation of free radicals which in turn alkylate biomolecules, resulting in parasite death (O'Neill et al., 2010). By killing young gametocytes, the artemisinins reduce both the number of circulating parasites and the number of parasites that develop and mature. In this manner, the artemisinins rapidly reduce parasitaemia compared to other agents such as quinine which target mature parasites (White, 2008). Other agents such as the antibiotics: azithromycin, clindamycin, tetracyline, and doxycycline **(1.18)** are also used in the treatment of malaria (Williams and Lemke, 2002).

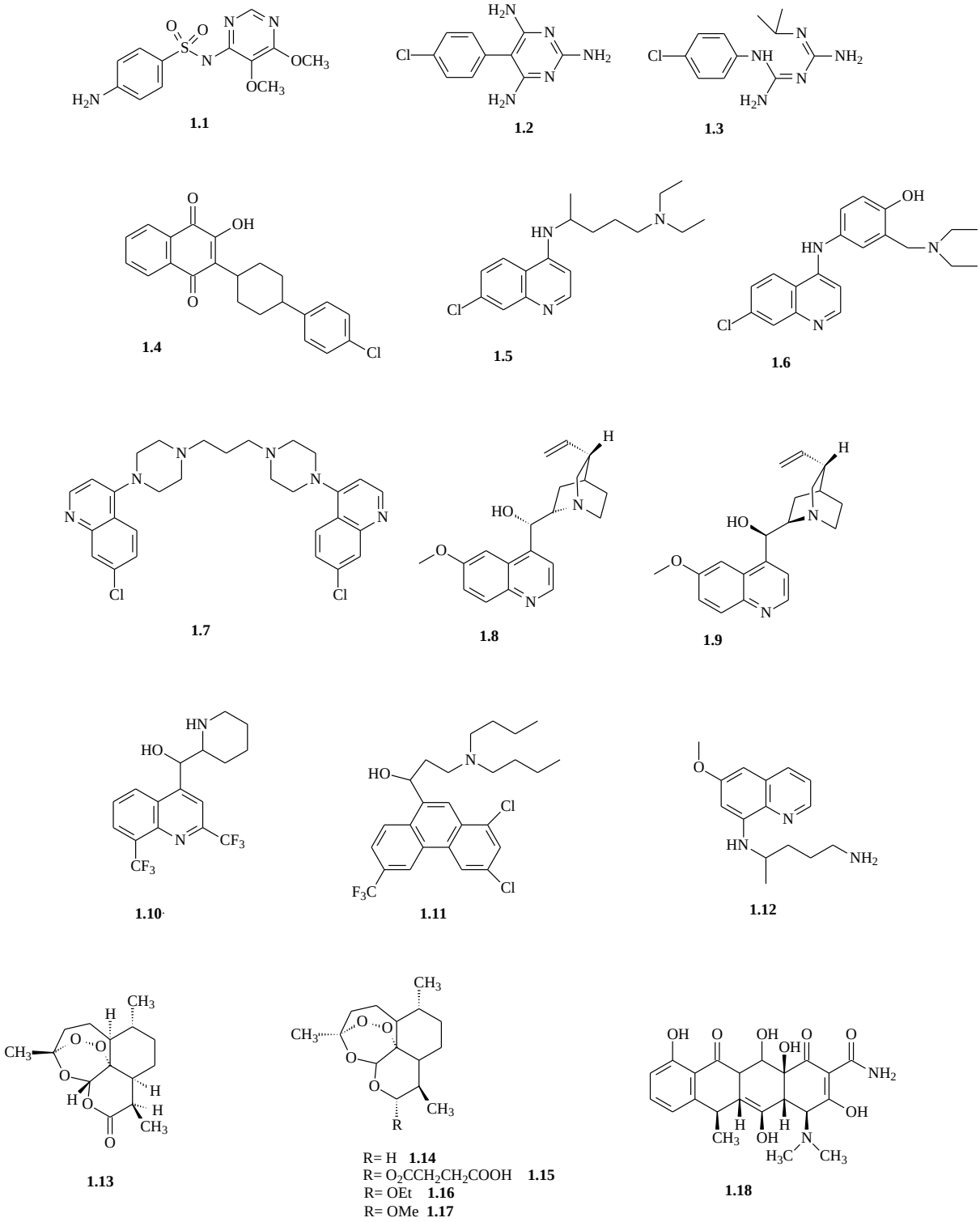


Figure 1.3 Some of the currently used antimalarial agents
(World Health Organisation, 2010b; Williams and Lemke, 2002)

1.1.2 The discovery and development of important antimalarial agents

Most antimalarial agents have been available for a long time (Olliaro, 2001). The first compound documented to be used as an antimalarial agent was quinine **(1.9)** (Talisuna et al., 2004). This compound, which was extracted from the bark of the cinchona tree was used to treat malaria from as early as 1632 (Talisuna et al., 2004). Quinine and its stereoisomer; quinidine, were finally isolated and identified by French chemists in 1820 (Greenwood, 2001). Quinine was still the only known antimalarial by the 19th century, however, its use as an antimalarial agent was followed by the first synthetic antimalarials; primaquine **(1.12)** and quinacrine which were produced after 1918 (Talisuna et al., 2004).

The synthesis of chloroquine **(1.5)** in 1934 and its development for use as an antimalarial agent after the Second World War (1945) was an important milestone in the treatment of malaria (Greenwood, 1995; Greenwood, 2001). Due to its superior antiplasmodial activity, this agent went on to replace agents used before it in the treatment of malaria (Greenwood, 1995; Greenwood, 2001). In addition to this, chloroquine was found to be highly effective, with good absorption, distribution and safety profiles with the added advantage of being relatively inexpensive (Williams and Lemke, 2002). Unfortunately, the use of this agent was hampered by the development and spread of plasmodial resistance to it in 1960 (Olliaro, 2001; Greenwood, 1995). Although this marked another step backwards in the fight against this disease, the emergence of chloroquine resistance was followed by the discovery of potent antimalarial compounds from the extracts of the sweet wormwood; *Artemisia annua*, by a group of Chinese Scientists in the 1970's (White, 2008). The most potent of these compounds was characterised, identified and announced to the world as artemisinin **(1.13)** in 1979. Despite the fact that the active component of *A. annua* was identified in the late 1970's the use of an aqueous infusion of this plant as a tea, had been documented as treatment for non-specific chills and fevers in China since the 16th century (White, 2008; Cui and Su, 2009). The discovery of artemisinin; which has been described as one of the most potent and effective antiplasmodial agent to be discovered, led to the synthesis of several analogues of this compound (Balint, 2005). The use of artemisinin has been widely replaced by some of its more potent derivatives such as dihydroartemisinin **(1.14)**, artesunate **(1.15)** and artemether **(1.16)** (White et al., 1999). The artemisinins were found to be superior to other antimalarial agents due to their higher potency, more rapid onset of action in addition to lower toxicity profiles when compared to other antimalarial agents (White et al., 1999; Balint, 2005).

The use of artemisinin derivatives in combination with other anti-malarial agents in artemisinin-based combination therapy was initially introduced in an effort to delay the development of parasitic resistance to the artemisinin derivatives (O'Neill et al., 2010). However, the emergence of plasmodial resistance to these agents has been documented at the Thai-Cambodian border - a historic region for this disease with the first signs of *P. falciparum* resistance to chloroquine being documented there (O'Neill et al., 2010; Talisuna et al., 2004; World Health Organisation, 2010b). The loss of the artemisinin group of antimalarial agents to parasite resistance could severely hamper global progress against malaria (World Health Organisation, 2011a). In addition to this, the highly adaptive nature, complex life cycle and large genome of the *Plasmodium* parasite has resulted in significant challenges in the successful development of a vaccine against malaria (Ravichandran et al., 2007; Plowe et al., 2009). However, recent reports of a malaria vaccine, RTSS/A0S2A that has proved to be safe and efficacious in phase I and II b clinical trials have emerged (Sacarlal et al., 2009). The fact that there is no registered vaccine against malaria despite longstanding efforts to develop one and the potential loss of the artemisinin derived antimalarials to resistance makes the development of new chemotherapeutic agents for this disease, of paramount importance (Ziegler et al., 2002; Laurent and Pietra, 2006; Kreidenweiss et al., 2006).

1.1.3 Natural products in the treatment of malaria

Natural products have played an essential part in drug discovery and development, especially in the treatment of malaria (Mancini et al., 2009). This is illustrated by the emergence of significant antiplasmodial agents from natural sources such as quinine **(1.9)** and artemisinin **(1.13)** (Wright et al., 1996). In addition to this, many of the currently used antimalarial agents are based on compounds from natural sources (Ziegler et al., 2002; Laurent and Pietra, 2006). Following the synthesis of chloroquine and the development of resistance to it, the discovery of artemisinin resulted in a renewed interest in the screening of natural sources for antimalarial agents (Laurent and Pietra, 2006). This serves to highlight the fact that the chemical diversity in biological systems is important in the identification and development of lead compounds for this disease (Ziegler et al., 2002). The marine environment has shown itself to be a rich source of numerous chemical entities of novel and distinct chemotypes, many of which have not been reported from the terrestrial environment (Paterson and

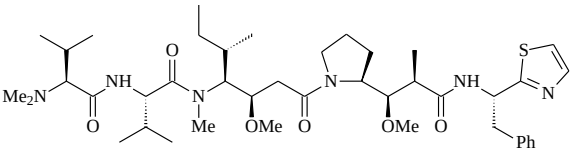
Findlay, 2008; Donia and Hamann, 2003). Although the marine environment has been less studied compared to the terrestrial environment, it has the potential to provide lead compounds for use as antiplasmodial agents (Donia and Hamann, 2003; Paterson and Findlay, 2008; Young-Won Chin et al., 2006; Faulkner, 2000a).

1.2 The Marine environment

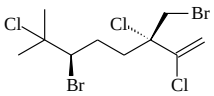
The marine environment displays great diversity in terms of both the living organisms which inhabit it and in the range of secondary metabolites that they produce (Ravichandran et al., 2007; Pomponi, 1999). Compared to the biodiversity displayed amongst terrestrial organisms, the marine environment has the greatest biodiversity (Donia and Hamann, 2003). Recorded as the habitat of 34 of the 36 phyla of all living organisms, this environment, has over 300,000 described species of plants and animals (Pomponi, 1999; Ravichandran et al., 2007). The adaptation of these marine organisms to the harsh conditions of this environment has resulted in the production of an array of chemical defence strategies to enable their survival (Ham et al., 2010; Park et al., 2008; Ravichandran et al., 2007; Paterson and Findlay, 2008). These chemical defences; seen as secondary metabolites have presented natural products chemists with a collection of unique compounds that display a wide range of biological activities and potential medicinal applications (Ham et al., 2010; Park et al., 2008).

As diseases continue to develop, evolve and present unending challenges for scientists, the marine environment provides a fertile ground for the discovery of potential medicinal agents (Donia and Hamann, 2003). The possible uses of these isolated metabolites has fuelled an interest in compounds of marine origin (Blunt et al., 2010; Faulkner, 2000b). Since the identification of the first natural products from marine organisms, chemists have been intrigued by compounds which display vast structural and complex diversity isolated from this environment (Lane and Moore, 2011). The marine environment has thus far yielded a registered drug, Ziconotide, the synthetic version of a ω-conotoxin peptide isolated from the venom of the cone snail *Conus magnus* (Young-Won Chin et al., 2006). This compound is used in the treatment of chronic pain (Young-Won Chin et al., 2006). Despite the fact that not many compounds of marine origin have resulted in registered pharmaceutical products, several compounds of marine origin such as compounds **1.19**- **1.26** (figure 1.3), have entered clinical trials; mainly for the treatment of cancer, while others have provided lead compounds

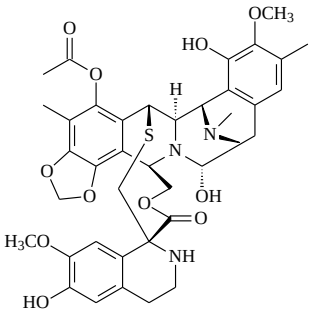
for new drug candidates (Faulkner, 2000a; Donia and Hamann, 2003; Butler, 2005; Young-Won Chin et al., 2006).



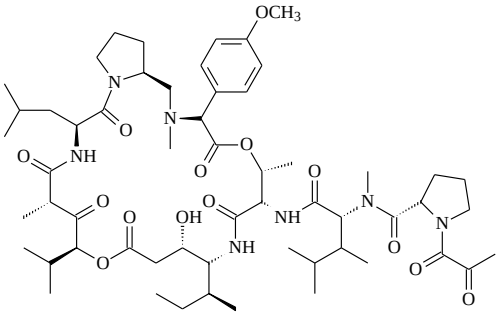
Dolastatin 10 **(1.19)**



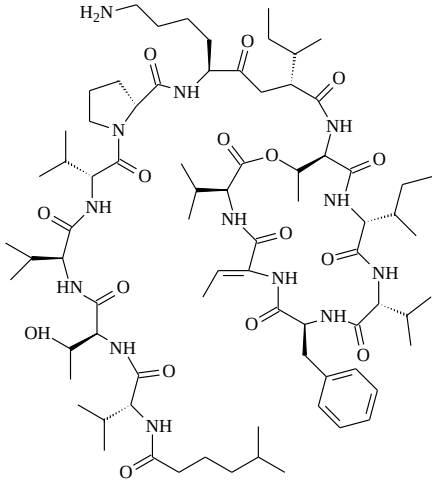
Halomon **(1.21)**



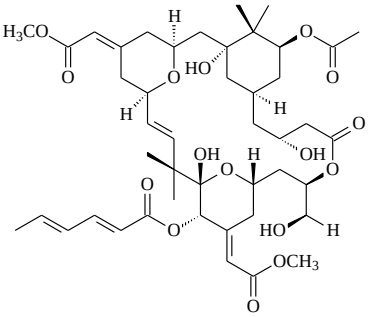
Ecteinascidin- 743 **(1.20)**



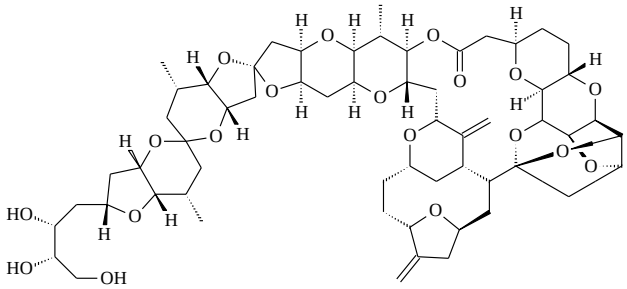
Aplidin **(1.22)**



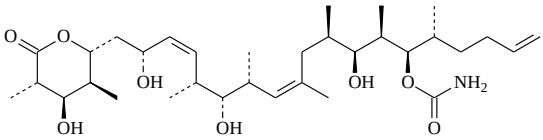
Kahalilide F **(1.23)**



Bryostatin 1 **(1.25)**



Halichondrin B **(1.25)**



Discodermolide **(1.26)**

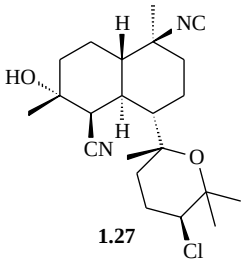
Figure 1.4 Marine derived bioactive compounds from natural products that have either entered in clinical trials or provided lead compounds for new drugs (Donia and Hamann, 2003; Faulkner, 2000c; Konig et al., 2006; Butler, 2005)

1.2.1 Antiplasmodial agents from the marine environment

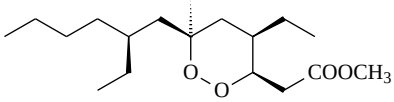
Several compounds of marine origin have been identified as potential antiplasmodial agents (Ravichandran et al., 2007; Wright et al., 1996). Kalihinol A **(1.27)** from a sponge of the *Acanthella* species (Miyaoka et al., 1998) as well as plakortins such as dihydroplakortin **(1.28)** isolated from *Plakortis simplex* are amongst some of these metabolites (Fattorusso et al., 2002). A group of alkaloids known as the manzamines have been isolated from several sponges of the *Pellina*, *Pachypellina*, *Xestospongia*, *Ircina*, and *Amphimedon* species. The manzamines have shown significant antiplasmodial activity (Ang et al., 2000). Of these secondary metabolites, manzamine A **(1.29)** has been described as the most promising compound possesing antiplasmodial activity to be isolated from the marine environment (Ang et al., 2000; Mendiola et al., 2006; Wright et al., 1996). Thus far, the complete synthesis of manzamine A has been reported (Wright et al., 1996). From the marine red alga *Laurencia papillosa*, two aromatic compounds, p-hydroxy benzaldehyde **(1.30)** and *p*-methoxybenzyl alcohol **(1.31)** have been isolated (Wright et al., 1996). Of these compounds, *p*-hydroxyl benzaldehyde was reported to have antiplasmodial activity (Wright et al., 1996). Despite the fact that the activity of this compound was relatively weak compared to other compounds from the same organism, it had good selectivity for the *Plasmodium* parasite (Wright et al., 1996). The sponge *Acanthella clethra* has also yielded several metabolites with unique structures and good antiplasmodial activity (Gross and Konig, 2006). Of these metabolites, axisonitrile-3 **(1.32)** and diisocyanoadociane **(1.33)** have been shown to exhibit the most potent activity (Gross and Konig, 2006; Wright et al., 1996). The main advantages of these compounds over the rest of the metabolites are the high activity and low cytotoxic profile of axisonitrile-3 and the greater antiplasmodial activity of diisocyanoadociane despite its high cytotoxic activity (Angerhofer et al., 1992; Gross and Konig, 2006). Two phloeodictynes **1.34** and **1.35** with good antiplasmodial activity and selectivity indices have also been reported from the Caledonian sponge; *Phloeodictyon sp* (Mancini et al., 2004; Laurent and Pietra, 2006). Since the discovery of the antiplasmodial potential of these compounds, the total synthesis of some of the phloeodictynes has been reported (Laurent and Pietra, 2006).

Metabolites from marine microorganisms have also provided potential antiplasmodial agents (Wright, 2005; Wright and Lang-Unnasch, 2005). The screening of several metabolites from marine fungi afforded the identification of 6*R*,12*R*,14*R*-colletoketol **(1.36)** isolated from *Varicosporina ramulosa*, a fungus derived from brown algae of the genus *Cystoseira* as well

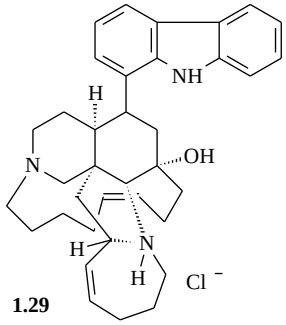
as pycnidione **(1.37)** which was isolated from fungi of the *Phoma* species (Wright and Lang-Unnasch, 2005; Holler, 1999). Results from this screening showed that marine products of fungal origin are a potential source of antimalarial lead compounds and that structural modification of these metabolites could result in an increase in antiplasmodial activity accompanied by a decrease in the cytotoxicity profiles of these metabolites (Wright and Lang-Unnasch, 2005).



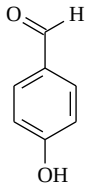
1.27



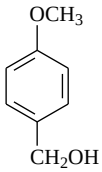
1.28



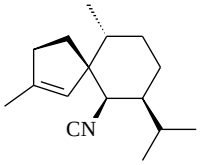
1.29



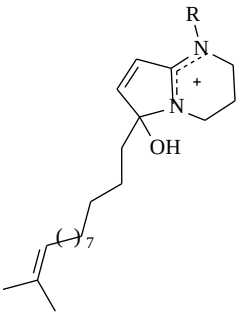
1.30

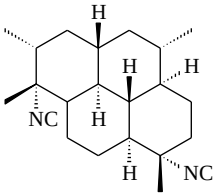


1.31

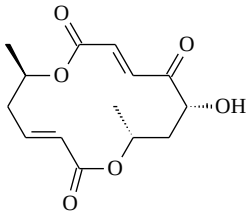


1.32



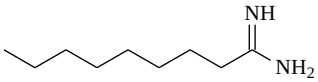


1.33

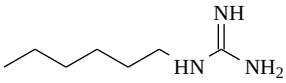


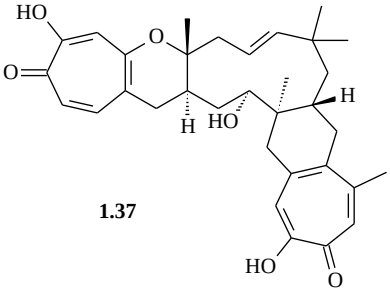
1.36

1.34 R =



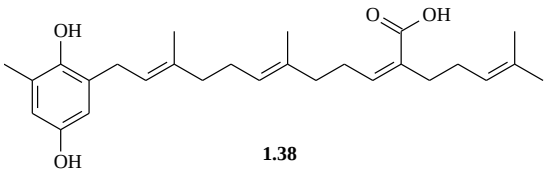
1.35 R =



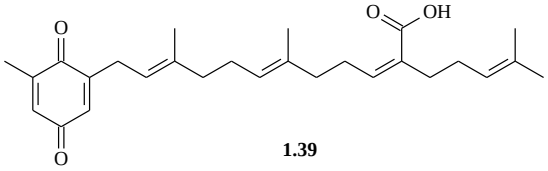


1.37

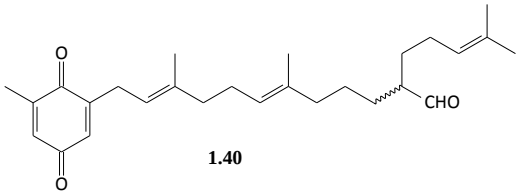
Four compounds from *Sargassum heterophyllum* have been reported to exhibit antiplasmodial activity (Afolayan et al., 2008). These compounds are sargahydroquinoic acid **(1.38)**, sargaquinoic acid **(1.39)**, sargaquinal **(1.40)** and fucoxanthin **(1.41)** (Afolayan et al., 2008). Of these metabolites, fucoxanthin and sargaquinal showed good antiplasmodial activity with IC_{50} values of 1.5 µM and 2.0 µM respectively, while sargaquinoic acid and sargahydroquinoic acid were found to possess moderate antiplasmodial activity with IC_{50} values of 12.0 µM and 15.2 µM respectively against the D10 chloroquine sensitive strain of *Plasmodium falciparum* (Afolayan et al., 2008). The mechanisms by which these compounds bring about their antiplasmodial activity and the pharmacophoric groups responsible for such activity are unknown. In addition to this, the activity of these compounds against chloroquine resistant strains of *P. falciparum* is still to be established.



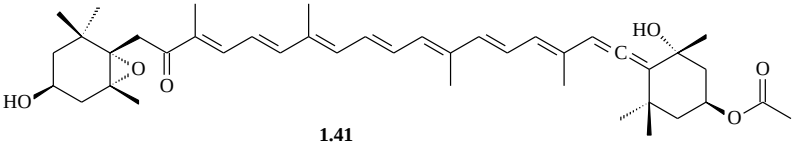
1.38



1.39



1.40



1.41

1.3 Research objectives

The main aim of this research project was to synthesise several substituted derivatives of the tetraprenylated toluhydroquinone, sargahydroquinoic acid **(1.38)** using the natural product as the starting material. This was done in an effort to identify the pharmacophoric groups responsible for the antiplasmodial activity of the tetraprenylated toluquinones from *S. heterophyllum*.

In addition to this, the project aimed to:

i. Determine the most appropriate time and site for the collection of *S. heterophyllum*
ii. Determine the most efficient extraction technique for the extraction of selected tetraprenylated metabolites from *S. heterophyllum*
iii. Isolate sufficient quantities of sargahydroquinoic acid to enable the chemical modification of this compound
iv. Assess the antiplasmodial activity of the synthesized derivatives against a chloroquine resistant strain of *Plasmodium falciparum*

References

Afolayan, A.F., Bolton, J.J., Lategan, C.A., Smith,P.J., and Beukes,D.R. (2008). Fucoxanthin, Tetraprenylated Toluquinone and Toluhydroquinone Metabolites from *Sargassum heterophyllum* Inhibit the *in vitro* Growth of the Malaria Parasite *Plasmodium falciparum*. Z Naturforsch 63c:848-852.

Ang, K.K.H., Holmes, M.J., Higa, T., Hamann, M.T., and Kara, U.A. (2000). *In vivo* Antimalarial Activity of the Beta-Carboline Alkaloid Manzamine A. Antimicrobial agents and Chemotherapy 44:1645-1649.

Angerhofer, C.K., Pezzuto, J.M., Konig, G.M., Wright, A.D., and Sticher, O. (1992). Antimalarial activity of sesquiterpenes from the marine sponge *Acanthella klethra*. Journal of Natural Products 55:1789.

Balint, G.A. (2005). Artemisinin and its derivatives: an important new class of antimalarial agents. Pharmacology and Therapeutics 90:261-265.

Blunt, J.W., Copp, B.R., Munro, M.H.G., Northcote, P.T., and Prinsep, M.R. (2010). Marine natural products. Natural Products Reports 27:165-237.

Butler, M.S. (2005). Natural products to drugs: natural product derived compounds in clinical trials. Natural Products Reports 22:162-195.

Chima, R.I., Goodman, C.A., and Mills, A. (2003). The economic impact of malaria in Africa: a critical review of the evidence. Health Policy 63:17-36.

Chin, Y-C., Balunas, M.J., Chai, H.B., and Kinghorn, D. (2006). Drug Discovery From Natural Sources . The American Association of Pharmaceutical Scientists Journal 8:239-253.

Cui, L. and Su, X. (2009). Discovery, mechanisms of action and combination therapy of artemisinin. Expert Reviews of Anti-infective Therapy 7:999-1013.

Donia, M. and Hamann, M.T. (2003). Marine natural products and their potential applications as anti-infective agents. The Lancet- Infectious Diseases 3:338-348.

Fattorusso, E., Parapini, S., Campagnuolo, C., Basilico, N., Taglialatela-Scafati, O., and Taramelli, D. (2002). Activity against *Plasmodium falciparum* of cycloperoxide compounds obtained from the sponge *Plakortis simplex*. Journal of Antimicrobial Chemotherapy 50:883-888.

Faulkner, D.J. (2000a). Highlights of marine natural products chemistry (1972-1999). Natural Products Reports 17:1-6.

Faulkner, D.J. (2000b). Marine natural products. Natural Products Reports 17:7-55.

Faulkner, D.J. (2000c). Marine pharmacology. Antonie van Leewenhoek 77:135-145.

Foote, S.J. and Cowman, A.F. (1994). The mode of action and the mechanism of resistance to antimalarial drugs. Acta Tropica 56:157-171.

Greenwood, D. (1995). Conflicts of interest: the genesis of synthetic antimalarial agents in peace and war. Journal of Antimicrobial Chemotherapy 36:857-872.

Greenwood, D. (2001). History of Antimalarial Agents. John Wiley & Sons, Ltd.

Gross, H. and Konig, G.M. (2006). Terpenoids from marine organisms: unique structures and their pharmacological potential. Phytochemistry Reviews 5:115-141.

Ham, Y.M., Kim, K.-N., Lee, W.J., Lee, N.H., and Hyun, C.G. (2010). Chemical Cnstituents from *Sargassum micracanthum* and antioxidant activity. International Journal of Pharmacology 6:147-151.

Holler, Ulrich. Isolation , biological activity and secondary metabolite investigations of marine derived fungi and selected host sponges. PhD disertation, University of Braunscheig,Braunscheig, German. 1999.

Konig, G.M., Kehraus, S., Seibert, S.F., Abdel-Lateff, A., and Muller, D. (2006). Natural Products from Marine Organisms and Their Associated Microbes. ChemBioChem 7:229-238.

Kreidenweiss, A., Mordmulle, B., Krishna, S., and Kremsner, P.G. (2006). Antimalarial Activity of a Synthetic Endoperoxide (RBx-11160/OZ277) against *Plasmodium*

falciparum Isolates from Gabon. Antimicrobial agents and Chemotherapy 50:1535-1537.

Lane, A.L. and Moore, B.S. (2011). A sea of biosynthesis: marine natural products meet the molecular age. Natural Products Reports 28:411-428.

Laurent, D. and Pietra, F. (2006). Antiplasmodial Marine Natural Products in the Perspective of Current Chemotherapy and Prevention of Malaria. A Review. Marine Biotechnology 8:433-447.

Mancini, I., Guella, G., and Defant, A. (2009).Synthesis of Marine Natural Products with Antimalarial Activity. Mini Reviews in Medicinal Chemistry. 8:1265-1284

Mancini, I., Guella, G., Sauvain, M., Debitus, C., Duigou, A.G., Ausseil, F., Menou, J.L., and Pietra, F. (2004). New 1,2,3,4-tetrahydropyrrolo[1,2-a]pyrimidinium alkaloids (phloeodictynes) from the New Caledonian shallow-water haplosclerid sponge *Oceanapia fistulosa*. Structural elucidation from mainly LC-tandem-MS-soft-ionization techniques and discovery of antiplasmodial activity. Organic and Biomolecular Chemistry 2:783-787.

Mather, M.W., Darrouzet, E., Valkova-Valchanova, M., Cooley, J.W., McIntosh, M.T., Daldal, F., and Vaidya, A.B. (2005). Uncovering the Molecular Mode of Action of the Antimalarial Drug Atovaquone Using a Bacterial System. Journal of Biological Chemistry 280:27458-27465.

Mendiola, J., Hernandez, H., Sariego, I., Rojas, L., Otero, A., Ramirez, A., de los Angeles Chavez, M., Payrol, J.A., and Hernandez, A. (2006). Antimalarial activity from three ascidians: an exploration of different marine invertebrate phyla. Transactions of the Royal Society of Tropical Medicine and Hygiene 100:909-916.

Michalakis, Y. and Renaud, F. (2009). Malaria: Evolution in vector control. Nature 462:298-300.

Miyaoka, H., Shimomura, M., Kimura, H., Yamada, Y., Kim, H., and Wataya, Y. (1998). Antimalarial activity of kalihinol A and new relative diterpenoids from the Okinawan sponge *Acanthella* sp. Tetrahedron 54:13474.

O'Neill, P.M., Barton, V.E., and Ward, S.A. (2010). The Molecular Mechanism of Action of Artemisin - The Debate Continues. Molecules 15:1705-1721.

Olliaro, P. (2001). Mode of action and mechanisms of resistance for antimalarial drugs. Phamacology & Therapeutics 89:207-219.

Park, B.G., Shin, W.S., Um, Y., Cho, S., Park, G.M., Yeon, D.S., Kwon, S.C., Ham, J., Choi, B.W., and Lee, S. (2008). Selective vasodilatation effect of sargahydroquinoic acid, an active constituent of *Sargassum micracanthum*, on the basilar arteries of rabbits. Bioorganic & Medicinal Chemistry Letters 18:2624-2627.

Paterson, I. and Findlay, A.D. (2008). Total synthesis of cytotoxic marine macrolides: Callipeltoside A, Aurisides A and B, and Dolastatin 19. Pure and Applied Chemistry 80:1773-1782.

Plowe, C.V., Alfonso, P., and Hoffman, S.L. (2009). The potential Role of Vaccines in the Elimination of *Falciparum* Malaria and the Eventual Eradication of Malaria. The Journal of Infectious Diseases 200:1646-1649.

Pomponi, S.A. (1999). The bioprocess-technological potential of the sea. Journal of Biotechnology Volume 70:5-13.

Ravichandran, S., Kathiresan, K., and Balaram, H. (2007). Anti-malarials from marine sponges. Biotechnology and Molecular Biology Review 2:33-38.

Sacarlal, J., Aide, P., Aponte, J.J., Renom, M., Leach, A., Mandomando, I., Lievens, M., Bassat, Q., Lafuente, S., Macete, E., Vekemans, J., Guinovart, C., Sigauque, B., Sillman, M., Milman, J., Dubois, M.C., Demoitie, M.A., Thonnard, J., Menendez, C., Ballou, W.R., Cohen, J., and Alonso, P.L. (2009). Long-Term Safety and Efficacy of the RTS,S/AS02A Malaria Vaccine in Mozambican Children. Journal of Infectious Diseases 200:329-336.

Srivastava, I.K., Rottenberg, H., and Vaidya, A.B. (1997). Atovaquone, a Broad Spectrum Antiparasitic Drug, Collapses Mitochondrial Membrane Potential in a Malarial Parasite. Journal of Biological Chemistry 272:3961-3966.

Talisuna, A.O., Bloland, P., and D'Alessandro, U. (2004). History, Dynamics, and Public health Importance of Malaria Parasite Resistance. Clinical Microbiology Reviews 17:235-254.

White, N.J. (2008). Qinghaosu (Artemisinin): The Price of Success. Science 320:330-334.

White, N.J., Nosten, F., Hien, T.T., Watkins, W.M., and Olliaro, P. (1999). Averting a malaria disaster. The Lancet 354:1389-1390.

Williams, D.A. and Lemke, T.L. (2002). Antiparasitic Agents . Foye's Principles of Medicinal Chemistry.Philadelphia: Lippincott Williams and Wilkins. 867-890.

World Health Organisation. Guideines for the treatment of Malaria , 2nd Edition. 2010a. Geneva , Switzerland, WHO Press.

World Health Organisation. World Malaria Report 2010. WHO. 2010b. Geneva, Switzerland, WHO Press.

World Health Organisation. Consideration of Mass Drug Administratio for the containment of artemisinin resistant malaria in the Greater Mekong Subregion. WHO. 2011a.

World Health Organisation. Malaria, countries or areas at risk of transission 2010. gamapserverver.who.int/mapLibrary/Files/Maps/Global_Malaria_2010.png . 2011b.

Wright, A.D., Konig, G.M., Angerhofer, C.K., Greenidge, P., Linden, A., and Desqueyroux-Faundez, R. (1996). Antimalarial activity:the search for marine-derived natural products with selective antimalarial activity. Journal of Natural Products 59:710-716.

Wright, A.D. and Lang-Unnasch, N. (2005). Potential Antimalarial Lead Structures from Fungi of Marine Origin. Planta Medica 71:964-966.

Wright, C.W. (2005). Plant derived antimalarial agents: New leads and challenges. Phytochemistry Reviews 4:66-61.

Ziegler, H.L., Staerk, D., Christensen, J., Hviid, L., Hagerstrand, H., and Jaroszewski, J.W. (2002). In *vitro Plasmodium falciparum* Drug Sensitivity Assay: Inhibition of Parasite Growth by Incorporation of Stomatocytogenic Amphiphiles into the Erythrocyte Membrane. Antimicrobial agents and Chemotherapy 46:1441-1446.

Chapter 2

Extraction and Isolation of Tetraprenylated Toluquinones and Fucoxanthin from *Sargassum heterophyllum*

2.1 Introduction

Macro-algae produce a variety of secondary metabolites, the natural functions of which have been widely investigated (Hellio et al., 2004; da Gama et al., 2002). Findings from these investigative studies have shown that secondary metabolites play a role in chemical mediation as a defence mechanism against herbivores and microorganisms in their immediate environment (Simpson, 1987; Hellio et al., 2004; Da Gama et al., 2002; Hay et al., 1987; Paul and Van Alstyne, 1992; Kato et al., 1975). In the past, secondary metabolites have been regarded to be 'waste products' of living organisms with no specific functions (Verpoorte, 1998; Croteau et al., 2000). However, increasing evidence into the varying roles of these compounds has been extensively presented. Despite this previous opinion on secondary metabolites, organic chemists have studied the chemical properties of these phytochemicals from as early as the 1850s (Maplestone et al., 1992; Stone and Williams, 1992; Da Gama et al., 2002).

2.1.1 *Sargassum* species

Sargassum species are a species of algae occurring widely in warm and temperate waters of tropical and sub-tropical oceans (Dar A et al., 2007; Gillespie and Critchley, 1999). Comprising of 150 species, *Sargassum* species, commonly known as gulf-weed or sea holly belong to the order Fucales, subclass Cyclosporeae and class Phaeophyceae (Dar A et al., 2007). The chemical composition of *Sargassum* species has been studied extensively (Reddy and Urban, 2009; Blunt et al., 2008). This has resulted in the isolation of, phloroethols (e.g. **2.1**) (Glombitza et al., 1997), flavanoids (e.g. **2.2**), dipeptides (e.g. **2.3**), coumarins (e.g. **2.4**), and sterols (e.g. **2.5**) (Harvey and Kennicutt , 1992; Liu et al., 2009).

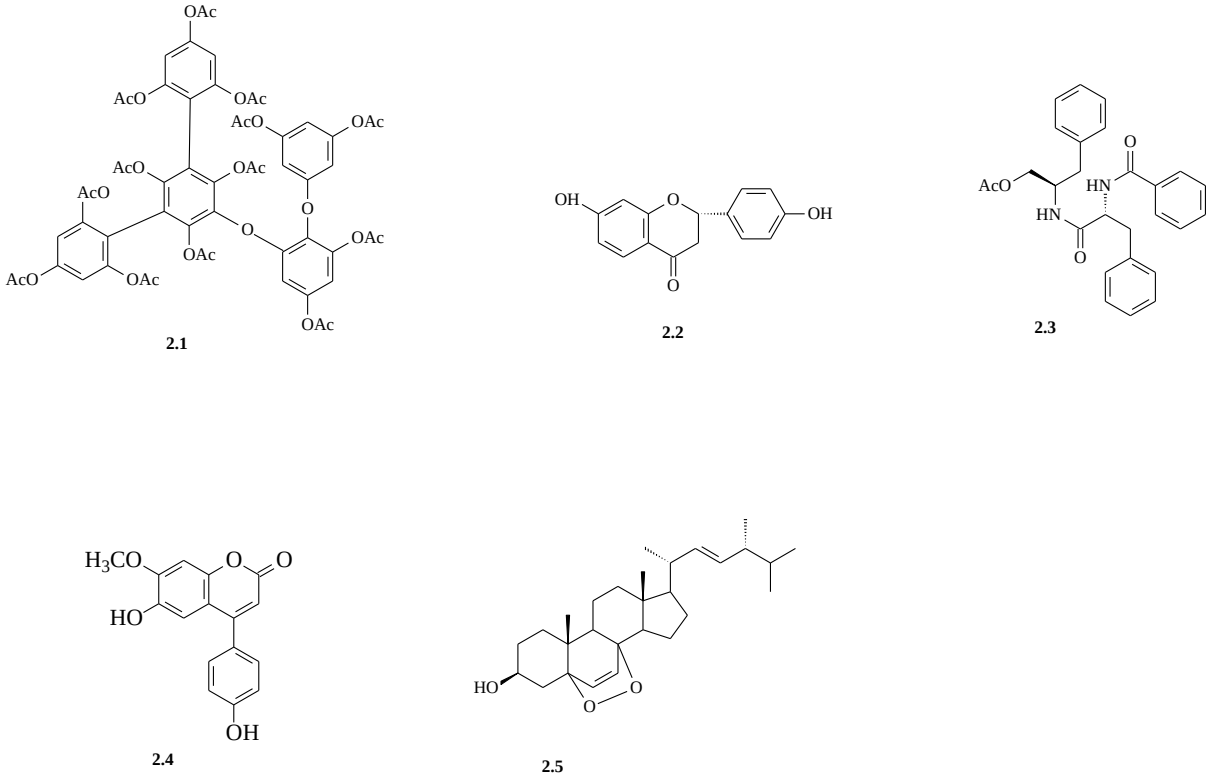


Figure 2.1 Selected structural classes of metabolites isolated from *Sargassum* species

Polyprenylated metabolites such as; fallahydroquinone **(2.6)**, fallaquinone **(2.7)** and fallachromenoic acid **(2.8)** have been isolated from the Southern Australian alga *Sargassum fallax* together with the prenylated toluquinones and toluhydroquinone ; sargaquinone **(2.9)** sargahydroquinoic acid **(1.38)** and sargaquinoic acid **(1.39)** (figure 2.2) (Reddy and Urban, 2009). These compounds, are generally composed of a polyprenyl chain connected to a hydroquinone ring moiety (Reddy and Urban, 2009) and have been isolated from coelenterates, fish, sponges and tunicates, amongst other marine organisms (Jung et al., 2008; Jang et al., 2005). Prenylated toluquinones isolated from *Sargassum* species usually have the same structural backbone, differing mainly in the arrangement of substituents in the linear terpene moiety (Reddy and Urban, 2009).

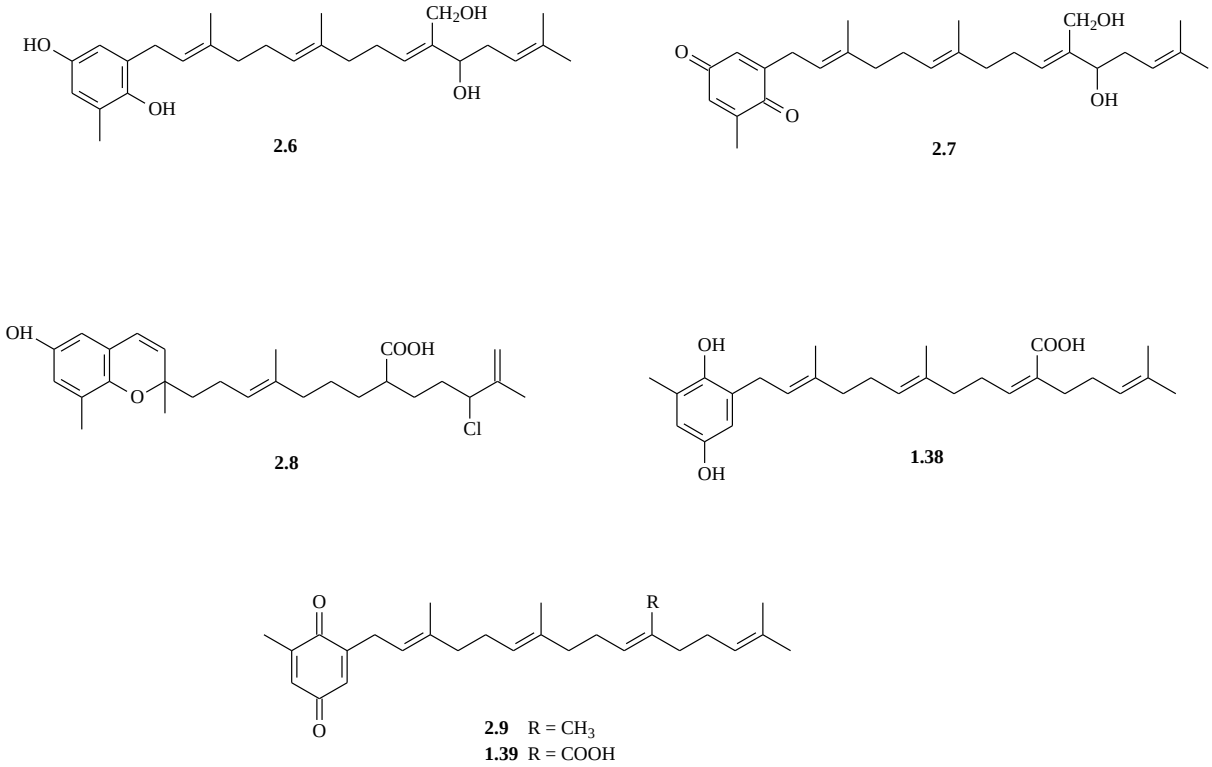


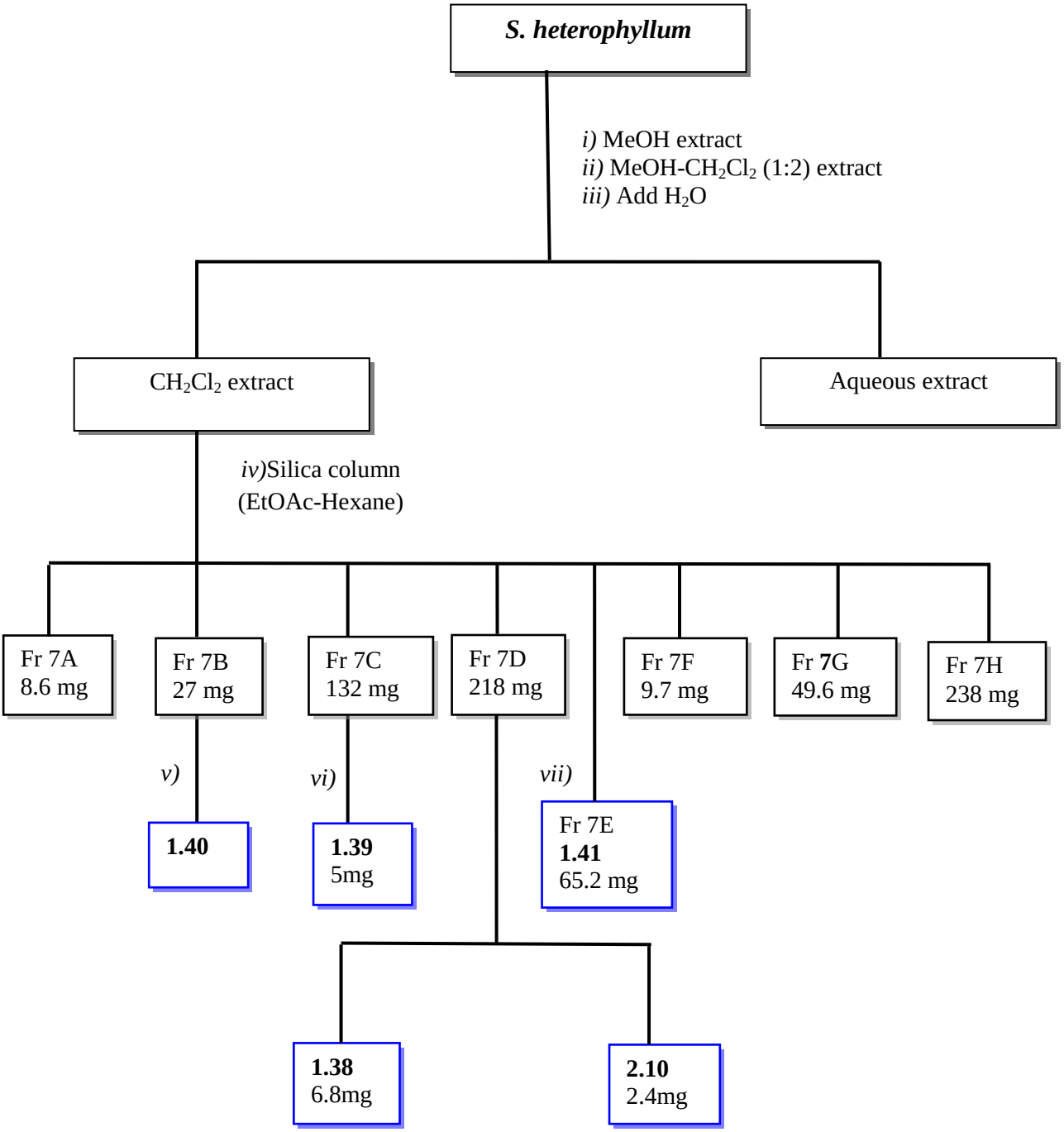
Figure 2.2 Prenylated metabolites isolated from *Sargassum* species

We have previously reported on the antiplasmodial activity of prenylated toluquinols from *S. heterophyllum* (Afolayan et al., 2008). The objectives of the research presented in this chapter were to: isolate selected natural products as reference compounds for further studies of their antiplasmodial activity and to obtain sufficient amounts of sargahydroquinoic acid **(1.38)** to be used as the starting material for the chemical modification of this compound.

2.2 Results and Discussion

2.2.1 Extraction and isolation of compounds

Specimens of *S. heterophyllum* (PA071b) were collected from Port Alfred on the south eastern coast of South Africa on 21 September 2007. The alga was extracted with MeOH, followed by MeOH-CH_2Cl_2 (1:2). The extracts were pooled and separated into aqueous and organic phases by the addition of deionised water. Concentration of the organic phase under reduced pressure gave a dark green residue. A portion of this residue was fractionated by gradient elution on a silica gel column using solvents of differing compositions of EtOAc and Hexane resulting in seven fractions (7A-G). Selected fractions, containing ^{1}H NMR signals of interest, were further purified by HPLC or silica gel column chromatography to give compounds **1.38** to **1.41** in addition to compound **2.10** (scheme 2.1).



Scheme 2.1 Isolation of compounds **1.38**, **1.39**, **1.40**, **1.41** and **2.10** from *S. heterophyllum* *v)* Silica gel column chromatography, solvent Hexane: EtOAc (9:1) *vi)* Normal phase HPLC, Hexane-EtOAc (80:20) *vii)* Reversed Phase HPLC MeOH: H_2O (90:10).

2.2.2 Characterization of isolated compounds

Not surprisingly, the ^{1}H NMR spectra of compounds **1.38-1.40** were very similar. Key signals in these spectra included aromatic signals between δ 6.50 and δ 6.26 ppm (table 2.1). In addition to these signals, all three compounds had more than one signal between δ 5.38 and δ 5.10 ppm indicating the presence of unsaturated hydrocarbon moieties. The ^{1}H NMR spectrum of compound **1.38** (figure 2.3) showed two aromatic singlets at δ 6.50 ppm and δ 6.46 ppm (H-3 and H-5) along with three groups of olefinic methine triplets at δ 6.0 ppm (H-10'), δ 5.27 ppm (H-2') and δ 5.1 ppm (H-6' and H-14'). Two methylene signals at δ 3.29 ppm (H-1') and δ 2.58 ppm (H-9') were also visible in the ^{1}H NMR spectrum of this compound.

Two key differences between the ^{1}H NMR spectra of **1.38** and **1.39** were observed. Firstly, the aromatic protons in **1.39** were more deshielded occurring at δ 6.55 and δ 6.47 ppm compared to δ 6.50 and δ 6.46 ppm compared in compound **1.38** (figure 2.4). In addition to this, the methylene proton signal in **1.38** appeared at δ 3.29 ppm compared to δ 3.13 ppm in **1.39**. The ^{13}C NMR spectrum of these two compounds showed a total of 27 carbons with three carbonyl signals for **1.39** occurring at δ 188.8, δ 187.9 and δ 172.7 ppm while that of compound **1.38** revealed only one carbonyl signal carbonyl signal at δ 172.4 ppm (figure 2.5). The carbonyl signals at δ 172 ppm in both compounds were characteristic of a carboxylic acid moiety while the other carbonyl functional groups in **1.39** corresponded to those found in benzoquinones. Comparison of spectral data for these compounds with that of metabolites previously isolated from *Sargassum* species in literature allowed for the identification of these compounds as sargahydroquinoic acid **(1.38)** and sargaquinoic acid **(1.39)**. Both these metabolites have been previously isolated from *S. heterophyllum* (Afolayan et al., 2008) and *S. thunbergii* (Seo et al., 2004) amongst other species of the *Sargassum* genus.

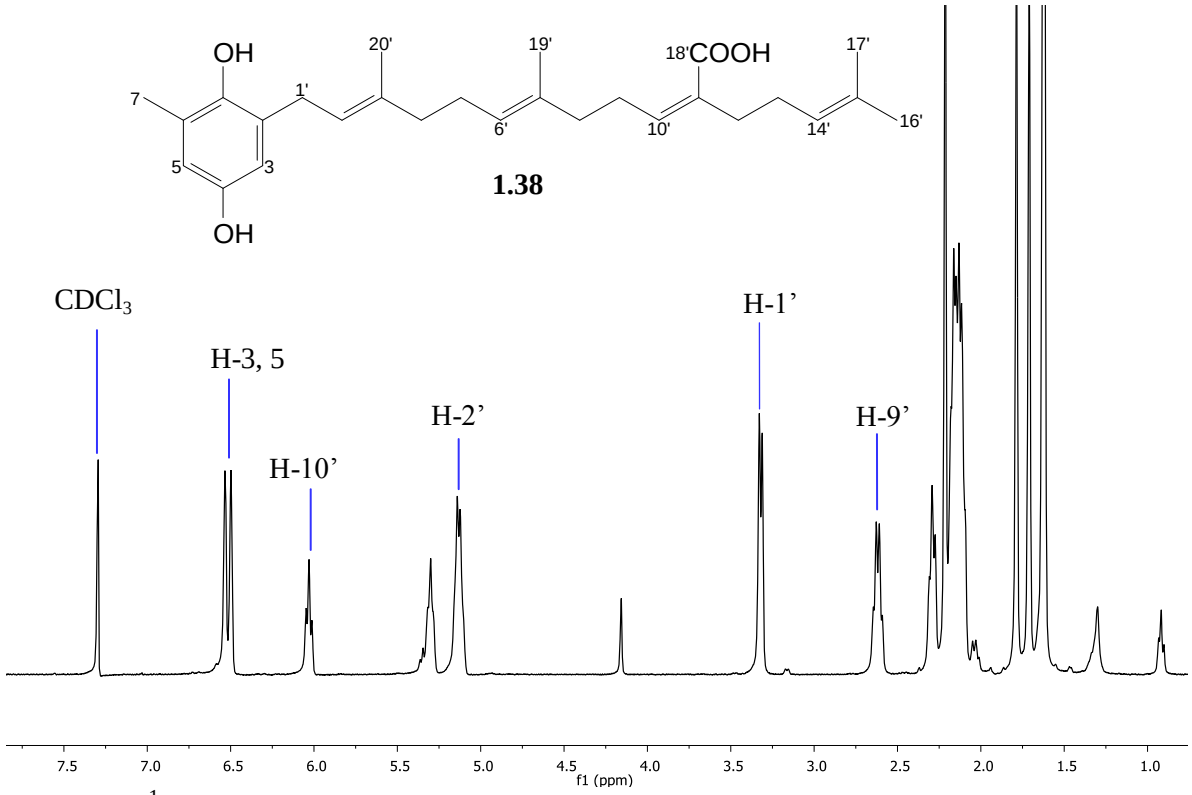


Figure 2.3 ^{1}H NMR spectrum of compound **1.38** (400 MHz, $CDCl_3$)

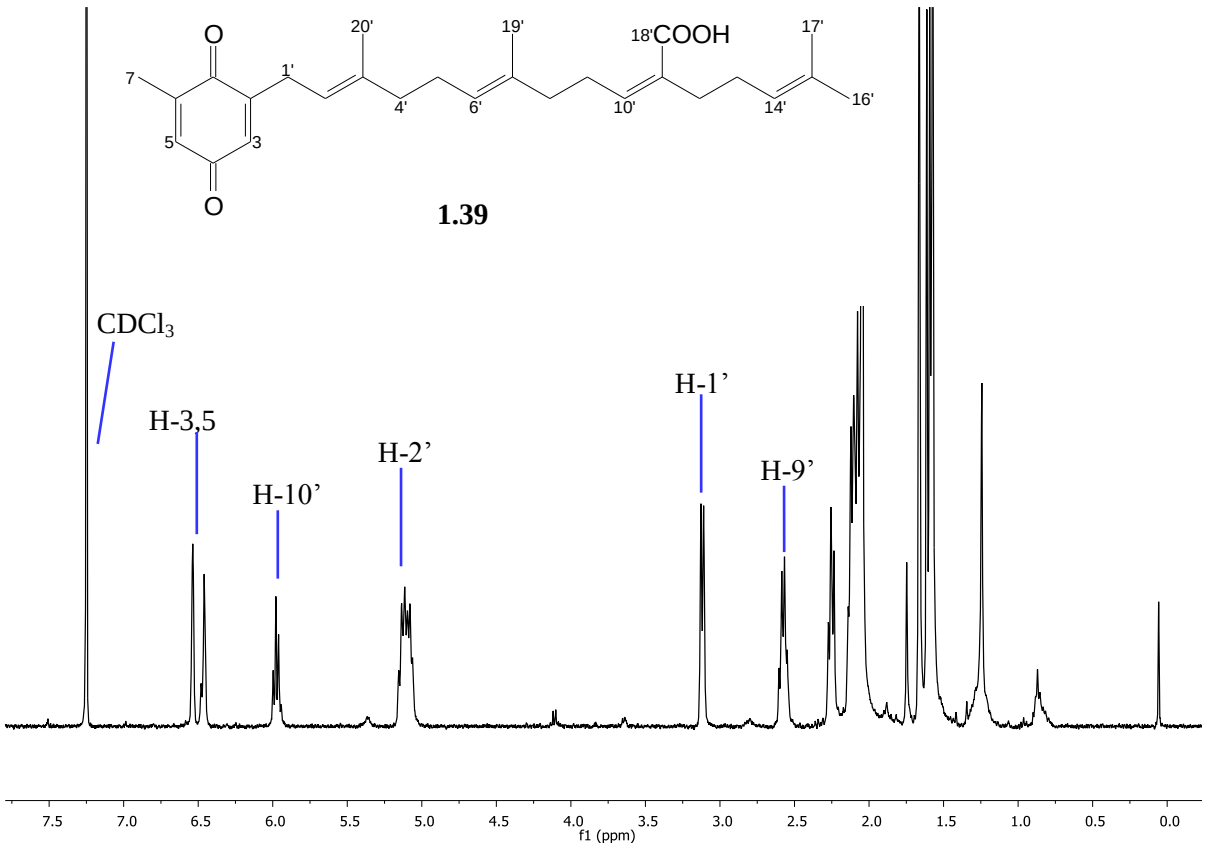


Figure 2.4 ^{1}H NMR spectrum of compound **1.39** (400 MHz, $CDCl_3$)

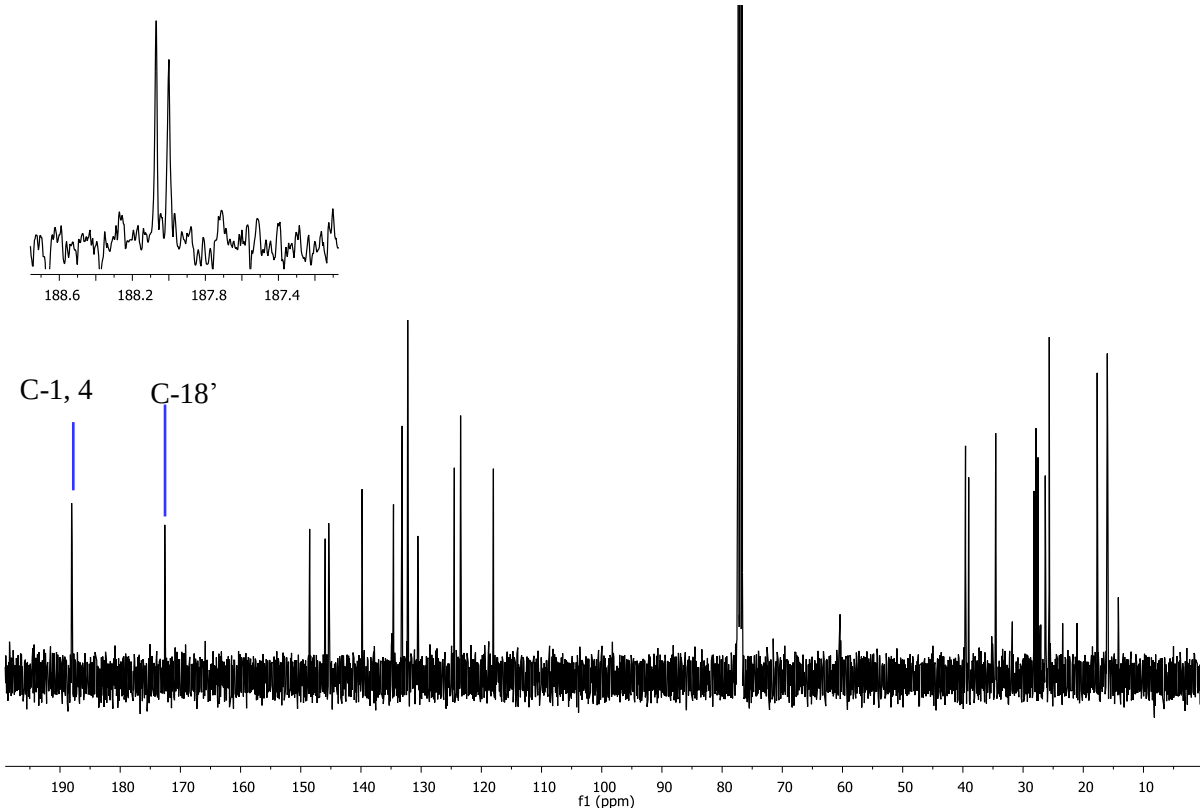


Figure 2.5 ^{13}C NMR spectrum of compound **1.39** (400 MHz, $CDCl_3$) with the insert showing an expansion of the region between δ 188.6 and δ 187.4 ppm

Table 2.1 ^{1}H and ^{13}C NMR (400 and 100 MHz) data for compounds **1.38**, **1.39**, **1.40** and **2.10** recorded in $CDCl_3$.

	1.38		**1.39**		**1.40**		**2.10**	
Carbon no.	δ_C	δ_H, mult, J_{Hz}	δ_C	δ_H, mult, J_{Hz}	δ_C	δ_H, mult, J_{Hz}	δ_C	δ_H, mult, J_{Hz}
1	146.4		188		187.96		145.6	
2	125.5		145.9		145.9		121.3	
3	115.4	6.50, br s	133.1	6.55, s	132.3	6.54, s	110.3	6.32, d, 2.5
4	148.7		187.9		187.91		148.5	
5	113.9	6.46, br s	132.2	6.47, s	132.1	6.43, s	117.1	6.47 ,d, 2.4
6	127.6		148.5		148.4		126.3	
7	16.1	2.18, s	17.7	2.06, m	16	1.67	15.7	2.13, s
1’	29.9	3.29 , d, 6.90	27.5	3.13, d, 7.3	27.3	3.12, d, 7.2	122.9	6.26, s
2’	121.7	5.27, t, 6.47	117.9	5.12, dt	118.2	5.10, dt, 6.8	130.7	5.57, d, 9.8
3’	138.2		139.8		139.9		77.8	
4’	39.5	2.07, m	39.6	2.08, m	39.5	2.06, m	40.8	1.68, s
5’	26.0	2.11, m	26.3	2.09, m	26.4	2.06, m	22.6	2.06, m
6’	124.2	5.10, d , 6.70	124.5	5.12, m	125.5	5.10, dt, 6.8	124.9	5.12, dt, 19, 6.4
7’	134.7		134.6		133.6		134.3	
8’	39.0	2.07, m	39	2.09, m	39.4	2.24, m	39.1	2.06, m
9’	28.3	2.58, q, 7.0	28.2	2.59, q, 7.5	27.5	2.06, m	28.1	2.59, q, 7.3
10’	145.5	6.00, t, 6.90	145.4	6.00, t, 7.2	154.9	5.38, m	144.9	5.98, t, 7.2
11’	130.5		130.6		132.1		130.5	
12’	34.5	2.26, t , 7.1	34.5	2.26, t	27.3	2.06, m	34.5	2.27, t, 7.4
13’	27.8	2.11, m	27.9	2.11, m	25.7	1.67, s	27.9	2.13, s
14’	123.4	5.10, d, 6.70	123.4	5.12, m	123.6	5.10, t, 6.8	123.4	5.12, dt, 19, 6.4
15’	132.3		132.2		133.2		132.3	
16’	25.7	1.68, s	25.6	1.67, s	25.2	1.67, s	25.7	1.68, s
17’	17.7	1.58, s	16.1	1.58, s	17.7	1.57, s	17.7	1.58, s
18’	172.4		172.7		205.4	9.55, s	172.9	
19’	16.0	1.59, br s	16	1.60, s	16	1.62, s	15.5	1.58, s
20’	16.1	1.75, s	16.1	1.62, s	16.1	1.62, s	25.9	1.68, m

The ^{1}H NMR spectrum of compound **1.40** was similar to that of sargaquinoic acid (**1.39**), except for the presence of a signal at δ 9.55 ppm. This signal, characteristic of an aldehyde was confirmed by the presence of a carbonyl signal at δ_C 205.4 ppm in the ^{13}C NMR spectrum of this compound (table 2.1). Compound **1.40** was identified as 2'*E*, 6'*E*, 10'*E* - sargaquinal, a compound that has been previously isolated from *S. heterophyllum* (Afolayan et al., 2008) and *S. serratifolium* (Kusumi et al., 1979).

There are discrepancies between the ^{13}C NMR data for 2'*E*, 6'*E*, 10'*E* - sargaquinal reported by Afolayan et al., and the data reported herein. The chemical shift of C-11' has been reported to resonate at δ 143.2 ppm by Afolayan et al., while this carbon resonates at δ 132.1 ppm in this study. The previously reported chemical shift for this carbon seems anomalous as the carbon atoms at the same position in other compounds of this series; namely sargahydroquinoic acid **(1.38)** and sargaquinoic acid **(1.39)** resonate between δ 130.5 and δ 132.1 ppm. In addition to this, the aldehyde signal (C-18') reported by Afolayan et al., resonates at δ 195.1 ppm while the same signal in compound **1.40** is at δ 205.4 ppm. As a result of this, there are differences in the ^{1}H NMR data due to the aldehyde signal (H-18'), with Afolayan et al., reporting the resonance of H-18' at δ 9.33 ppm while this proton is observed to resonate at δ 9.55 ppm in the present study. Unfortunately, the second reference in which 2'*E*, 6'*E*, 10'*E* - sargaquinal has been reported (Kusumi et al., 1979) does not contain ^{13}C NMR data and conclusive ^{1}H NMR data for this compound. To this effect none of these discrepancies affect the double bond geometry of this compound

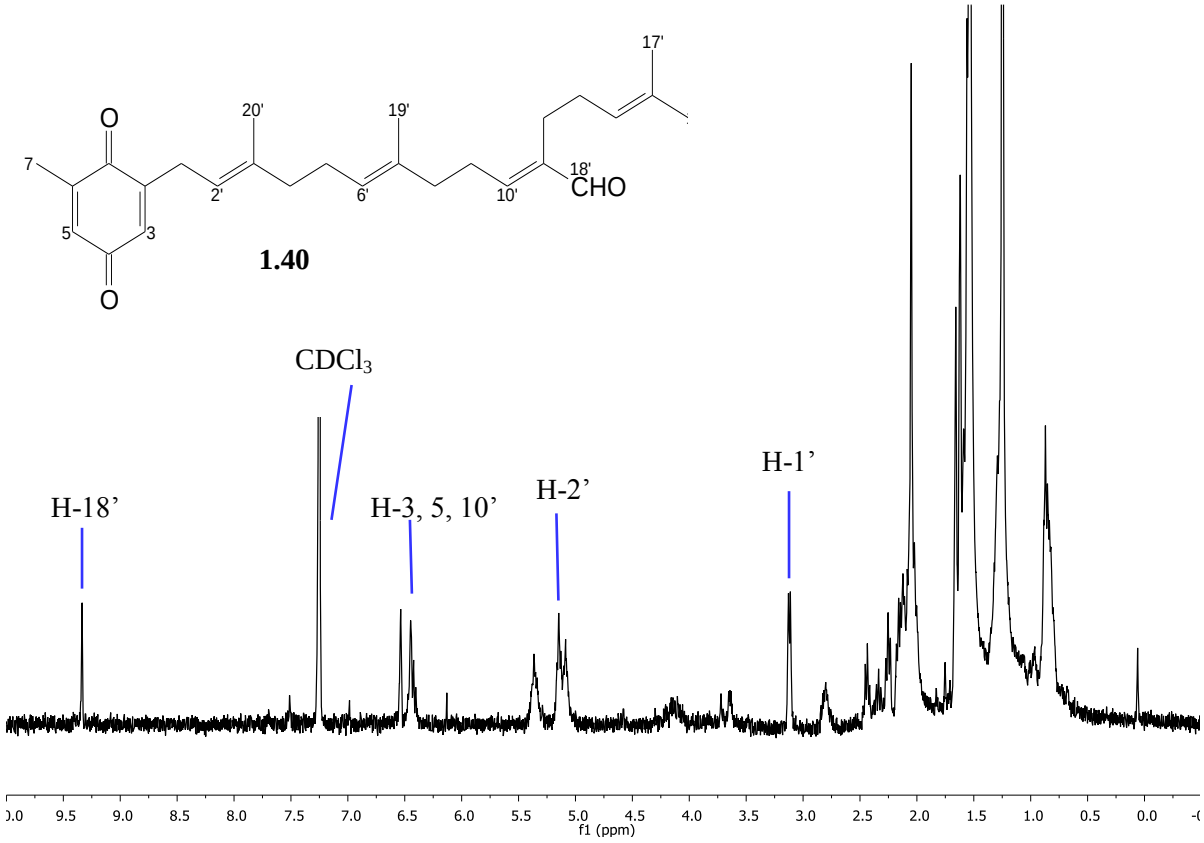


Figure 2.6 ^{1}H NMR spectrum of compound **1.40** (400 MHz, $CDCl_3$)

The ^{13}C NMR spectrum of compound **2.10** showed that it had the same number of carbons as compounds **1.38-1.40** with signals indicative of both the carboxylic acid (δ 172.9 ppm) and an aromatic ring (δ 148.5 ppm and δ 145.6 ppm) (table 2.1). However, the ^{1}H NMR spectrum of compound **2.10** was different to that of compounds **1.38-1.40**. While the two aromatic singlets were still present at δ 6.32 ppm (H-3) and δ 6.47 ppm (H-5) one of the olefinic triplets and the benzylic methylene at δ 3.29 ppm (H-1') had disappeared (figure 2.7). Instead, two new, mutually coupled olefinic signals at δ 6.26 (H-1') and δ 5.57 ppm (H-2') were apparent. A comparison of NMR spectral data for compound **2.10** with spectral data of the chromenols previously isolated from *Sargassum* species led to the identification of compound **2.10** as sargachromenol. This compound has been previously isolated from *S. micracanthum* (Ham et al., 2010).

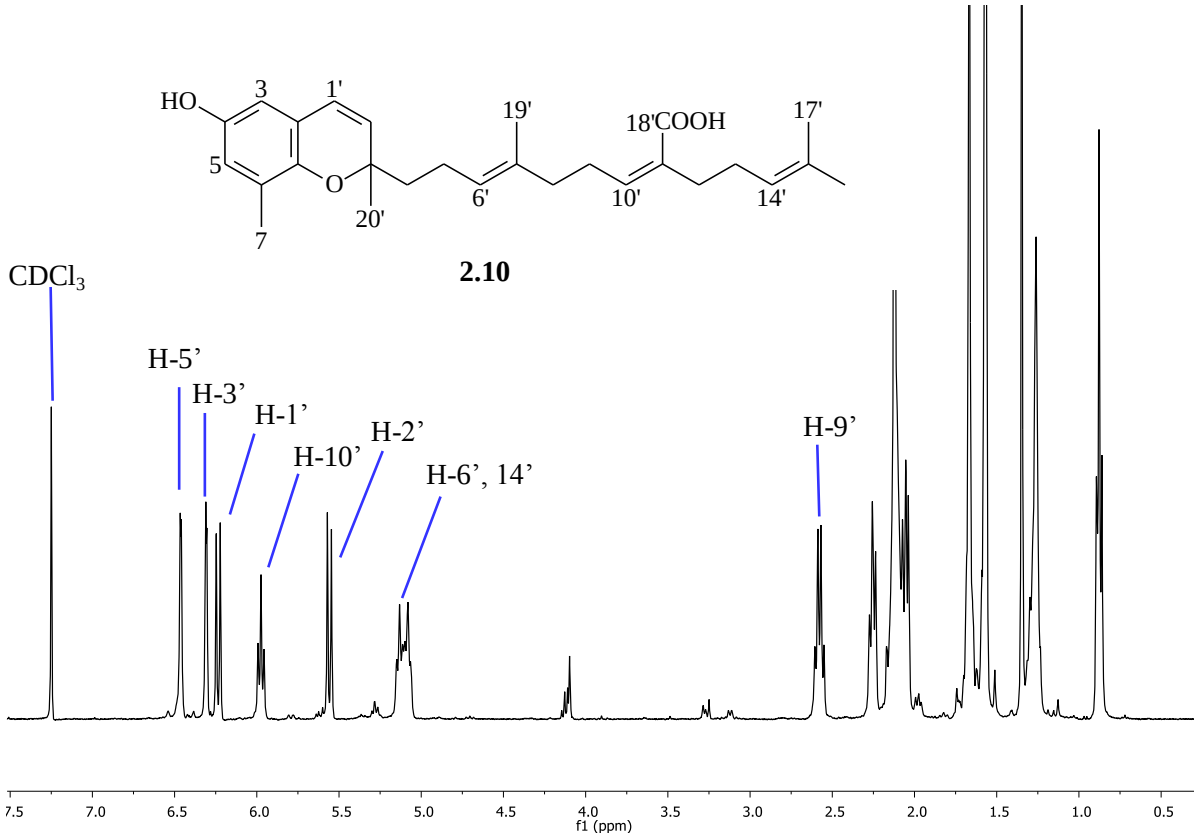


Figure 2.7 ^{1}H NMR spectrum of compound **2.10** (400 MHz, $CDCl_3$)

The ^{1}H NMR spectrum of **2.41** (figure 2.8) was different from the spectra of compounds **1.38**, **1.39**, **1.40** and **2.10**. It was characterised by a distinct doublet at δ 7.15 ppm in addition to a group of signals between δ 6.78 and δ 6.05 ppm. The ^{13}C NMR spectrum of this compound presented a set of resonances in different regions compared to those observed in compounds **1.38**, **1.39**, **1.40** and **2.10** (table 2.2). This, together with the fact that 42 resonances in the ^{13}C NMR spectrum of **2.14** as opposed to the 27 resonances observed in compounds **1.38**, **1.39**, **1.40** and **2.10** suggested that this molecule was of a different structural class (table 2.2). Comparison of ^{1}H and ^{13}C NMR data of this compound to other known compounds that had been previously isolated from *S. heterophyllum* (Afolayan et al., 2008) allowed for the positive identification of **1.41** as fucoxanthin.

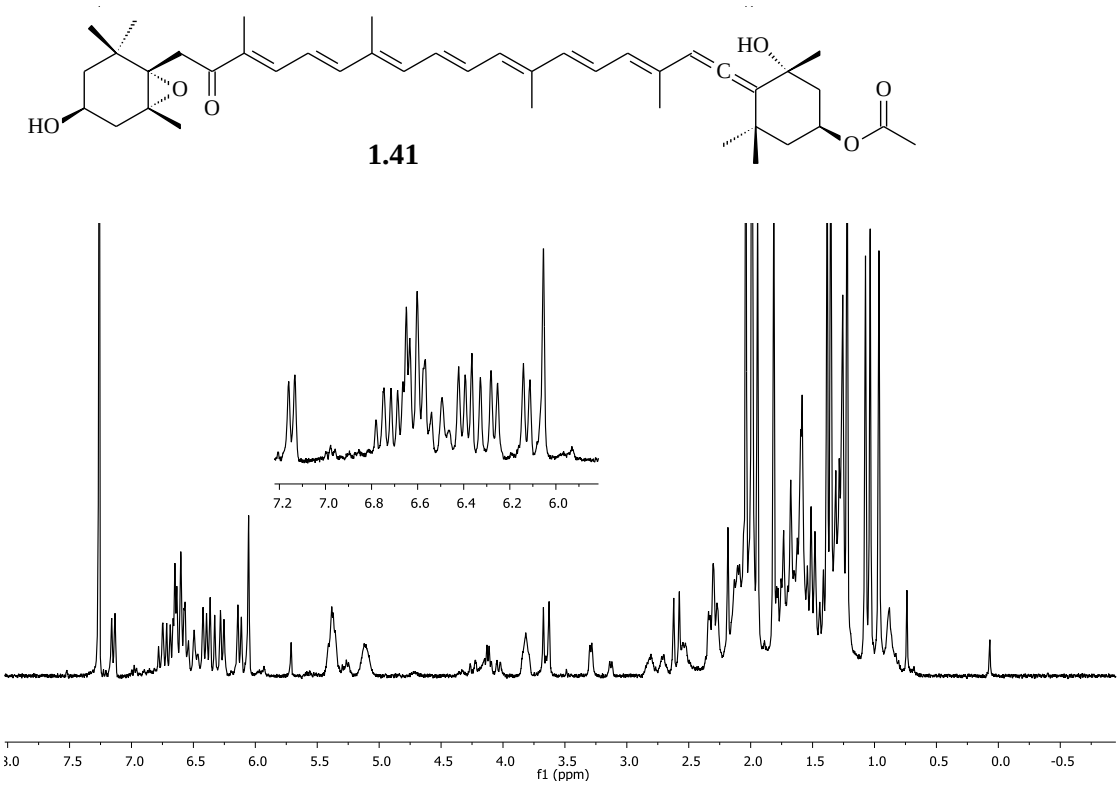


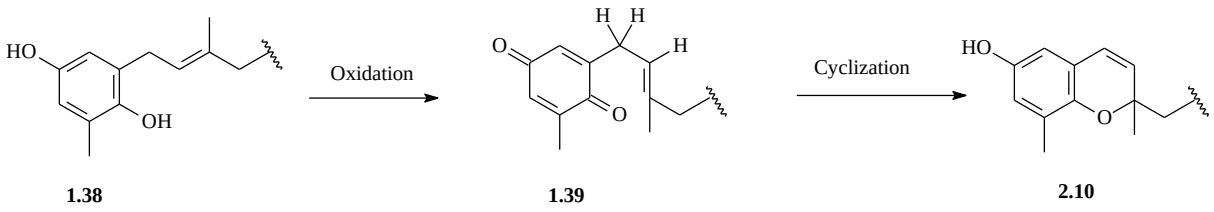
Figure 2.8 ^{1}H NMR spectrum of **1.41** (400 MHz, $CDCl_3$)

Table 2.2 ^{1}H and ^{13}C NMR (400 and 100 MHz, $CDCl_3$) data for compound **1.41**

Position No.	δ_C	δ_H	Carbon No.	δ_C	δ_H
1	35.76		**11**	123.36	6.54
1'	35.14		**11'**	125.66	6.58
2	47.1		**12**	144.99	6.66
2'	45.43	1.48	**12'**	137.1	6.33
3	64.3	3.82	**13**	135.4	
3'	68	5.37	**13'**	18.05	
4	41.65	1.73	**14**	136.6	6.39
4'	45.23	1.51	**14'**	132.15	6.25
5	66.12		**15**	129.4	6.63
5'	72.67		**15'**	132.47	6.75
6	67.08		**16**	25.03	1.03
6'	117.53		**16'**	21.36	1.81
7	40.8	2.58	**17**	28.1	0.96
7'	202.34	3.63	**17'**	32.06	1.07
8	197.84		**18**	21.14	1.22
8'	103.38	6.05	**18'**	31.26	1.35
9	134.54		**19**	11.8	1.94
9'	132.47		**19'**	13.99	1.81
10	139.1	7.13	**20**	12.74	1.99
10'	128.5	6.11	**20'**	12.89	1.99

The purification of the CH_2Cl_2 extract of *S. heterophyllum* yielded five distinct compounds. All these compounds have been identified as metabolites occurring in *Sargassum* species. Sargachromenol (**2.10**) has also been isolated from the fruit of a terrestrial Amazonian tree *Iryanthera juruensis* (*Myristicaceae*) (Silva et al., 2001). Most of the metabolites isolated and characterised in this study were polyprenylated toluquinones, with the metabolite of highest abundance being the tetraprenylated toluhydroquinone; sargahydroquinoic acid **(1.38)**. The presence of these compounds in *Sargassum* species is expected, as algae in the order; Fucales are known to produce phenolic compounds as their most dominant secondary metabolites which may amount to up to 30% of the algal dry mass (Plouguerne et al., 2006). During the purification of metabolite **1.38** we observed its slow oxidation to **1.39**, its benzoquinone analogue. In addition to this we also observed the conversion of **1.39** to **2.10** (scheme 2.2). This *in vitro* transformation of hydroquinones into quinones which are in-turn converted to

chromanols in *Sargassum* metabolites has been reported in the literature literature (Perez-Castorena et al., 2002; Kusumi et al., 1979).



1.38 **1.39** **2.10**

Scheme 2.2 Reaction scheme showing the conversion of sargahydroquinoic acid **(1.38)** to sargachromenol **(2.10)**

2.3 Experimental

2.3.1 General Experimental

All extraction solvents were of analytical grade, purchased from commercial suppliers (Merck, Germany) and used without further purification. Silica gel (40-63 μm particle size), (Merck, Germany) was used for column chromatography. HPLC was carried out on a Waters 1525 HPLC system fitted with a waters 2487 dual wavelength UV detector set at 254 and 294nm. Normal Phase HPLC was carried out on a Phenomenex Luna C_{18} column (10mm × 250 mm, 10 μm), while a Whatman Partisil 10 semi-preparative column (10mm × 500 mm, 10 μm) was used for reversed phase HPLC. NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer and referenced to $CDCl_3$ solvent signals ($^1H_\delta$ 7.26 and $^{13}C_\delta$ 77.0).

2.3.2 Plant Material

Specimens of *S. heterophyllum* (PA071b) were collected from Port Alfred on the south east coast of South Africa on 21 September 2007. The alga was transported to the laboratory in ice and frozen immediately. The samples were authenticated by comparison with voucher specimens in the Division of Pharmaceutical Chemistry, Rhodes University, Grahamstown, South Africa. The alga was stored at - 4ºC until it was extracted.

2.3.3 Extraction and isolation

The frozen alga (38.77 g, extracted dry weight) was allowed to thaw after which it was soaked in methanol for one hour. The methanol was removed and the alga immersed in a mixture of MeOH- CH_2Cl_2 (1:2) sufficient to cover it. The alga was then extracted by heating at 40°C for 30 minutes. This was repeated twice with solvent of the same composition. Extracts were pooled and separated into aqueous and organic phases by the addition of deionised water. Concentration of the organic phase under reduced pressure gave a dark green residue (3.87 g) whilst the aqueous phase gave a pale green residue (1.16 g). These fractions were then analysed by ^1H NMR. A portion of the organic fraction (1.09 g) was fractionated by gradient elution on a silica gel column (10 g) using solvents of differing Hexane : EtOAc compositions to give seven fractions as follows : Fr 7A (H:E, 10:0, 8.6mg), Fr 7B (H:E, 9:1, 27mg), Fr 7C (H:E , 8:2, 132mg), Fr 7D (H:E, 6:4, 218mg), Fr 7E (H:E, 4:6,65mg), Fr 7F (H:E, 2:8, 9.7mg) and Fr 7G (H:E ,0:10, 50mg) followed by MeOH:

EtOAc (1:1), Fr 7H (238mg). Some of these fractions were further purified by HPLC or silica gel chromatography.

Purification of crude fractions

Fraction **7B** (27 mg).19 mg of this fraction was purified by silica gel column chromatography using a system solvent of Hexane: EtOAc (9:1) to give 1.7 mg (8.95% extracted yield) of **1.40.**

Fraction **7C** (132 mg). 40 mg of this fraction was purified by normal phase HPLC, Hexane-EtOAc (80:20) at a flow rate of 4 ml/min to give 15 mg (37.5%) of **1.39** with a retention time of 12 min.

Fraction **7D** (218 mg). 20 mg of this fraction was purified by reverse phase HPLC using a system solvent of MeOH: H_2O (90:10) at a flow rate of 2 ml/min to give compounds **1.38** (6.8 mg, 34%) and **2.10** (2.4 mg, 12%) with retention times of 12 and19 min respectively.

Compound **1.41** was characterised from PA071b-**7E** without further purification.

2.3.4 Compounds

^{1}H and ^{13}C NMR data for sargahydroquinoic acid **(1.38)**, sargaquinoic acid **(1.39),** 2'E, 6'E, 10'E – sargaquinal **(1.40)** and sargachromenol **(2.10) –** see table 2.1

^{1}H and ^{13}C NMR data for fucoxanthin **(1.41) –** see table 2.2

Supplementary information

See supplementary information for 2D NMR data for sargahydroquinoic acid **(1.38)**, 2'*E*, 6'*E*, 10'*E* – sargaquinal **(1.41)** and sargachromenol **(2.10)**

References

Afolayan, A.F., Bolton, J.J., Lategan, C.A., Smith, P.J., and Beukes, D.R. (2008). Fucoxanthin,tetraprenylated toluquinone and toluhydroquinone metabolites from *Sargassum heterophyllum* Inhibit the in vitro Growth of the Malaria Parasite *Plasmodium falciparum*. Zeitschrift für Naturforschung B 63:848-852.

Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H.G., Northcotec, P.T., and Prinsep,M. (2008). Marine Natural Products. Natural Products Reports 25:35-94.

Croteau, R., Kutchan, T.M., and Lewis, N.G. (2000). Natural Products (Secondary Metabolites). In Buchanan, B., Gruissem, W., and Jones, R., eds. Biochemistry & Moecular Biology of Plants.CA,USA: American Society of Plant Biologists. 1250-1318.

Da Gama, B.A.P., Pereira, R.C., Carvalho, A.G.V., Coutinho, R., and Yoneshigue-Valentin, Y. (2002). The Effects of Seaweed Secondary Metabolites on Biofouling. Biofouling 18:13-20.

Dar, A., Baig, H.S., Saifullah S.M., Ahmad, V.U., Yasmeen, S., and Nizamuddin M. (2007). Effect of seasonal variation on the anti-inflammatory activity of Sargassum wightii growing on the N. Arabian Sea coast of Pakistan. Journal of Experimental Marine Biology and Ecology1-9.

Gillespie, R.D. and Critchley, A.T. (1999). Phenology of *Sargassum* spp. (Sargassaceae, Phaeophyta) from Reunion Rocks, KwaZulu-Natal, South Africa. Hydrobiologia 398-399:201-210.

Glombitza, K.W., Keusgen, M., and Hauperich, S. (1997). Fucophlorethols from the brown algae *Sargassum spinuligerum* and *Cystophora torulosa*. Phytochemistry 46:1417-1422.

Ham, Y.M., Kim, K.-N., Lee, W.J., Lee, N.H., and Hyun, C.G. (2010). Chemical Constituents from *Sargassum micracanthum* and antioxidant activity. International Journal of Pharmacology 6:147-151.

Harvey, H.R. and Kennicutt, M.C. (1992). Selective alteration of *Sargassum* lipids in anoxic sediments of the Orca Basin. Organic Geochemistry 18:181-187.

Hay, M.E., Duffy, J.E., Pfister, C.A., and Fenical, W. (1987). Chemical Defense Against Different Marine Herbivores: Are Amphipods Insect Equivalents? Ecology 68:1567-1580.

Hellio, C., Marechal, J.P., Veron, B., Bremer, G., Clare, A., and Le Gal, Y. (2004). Seasonal Variation of Antifouling Activities of Marine Algae from the Brittany Coast (France). Marine Biotechnology 6:67-82.

Jang, K.H., Lee, B.H., Choi, B.W., Lee, H.S., and Shin, J. (2005). Chromenes from the brown alga *Sargassum siliquastrum*. Journal of Natural Products 68:716-723.

Jung,M., Jang,K.H., Kim,B., Lee,B.H., Choi,B.W., Oh,K.-B., and Shin,J. (2008). Meroditerpenoids from the Brown Alga *Sargassum siliquastrum*. Journal of Natural products 71:1714-1719.

Kato , T., Kumanireng, A.S., Ichinose, I., Kitahara, Y., Kakinuma, Y., and Kato, Y. (1975). Structre and synthesis of active component from a marine alga ,*Sargassum tortile* which induces the settling of swimming larvae of *Coryne uchidai*. Chemistry Letters 335-338.

Kusumi, T., Ishitsuka, M., Kinoshita, T., Kakisawa, H., and Shibata, Y. (1979). Structures of new plastoquinones from the brown alga *Sargassum serratifolium*. Chemistry Letters 277-278.

Liu, X., Wang, C.Y., Shao, C.L., Wei, Y.X., Wang, B.G., Sun, L.L., Zheng, C.J., and Guan, H.S. (2009). Chemical constituents from *Sargassum pallidum* (Turn.) C. Agardh. Biochemical Systematics and Ecology 37:127-129.

Maplestone, R.A., Stone, M.J., and Williams, D.H. (1992). The evolutionary role of secondary metabolites - a review. Gene 115:151-157.

Paul, V.J. and Van Alstyne, K.L. (1992). Activation of chemical defenses in the tropical green algae *Halimeda* sp. Journal of Experimental Marine Biology and Ecology 160:191-203.

Perez-Castorena, A., Arciniegas, A., Apan, M.T.R., Villasenor, J.l., and de Vivar, A. (2002). Evaluation of the anti-inflammatory and antioxidant activities of the plastoquinones derivatives isolated from *Roldana barba-johanis*. Planta Medica 68:645-647.

Plouguerne , E., Le Lann , K., Connan , S., Desandes , J.E., and Stiger-Pouvreau , V. (2006). Spatial and seasonal variation in density , reproductive status , lenght and phenolic content of the invasive brown macroalga *Sargassum muticum* (Yendo) Fensholt along the coast of Western Brittany (France). Aquatic Botany 85:337-344.

Reddy, P. and Urban, S. (2009). Meroditerpenoids from the southern Australian marine brown alga *Sargassum fallax*. Phytochemistry 70:250-255.

Seo, Y., Lee, H.-J., Park, K., Kim, Y., Ahn, J., Yoo, J., and Lee, B. (2004). Peroxynitrite-scavenging constituents from the Brown alga *Sargassum thunbergii*. Biotechnology and Biopress Engineering 9:212-216.

Silva, D.H.S., Pereira, F.C., Zanoni, M.V.B., and Yoshida, M. (2001). Lipophyllic antioxidants from *Iryanthera juruensis* fruits. Phytochemistry 57:437-442.

Simpson, T.J. (1987). Applications of multinuclear NMR to structural and biosynthetic studies of polyketide microbial metabolites. Chemical Society Reviews 16:123-160.

Stone, M.J. and Williams, D.H. (1992). On the evolution of functional secondary metabolites (natural products). Molecular Microbiology 6:29-34.

Verpoorte, R. (1998). Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. Drug Discovery Today 3:232-238.

Chapter 3

Geographical and Seasonal Variation of *S. heterophyllum* and *S. elegans*

3.1 Introduction

Secondary metabolites have been reported to vary both quantitatively and qualitatively, even within the same species (Kamiya et al., 2010; Plouguerne et al., 2006; Vallim et al., 2007). These variations have been attributed to several factors, some of which have been shown to vary geographically, while others have been associated with changes occurring over time within the same geographical location (Chennubhotla et al., 1982; Vallim et al., 2007; Targett et al., 1992; Ang, 1984). Such variations have the potential to slow down the search for medicinal compounds from natural sources. For the development of lead compounds from natural products, it is important to ensure an adequate supply of the natural product. The main purpose of this study was to identify the most important factors that may affect the yield of sargahydroquinoic acid **(1.38)** and related metabolites from *Sargassum heterophyllum* with the specific aim of identifying the most suitable site and time for collecting the alga.

3.1.1 Variation of secondary metabolites- a brief overview

In addition to physical defence mechanisms, marine organisms have also developed chemical defence mechanisms against other organisms in their immediate environment (Hellio et al., 2004). The development of secondary metabolites as a chemical means of anti-fouling is one example of these chemical defences (Hellio et al., 2004). The fluctuation of these anti-fouling metabolites with varying fouling conditions associated with seasonal changes throughout the year has been observed (Hellio et al., 2004; Marechal et al., 2004). In turn, fouling and other conditions related to seasonal changes are associated with physical and genetic factors (Hellio et al., 2004; Culioli et al., 2002; Marechal et al., 2004; Ursi et al., 2003;Valls and Piovetti, 1995). Preliminary studies in brown algae have shown geographical and seasonal variation of phenolic metabolites in organisms of the same genus (Plouguerne et al., 2006; Dar et al., 2007). Seasonal changes in themselves bring about changes in several environmental factors such as temperature, pH, salinity, nutrient concentrations and light; which may also vary with differing geographical locations (Hellio et al., 2004; Culioli et al., 2002; Marechal et al.,

2004; Ursi et al., 2003; Valls and Piovetti, 1995).These conditions have an impact on the biosynthesis of secondary metabolites (Dar et al., 2007; Chennubhotla et al., 1982).

3.1.2 Taxonomy of the *Sargassum* Genus

Six species of the *Sargassum* genus have been found to occur in South Africa. These include *S. crassifolium*, *S. elegans*, *S. heterophyllum*, *S. lendigerum*, *S. longifolium* and *S. obovatum* (Seagrief, 1984). This genus has been recorded to be taxonomically complicated as it has a divided classification system (Mattio and Payri, 2011; Mattio et al., 2009).There does not seem to be an agreement on the number of *Sargassum* species in the world since Womersley (1987) reported 150-200 species, while Yoshida (1989) documented about 500 species and most recently Silva (1996) reported 239 species (Yoshida, 1989; Womersley, 1987; Silva et al., 1996; Gillespie and Critchley, 2001). Currently, four *Sargassum* subgenera are recognised, these are the subgenera: *Arthrophycus*, *Bactrophycus*, *Sargassum* and *Phyllotrichia* (Mattio and Payri, 2011). These subgenera are further divided into 12 sections each with further subdivisions (Mattio and Payri, 2011). In addition to this, Mattio and Payri (2011), suggest that the subgenera *Arthrophycus* and *Bactrophycus* be merged (with the addition of subgenus *Arthrophycus* to subgenus *Bactrophycus*) and that subgenus *Phyllotrichi*a be transferred to the subgenus *Sargassopsis* which has recently been reinstated (Mattio and Payri, 2011; Draisma et al., 2010).

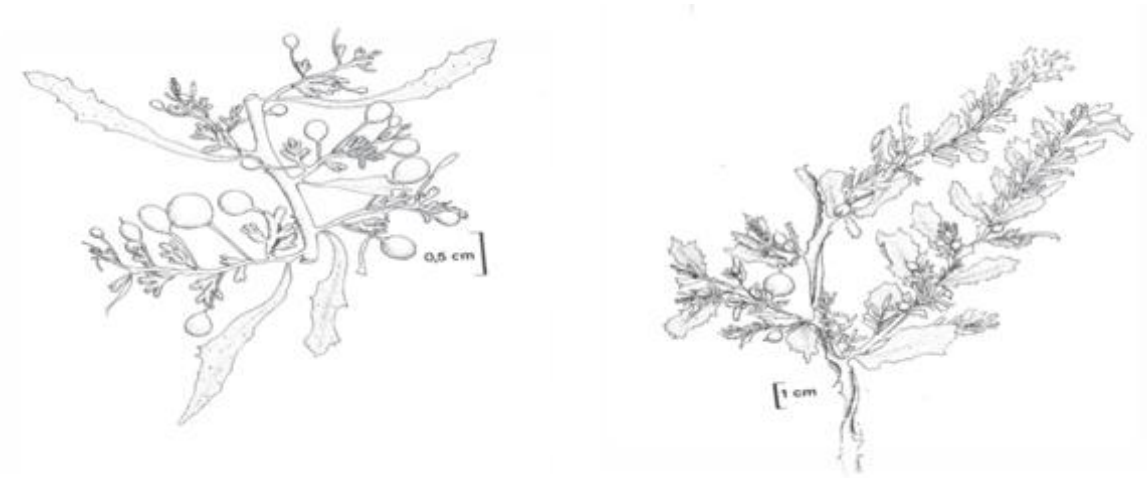
3.1.2.1 Classification in the *Sargassum* Genus

The classification of *Sargassum* species is based on several morphological features with subgenera being differentiated chiefly by the arrangement of their axes (Mattio and Payri, 2011). However, the subgenera *Bactrophycus* and *Arthrophycus* are distinguished by their receptacle morphology (Mattio and Payri, 2011; Yoshida, 1989). Despite this, the morphological characteristics used for distinguishing other subdivisions of this genus are not clear (Mattio and Payri, 2011). The temporal, intraspecific and intra-geographical variation of species within this genus has been identified as the main cause for the taxonomic problems of this genus (Gillespie and Critchley, 2001; Silva et al., 1996). Due to their polymorphic nature and phenotypic plasticity, it is possible that current species of this genus have been found

synonymous with other species when investigated (Mattio and Payri, 2011). Other reasons for the current taxonomical challenges in this genus include possible erroneous identification and classification as well as difficulties in retrieving original collected specimens some of which may have been accidentally destroyed (Mattio and Payri, 2011; Gillespie and Critchley, 2001).

We have commonly encountered two of the *Sargassum* species occurring on the eastern coast of South Africa. These species include *S. elegans* which belongs to the subgenus *Sargassum* and *S. heterophyllum* of the subgenus *Arthrophycus*. *S. elegans* plants are generally 30-50 cm in height, lax and olive-brown in colour, while *S. heterophyllum* plants are about 50 cm tall, coarse, rigid and yellowish in colour with brown streaks when fresh (Stegenga et al., 1997). Primary branches of both species are long and can be up to 2 mm in diameter, while foliose appendages decrease in size from the stipe to the most distal parts of the alga. These leafy appendages are almost linear, attaining sizes up to 5 cm in length and 5 mm in diameter; bearing leaves with a distinct midrib and smooth to jaggered edges (Stegenga et al., 1997). Members of both species also often have receptacles which may occur in several forms in the axils of leafy laterals (Stegenga et al., 1997). The basis of differentiation between these two species is their morphological differences (Stegenga et al., 1997). Branches of *S. elegans* are circular in cross section with branched and upright receptacles while the laterals of *S. heterophyllum* are triangular in section with simple, compressed or triangular receptacles. Holdfasts are generally small flat and circular for *S. elegans* plants while they are conical in shape in specimens of *S. heterophyllum* (Stegenga et al., 1997). The chemical composition of each of these two species is of particular interest to us, given the taxonomic problems of this genus.

Figure 3.1a Photograph of *S. elegans* (adapted from http://www.algaebase.org/search/images/detail/?img_id=12192 .Accessed 18/01/2012)



S. elegans *S. heterophyllum*

Figure 3.1b Diagrams of *S. elegans* and *S. heterophyllum* (Stegenga et al., 1997)

From the *Sargassum* species of Southern Africa, it is also clear that there appears to be significant variations within species of this genus (Stegenga et al., 1997). Certain

morphological differences have been observed in species from differing locations within this region. For example, both the significantly dentate leafy appendages of specimens from False Bay on the western coast of Southern Africa (figure 3.2) which seldom exceed a length three times longer than their width and specimens from the eastern coast of the same region; with smooth edged leafy appendages up to 10 times longer than their width, have been classified as *S. heterophyllum* (Stegenga et al., 1997). It is for this reason that Stegenga et al., (1997) recommend that *S. incisifolim* be recognised as the algae found in the western cape, while Seagrief (1984) listed *S. incisifolium* and *S.ilicifolium* as synonyms for *S. heterophyllum* occurring along the eastern coast of South Africa (Seagrief, 1984).

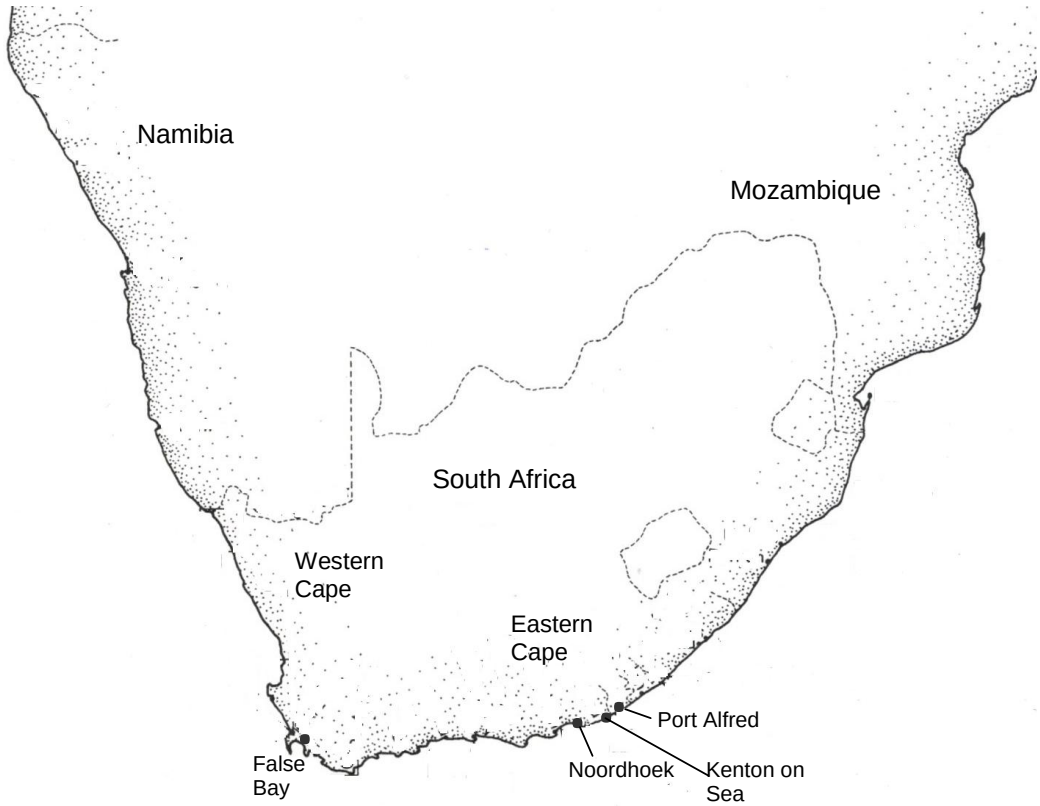


Figure 3.2 Map of South Africa's coastline (adapted from Seaweeds of the South African West Coast (Stegenga et al., 1997))

Crude extracts of *S. heterophyllum* and *S. elegans* were obtained from algae collected at three different sites on the eastern cost of South Africa namely: Port Alfred (PA), Noordhoek (NDK) and Kenton on Sea (KOS). ^{1}H NMR spectra of crude extracts from algae collected at each site were evaluated for qualitative differences and compared with each other for both intra- and inter-site variation as well as for seasonal variation. Crude extract profiles obtained from the different species were also compared. In an effort to eliminate variations in extracted metabolites due to the use of different solvents, the selection of extraction solvents was evaluated by investigating the types of metabolites extracted by various solvent systems. Preliminary investigations of the potential change in metabolite profile between collection and extraction were also carried out.

3.2 Results and Discussion

At the outset of this study we recognised that extracts of *S. elegans* and *S. heterophyllum* were identical. Where possible the two species were collected separately. However, during the study we also found that only one of the two species often occurs at specific collection sites during specific seasons.

3.2.1 Evaluation of extraction solvents

Before investigating the geographical and seasonal variation of metabolite profiles, preliminary studies to determine the effect of extraction solvents on the metabolite profiles were performed. The ^{1}H NMR profiles of acetone and EtOH extracts of *S. elegans* were compared to profiles obtained from our standard CH_2Cl_2- MeOH (2:1) extraction protocol for freshly collected algae. Fresh algae were rinsed with deionised water and blotted dry. Stipes were separated from the rest of the plant which was then size reduced into fine particles using liquid nitrogen and a mortar and pestle. Approximately 2 mL of the size reduced algae was placed in a centrifuge tube into which 9 mL of extraction solvent was added. The algae in extraction solvent was sonicated for 5 minutes and subsequently centrifuged at high speed for 5 minutes. 3 mL of the supernatant's lower layer was collected, concentrated under reduced pressure and analyzed by ^{1}H NMR spectroscopy.^{1}H NMR spectra of *S. elegans* extracted with all three solvent systems did not show major qualitative differences. From the spectra, the major metabolites that could be identified were sargahydroquinoic **(1.38)** and sargaquinoic acid **(1.39)**. These metabolites were identified by their signals in the aromatic region (figure 3.3).

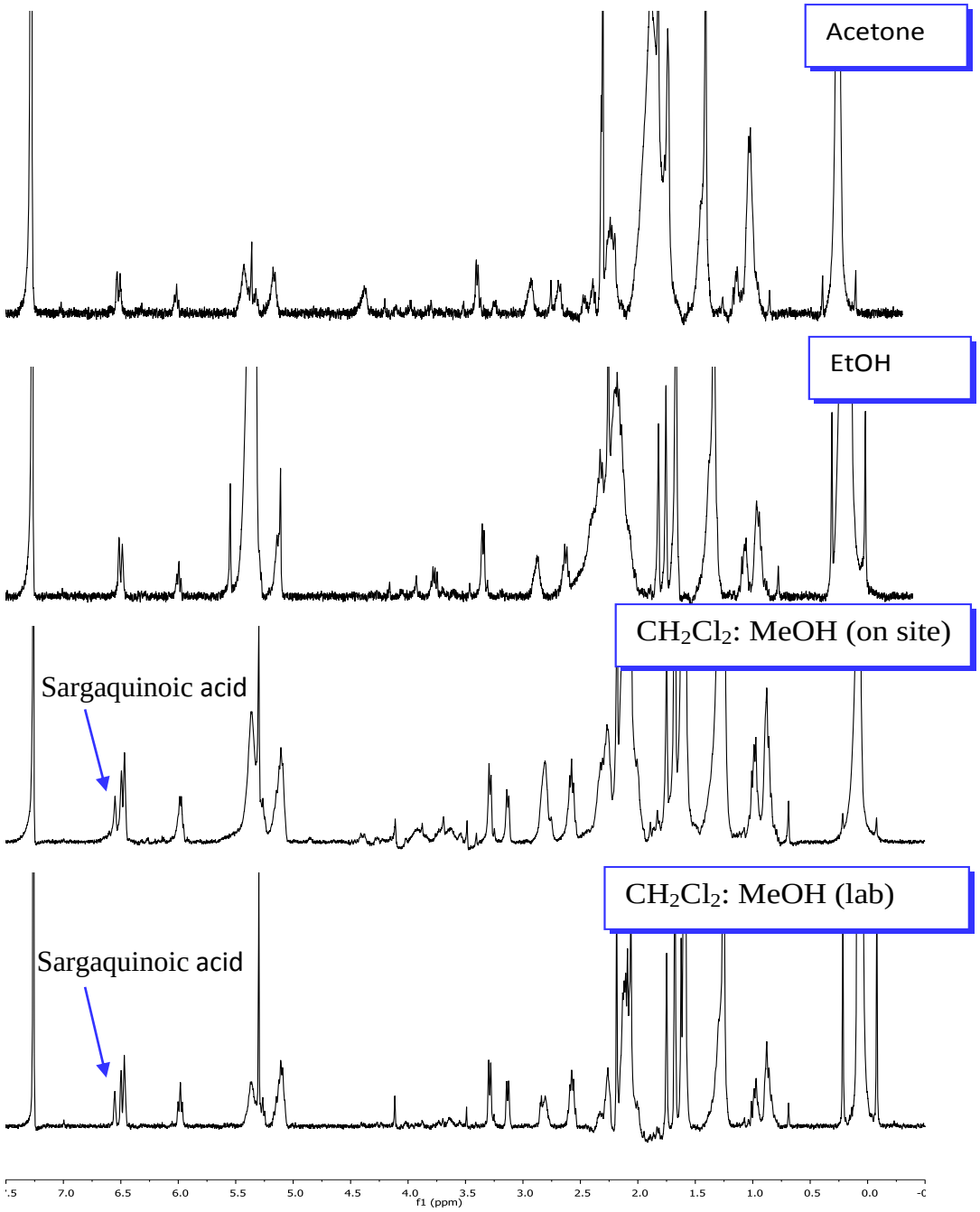


Figure 3.3 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* (PA100331) obtained from extraction with different solvents at the site of collection and with CH_2Cl_2-MeOH (2:1) at the laboratory

3.2.2 Investigation of metabolite profile changes between collection site and extraction at the laboratory

Possible alterations in the metabolite profiles of *S. elegans* between collection and transportation of algae from the collection site to the laboratory were investigated. *S. elegans* (PA100331) was size reduced as outlined above at the site of collection. From the same homogenous mixture of size reduced algae, two portions of approximately 2 mL each were placed into two separate centrifuge tubes. One tube was left without solvent while 9 mL of CH_2Cl_2-MeOH (2:1) was placed in another tube. Upon arrival at the laboratory (about 2 hours later) 9 mL of solvent (CH_2Cl_2-MeOH (2:1)) was placed into the centrifuge tube containing only size reduced algae. Both samples were sonicated for 5 minutes and centrifuged at constant speed for another 5 minutes. 3 mL of the liquid extract was withdrawn from the supernatant's lower layer, concentrated under reduced pressure and the crude extract analyzed by ^{1}H NMR spectroscopy. Spectra of algae extracted with CH_2Cl_2- MeOH (2:1) both at the site of collection and at the laboratory (figure 3.3) were also similar to spectra obtained from the preliminary evaluation of extraction solvents. These results led to the selection of CH_2Cl_2-MeOH (2:1) as the solvent of extraction for the rest of the study. These results also showed that algae did not undergo any chemical changes during transportation between the collection site and the laboratory where it was extracted two hours after collection.

3.2.3 Geographical and seasonal variation

Three collections were undertaken in each season: one in autumn (March), the other in winter (July) and the last collection in spring (September). *S. heterophyllum* was collected from two sites while *S. elegans* was collected from three sites along the eastern coast of South Africa. *S. heterophyllum* collections were made from Noordhoek on the 28th of March and on the 27th of July and from Port Alfred on the 30th of July and on the 24th of September (Table 3.1). *S. elegans* was collected from Noordhoek on the 28th of March, from Kenton on Sea on the 29th of March and from Port Alfred on the 31st of March and on the 24th of July. All collections were undertaken within 2010. Collections from all three sites were carried out from pools which were about 5 m apart; at least two specimens were collected from each pool.

To determine the intra-site variation of metabolites within each species, ^{1}H NMR profiles of specimens collected from different pools at each site within the same month were compared. Inter-site variation was assessed by the comparison of ^{1}H NMR profiles of the same species collected from different sites in the same season. Seasonal variation of metabolites was evaluated by comparing metabolite profiles collected at the same site within different months; firstly within species and then between species.

Table 3.1 Summary of collection sites, collected species and dates of collection

Code	**Site**	**Month of Collection**	**Species**
PA100331	PA	March	*S. elegans*
PA100727-11a & 12a	PA	July	*S. heterophyllum*
PA100924-9c	PA	September	*S. heterophyllum*
PA100924-10c & 11c	PA	September	*S. elegans*
NDK100328-4 & 2	NDK	March	*S. heterophyllum*
NDK100328-1 & 5	NDK	March	*S. elegans*
NDK100727-4b	NDK	July	*S. heterophyllum*
KOS100329-5	KOS	March	*S. elegans*

3.2.3.1 Intra- and Inter-site variation

Intra-site variation studies carried out amongst three separate sites in all three seasons i.e at PA – in March (figure 3.3), July (figure 3.4 a) and September (figures 3.4 b and c), at NDK in March (figures 3.5 a and b) and in July (figure 3.5 c) and at KOS in March (figure 3.6 a), yielded identical results for both species.

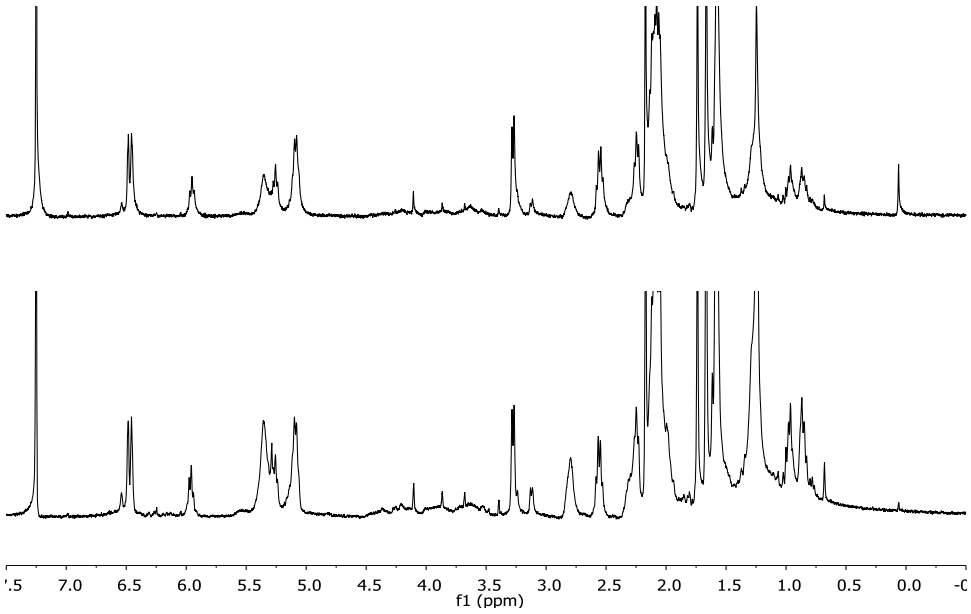


Figure 3.4 a ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of crude *S. heterophyllum* extracts (PA100727-11a and 12a) collected from different pools at Port Alfred in July

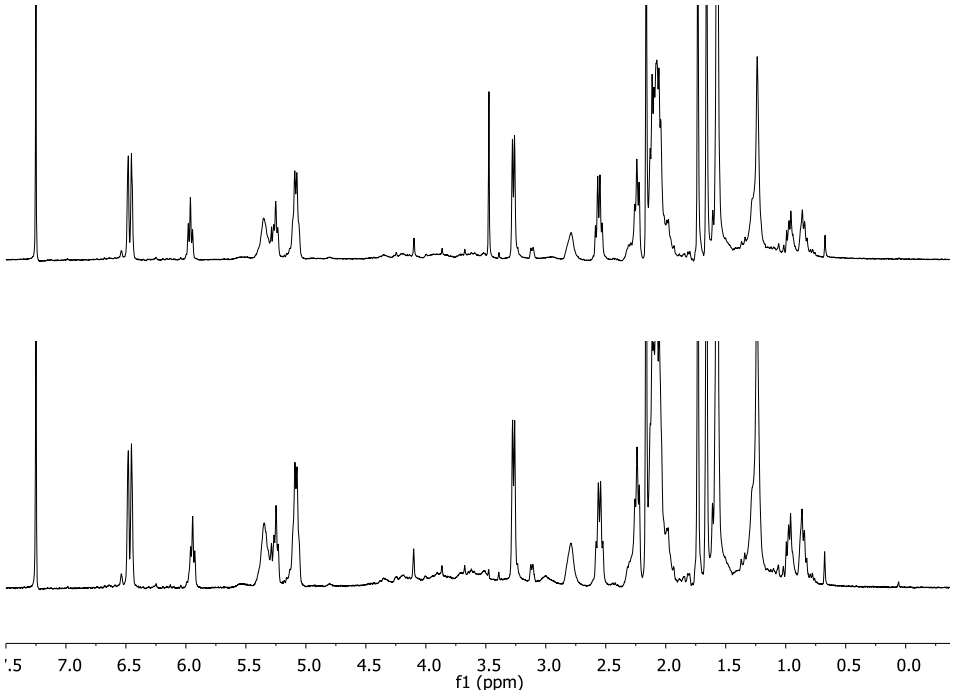


Figure 3.4 b ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts (PA100924-9c) collected from different pools at Port Alfred in September

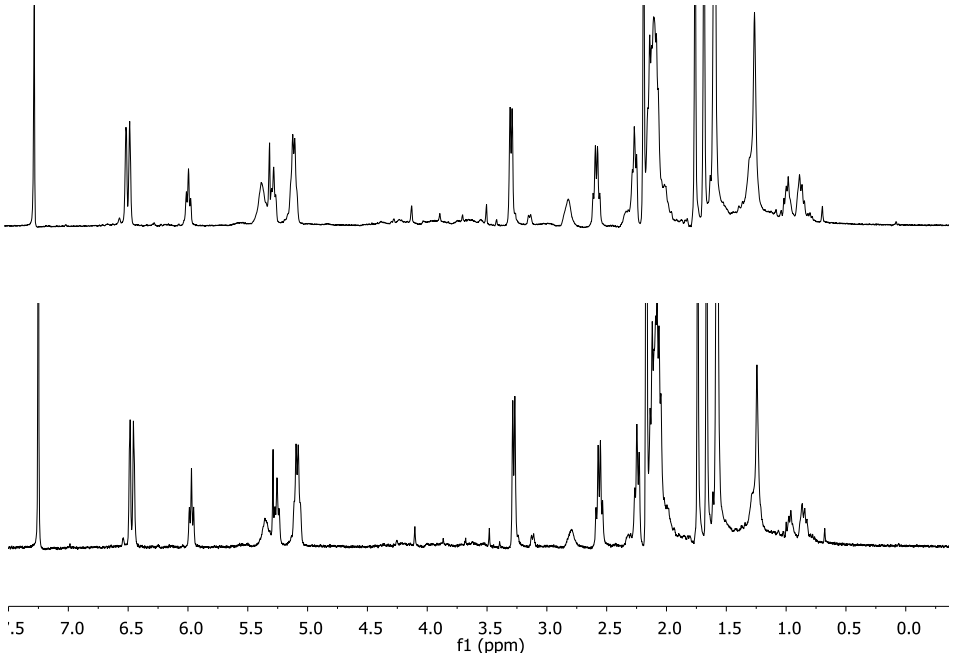


Figure 3.4 c ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts (PA100924-10c & 11c) collected from different pools at Port Alfred in September

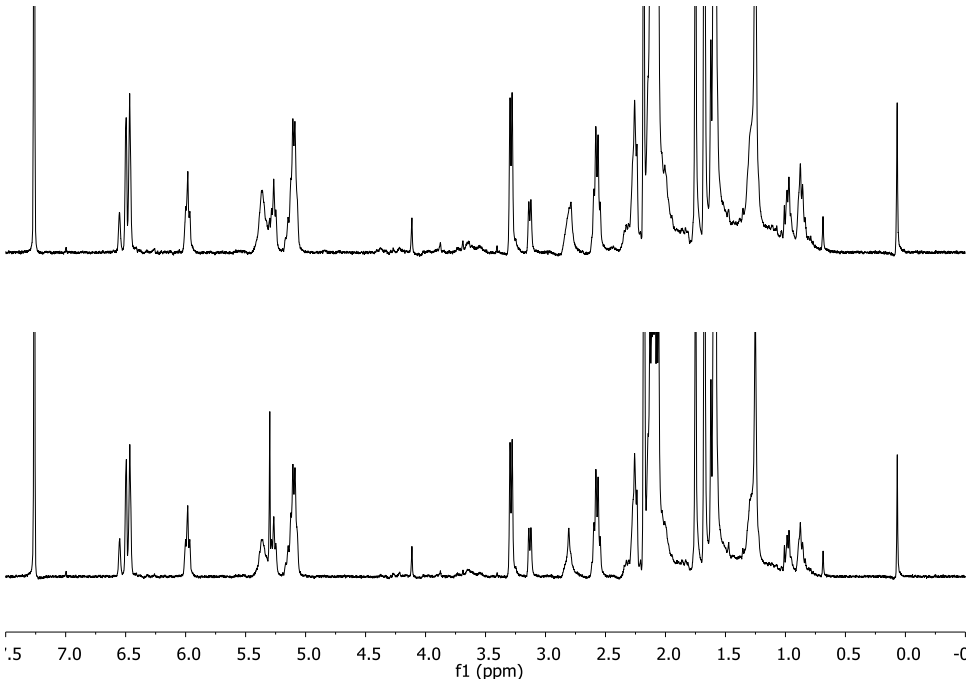


Figure 3.5 a ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts (NDK100328-4 &2) collected from different pools at Noordhoek in March

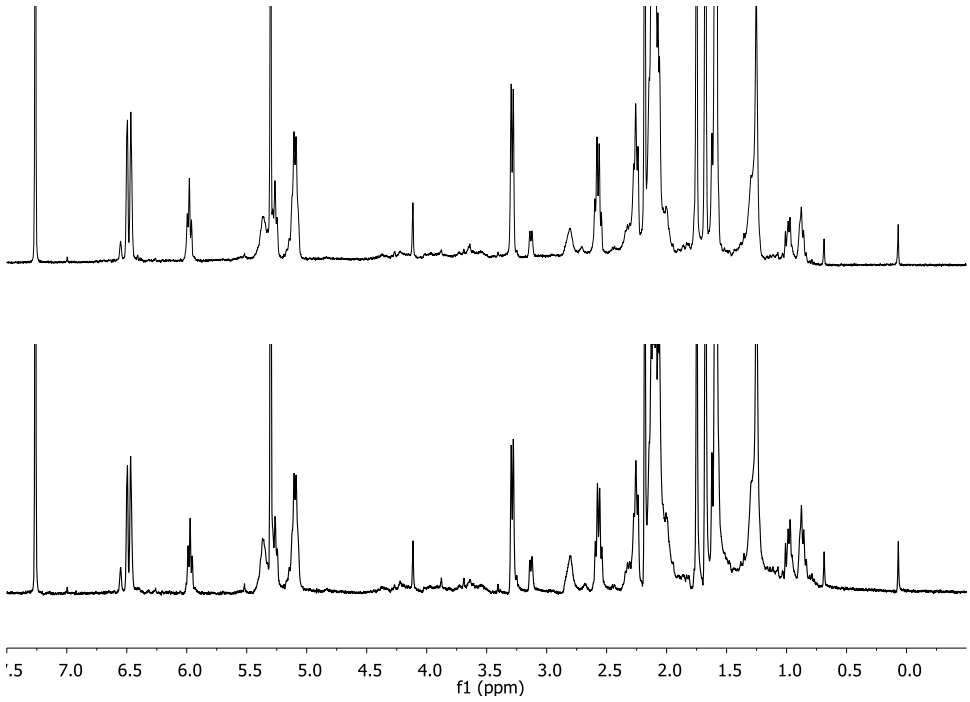


Figure 3.5 b ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts (NDK100328-1 & 5) collected from different pools at Noordhoek in March

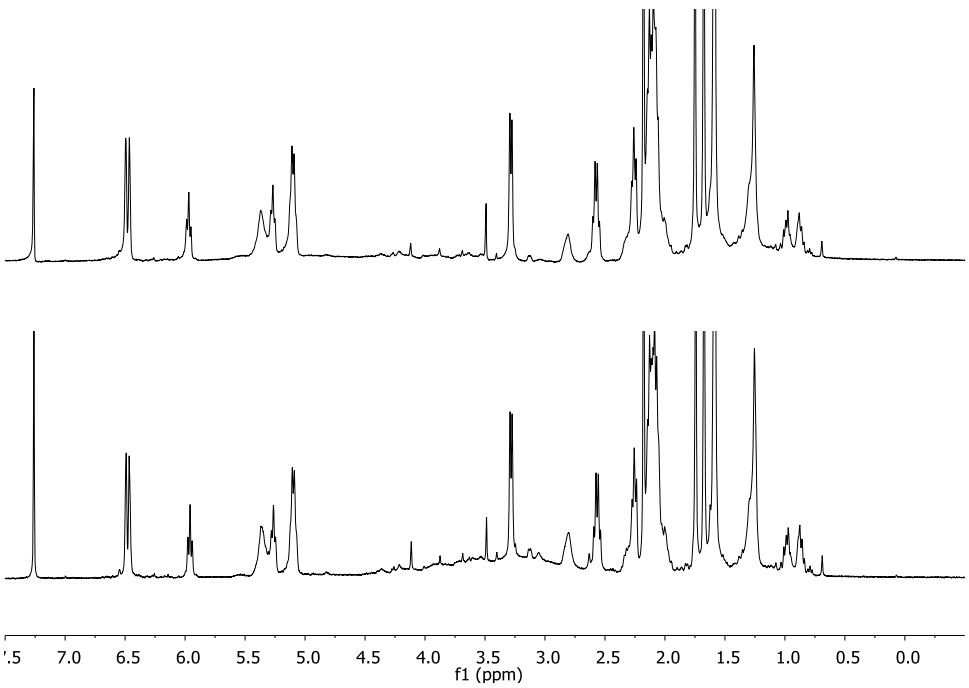


Figure 3.5 c ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts (NDK100727-4b) collected from different pools at Noordhoek in July

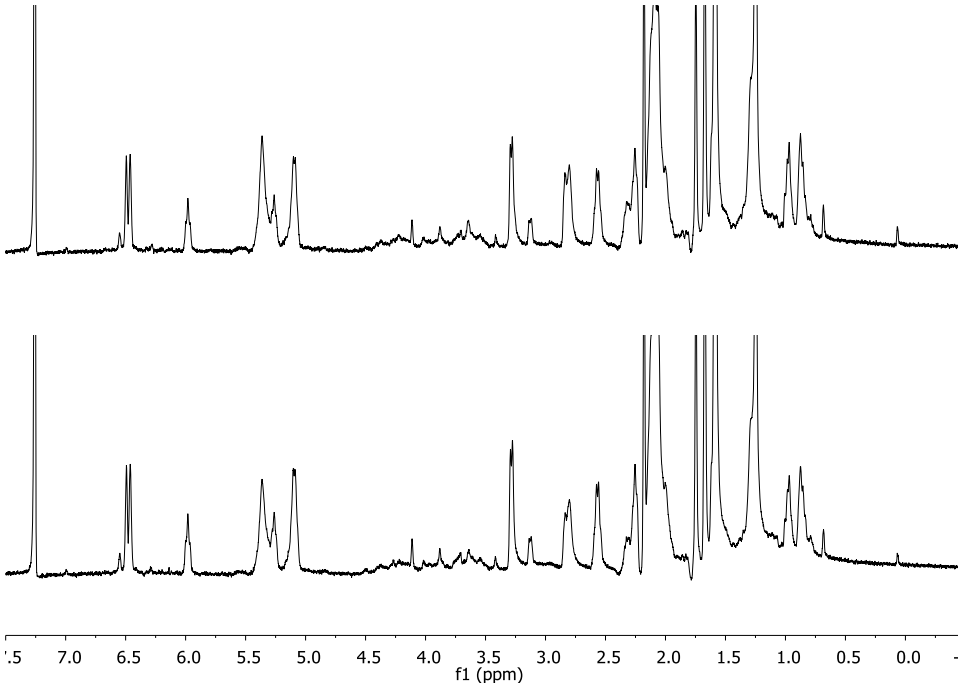


Figure 3.6 a ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts (KOS100329-5) collected from different pools at Kenton on Sea in March

These results allowed for the comparison of *Sargassum* metabolite profiles obtained from specimens collected at different sites within the same month. Results from this comparison showed that there was no qualitative variation of metabolites between different sites in the same season (figure 3.7).

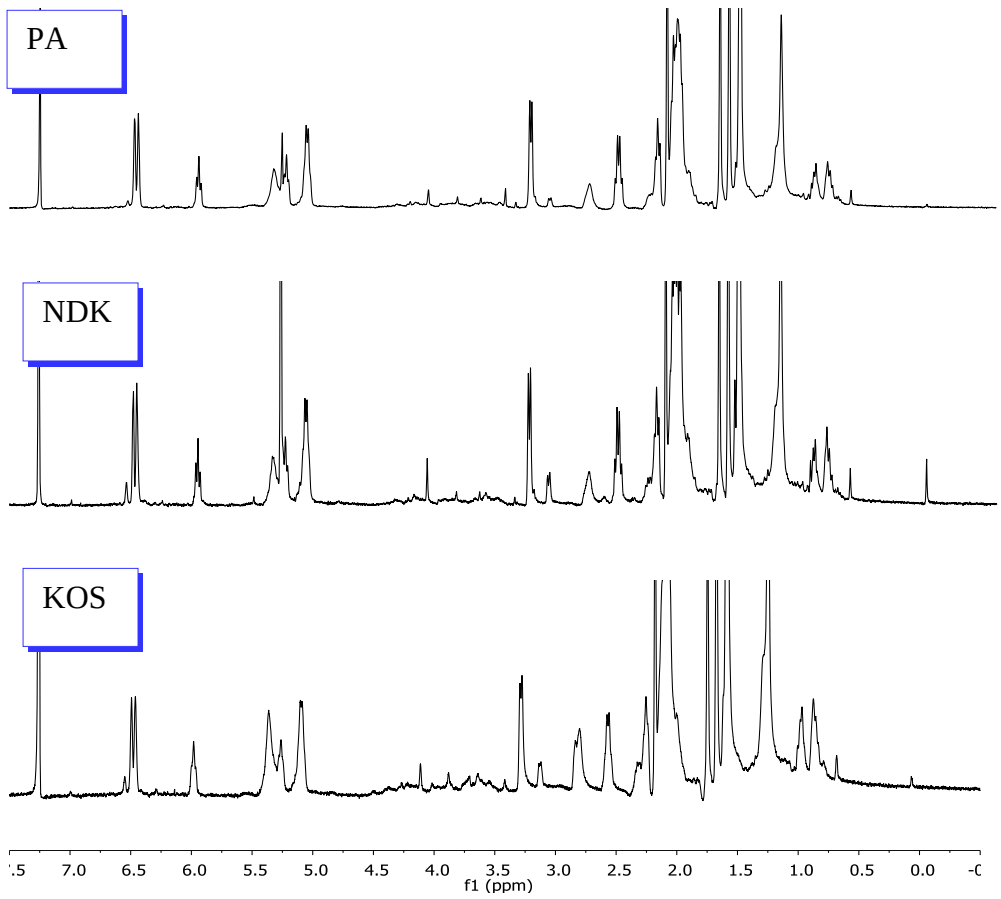


Figure 3.7 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. elegans* extracts collected from different sites in March (PA- Port Alfred, NDK- Noordhoek, KOS- Kenton on Sea)

3.2.3.2 Seasonal Variation

Seasonal variations of metabolites were evaluated by comparing metabolite profiles of algae collected at the same site within different months firstly within species then between species. This was done for *S. heterophyllum* collected from Port Alfred (PA) in July and September (figure 3.8) and for the same species collected from Noordhoek (NDK) in March and July. Similar comparisons were also made for *S. elegans* collected from Port Alfred in March and September. Again no significant differences were noted between all metabolite profiles other than the appearance of sargaquinoic acid **(1.39)** from the air oxidation of sargahydroquinoic acid **(1.38)**.

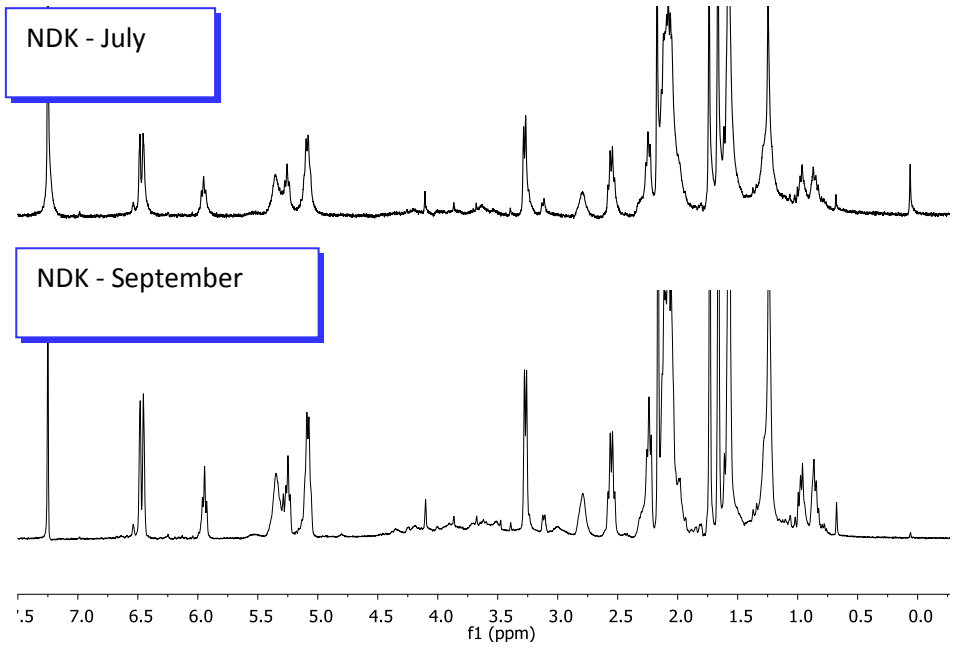


Figure 3.8 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* extracts collected from Noordhoek (NDK) in July and September

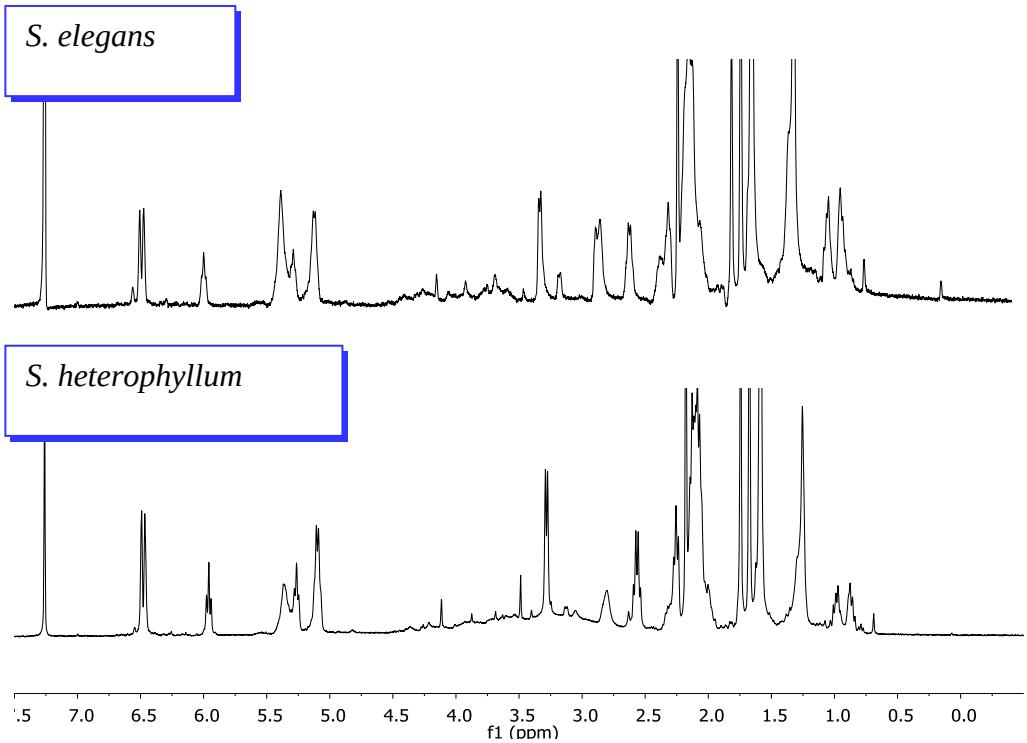


Figure 3.9 ^{1}H NMR (400 MHz, $CDCl_3$) spectra of crude *S. heterophyllum* and *S. elegans* extracts collected from Noordhoek in March

This preliminary investigation of the qualitative geographic and seasonal variation of secondary metabolites from *S. heterophyllum* and *S. elegans* showed that there is no variation amongst metabolites of both *S .heterophyllum* and *S. elegans* species collected at the sampled sites. This was the case with samples collected at two of the three sites namely; Noordhoek and Port Alfred (figure 3.9). Unfortunately no specimens of *S. heterophyllum* were found at Kenton on Sea when sampling was carried out at this site in March. Results from this study suggest that the two species under investigation are chemically in-distinct. The present study was successful in finding that the collection of *S. heterophyllum* from any three of the sampled sites in the sampled months will qualitatively yield the same metabolites. This was important for the future collection of algae for metabolites required for the rest of this research study.

3.3 Experimental

3.3.1 General Experimental

All solvents used were of analytical grade and were used without further purification from commercial sources. Organic extracts were dried under reduced pressure. ^{1}H-NMR spectra were acquired on Bruker either a 400 MHz or 600 MHz spectrometer. Deuterated chloroform ($CDCl_3$) was used for all ^{1}H-NMR determinations. All experiments were carried out in duplicate.

3.3.2 Plant Material

Specimens of both *Sargassum* species were collected by hand on three different sites along the eastern coast of South Africa: Port Alfred (PA), Kenton on Sea (KOS) and Noordhoek (NDK). *S. heterophyllum* was collected from NDK and PA in March and July respectively while *S. elegans* was collected from all three sites in March and from PA in July. These sites were selected mainly due to their proximity to the laboratory as well as their ease of access.

S. heterophyllum and *S. elegans* were differentiated based on morphological differences. Algae with conical holdfasts as well as triangular branches were classified as *S. heterophyllum* while specimens with circular holdfasts and branches in addition to branched receptacles were classed as specimens of *S. elegans*.

3.3.3 Optimization of extraction solvents

Several plants of *S. elegans* (PA100331) were washed with deionised water and blotted dry. The stipes were removed, placed in a pre-chilled mortar and crushed with liquid nitrogen. Approximately 2 mL of biomass was placed in a centrifuge tube and made up to 10 mL with one of the following solvents: EtOH, acetone and a mixture of CH_2Cl_2-MeOH (2:1) one centrifuge tube of crushed algae was left without solvent. Upon arrival at the laboratory (2 hours later) samples were sonicated for 5 minutes and centrifuged at constant speed for another 5 minutes. 3 mL of the liquid extract was withdrawn and concentrated under reduced pressure. The crude extract was then analyzed by ^{1}H NMR spectroscopy.

3.3.4 Intra-site and Inter-site differences and Geographical variation

Frozen individual specimens were allowed to thaw at room temperature and subsequently extracted in duplicate as outlined above.

3.3.5 ^{1}H-NMR analysis

Concentrated crude extracts were dissolved in $CDCl_3$ and ^{1}H NMR data acquired on a Bruker 400 MHz spectrometer. The same volume (500 µL) of $CDCl_3$ was used for the analysis of each extract.

References

Algae base. *Sargassum elegans* Suhr. http://www.algaebase.org/search/images/detail/?img_id=12192. 2012

Ang, P.O. (1984). Preliminary study on the alginate contents of *Sargassum* spp. in Balibago, Calatagan, Philipines. Hydrobiologia 116/117:547-550.

Chennubhotla, V.S.K., Kaliaperumal, N., Kalimuth, S., Selvaraj, M., Ramalingam,J.R., and Najmuddin, M. (1982). Seasonal Changes in Growth & Alginic Acid & Mannitol Contents in *Sargassum ilicifolium* (Turner) J. Agardh & *S. myriocystum* J.Agardh. Indian Journal of Marine Sciences 11:195-196.

Culioli, G., Ortalo-Magne, A., Richou, M., Valls, R., and Piovetti, L. (2002). Seasonal variations in the chemical composition of *Bifurcaria bifurcata* (Cystoseiraceae). Biochemical Systematics and Ecology 30:61-64.

Dar, A., Baig, H.S., Saifullah, S.M., Ahmad, V.U., Yasmeen, S., and Nizamuddin, M. (2007). Effect of seasonal variation on the anti-inflammatory activity of *Sargassum wightii* growing on the N. Arabian Sea coast of Pakistan. Journal of Experimental Marine Biology and Ecology 351:1-9.

Draisma, S.G.A., Ballesteros, E., Rousseau, F., and Thibaut, T. (2010). DNA Sequence Data Demonstrate The Polyphyly Of The Genus Cystoseira And Other *Sargassaceae* Genera (Phaeophyceae). Journal of Phycology 46:1329-1345.

Gillespie, R.D. and Critchley, A.T. (2001). Assesment of spatial and temporal variability of three *Sargassum* species (Fucales, Phaeophyta) from KwaZulu-Natal, South Africa. Phycological Research 49:241-249.

Hellio, C., Marechal, J.P., Veron, B., Bremer, G., Clare, A., and Le Gal, Y. (2004). Seasonal Variation of Antifouling Activities of Marine Algae from the Brittany Coast (France). Marine Biotechnology 6:67-82.

Kamiya, M., Nishio, T., Yokoyama, A., Yatsuya, K., Nishigaki, T., Yoshikawa, S., and Ohki, K. (2010). Seasonal variation of phlorotannin in *Sargassacean* species from the coast of the Sea of Japan. Phycological Research 58:53-61.

Marechal, J.P., Hellio, C., Culioli, G., Thomas-Guyon, H., Callow, M., Clare, A., and Ortalo-Magne, A. (2004). Seasonal variation in antifouling activity of crude extracts of the brown alga *Bifurcaria bifurcata* (Cystoseiraceae) against cyprids of *Balanus amphitrite* and the marine bacteria *Cobetia marina* and *Pseudoalteromonas haloplanktis*. Journal of Experimental Marine Biology and Ecology 313:47-62.

Mattio, L. and Payri, C. (2011). 190 Years of *Sargassum* Taxonomy, Facing the Advent of DNA Phylogenies. The Botanical Review 77:31-70.

Mattio, L., Payri, C.E., and Verlaque, M. (2009). Taxonomic revision and geographic distribution of the subgenus *Sargassum* (Fucales, Phaeophyceae) in the western and central Pacific Islands based on morphological and molecular analyses. Journal of Phycology 45:1213-1227.

Plouguerne, E., Le Lann, K., Connan, S., Desandes, J.E., and Stiger-Pouvreau, V. (2006). Spatial and seasonal variation in density, reproductive status, length and phenolic content of the invasive brown macroalga *Sargassum muticum* (Yendo) Fensholt along the coast of Western Brittany (France). Aquatic Botany 85:337-344.

Seagrief, S.C. (1984). A catalogue of South African green, brown and red marine algae. Pretoria, South Africa: Botanical Research Institute, Department of agriculture.

Silva, P.C., Basson, P.W., and Moe, R.L. (1996). Catatogue of the Benthic Marine Algae of the Indian Ocean. California: University ot California Press, California.

Stegenga, H., Bolton, J.J., and Anderson, R.J. (1997). Seaweeds of the South African West Coast. Cape Town : Creda Press.

Targett, N.M., Loren, D.C., Anne, A.B., and Tanner, C.E. (1992). Biogeographic Comparisons of Marine Algal Polyphenolics: Evidence against a Latitudinal Trend. Oecologia 89:464-470.

Ursi, S., Pedersen, M., Plastino, E., and Snoeijs, P. (2003). Intraspecific variation of photosynthesis, respiration and photoprotective carotenoids in *Gracilaria birdiae* (Gracilariales: Rhodophyta). Marine Biology 142:997-1007.

Vallim, M.A., Teixeira, V.L., and Perez-Castorena, A. (2007). Feeding-deterrent properties of diterpenes of *Dictyota mertensii* (Phaeophyceae, Dictyotales). Brazilian Journal of Oceanography 55:223-229.

Valls, R. and Piovetti, L. (1995). The chemistry of the *Cystoseiraceae* (Fucales: Pheophyceae): Chemotaxonomic relationships. Biochemical Systematics and Ecology 23:723-745.

Womersley, H.B.S. (1987). The Marine Benthic Flora of Southern Australia II. Adelaide: South Australia Government Printer.

Yoshida, T. (1989). Taxonomy of *Sargassum*. Korean Journal of Phycology 4:107-110.

Chapter 4

A Comparison of four different techniques for the extraction of metabolites from *S. heterophyllum* using qH NMR

4.1 Introduction

After determining the most suitable times and sites of collection for *S. heterophyllum*, gram quantities of sargahydroquinoic acid **(1.38)** were required to further explore the biological activity of this secondary metabolite. The extraction of compounds from plant matter is the first step towards the analysis of metabolites from natural products. It is thus regarded as a fundamental process in the study of natural products (Mandal V. et al., 2007). The preparation of plant material before extraction is equally important as it has the potential to affect the metabolites isolated from plant material. The main purpose of this part of the study was to optimize the extraction of sargahydroquinoic acid **(1.38)** from *S. heterophyllum*. The extraction of this and other metabolites using different extraction techniques was investigated and the extraction capacity of each method quantitatively assessed by NMR.

4.1.1 Overview of commonly used extraction techniques

Different methods have been employed in the extraction of natural products (Cravatto, 2008; Raynie, 2006). These techniques play a significant role in both analytical and natural products chemistry as they influence the recovery and stability of the extracted metabolites (Raynie, 2006). The careful selection and optimisation of extraction methods to suit their particular application is important to prevent chemical alteration of extracted metabolites (Cravatto, 2008). Some of the commonly used extraction methods include soxhlet, maceration, percolation, ultrasonic and microwave assisted extraction (Cravatto, 2008; Shen and Shao, 2005). Unfortunately, some of these methods of extraction are time consuming and expensive in that they require significant volumes of solvents (Shen and Shao, 2005). In addition to this, several solvents used in some extraction techniques are harmful to the environment and expensive to purchase and dispose of (Shen and Shao, 2005). These disadvantages have been the focus of the development of current and new extraction techniques (Raynie, 2006).

The most commonly used methods of solid-liquid extraction are based on the diffusion of metabolites from the plant matrix to the surrounding extraction solvent. It is for this reason that factors that affect diffusion such as temperature, duration of extraction, the size of plant material, solvent recycling and agitation have been explored in an effort to improve extraction yields (Mandal V. et al., 2007; Cravatto, 2008; Cravotto et al., 2004; Raynie, 2006). A few of the commonly used solid-liquid extraction techniques are discussed below.

4.1.1.1 Extraction by maceration

Extraction of plant material by maceration has been documented to be the simplest and most economical method of extraction (Cravatto, 2008). Extraction by this method involves immersing plant material in extraction solvent and allowing metabolites to diffuse out of the plant material into the surrounding solvent (Mandal V. et al., 2007). Stirring during extraction has been shown to be important in preventing saturation of solvent closest to the plant material with extract. Such solvent saturation adversely affects extraction yields by impeding diffusion (Cravatto, 2008). Heating the solvent and plant material during extraction has proved to be useful; however, caution has to be exercised when selecting both the temperature and method of heating, especially where thermo-labile compounds are to be extracted (Cravatto, 2008). In addition to agitation and heating, the type and amount of solvent as well as the duration of extraction are key factors that affect extraction yields using this technique (Mandal V. et al., 2007; Cravatto, 2008; Cravotto et al., 2004; Raynie, 2006).

4.1.1.2 Soxhlet extraction

A Significant disadvantage of soxhlet extraction includes the lengthy extraction periods associated with this technique (Mandal V. et al., 2007; Cravatto, 2008). Despite this disadvantage, this method makes use of important mechanisms such as solvent recycling and penetration into the solid matrix to facilitate high dissolution of metabolites from the plant material - often resulting in high extraction yields (Paik M.J. et al., 2004; Kim and Verpoorte, 2009). It is for these reasons that extraction yields using this technique are dependent mainly on extraction times and solvent volumes in addition to other factors affecting diffusion as listed above.

4.1.1.3 Ultra Sound Assisted Extraction (USAE)

Sonication of plant material during extraction facilitates the swelling and hydration of plant matrices resulting in the enlargement of cell wall pores (Cravatto, 2008; Kim and Verpoorte, 2009). This disruption of cell wall structure increases the rate at which metabolites are transferred from the plant matrix into the surrounding solution (Cravatto, 2008; Kim and Verpoorte, 2009; Vinatoru M., 2001). In addition to the factors affecting diffusion, the extraction yields attained by this method are dependent on the type of solvent used in addition to the frequency at which extraction is carried out. Generally, the use of lower frequencies during extraction (18-40 KHz) results in higher extraction yields compared to compared to extraction under higher frequencies (400-800 KHz) (Cravatto, 2008).

4.1.1.4 Microwave Assisted Extraction (MAE)

Microwave extraction is based on the capacity of materials to absorb microwave energy and convert it to heat (Cravatto, 2008). Unlike conductive heating used in maceration and soxhlet extraction, microwave heating has its advantage in that it heats the entire sample simultaneously with little or no loss of heat to the environment (Cravatto, 2008). Microwave heating disrupts weak hydrogen bonds in the plant matrix by promoting the rotation of molecular dipoles and in so doing disrupts cell walls (Cravatto, 2008; Raynie, 2006). This effect, by which MAE functions, relies on both the characteristics of the solvent and the plant matrix from which compounds are to be extracted (Cravatto, 2008). In some cases, the microwaves disrupt weak hydrogen bonds in the plant matrix whilst the solvent remains cold. This is the case with solvents with low dielectric constants and has great advantages for the extraction of thermo-labile compounds (Cravatto, 2008). In addition to the microwave absorbing characteristics of the solvent, this method of extraction is also dependent on the power at which the extraction is carried out. A higher power invariably results in an increased temperature which may increase the extraction yield (Mandal V. et al., 2007). However, the power at which extractions are carried out should also be selected cautiously to avoid high temperatures, which could result in the degradation of thermo-labile metabolites. An interesting approach to improving both MAE and soxhlet extraction involves using the solvent recycling characteristics of soxhlet extraction while using microwaves as the source of heat (Priego-Capote and Luque de Castro, 2004).

Both ultrasound assisted and microwave assisted extraction have the potential to significantly reduce extraction times whilst positively influencing extraction yields. It is for this and other reasons that these two extraction techniques have been recognised as efficient methods of extraction (Cravatto, 2008; Cravotto et al., 2004).

Once the method of extraction has been selected, there are several important factors that need to be considered for the optimal extraction of any target compound or group of compounds from natural sources (Kim and Verpoorte, 2009). These include the choice of solvent, ratio of solvent to biomass, duration of extraction as well as the temperature at which the extraction is to be carried out (Kim and Verpoorte, 2009).

4.1.2 The selection of extraction solvents

The choice of extraction technique may influence other extraction parameters such as the selection of extraction solvents (Kim and Verpoorte, 2009). For example, microwave heating of certain solvents may result in an abrupt increase in the internal temperature of a solution and this may lead to explosions which pose safety risks to researchers. On the other hand, certain solvents such as hexane cannot be heated by microwaves as they are transparent to them, while other solvents such as EtOH have good microwave absorbing capacities allowing for rapid heating of both the sample and the extraction solvent which may result in higher extraction yields (Mandal V. et al., 2007; Kim and Verpoorte, 2009; Letellier et al., 1999). The use of solvents such as hexane and chloroform is particularly useful for the extraction of thermo-labile compounds. Due to their transparency to microwave heating, these solvents allow for the selective heating of the plant matrix which facilitates the release of compounds from plant material with relatively less degradation of thermo-labile substances (Mandal V. et al., 2007). The physico-chemical characteristics of solvents such as polarity and selectivity for the analyte as well as the solvent's potential toxicity to the user and its inertness towards metabolites being extracted are also important factors to be considered when selecting solvents for extraction (Kim and Verpoorte, 2009). Solvent polarity is an essential factor in extraction as most metabolites are extracted by the 'like-dissolves-like' principle (Kim and Verpoorte, 2009). However, a variety of other factors may also enhance or retard extraction by this principle (Kim and Verpoorte, 2009). Where no published data with respect to

extraction solvents for certain metabolites exists, preliminary experiments are essential to determine the suitability of a solvent for extraction (Kim and Verpoorte, 2009).

Table 4.1 Physical properties of extraction solvents used in the current study (Lide, 2003)

Solvent	Dielectric Constant (ε)	Boiling Point (ºC)	Dipole Moment (μ Debye units)
Dichloromethane	9.08	40	1.60
Methanol	33	65	1.70
Ethanol	24.30	78	1.69
Ethyl acetate	6.02	77	1.78
Water	78.54	100	1.85
Hexane	1.89	69	0.00

Table 4.2 Solvents used to extract specific metabolites from *Sargassum* sp.

Metabolite	Solvent	Reference
Sargaquinoic acid	MeOH	(Kusumi et al., 1979; Perez-Castorena et al., 2002; Seo et al., 2004)
	$CHCl_3$	(Tang et al., 2002)
	EtOH	(Segawa and Shirahama, 1987)
	MeOH-CH_2Cl_2	(Afolayan et al., 2008; Seo et al., 2006)
	80% EtOH	(Ham et al., 2010)
	MeOH-CH_2Cl_2 (3:1)	(Reddy and Urban, 2009)
Sargahydroquinoic acid	MeOH	(Kusumi et al., 1979; Perez-Castorena et al., 2002; Seo et al., 2004)
	EtOH	(Segawa and Shirahama, 1987)
	MeOH-CH_2Cl_2	(Afolayan et al., 2008; Jung et al., 2008; Seo et al., 2006)
	MeOH-CH_2Cl_2 (3:1)	(Reddy and Urban, 2009)

4.1.3 Other factors affecting extraction yields

Regardless of the type of solvent used during extraction, only a certain amount of metabolites can be dissolved in a given amount of extraction solvent. It is for this reason that the ratio of solvent to plant biomass is also an important factor to be considered before extraction begins (Kim and Verpoorte, 2009). Generally, an increase in the amount of solvent results in an increase in the amount of analyte extracted due to the establishment of a steep diffusion gradient down which solutes may diffuse from the plant material into the surrounding solvent (Kim and Verpoorte, 2009). However, this may not be the case for techniques such as microwave assisted extraction in which high solvent volumes may result in low extraction yields due to insufficient stirring of extraction solvent by microwaves (Mandal V. et al., 2007). This may be overcome by introducing mechanical agitation by using magnetic stirrers during extraction. The rate at which metabolites leave the plant matrix is also an important factor in attaining good extraction yields (Kim and Verpoorte, 2009). Where dissolution rates are low, the addition of energy into the extraction vessel e.g. by providing mechanical agitation, convectional currents or ultrasonication may increase extraction yields (Kim and Verpoorte, 2009). Dissolution is also impeded by metabolite–matrix interactions. In certain cases, the matrix may exhibit adsorbent properties which may result in low extraction yields despite adequate agitation (Kim and Verpoorte, 2009).

As illustrated by the discussion above; many factors affect the optimisation of any given extraction technique. Such optimisation experiments require thorough investigations of these factors if they are to provide conclusive results. The techniques by which various extraction conditions and methods are quantitatively assessed are also of equal importance.

4.2 Quantification of metabolites from natural sources.

Several techniques for the quantitative analysis of metabolites from natural sources exist. These techniques such as HPLC and GC, IR, MS and NMR are important in the analysis of bioactive components from natural sources (Bilia et al., 2001). Each of these techniques has its own advantages and disadvantages; however, the ideal technique for the quantification of

metabolites from natural sources in metabolite profiling should be simple, efficient, suitable for the metabolites under investigation, adequately sensitive and reproducible.

Quantitative NMR (qNMR) is a useful analytical technique as it has the potential to detect several metabolites in a relatively short time with little sample preparation. This technique has found widespread application in the identification and quantification of metabolites within complex biological samples (Viant, 2007; Harshey, 2003).

4.3 NMR as a method of quantification

NMR is one of the most widely used analytical methods in academic and industrial research (Malz and Jancke, 2005). Unlike many synthetic compounds, natural products are often formed in complex biological pathways occurring in the presence of many other compounds (Pauli et al., 2005). By allowing for simultaneous qualitative and quantitative analysis, NMR is valuable in the analysis of metabolites in their complex systems, as it removes the need for separation and other time consuming processes that are necessary for the use of other quantitative techniques (Pauli et al., 2005). The development of NMR as a tool for quantification has been accelerated by the significant increase in sensitivity of this method of analysis due to the use of high-field NMR spectrometers and modern software packages that facilitate accurate and precise data processing (Pauli et al., 2005). In addition to this, the ability of this technique to quantitatively determine relative amounts of different compounds enables it to be used in the quantitative analysis of molecules in mixtures (Malz and Jancke, 2005). This has important applications in natural products studies where crude extracts need to be screened in an effort to quantitate one or more metabolites.

The advantages of qNMR as an analytical tool include its precision, reproducibility, non-destructive nature and its ability to determine both qualitative and quantitative information on analytes (Pauli et al., 2005; Viant, 2007; Harshey, 2003; Verpoorte et al., 2007). The non-destructive nature of this method is an added advantage in natural products laboratories where plant derived metabolites are valuable and scarce, making sacrificial analysis undesirable (Pauli et al., 2005). In addition to this, qNMR is a reliable technique which can be used in the analysis of NMR visible compounds such as ursolic acid which lack a chromophore or other physicochemical properties necessary for chromatographic detection (Pauli et al., 2005).

NMR has been used in the total identification and quantification of metabolites in biological systems, a field known as metabolomics (Verpoorte et al., 2007) Since many organisms produce the same primary metabolites, ideal metabolomic methods are those which are able to easily identify and quantify these metabolites with high reproducibility (Verpoorte et al., 2007) Despite the fact that many of the techniques used in metabolomics have their own advantages and disadvantages – the above mentioned advantages of NMR make it suitable for use in metabolomic studies (Hagel and Facchini 2008; Verpoorte et al., 2007) . The suitability of this method in metabolomics studies facilitates its use in the quantification of selected metabolites provided that certain criteria are met.

However, this technique is expensive due to the high cost of NMR spectrometers (Malz and Jancke, 2005). Another disadvantage of qNMR in comparison to other methods of quantitative analysis is its poor sensitivity (Wawer et al., 2008). The limits of detection for ^{1}H NMR on a high field spectrometer such as a 600 MHz spectrometer are around 10^{-9} while the same limits for IR and MS based techniques are about 10^{-14} and 10^{-18} respectively (Wawer et al., 2008). This disadvantage of qNMR stems from the fact that NMR sensitivity depends on the magnetic field strength, the nucleus under observation and the pulse sequence employed. These disadvantages may be reduced by increasing the number of scans during data acquisition, ensuring precise shimming as well as by improving sample concentration with the use of micro-coil technology (Wawer et al., 2008).

4.3.1 Applications of qNMR

QNMR has found applications in the quantification of compounds in mixtures, complex matrices and drug formulations and in the validation of reference compounds e.g. purity analyses, in biosynthetic, stability as well as reaction kinetic studies (Pauli et al., 2005; Diercks T. et al., 2001). This technique has the potential to play an important role in the regulatory analysis of pharmaceutical preparations of natural and synthetic drugs (Pauli et al., 2005). The ability of qNMR to be used in stability and kinetic studies is of significance to the pharmaceutical industry, while its use in the quantification of one or more components from complex mixtures is of great importance to the study of natural products (Pauli et al., 2005). This technique's potential to quantitate secondary metabolites has been utilised successfully

by various groups. A few examples of the use of qNMR in the quantification of compounds of natural origin are summarised in table 4.3 below.

Table 4.3 Examples of natural product based studies in which qNMR was utilised as the method of analysis (Pauli et al., 2005)

Analyte(s)	Natural Source	Reference
Cannabinoids	Cannabis *(Cannabis Sativa)*	(Choi et al., 2004)
Capsaicin Gingerol Piperine	Chilli (*Capsicum spp*) Ginger *(Zingiber Officinalis)* Black pepper (*Pipernigrum L.*)	(Catchpole O.J. et al., 2003)
Bilobalide, Gingkolides A,B,C	*Ginkgo biloba* leaves	(Choi et al., 2003)

4.3.2 Quantitative ^{1}H NMR

4.3.2.1 Principles of NMR quantification

The key principles of qNMR reside in the fact that the area under an NMR signal, is directly proportional to the number of nuclei generating the signal (Rundlöfa et al., 2010; Pauli et al., 2005). Hence, signal integration enables the determination of the ratio of components in a mixture (Pauli et al., 2005; Rundlöfa et al., 2010). Signal intensities (peak height) may also be used in a similar manner. However, the use of signal intensities as a measure of relative quantities is only valid when the NMR signal is represented by a single Lorentzian line, which rarely occurs (Pauli et al., 2005). It is for this reason that integration of the area under the NMR signal is the most commonly used method of signal analysis in qNMR (Holzgrabe and Malet-Martino, 2011).

The NMR active nuclei with highest natural abundance are ^{1}H, ^{19}F, ^{31}P and ^{15}N respectively. Due to the lower abundance of the other NMR susceptible nuclei such as ^{13}C (1.1% relative abundance) compared to ^{1}H (~100% abundance), the quantitative information obtained from these nuclei would be more difficult to obtain compared to that obtained from the more

abundant nuclei (Rundlöfa et al., 2010; Malz, 2008; Pauli et al., 2005). The use of other NMR susceptible nuclei such as ^{19}F, ^{15}N and ^{31}P is limited to molecules containing these nuclei (Pauli et al., 2005). Of all the abundant NMR susceptible nuclei, only ^{1}H is present in most organic molecules hence, ^{1}H nuclei are the basis of most qNMR applications. Despite complex resonances of signals from several overlapping metabolite peaks in the ^{1}H NMR spectra of mixtures, the development of NMR analysis software has enabled the de-convolution of overlapping peaks to facilitate quantification from selected signals (Weljie et al., 2006; Dumas et al., 2002). In an effort to improve on the distribution of signals in the spectrum, 2D NMR experiments may be employed (Pauli et al., 2005). This is often the case with complex samples. However, these techniques e.g. COSY, HSQC and HMBC require longer acquisition times as well as more complex data processing compared to ^{1}H NMR experiments (Pauli et al., 2005).

4.3.3 Important aspects of qNMR analysis

The two most important factors governing the successful use of qNMR include the appropriate selection of suitable quantitative conditions and post-acquisition processing parameters (Csaba, 1992).

4.3.3.1 Quantitative experimental conditions

The careful selection and optimisation of NMR parameters such as relaxation delay (D1) and acquisition time is essential for the accurate quantification of compounds by qNMR (Pauli et al., 2005; Pauli, 2001; Pinciroli et al., 2004). The fundamental pulse scheme for obtaining 1D proton NMR data involves the following three primary parameters: relaxation delay, pulse width and acquisition time of the time domain data (Pauli et al., 2005).

The relaxation delay (D1), represents the amount of time taken for excited protons to return to ground state after pulsing (Pauli et al., 2005; Holzgrabe et al., 1998). The relaxation delay is a property of the nucleus in a specific magnetic environment and is affected by the relaxation properties of other components in the mixture under investigation (Pauli et al., 2005). Ideally, a sufficiently long D1 should be used to allow for equilibrium magnetization to be attained before the next pulse cycle begins (Pauli et al., 2005). It is often not feasible to

allow for the complete relaxation of all nuclei in a sample as long relaxation delays inevitably result in longer experimental times. It is thus common practice to determine the relaxation delay based on the relaxation properties of selected signals of target analytes when dealing with mixtures. The acquisition of NMR spectra under identical relaxation times should result in good quantification as each component of the mixture will be subject to identical relaxation phenomena (Weljie et al., 2006). The acquisition time is usually selected on the basis of the spectral width in addition to the level of digital resolution required for that spectral width (Pauli et al., 2005). Usually, acquisition for ^{1}H NMR spectra is selected such that spectral resolution is approximately 0.25 Hz or better (Pauli et al., 2005).

4.3.3.2 Post-acquisition data processing

While the acquisition time is set prior to the NMR experiment, improvement of the digital resolution can be achieved by post acquisition data processing techniques such as zero-filling or linear prediction (Pauli et al., 2005). Once NMR spectra have been acquired, the use of appropriate processing techniques is essential. Phasing and baseline correction should be performed prior to integration as both these functions ensure that quantities represented by the various integrals will have minimal distortions, resulting in both accurate and reproducible quantification (Pauli et al., 2005). These post acquisition processes can be applied either manually or automatically. Where manual processing is employed, integration will vary from operator to operator (Holzgrabe and Malet-Martino, 2011).

Ideally, integration limits should be set to a range covering 64 times the width at half signal height as this ensures that 99% of the entire signal intensity is obtained (if the signal is a Lorentzian line) (Holzgrabe and Malet-Martino, 2011). This is a potential challenge firstly because NMR signals are not normally represented by Lorentzian lines and secondly because such a range may overlap with adjacent signals hence, a compromise has to be made (Holzgrabe and Malet-Martino, 2011). The accuracy of the integration procedure is also dependent on the signal to noise ratio (S/N) (Holzgrabe and Malet-Martino, 2011). Significant precision (standard deviation < 1%) has been attained for ^{1}H NMR with minimal signal to noise ratios (S/N > 250:1) (Holzgrabe and Malet-Martino, 2011).

4.3.4 Reference compounds for quantification

As for other methods of quantitative analysis, qNMR also requires the use of calibrated standards (Pauli et al., 2005; Holzgrabe et al., 1998; Henderson, 2002). Two main methods of calibration exist; these include external and internal calibration in which a pre-determined amount of an internal standard is added to the analyte or mixture for analysis (Pauli et al., 2005; Holzgrabe et al., 1998; Henderson, 2002). External calibration is used when contamination of the analyte needs to be avoided. To achieve this, a special type of NMR tube with a coaxial capillary tube is used. The calibration standard is placed in the coaxial tube which is then inserted into the NMR tube containing the compound(s) to be analysed (Pauli et al., 2005; Holzgrabe et al., 1998; Henderson, 2002). Alternatively, two NMR tubes of analyte and standard are used and spectra of each determined separately (Pauli et al., 2005; Holzgrabe et al., 1998; Henderson, 2002). Recent technology has enabled quantification using electronically generated signals in the NMR spectrum (Silvestre V. *et al.*, 2001). This can be achieved by making use of the ERETIC (electronic reference to access *in vivo* concentrations) or PULCON (pulse length based concentration determination) methods which remove the need for using a separate compound as an internal standard for quantification (Holzgrabe and Malet-Martino, 2011; Silvestre V. et al., 2001). However, where one is used, an ideal internal standard for qNMR should be readily available in high purity, inexpensive, stable, chemically inert and should not be volatile or hygroscopic to avoid changes during handling which may adversely affect quantification. In addition to this, a good standard for qNMR should have a short relaxation time and a relatively simple NMR spectrum with unique signals which are well removed from those of the target analyte(s) (Pauli et al., 2005; Rundlöfa et al., 2010).

4.3.5 Quantitative Analysis

For the analysis and eventual quantification of compounds by qNMR, one of two approaches may be used (Pauli et al., 2005; Holzgrabe et al., 1998; Malz and Jancke, 2005).

a. Relative Method

This is essentially the determination of ratios of components in compound mixtures. The molar ratio n*x*/n*y* of two components X and Y may be calculated using the integrals of sufficiently separated signals as follows:

$$\frac{nx}{ny} = \frac{Ix}{Iy} \cdot \frac{Ny}{Nx} \qquad (1)$$

Where *nx* is the number of moles of analyte x, *I* is the integral of the selected NMR signal and *Nx* is the number of nuclei contributing to the integral of x.

b. Absolute Method

Two different absolute methods may be employed for the quantitative analysis of content or concentration. (Pauli et al., 2005; Holzgrabe and Malet-Martino, 2011; Malz and Jancke, 2005)

(i) The 100% method in which all compounds appearing in the spectrum are known and can be both unambiguously assigned structurally and quantitatively measured.

(ii) Calculation of the main component P*x* with the use of a standard of known concentration P*std* as follows :

$$Px = \frac{Ix}{Iy} \times \frac{Nstd}{Nx} \times \frac{Mx}{Mstd} \times \frac{mstd}{m} \times Pstd \qquad (2)$$

Where: *Mx* and *Mstd* are the molecular weights of analyte (m) and standard (std), m and *mstd* are the weights of analyte and standard respectively, *Nx* and *Nstd* are the number of contributing nuclei of the relevant signal and *Px* and *Pstd* are the concentrations of standard and analyte respectively.

The main objective of the work presented in this chapter was to develop the most appropriate method for the extraction of sargahydroquinoic acid (**1.38**) from *S. heterophyllum*. The methods investigated in this study include maceration, soxhlet, ultrasound and microwave assisted extraction. The amount of sargahydroquinoic acid and other tetraprenylated metabolites extracted by each technique was quantified by qNMR.

4.4 Results and Discussion

4.4.1 Effect of drying method on the metabolite profile of *S. heterophyllum*

In chapter three it was shown that there is almost no intra-site variation of metabolites in freshly collected *S. heterophyllum*. Here we investigated the effect of different drying methods on the metabolite profiles of *S. heterophyllum.* A portion of *S. heterophyllum* collected from Noordhoek was size reduced while another portion was air-dried in a fume cupboard for two weeks. Of the size reduced algae, a portion was freeze dried for 24 h while the rest was also air dried for two weeks. The three batches of algae were extracted by ultrasound assisted extraction with DCM-MeOH (2:1) as the extraction solvent. Comparison of spectra obtained from this section of the study (figure 4.1) showed no qualitative differences between algae prepared by the three different methods. This was somewhat surprising since we have previously observed the relatively rapid conversion of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)** during purification. Freeze drying as the method of biomass preparation was selected for this study due to the shorter time period associated with freeze drying algae compared to air drying.

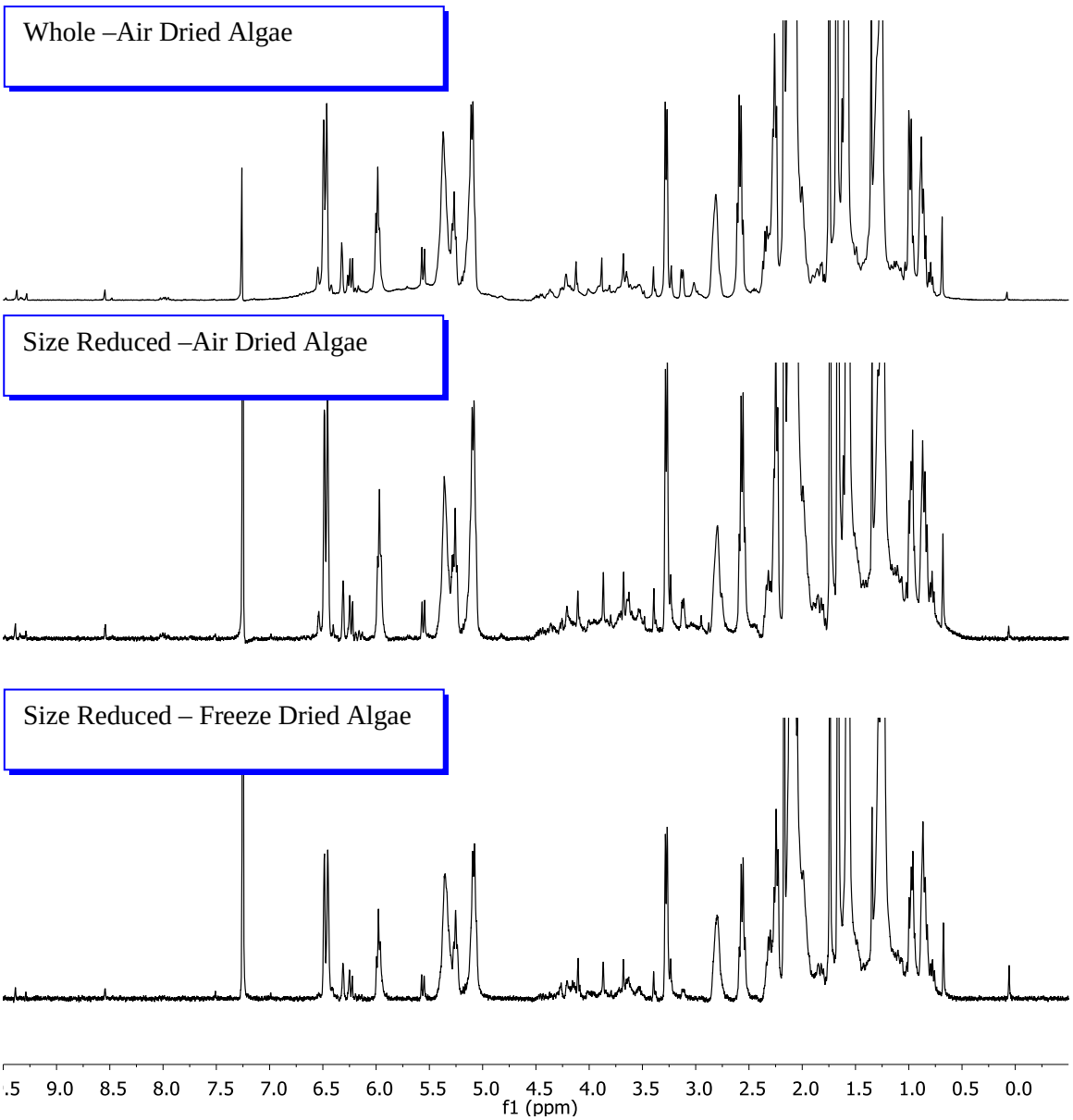


Figure 4.1 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of crude extracts obtained from *S. heterophyllum* dried by different methods

4.4.2 Development of a quantitative NMR method for the analysis of crude extracts of *S. heterophyllum*

4.4.2.1 Selection of protons of interest

The tetraprenylated toluquinones; sargahydroquinoic acid **(1.38)** and sargaquinoic acid **(1.39)** have relatively simple ^{1}H NMR spectra (figure 4.2). However, in the presence of other *S. heterophyllum* metabolites most of these signals are obscured. Despite this, a doublet due to the methylene protons (H-1') at δ 3.3 ppm in sargahydroquinoic acid and δ 3.1 ppm in sargaquinoic acid, are characteristic signals of these compounds which are visible even in the spectra of crude extracts. Sargachromenol **(2.10)**, also a metabolite of *S. heterophyllum* has relatively unobscured signals such as the doublet at δ 5.6 ppm (figure 4.2) due to an olefinic methine proton (H-2').

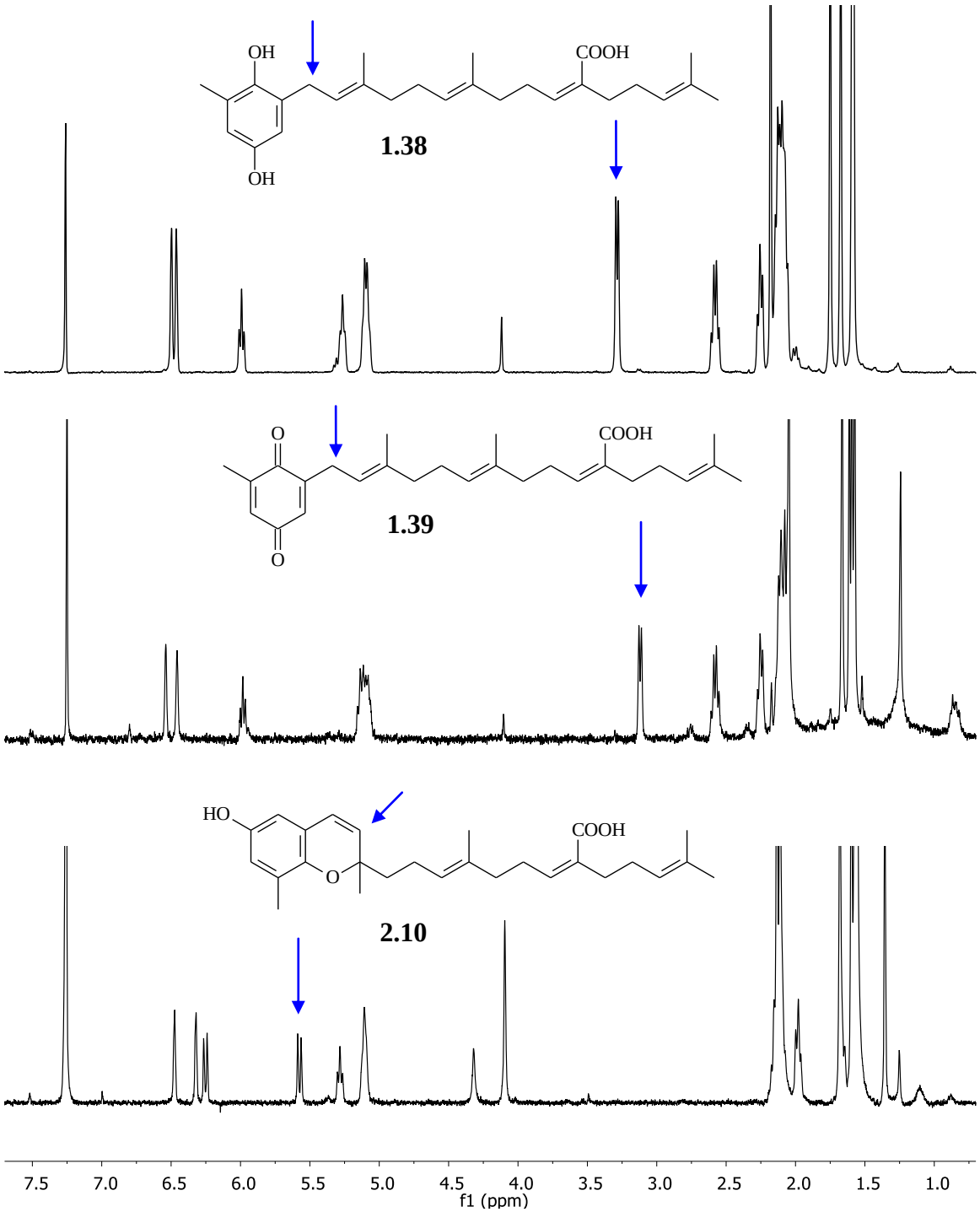


Figure 4.2 ^{1}H NMR spectra (400 MHz, $CDCl_3$) of sargahydroquinoic acid **(1.38)**, sargaquinoic acid **(1.39)** and sargachromenol **(2.10)** showing protons selected for integration in qNMR

4.4.2.2 Selection of an internal standard for quantification

The internal standard for quantification was selected from a literature search of internal standards used for the qNMR analysis of natural products. From this search, pyridine, *N, N*-dimethylformamide, dichlorophthalonitrile and benzene where selected and investigated as potential internal standards for the current study. N,N-dimethylformamide (DMF) was selected as the internal standard because of its relatively simple ^{1}H NMR spectrum which contains an aldehyde signal at δ 8.0 ppm. This and other signals in the ^{1}H NMR spectrum of DMF are well removed from the selected signals of the target analytes (figure 4.3). The relatively high boiling point of DMF was an added advantage for its use as an internal standard as it meant that the internal standard was unlikely to evaporate during sample preparation, thus preventing inaccurate quantification. After the selection of the internal standard, the suitability of all selected signals for quantification was assessed. This was done by obtaining an HSQC spectrum of one of the crude extraction mixtures to be analysed (figure 4.4). This spectrum showed no overlap between the selected signals and other signals in the crude spectrum.

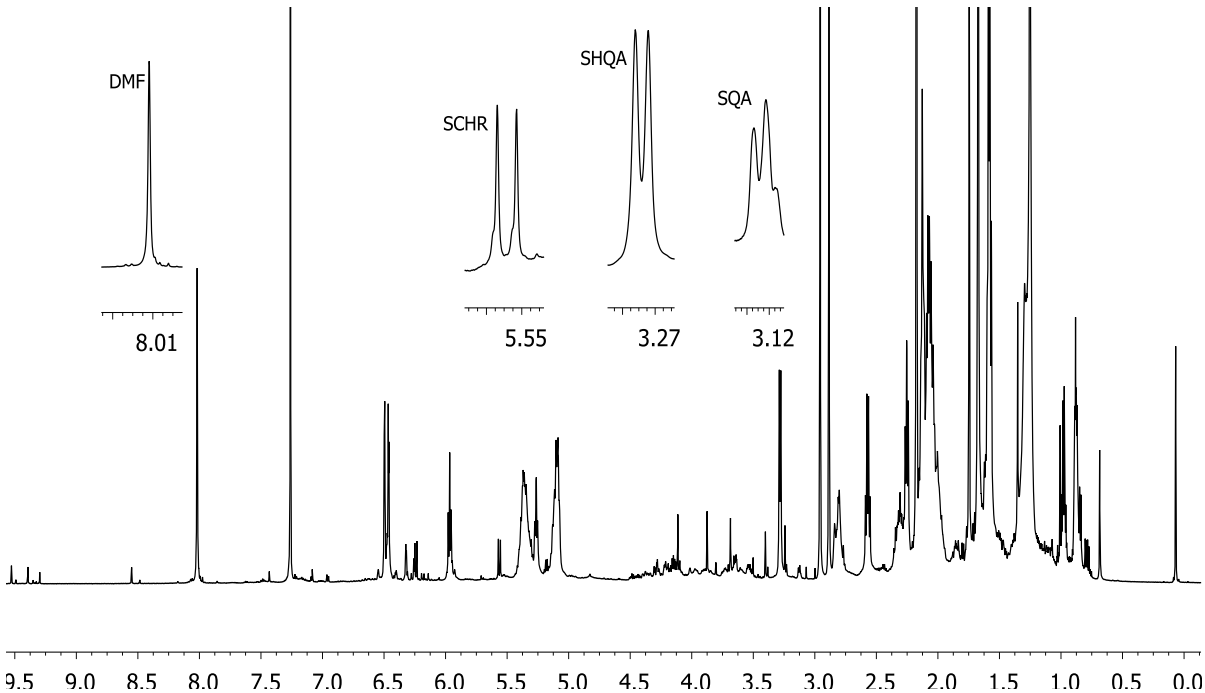


Figure 4.3 ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of crude *S. heterophyllum* with internal standard (DMF- N,N-dimethylformamide, SCHR- sargachromenol **(2.10)**, SHQA- sargahydroquinoic acid **(1.38)** and SQA- sargaquinoic acid **(1.39)**)

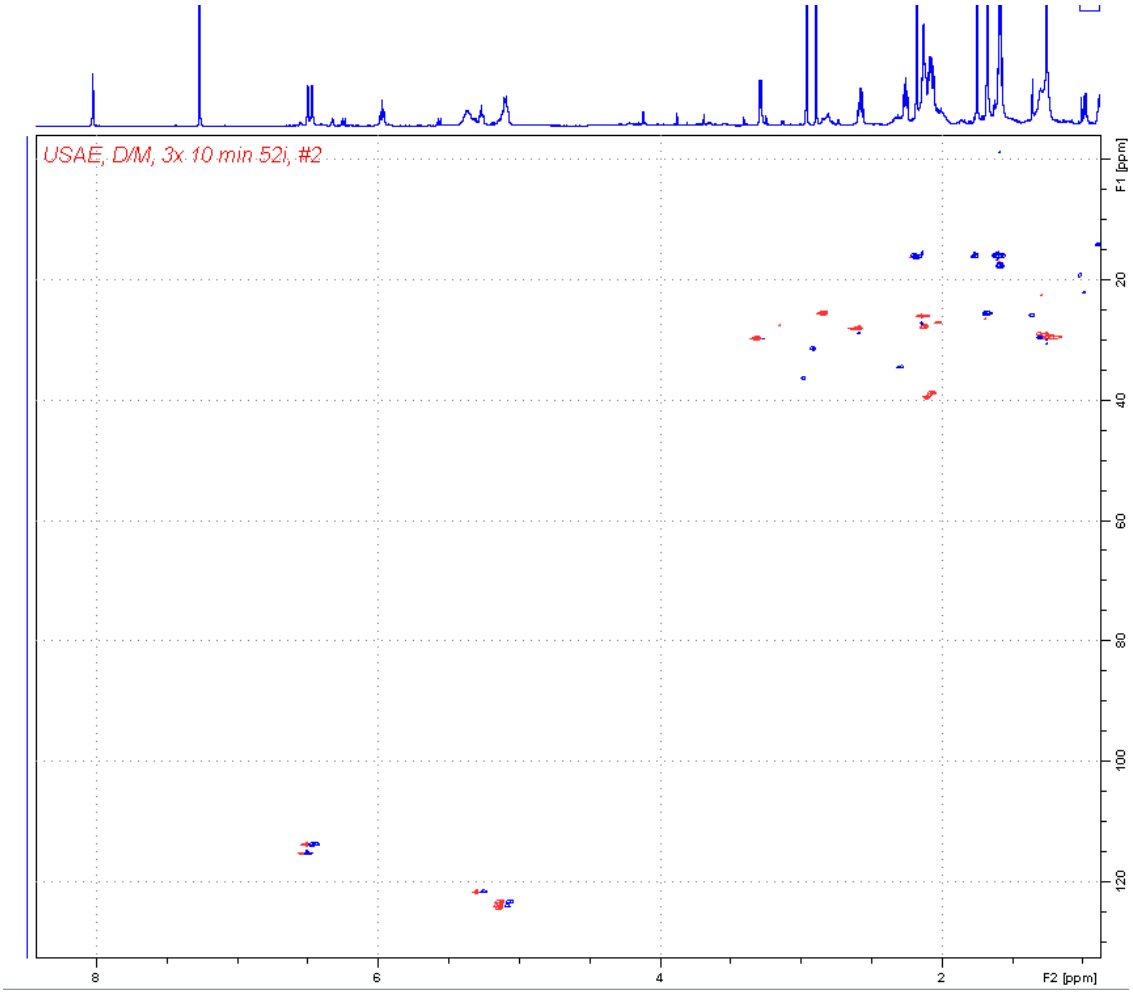


Figure 4.4 HSQC spectrum (600 MHz, $CDCl_3$) of crude *S. heterophyllum* with DMF

4.4.2.3 Quantification of metabolites in *S. heterophyllum*

Pre-weighed extracts were dissolved in 700 µL $CDCl_3$ containing 0.1% v/v DMF as the internal standard. Each extract was sonicated for 30 seconds and filtered into a 5 mm diameter NMR tube. NMR spectra were acquired on a 600 MHz Bruker Avance spectrometer, using 32 scans and a relaxation delay of 10 seconds. Other studies using DMF as an internal standard for qNMR have used a D1 of 12.5 seconds (Catchpole O.J. et al., 2003) however, the number of scans and D1 were selected to give reasonable experimental times. A D1 of 10 seconds was also selected based on T1 values of the signals of interest to this study (table 4.4). The selected D1 allowed for the total relaxation of the selected protons in all target compounds however, it did not allow for the total relaxation of the selected proton of the internal standard.

Table 4.4 T1 values of selected protons

Compound	**Signal of interest** (δ_H)	**T1** (seconds)
N,N-dimethylformamide	7.99	5.67
Sargahydroquinoic acid **(1.38)**	3.27	0.83
Sargaquinoic acid **(1.39)**	3.12	0.97
Sargachromenol **(2.10)**	5.57	1.81

A macro (table 4.5) with preset parameters was developed to locate and integrate peaks of interest. This macro was developed based on the integration of target peaks from varying points. For the internal standard, integration of the signal of interest in a mixture of crude sample was performed from various points. It was shown that integration with or without spinning sidebands yielded a difference of less than 0.2 % between the integral with and the integral without side bands (table 4.6). The same process was carried out for all the other signals and limits yielding more than 98% of the signal area were selected. NMR analysis of DMF showed no impurities, hence, no allowance was made for impurities during the final quantification calculations.

Table 4.5 Range of chemical shifts used in the integration of selected NMR signals

Compound	**Integration Range**	**(δ_H ppm)**
N,N dimethylformamide (DMF)	8.1495	7.8766
Sargahydroquinoic acid **(1.38)**	3.34730	3.1971
Sargaquinoic acid **(1.39)**	3.1480	3.1020
Sargachromenol **(2.10)**	5.5982	5.5330

Table 4.6 Integration limits used to select the most suitable integration limits for DMF

Integration Range	**Integral**
8.19190 - 7.83291 (with spinning sidebands)	1.00000
8.14953 - 7.87662 (without spinning sidebands)	0.98738
8.09909 -7.94934	0.97911
8.06937 -7.95906	0.97441

The signal-to-noise ratios of the selected signals ranged from 844:1-33:1 for the sample containing the least amount of sargahydroquinoic acid and from 895:1-122:1 for the extract containing the highest amount of sargahydroquinoic acid (table 4.7). The amounts of sargahydroquinoic acid (SHQA), sargaquinoic acid (SQA) and sargachromenol (SCHR) obtained by each extraction technique were calculated using the relative method as outlined in equation (1). The quantities of each metabolite extracted under various extraction conditions are listed in table 4.8.

Table 4.7 Signal to noise ratios (S/Ns) calculated from peaks of each metabolite

Analyte	**S/N of sample containing**	
	highest amount of SHQA (1.38)	**least amount of SHQA (1.38)**
N,N-dimethylformamide	895	844
Sargahydroquinoic acid **(1.38)**	714	430
Sargaquinoic acid **(1.39)**	122	33
Sargachromenol **(2.10)**	144	42

4.4.3 Assessment of various extraction techniques

4.4.3.1 Ultrasound Assisted Extraction

Freeze-dried *S. heterophyllum* (1 g) was sonicated in solvent (10 mL) while the temperature was maintained at 25°C. The mixture was filtered and the solid material washed with an additional 10 mL of solvent. The crude extract was concentrated under reduced pressure and analysed by ^{1}H NMR spectroscopy.

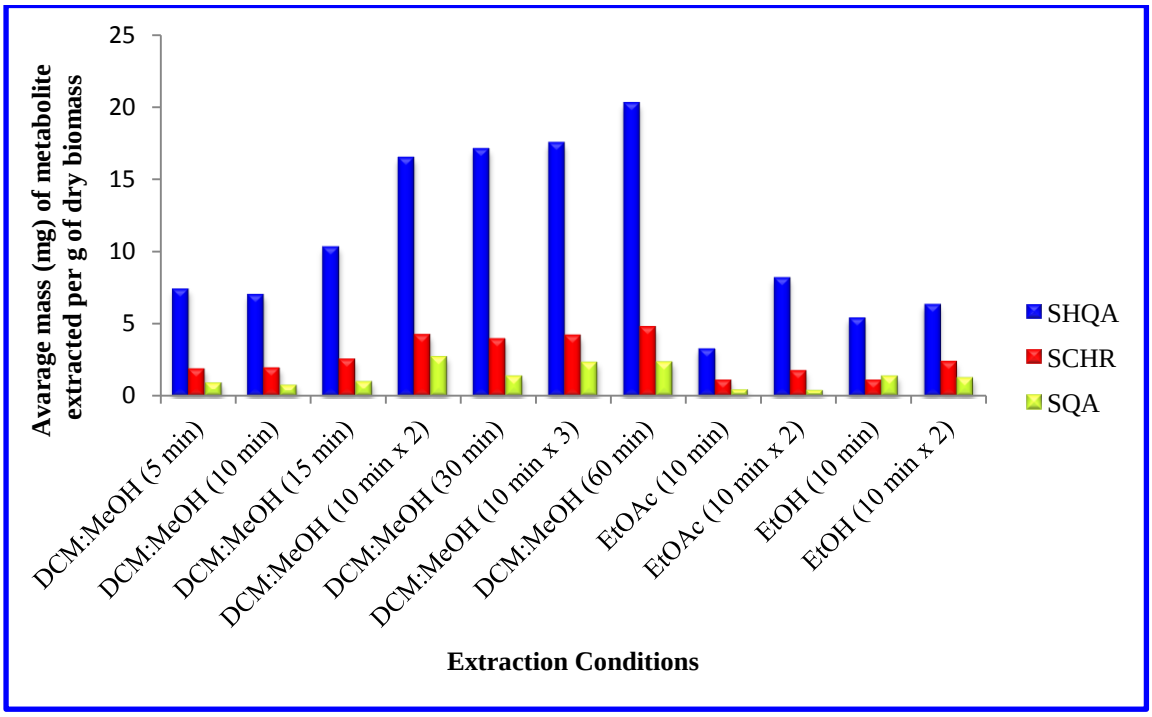


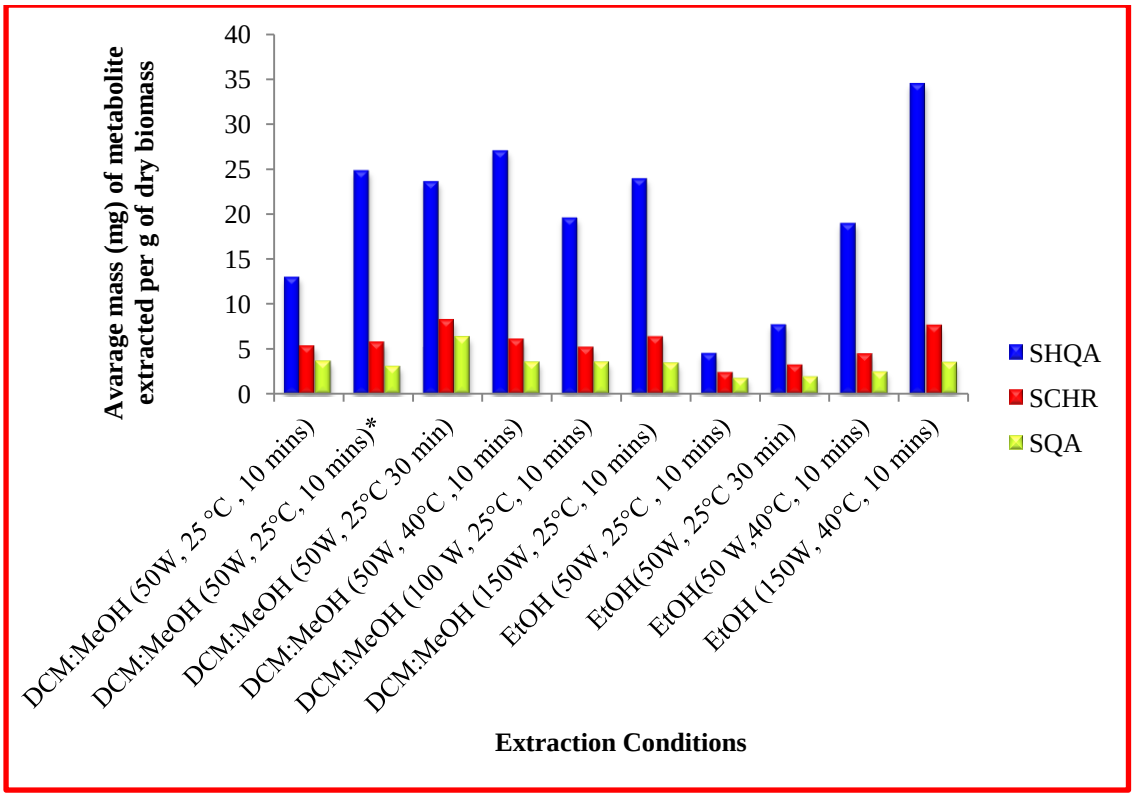
Figure 4.5 Amount of sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) extracted using USAE

Of the three solvent systems explored using USAE, DCM-MeOH yielded the highest mass of both crude extract and metabolites extracted per gram of biomass (figure 4.5). Not surprisingly, a steady increase in the mass of extracted metabolites was observed with an increase in extraction time or when the alga was extracted more than once. These results are in accordance with expected trends as longer extraction periods allow for higher amounts of metabolites to leave the plant matrix. Replacing extraction solvent steepens dissolution gradients between the plant material and the surrounding solvent. Interestingly, the masses of sargahydroquinoic acid extracted with two portions of DCM-MeOH for 10 minutes each (20 minutes) and one portion DCM-MeOH for 30 minutes, were almost the same. The use of

three portions of solvent for 10 minutes yielded almost similar results to extracting for 30 minutes with one portion of solvent.

4.4.3.2 Microwave Assisted Extraction

Freeze-dried alga (0.5 g) was placed in a glass 10 mL capped microwave vial and extracted in a microwave in 5 mL of solvent. The mixture was filtered and washed with an additional 5 mL of solvent. The crude extract was concentrated under reduced pressure and analysed by ^{1}H NMR spectroscopy.



*Extraction performed with agitation

Figure 4.6 Amount of sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) using MAE

The effects of agitation, extraction time, microwave power and temperature were explored using microwave assisted extraction. The introduction of agitation while extracting with DCM-MeOH at 25°C and 50 W resulted in an increase in the amount of sargahydroquinoic acid extracted by 11.8 mg (figure 4.6). An increase in the extraction time at 50 W resulted in an increase in the quantities of all three metabolites extracted per gram of biomass. However, the use of agitation while extracting at 50 W and 25°C for 10 minutes resulted in the extraction of more sargahydroquinoic acid, compared to extracting at the same power and temperature for 30 minutes without agitation. It was also interesting to note that an increase in temperature from 25 to 40ºC yielded similar results to increased extraction time. Hence, simply increasing the temperature from 25 to 40ºC has the potential to cut extraction time by 20 minutes, at a power of 50 W with DCM-MeOH as the extraction solvent. An increase in the extraction power from 50 to 100 W also resulted in an increase in the amounts of all three metabolites extracted. The same trends were also observed for the extraction of all three metabolites using EtOH. The highest amount of sargahydroquinoic acid extracted using this technique was attained using EtOH by increasing both extraction temperatures and extraction power from 25°C to 40°C and 50 W to 150 W respectively. These conditions were not attempted with the DCM-MeOH for safety reasons. The highest amounts of sargaquinoic acid and sargachromenol were obtained from extraction with DCM-MeOH for 30 minutes at 25°C and 50 W. Although not many parameters were investigated with EtOH as the extraction solvent, the extraction of biomass with this solvent for 10 minutes at 150 W and 40°C yielded the highest amount of crude extract and sargahydroquinoic acid. It is clear that this method of extraction has good potential for efficient, fast, easy and environmental friendly extraction.

4.4.3.3 Maceration and Soxhlet Extractions

Maceration Extraction

Freeze-dried alga was placed into a 50 mL round bottomed flask fitted with a reflux condenser. The material was extracted in 10 mL of solvent for 30 minutes over a temperature controlled water bath. Extractions were carried out between 37.5 and 38ºC. The mixture was filtered and the solid material washed 10 mL of solvent. The crude extract was concentrated under reduced pressure and analysed by ^{1}H NMR spectroscopy.

Soxhlet Extraction

A classical soxhlet apparatus was used in which 5 g of freeze-dried alga was extracted with 200 mL of solvent for 5 hours. The mixture was filtered and the solid plant material was washed with 20 mL of solvent. The crude extract was concentrated under reduced pressure and analysed by ^{1}H NMR spectroscopy.

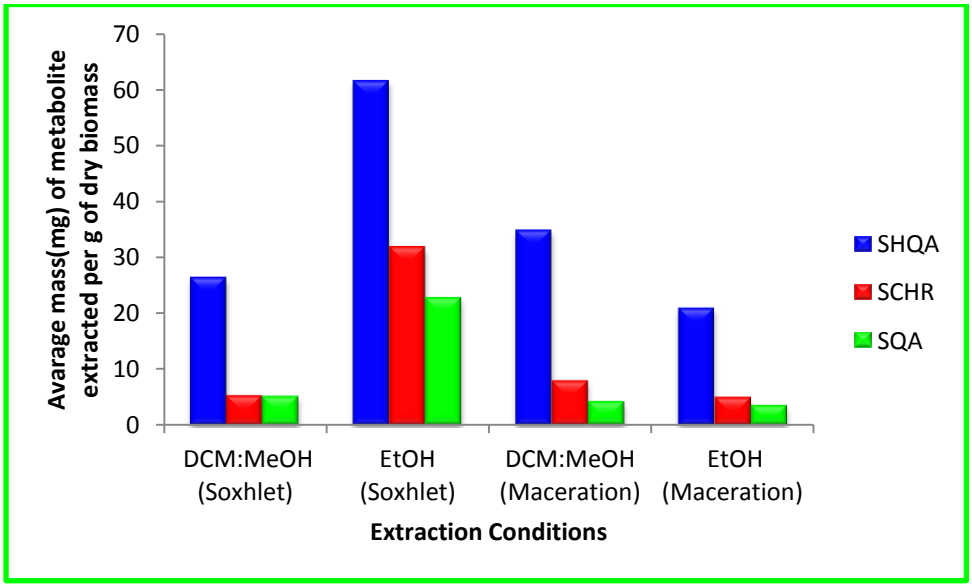


Figure 4.7 Amount of sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) extracted by maceration and soxhlet extraction

A comparison of results from maceration and soxhlet extraction yielded interesting results in that DCM-MeOH gave the highest yield of sargahydroquinoic acid for extraction by maceration while EtOH gave the highest yield for soxhlet extraction (figure 4.7). Where the optimisation of extracted metabolites is concerned, extraction by soxhlet using the more environmentally benign EtOH as the extraction solvent yielded the highest amounts of all

three metabolites. However, in interpreting these results important factors need to be taken into account. It was expected that soxhlet extraction would yield more material firstly because the solvent: biomass ratio employed in this technique (1:40) was much larger than the same ratio in maceration extraction (1:10) and secondly soxhlet extraction was performed for 5 hours while maceration was only performed for 30 minutes. In addition to this, soxhlet extraction is generally expected to yield better extraction results compared to maceration as the former technique makes use of solvent recycling to increase analyte diffusion rates. A more rational comparison of the two techniques would entail using the same solvent: biomass ratios and extraction periods for both extraction techniques.

Results from this study showed that the extraction of *S. heterophyllum* by soxhlet extraction using EtOH yields the highest amounts of sargahydroquinoic acid compared to the other extraction methods and conditions explored herein. The use of this solvent in microwave assisted extraction also shows great potential to yield high amounts of this metabolite. This could be due to the microwave absorbing characteristics of EtOH which are higher than those of all the other solvents used in this study.

We have been able to optimise extraction techniques available to us for the extraction of sargahydroquinoic acid **(1.38)** from *S. heterophyllum*. In addition to this, the current study has enabled us to identify new extraction conditions that will facilitate environmentally friendly extraction while generating significantly higher amounts of target metabolites from *S. heterophyllum*. It would have been useful to compare results obtained from the qNMR study with those compared from gravimetric experiments; however, the rapid oxidation of pure sargahydroquinoic acid would have presented significant challenges for accurate gravimetric quantification of this metabolite. Despite the relatively high standard deviations obtained in this study qNMR allowed for the simple and efficient quantification of analytes extracted by various techniques.

Table 4.8 Masses (mg /g of dry biomass) of crude extract, sargahydroquinoic acid (SHQA), sargachromenol (SCHR) and sargaquinoic acid (SQA) extracted by each extraction technique

Ultrasound Assisted Extraction (USAE)

		Average amount (mg/g)							
Solvent	Time	Crude extract	SD	SHQA	SD	SCHR	SD	SQA	SD
DCM:MeOH	5 min	16.08	0.76	7.42	1.53	1.87	0.02	0.93	0.69
DCM:MeOH	10 min	18.32	4.33	7.04	1.69	1.94	0.01	0.75	0.21
DCM:MeOH	15 min	21.16	3.79	10.33	2.90	2.53	0.02	0.99	0.33
DCM:MeOH	30 min	28.56	1.31	17.09	8.04	3.92	0.05	1.39	0.53
DCM:MeOH	60 min	36.22	2.04	20.27	1.22	4.73	0.00	2.33	0.62
DCM:MeOH	10 min x 2	35.56	3.17	16.5	2.19	4.21	0.03	2.7	0.62
DCM:MeOH*	10 min x 3	42.26	1.01	17.52	1.85	4.15	0.01	2.31	0.00
EtOH	10 min	11.91	2.09	3.3	2.83	1.13	0.01	0.46	0.01
EtOH*	10 min x 2	12.73	0.28	8.19	4.12	1.77	0.08	0.41	0.01
EtOAc	10 min	21	2.19	5.43	0.63	1.11	0.04	1.37	0.01
EtOAc	10 min x 2	17.3	5.11	6.35	3.44	2.38	0.05	1.28	0.01

Microwave Assisted Extraction (MAE)

Solvent	Time	Power (W)	Tempe-rature	Crude extract	SD	SHQA	SD	SCHR	SD	SQA	SD
DCM:MeOH	10 min*	50	25	37.38	5.30	13.02	25.78	5.28	0.06	3.65	0.03
DCM:MeOH	10 min	50 **	25	45.92	3.51	24.79	62.09	5.7	0.08	3.06	0.02
DCM:MeOH	10 min	100	25	35.73	9.54	19.58	38.48	5.14	0.03	3.517	0.01
DCM:MeOH	10 min	150	25	36.3	3.52	23.92	42.59	6.25	0.12	3.43	0.04
DCM:MeOH	10 min	50	40	47.42	0.94	27	11.20	6.01	0.02	3.52	0.01
DCM:MeOH	10 min x 3	50	25	47.17	20.13	23.6	79.56	8.13	0.20	6.28	0.08
EtOH	10 min	50	25	21.84	0.67	4.56	10.39	2.37	0.01	1.75	0.01
EtOH	10 min	50	40	26.74	6.16	7.74	25.65	3.21	0.02	1.91	0.01
EtOH	10 min x 3	50	25	48.68	12.66	18.98	19.37	4.41	0.01	2.47	0.01
EtOH	10 min	150	50	72.15	3.71	34.48	30.89	7.52		3.51	0.00

Soxhlet Extraction								
Solvent	Crude Extract	SD	SHQA	SD	SCHR	SD	SQA	SD
DCM:MeOH	302.93	5.08	26.48	26.48	5.31	0.00	5.20	0.02
EtOH*	537.3	18.96	61.61	75.6	31.7	0.7	22.7	0.25
Maceration								
Solvent	Crude Extract	SD	SHQA	SD	SCHR	SD	SQA	SD
DCM:MeOH*	69.3	0.91	34.94	0.3	7.98	0.01	4.32	0.00
EtOH*	59.6	3.43	20.97	0.3	5.06	0.01	3.59	0.03

* Extractions in which n = 2
**This extraction was performed with additional agitation
SD = Standard deviation

4.5 Experimental

4.5.1 Solvents and reference compounds

All solvents were of chromatographic grade (Merck, Germany) except for 95% ethanol which was redistilled before use. Deuterated Chloroform (99.8%), pyridine (99%) and *N*, *N*-dimethylformamide (99.9%) were all supplied by Merck, Germany, while di-chlorophthalonitrile (99 %) was obtained from Sigma- Aldrich, Germany. Sargahydroquinoic acid **(1.38)**, sargaquinoic acid **(1.39)** and sargachromenol **(2.10)** were isolated from *S. heterophyllum* as outlined in chapter 2 (section 2.3.3).

4.5.2 Plant material

Specimens of *S. heterophyllum* (NDK101124 - 4) were collected from Noordhoek near Port Elizabeth, South Africa on the 24th of November 2010. Algae was transported and stored as outlined in chapter 2, section 2.3.2.Some of the thawed algae was size reduced using liquid nitrogen in a mortar. Most of the crushed algae was freeze-dried on a Freezone ® 6 litre bench top freeze dryer system (Labconco, USA) for 24 hours while some of the size reduced algae together with the portion of whole algae was air dried in a fume hood for 14 days. The dry material was packed into air tight containers and stored at - 20°C until it was extracted.

4.5.3 Extraction of plant material

4.5.3.1 Qualitative assessment of algae preparation prior to extraction

Freeze dried, pre-sized reduced and whole air dried algae was extracted by ultrasound assisted sonication for 10 minutes as outlined below. ^{1}H NMR spectra of the crude extracts were acquired on a Bruker Avance 400 MHz spectrometer and compared for qualitative differences. Extraction and analysis of metabolite profiles in this section of the study was performed in duplicate.

4.5.3.2 Quantitative investigation of extraction methods

All extractions were performed in triplicate except where indicated, using the same batch of freeze-dried material. Details of the extraction conditions for each extraction are summarised in table 4.8.

Ultrasound assisted extraction (USAE)

USAE was performed in a Soniclean® US 2/5 sonicating bath (Ultrasonic Manufacturing Company, South Africa). Freeze-dried material (1 g) was placed into a 15 mL polypropylene centrifuge tube with a screw cap. The solvent (10 mL) was added and the mixture sonicated while the temperature was maintained at 25°C. The mixture was filtered through a Hirsch funnel and washed with 10 mL of solvent.

Microwave assisted extraction (MAE)

Freeze-dried material (0.5 g) was placed in a 10 mL glass capped microwave vial and extracted in a CEM Matthews Model Discover (700W) microwave (CEM Corporation, USA) in 5 mL of solvent. The mixture was filtered through a Hirsch funnel and washed with 5 mL of solvent.

Maceration (MAC)

Freeze-dried material (1 g) was placed in a 50 mL round bottomed flask fitted with a reflux condenser. The material was extracted in 10 mL of solvent for 30 minutes over a Grant temperature controlled circulating water bath (Grant Instruments, UK). Extractions were carried out between 37.5 and 38ºC. The mixture was filtered through a Hirsch funnel and washed with 10 mL of solvent.

Soxhlet Extraction (SOX)

A classical Soxhlet apparatus was used in which 5 g of freeze-dried biomass was extracted with 200 mL of solvent for 5 hours. The mixture was filtered through a Hirsch funnel and washed with 20 mL of solvent.

Evaporation of the filtered solvent extracts under vacuum (<40°C) using a Buchi rotovap (Buchi, Switzerland) gave the crude extract for each experiment. Crude extracts from each

extraction (4.1-21.6 mg) were weighed into vials which were stored at -4°C in a vacuum dessicator until they were analysed by NMR.

4.5.4 NMR Analysis

4.5.4.1 Sample Preparation

A solution of 0.1% v/v DMF in $CDCl_3$ was prepared by measuring 0.05 mL of DMF into a 50 mL volumetric flask. The DMF was made up to 50 mL with $CDCl_3$ and sonicated for five minutes. The contents of each vial were dissolved in 700 µL $CDCl_3$ (containing 0.1% v/v dimethylformamide as an internal standard). Each sample was then sonicated for 30 seconds and filtered into a 5 mm diameter NMR tube.

4.5.4.2 NMR apparatus and Parameters

^{1}H NMR spectra of the crude extracts were acquired using a Bruker Avance 600 MHz spectrometer and referenced to residual $CDCl_3$ solvent signal at δ 7.25 ppm. Automatic shimming was used on all samples.

Table 4.9 Acquisition parameters for the NMR analysis of all samples

NMR Parameter	Value
Temperature	300 K
Pulse angle	30º
Number of scans	32
Pulse width	11.55 µs
Pre acquisition delay (DE)	6.00 µs
Number of dummy scans	2
FID Resolution	0.11 Hz
Relaxation delay	10 seconds
Spectral width	72.11KHz
Acquisition time	4.54 seconds
Number of FID points	65.54 KHz

4.5.3.3 Post acquisition processing

MestreNova 6.0 (Mestrelab, Spain) was used for post acquisition processing. Before integration, each spectrum was automatically phase and baseline corrected.

Signal integration

A macro (table 4.5) with preset parameters was developed to locate and integrate peaks of interest. This macro was developed based on the integration of target peaks from varying points. For the internal standard, integration of the signal of interest in a mixture of crude sample was performed from various points. It was shown that integration with or without spinning sidebands yielded a difference of less than 0.2 % between the integral with side bands and the integral without (integrals one and two in table 4.6) The same process was carried out for all the other signals and limits yielding more than 98% of the signal area were selected.

References

Afolayan, A.F., Bolton, J.J., Lategan, C.A., Smith, P.J., and Beukes, D.R. (2008). Fucoxanthin,tetraprenylated toluquinone and toluhydroquinone metabolites from *Sargassum heterophyllum* Inhibit the *in vitro* Growth of the Malaria Parasite *Plasmodium falciparum*. Zeitschrift für Naturforschung B 63:848-852.

Bilia, A.R., Bergonzi, M.C., Mazzi, G., and Vincieri, F.F. (2001). Analysis of plant complex matrices by use of nuclear magnetic resonance spectroscopy: St. John's wort extract. Journal of Agricultural and Food Chemistry 49:2115-2124.

Catchpole, O.J., Grey, J.B., Perry, N.B., Burgess, E.J., Redmond, W.A, and Porter, N.G. (2003). Extraction of Chili, Black Pepper, and Ginger with Near-Critical CO2, Propane, and Dimethyl Ether: Analysis of the Extracts by Quantitative Nuclear Magnetic Resonance. Journal of Agricultural and Food Chemistry 51:4853-4860.

Choi, Y.H., Choi, H.K., Hazekamp, A., Bermejo, P., Schilder, Y., Erkelens, C., and Verpoorte, R. (2003). Quantitative Analysis of Bilobalide and Ginkgolides from Ginkgo biloba Leaves and Ginkgo Products Using ^{1}H-NMR. Chemical and Pharmaceutical Bulletin 51:158-161.

Choi, Y.H., Kim, H.K., Hazekamp, A., Erkelens, C., Lefeber, A.W.M., and Verpoorte, R. (2004). Metabolomic Differentiation of *Cannabis sativa* Cultivars Using 1H NMR Spectroscopy and Principal Component Analysis. Journal of Natural products 67:953-957.

Cravatto, G. (2008). Improved extraction of vegatable oils under high-intensity ultrasound and/or microwaves. Ultrasonics Sonochemistry 15:898-902.

Cravotto, G., Binello, A., Merizzi, G., and Avogadro, M. (2004). Improving solvent-free extraction of policosanol from rice bran by high-intensity ultrasound treatment. European Journal of Lipid and Science Technology 106:147-151.

Csaba, S.J. (1992). High-field NMR spectroscopy as an analytical tool for quantitative determinations: Pitfalls, limitations and possible solutions. Trends in Analytical Chemistry 11:332-344.

Diercks, T., Coles, M., and Kessler, H. (2001). Applications of NMR in drug discovery. Current Opinion in Chemical Biology 5:285-291.

Dumas, M.E., Debrauwer, L., Beyet, L., Lesage, D., Andre, F., Paris, A., and Tabet, J.C. (2002). Analyzing the Physiological Signature of Anabolic Steroids in Cattle Urine Using Pyrolysis/Metastable Atom Bombardment Mass Spectrometry and Pattern Recognition. The Journal of Analytical Chemistry 74:5393-5404.

Ham, Y.M., Kim, K-N., Lee, W.J., Lee, N.H., and Hyun, C-G. (2010). Chemical Constituents from *Sargassum micracanthum* and antioxidant activity. International Journal of Pharmacology 6:147-151.

Hagel, J.M. and Facchini, P.J (2008). Plant metabolomics: analytical platforms and integration within functional genomics. Phytochemistry Reviews 7: 479-497.

Harshey, R.M. (2003). Bacterial motility on a surface: many ways to a common goal. Annual Review of Microbiology 57:249-273.

Henderson, T.J. (2002). Quantitative NMR spectroscopy using coaxial inserts containing a reference standard: purity determinations for military nerve agents. Analytical Chemistry 74:191-198.

Holzgrabe, U., Diehl, B.W.K., and Wawer, I. (1998). NMR spectroscopy in pharmacy. Journal of Pharmaceutical and Biomedical Analysis 17:557-616.

Holzgrabe, U. and Malet-Martino, M. (2011). Analytical challenges in drug counterfeiting and falsification-The NMR approach. Journal of Pharmaceutical and Biomedical Analysis 55:679-687.

Jung, M., Jang, K.H., Kim, B., Lee, B.H., Choi, B.W., Oh, K-B., and Shin, J. (2008). Meroditerpenoids from the Brown Alga *Sargassum siliquastrum*. Journal of Natural Products 71:1714-1719.

Kim, H.K. and Verpoorte, R. (2009). Sample Preparation for Plant metabolomics. Phytochemical Analysis 21:4-13.

Kusumi, T., Ishitsuka, M., Kinoshita, T., Kakisawa, H., and Shibata, Y. (1979). Structures of new plastoquinones from the brown alga *Sargassum serratifolium*. Chemistry Letters 277-278.

Letellier, M., Budzinski, H., Charrier, L., Capes,S., and Dorthe, A.M. (1999). Optimization by factorial design of focused microwave assisted extraction of polycyclic aromatic hydrocarbons from marine sediment. Fresenius' Journal of Analytical Chemistry 364:228-237.

Lide, D.R. (2003). The Handbook of Chemistry and Physics. Florida: CRC Press LLC.

Malz, F. (2008). Quantitative NMR in the Solution State NMR. In Ulrike,H., Iwona,W., and Bernd,D., eds. NMR Spectroscopy in Pharmaceutical Analysis.Amsterdam: Elsevier. 43-62.

Malz, F. and Jancke, H. (2005). Validation of quantitative NMR. Journal of Pharmaceutical and Biomedical Analysis 38:813-823.

Mandal, V., Mohan, Y., and Hemalatha, S. (2007). Microwave Assisted Extraction – An Innovative and Promising Extraction Tool for Medicinal Plant Research. Pharmacognosy Reviews 1:7-18.

Paik, M.J., Park, J.E., Koo, W.H., Chung, G.H., Kim, J.H., and Kim, K.R. (2004). Modification of Soxhlet Extractor for Rapid and Effective Recovery of Phenolic Pollutants adsorbed on XAD-4 Resin. Chromatographia 60:693-698.

Pauli, G.F., Jaki, B.U., and Lankin, C.L. (2005). Quantitative ^{1}H NMR: Development and Potential of a Method for Natural Products Analysis. Journal of Natural Products 68:149.

Pauli, G.F. (2001). QNMR- A Versatile Concept for the Validation of Natural Products Reference Compounds. Phytochemical Analysis 12:28-42.

Perez-Castorena, A., Arciniegas, A., Apan, M.T.R., Villasenor, J.L., and de Vivar, A. (2002). Evaluation of the anti-inflammatory and antioxidant activities of the plastoquinones derivatives isolated from *Roldana barba-johanis*. Planta Medica 68:645-647.

Pinciroli, V., Biancardi, R., Visentin, G., and Rizzo, V. (2004). The Well-Characterized Synthetic Molecule: A Role for Quantitative ^{1}H-NMR. Organic Process Research and Development 8:381-384.

Priego-Capote, F. and Luque de Castro, M.D. (2004). Analytical uses of ultrasound I. Sample preparation. TrAC Trends in Analytical Chemistry 23:644-653.

Raynie, D.E. (2006). Modern Extraction Techniques. Analytical Chemistry 78:3997.

Reddy, P. and Urban, S. (2009). Meroditerpenoids from the southern Australian marine brown alga *Sargassum fallax*. Phytochemistry 70:250-255.

Rundlöfa, T., Mathiasson, M., Bekiroglu, S., Hakkarainen, B., Bowden, T., and Arvidsson, T. (2010). Survey and qualification of internal standards for quantification by 1H NMR spectroscopy. Journal of Pharmaceutical and Biomedical Analysis 53:645-651.

Segawa, M. and Shirahama, H. (1987). New plastoquinones from the brown alga *Sargassum sagamianum* var. yezoense. Chemistry Letters1365-1366.

Seo, Y., Lee, H-J., Park, K., Kim, Y., Ahn, J., Yoo, J., and Lee, B. (2004). Peroxynitrite-scavenging constituents from the Brown alga *Sargassum thunbergii*. Biotechnology and Biopress Engineering 9:212-216.

Seo, Y., Park, K., Kim, Y., Lee, H., Yoo, J., Ahn, J., and Lee, B. (2006). Isolation of tetraprenyltoluquinols from the brown alga *Sargassum thunbergii*. Chemistry and Pharmaceutical Bulletin 54:1730-1733.

Shen, J. and Shao, X. (2005). A comparison of accelerated solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco. Analytical and Bioanalytical Chemistry1003-1008.

Silvestre, V., Goupry, S., Trierweiler, M., Robins, R., and Akoka, S. (2001). Determination of substrate and product concentrations in lactic acid bacterial fermentations by proton NMR using the ERETIC method. Analytical Chemistry 73:1862-1868.

Tang, H., Yi, Y., Yao, X., Zhou, D., Lu, T., and Jiang, Y. (2002). Studies on bioactive steroid constituents from *Sargassum carpophyllum.* Chinese Pharmaceutical Journal 37:262-265.

Verpoorte, R., Choi, Y.H., and Kim, H.K. (2007). NMR-based metabolomics at work in phytochemistry. Phytochemistry Reviews 6:3-14.

Viant, M.R. (2007). Metabolomics of aquatic organisms: The new"omics" on the block. Marine Ecology Progress Series 332:301-306.

Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. Ultrasonics Sonochemistry 8:303-313.

Wawer, I., Holzgrabe, U., and Diehl, B.W.K. (2008). NMR Spectroscopy In Pharmaceutical Analysis.Amsterdam: Elsevier. 59-60.

Weljie, A.M., Newton, J., Mercier, P., Carlson, E., and Slupsky, C.M. (2006). Targeted Profiling: Quantitative Analysis of 1H-NMR Metabolomics Data. Analytical Chemistry 4430-4442.

Chapter 5

Structure Activity Relationships of Antiplasmodial and Cytotoxic Sargahydroquinoic acid Derivatives

5.1 Introduction

The isolation of several grams of SHQA acid allowed the modification of specific functional groups in this compound in order to assess their effect on antiplasmodial and cytotoxic activity. The tetraprenylated toluquinones that have been isolated from *S. heterophyllum* are all made up of the same structural backbone. However, some of these metabolites possess greater activity than others (Afolayan et al., 2008). To the best of our knowledge, the mechanism by which sargahydroquinoic acid and other tetraprenylated toluquinones from *S. heterophyllum* exert their antiplasmodial activity is unknown. In addition to this, the pharmacophoric groups responsible for this compound's biological activity are also unknown. The relative ease of acquiring these compounds from *S. heterophyllum* makes them good candidates for semi synthetic derivatisations.

5.1.1 The development of ideal antiplasmodial agents

Most antimalarial agents have not been developed from well-defined targets or mechanistic studies (Fidock et al., 2004; Laurent and Pietra, 2006). Some antimalarials such as quinine and to some extent artemisinin were identified from ethnopharmacology (Laurent and Pietra, 2006; Fidock et al., 2004). The discovery of these compounds from nature led to the development of synthetic drugs such as chloroquine and artesunate whose activity was based on the structures of compounds from natural origin. Other drugs, such as tetracycline and the folate antagonists were identified from their treatment of other ailments (Laurent and Pietra, 2006). With the increasing knowledge of the *plasmodium* parasite's biochemistry and its recently characterised genome, several targets for the development of chemotherapeutic agents have been identified and utilised for drug development (Fidock et al., 2004; Laurent and Pietra, 2006; Gardner et al., 2002; Bahl et al., 2003). Some of these targets include enzymes such as protein kinases, DOXP reductoisomerase and protein farnesyl transferases. (Fidock et al., 2004) Before the successful culture of the asexual cycle of *P. falciparum* in

human erythrocytes in 1976, screening for antimalarial agents was accomplished by using *in vivo* avian and rodent models (Kayser et al., 2003). These models used different strains of the *plasmodium* parasites such as *P. berghei*, *P. yoelii* and *P. chabaudi* (Kayser et al., 2003). However, none of these parasites cause disease in humans. In addition to this, there are essential differences between the biology and pharmacokinetics in mice and in humans (Kayser et al., 2003). Despite these limitations, the development of drug resistant strains of these parasites has been useful in the identification of new compounds against human malaria. Hence, rodent malaria has played an important role in the development of human antimalarial agents (Kayser et al., 2003). Since the mechanism of action of chloroquine was discovered, haemoglobin has become a popular target for drug discovery. This has led to the development of several assays based on this target (Laurent and Pietra, 2006; Francis and Sullivan, 1997; Wright et al., 2001).

In vitro toxicity assays against several *Plasmodium* parasite strains also play a critical role in the discovery and development of antimalarial agents (Desjardins et al., 1979; Ziegler et al., 2002). Pioneered by Desjardins in 1979, one of these assays is based on the parasite's uptake of radioactive substrates such as hypoxanthine, palmitate or ethanolamine in the presence of the test drug (Fidock et al., 2004). These *in vitro* assays involve the culturing of *P. falciparum* in human erythrocytes in different concentrations of the compound under investigation. Media containing low levels of non-radioactive substrate is used for initial parasite culture, after which radioactively labelled substrate is added to cells followed by cell harvesting and measurement of the amounts of radioactive substance taken up by the parasites (Laurent and Pietra, 2006). IC_{50} values are then determined from analysis of dose dependent curves (Fidock et al., 2004). These assays enable high throughput screening as only 1 - 2 mg of the compound under investigation is required for screening (Laurent and Pietra, 2006). The *in vitro* culture of the exoerythrocytic stages of *P. falciparum* in the liver using hepatocytes has also been accomplished (Kayser et al., 2003). However, these assays have not been used extensively in the evaluation of potential antimalarial agents (Kayser et al., 2003; Janse and Waters, 1995). Despite the disadvantages of relative high costs and complexity, the hypoxanthine assay has enabled the identification of both sensitivity and resistance of standard antimalarial agents to several *P. falciparum* strains (Kayser et al., 2003). Other simpler and cheaper assays such as colorimetric assays based on the detection of the reduction of nitro-blue tetrazolium to formazan by parasite enzymes have been developed.

However some of these assays are less sensitive than the hypoxanthine assay (Kayser et al., 2003; Basco et al., 1995).

The development of a good antiplasmodial agent not only depends on the agent's toxicity towards the *Plasmodium* parasite but also on the agent's toxicity to human cells (Mancini et al., 2009; Pink et al., 2005). An ideal antiplasmodial agent is one with higher antiplasmodial activity compared to cytotoxic activity. It has become common practice to compare antiplasmodial assay results to those obtained for the same compound in cytotoxicity assays (Laurent and Pietra, 2006). Cell lines such as KB, P-388, HeLa, A-549, MEL-28, and A-549, which depend on similar rates of division of parasite and tumour cells, are used for this comparison (Laurent and Pietra, 2006). The values of these two assays are compared to each other by calculating a parameter known as the selectivity index. This index is a ratio of a compound's cytotoxicity to its antiplasmodial activity (Laurent and Pietra, 2006). Ideally a compound's cytotoxicity should allow for a selectivity index greater than ten (Mancini et al., 2009).

5.1.2 Quinone bearing compounds as antiplasmodial agents

Several compounds which produce high levels of oxidative stress within the plasmodium infected erythrocyte have been identified (Lin et al., 1991;Vennerstrom and Eaton, 1988). These compounds include quinone bearing molecules such as quinones, hydroquinones, quinone methides and naphthoquinones in addition to other miscellaneous compounds such as thiosemicarbazone metal complexes and tetracyclines (Vennerstrom and Eaton, 1988; Figueiredo et al., 1998; Lin et al., 1991). Of these compounds, several naphthoquinones and quinones also have significant antiplasmodial activity against *P. falciparum* (Lin et al., 1991). An example of one such agent is the compound atavoquine, which is currently used as an antimalarial agent (Olliaro, 2001). Two mechanisms by which naphthoquinones exhibit these effects have been proposed. These include the generation of reactive oxygen species through oxidation/reduction cycling and the inhibition of the enzyme dihydroorotate dehydrogenase which is involved in the synthesis of nucleotides (Olliaro, 2001).

The main aim of this part of the project was to explore the antiplasmodial activity of a number of natural and synthetic analogues of sargahydroquinoic acid **(1.38)**. Specifically,

attention was focused on the hydroquinone and carboxylic acid moieties of this compound (figure 5.1). The antiplasmodial and cytotoxic activity of these semi-synthetic derivatives was also evaluated.

Hydroquinone Modifications

- Oxidation
- Methylation
- Acetylation
- Cyclization

Carboxylic Acid Modifications

- Esterification
- Reduction

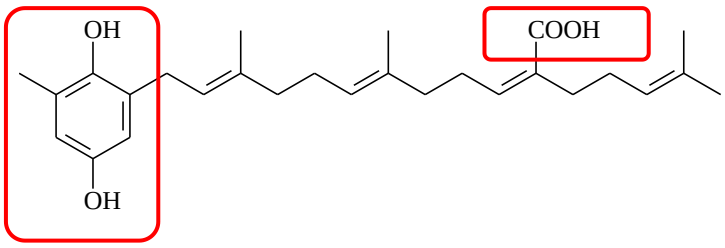
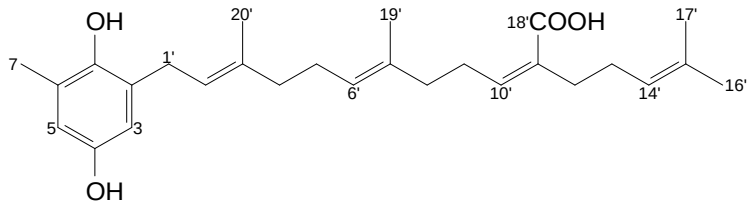


Figure 5.1 Proposed changes to the hydroquinone and carboxylic moiety of sargahydroquinoic acid moieties in order to assess their effect on antiplasmodial and cytotoxic activity **(1.38)**

5.2 Results and Discussion

5.2.1 Chemical Modification of Sargahydroquinoic acid

Sargahydroquinoic acid **(1.38)** is the major component of the DCM-MeOH extract of *S. heterophyllum*. Gram quantities of this natural product were isolated as described in section 2.3.2.



Sargahydroquinoic acid **(1.38)**

Oxidation of sargahydroquinoic acid (1.38) to sargaquinoic acid (1.39)

Although sargaquinoic acid **(1.39)** can be isolated from *S. heterophyllum*, the amount of **1.39** that was isolated was very low. During the course of this work, we observed the oxidation of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)** *in situ*. However, this process occurred slowly hence, we sought to establish a procedure that would provide higher yields of **1.39**. To this end, we explored a number of oxidation reactions in an attempt to optimise the formation of **(1.39)**. There is ample precedence in the literature for the air oxidation of hydroquinones to quinones (Kusumi et al., 1979; Perez-Castorena et al., 2002) and this was the first reaction attempted. Vigorous stirring of a solution of **1.38** in acetonitrile provided inconsistent results and was not explored further. However, both MnO_2 and Ag_2O converted sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)** essentially in quantitative yields in less than 8 hours. Further optimization of the procedure by using two instead of one molar equivalents of Ag_2O allowed for oxidation of **1.38** to **1.39** in significant yields, in less than 2 hours. The structure of the semi-synthetic sargaquinoic acid **(1.38)** was confirmed to be identical to that of the naturally occurring compound by comparison of NMR data with that of sargaquinoic acid isolated in section 2.3.3. The use of other oxidising agents for the conversion of **1.38** to **1.39** was also briefly explored (table 5.1).

Table 5.1 List of oxidising agents investigated for the conversion of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)**

Oxidizing agent	**Reaction Conditions**	**Isolated yield**
Air	ACN, rt, 12 h	30%
Oxone	ACN, rt,2 h	No Reaction
Ag_2CO_3	CH_2Cl_2, ($C_6H_{15}N$), rt, 5 h	20%
Ag_2O	$CHCl_3$,MeOH , rt, 2h /24h	70%
MnO_2	$CHCl_3$, MeOH, rt, 24 h	95%

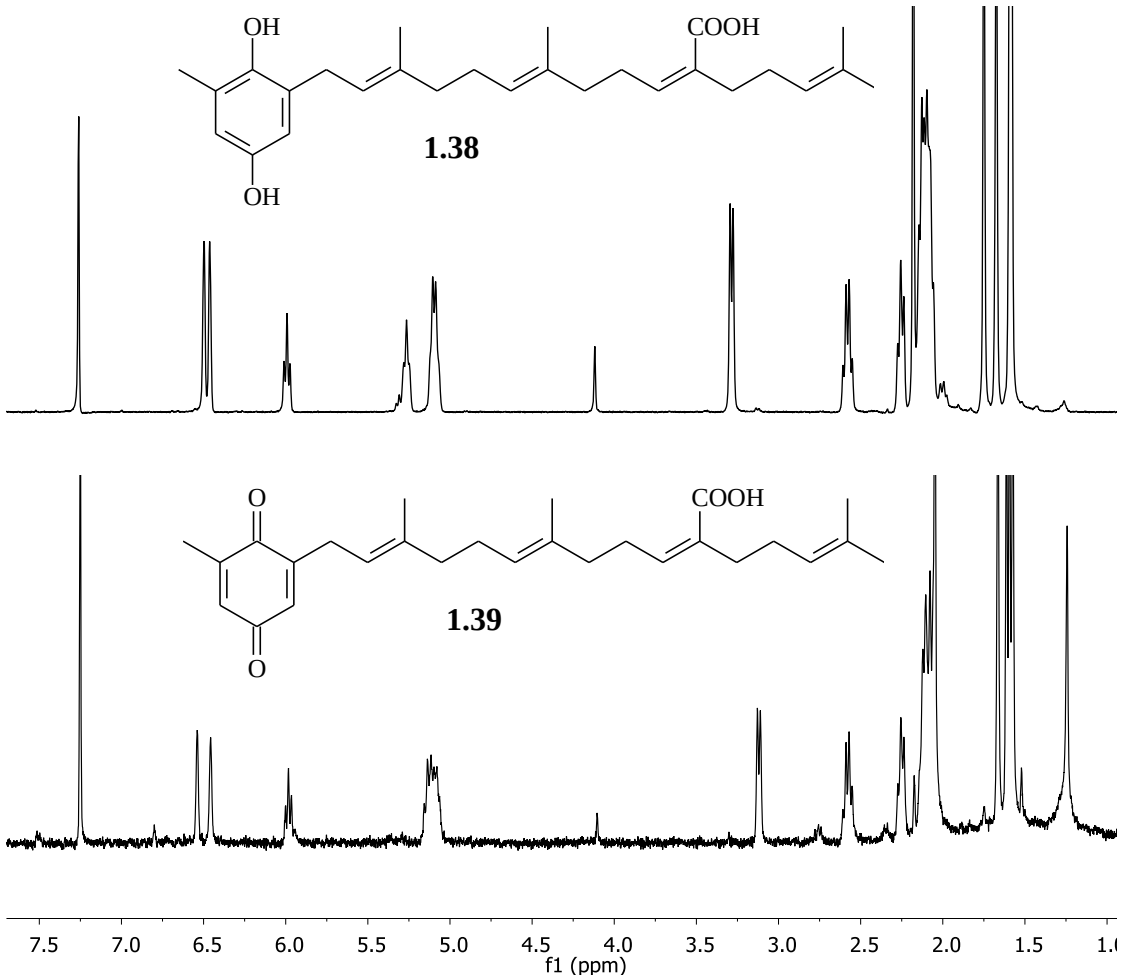


Figure 5.2 ^{1}H NMR spectra of sargahydroquinoic acid **(1.38)** and sargaquinoic acid **(1.39)** (400 MHz, $CDCl_3$)

The ^{1}H NMR spectrum of the crude product obtained from the Ag_2O catalysed oxidation of **1.38** (figure 5.3) indicated the presence of small amounts of an impurity characterised by the presence of de-shielded resonances between δ 8.00, δ 7.98 and δ 7.51 ppm. Targeted isolation and purification of this "impurity" allowed for the identification of an unexpected side product **(5.1)**. It was clear from the ^{1}H NMR spectrum of **5.1** (figure 5.4) that there had been a change in the aromatic part of sargahydroquinoic acid with the appearance of two additional, mutually coupled aromatic doublets at δ 8.00 (J = 7.9) and δ 7.51 (J = 7.9). In addition, one of the aromatic singlets had shifted downfield from δ 6.46 to δ 6.81 ppm

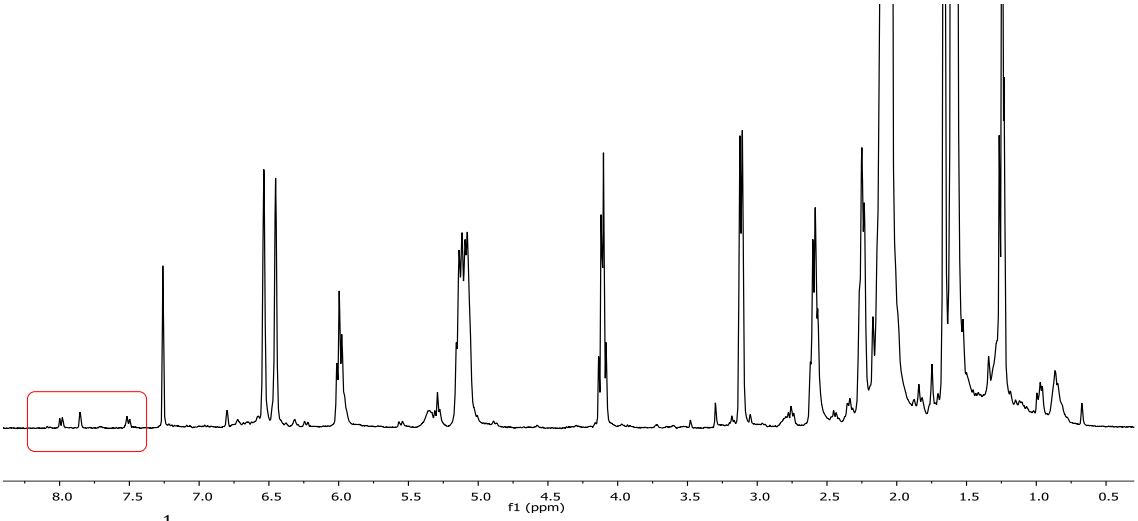


Figure 5.3 ^{1}H NMR spectrum of the crude product obtained from the Ag_2O catalysed oxidation of sargaquinoic acid (400 MHz, $CDCl_3$)

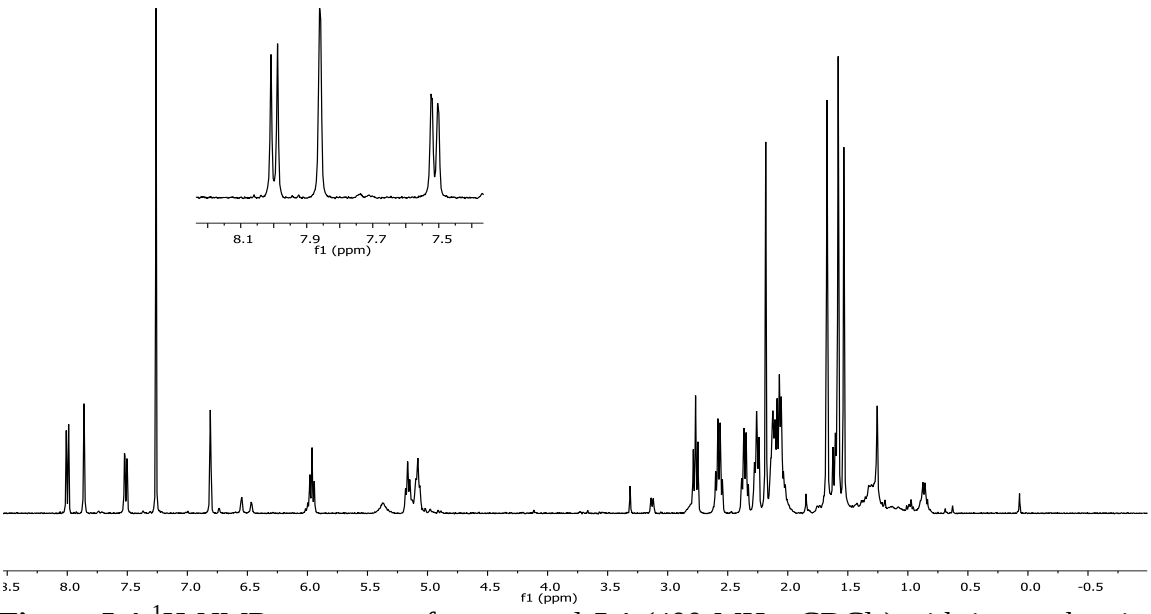


Figure 5.4 ^{1}H NMR spectrum of compound **5.1** (400 MHz, $CDCl_3$) with insert showing an expansion of the region between δ 8.1 and δ 7.4 ppm

Data from the ^{13}C NMR spectrum of compound **5.1** revealed no changes in the number of carbon atoms in the starting material **(1.38)** and in **5.1**. However, the ^{13}C NMR spectrum showed the presence of two quinone carbonyls at (δ 184.44 and δ 184.41) and the carboxylic acid moiety (δ 171.9). In addition to this, the DEPT-135 NMR spectrum indicated the loss of one methyl signal (δ 16.1, C-20') and a methylene signal (δ 121.7, C-2') and the appearance of two additional olefinic methine signals (δ133.8 (C-2) and δ125.9 (C-20)).

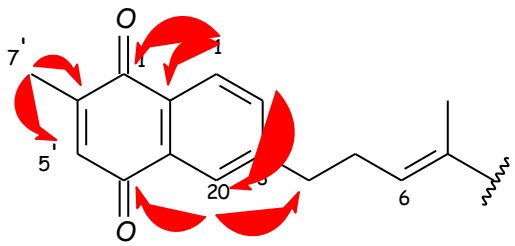
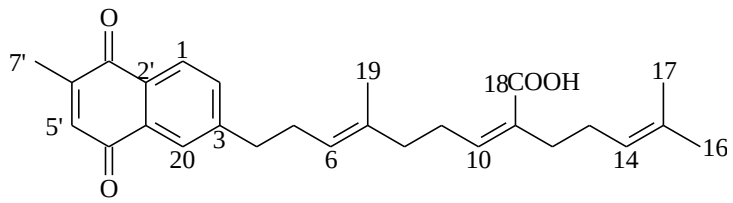


Figure 5.5 Key HMBC correlations for compound **5.1**

HMBC correlations from the doublet at δ_H 8.00 (H-1) to carbon signals at δ_C 126.7 (C-1') and δ_C 133.8 (C-2'); δ_H 7.51 (H-2) to the carbon signal at δ_C 125.9 (C-20) and δ_H 7.86 (H-20) to carbon signals at δ_C 36.17 (C-4) and δ_C 185.41 (C-4') allowed for the assignment of a naphthoquinone moiety. Spectroscopic data for **5.1** were almost identical to a compound previously isolated from the Taiwanese soft coral, *Nephthea chabrolii* (Sheu et al., 2004). The only difference was the geometry of the C-10 double bond. The geometry of the C10/11 double bond was assigned on the basis of comparison of proton chemical shifts of **5.1** to those of sargahydroquinoic acid **(1.38)**, sargaquinoic acid **(1.39)** and 2'*E*, 6'*E*, 10'*E* - sargaquinal **(1.40)** and the known compound previously isolated from *Nephthea chabrolii* (Sheu et al., 2004). To this effect, the linear portion of **5.1** is consistent with *Z* geometry compared to compounds **1.38** and **1.39**. For a *Z* geometry in the prenylated side chain the proton chemical shifts for H-10 and H-12 are approximately δ 5.96 and δ 2.26 ppm respectively while E geometry would show H-10 H-12 chemical shifts of 6.46 and δ 2.25 ppm.



5.1

Table 5.2 NMR spectroscopic data for compound **5.1** (600 and 100 MHz, $CDCl_3$)

Carbon No	δ_C	δ_C , mult	δ_H, mult, J_{Hz}	COSY	HMBC
1'	185.44	C	-		
2'	130.2	C	-		
3'	132.2	C			
4'	185.41	C	-		
5'	136	CH	6.81, s	H-7'	
6'	149	C	-		
7'	16.44	CH_3	2.18 s,	H-5'	C-6',C-5'
1	126.7	CH	8.00 d, 7.9	H-2	C-2',C-1'
2	133.8	CH	7.51 dd, 7.9	H-4,H-20	C-1', C-20
3	148.2	C	-		
4	36.17	CH_2	2.77, t, 7.6	H-5'	C-5,C-3
5	29.1	CH_2	2.36 m	H-4,H-6	C-4,C-6,C-7
6	123.2	CH	5.17	H-6	
7	136	C	-		
8	39.01	CH_2	2.08, m	H-9	C-6,
9	28.19	CH_2	2.57, m	H-10	C-8,C-10
10	145	CH	5.96 t, 7.3	H-9	C-8
11	130.6	C	-		
12	27.77	CH_2	2.26, m	H-13, H-14lr	C-13,C-14
13	28.19	CH_2	2.11, m	H-14	C-15,C-11
14	123.4	CH	5.17 ,t,7.0	H-13, H-14lr	
15	132.3	C			
16	25.63	CH_3	1.68, s	H-14lr	C-15,C-14
17	17.65	CH_3	1.59, s	H-14lr	C-16
18	171.9	C			
19	15.94	CH_3	1.58, s	H-6	C-6
20	125.9	CH	7.86, s	H-4	C-4',C-4

The conversion of a prenylated hydroquinone such as **1.38** to a naphthoquinone is unprecedented and presents novel synthesis of these compounds. These compounds are usually synthesised by Diels-Alder type reactions between *p*-benzoquinones and dienes or by the prenylation of halogenated naphthoquinone moieties (Alonso et al., 2004; de Koning et al., 2003; Couladouros et al., 1996). In view of the prominence of the naphthoquinone pharmacophore in antimalarial natural products the optimized chemical modification of **1.38** to **5.1** was briefly investigated. The importance of time, temperature, catalyst and solvent were all explored. At this point, a yield of 63% has been obtained by stirring the reaction vessel contents as outlined above for seven days. The following reaction scheme has been proposed for the conversion of **1.38** to **5.1**.

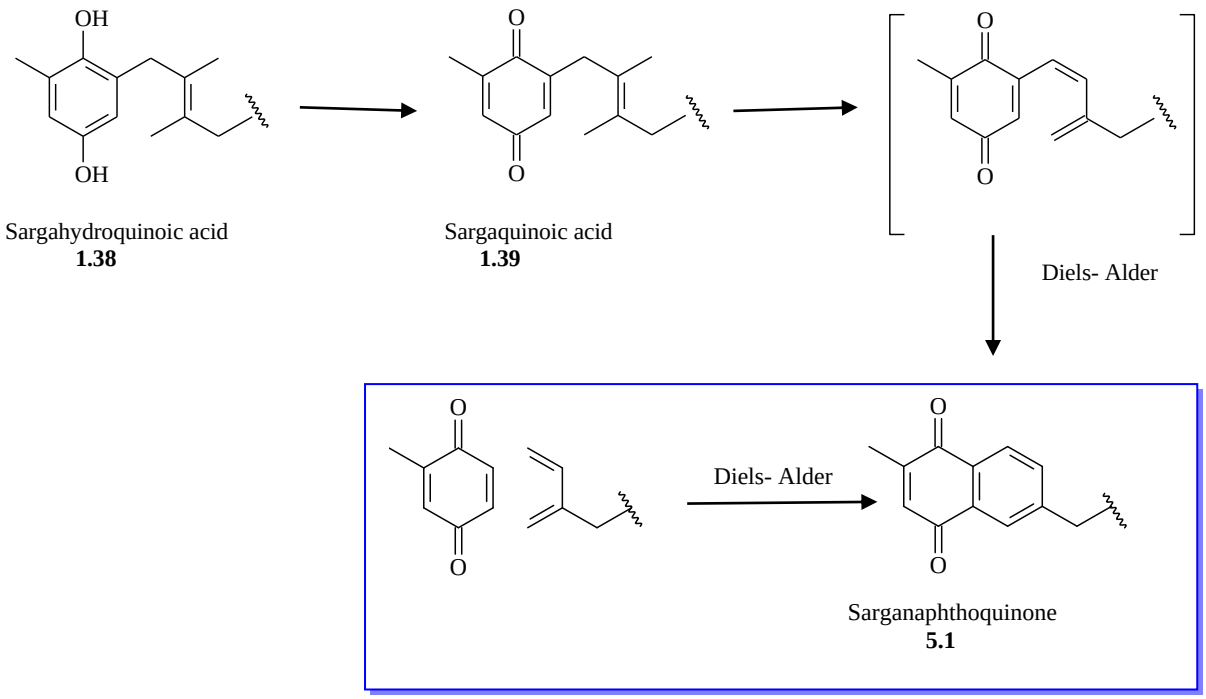


Figure 5.6 Proposed scheme for the conversion of **1.38** to **5.1**

Methylation and acetylation of sargahydroquinoic acid (1.38)

Methylation of alcohols may be achieved by Williamson ether syntheses using reagents bearing the required methyl group such as CH_3I and $(CH_3O)_2$ SO_2 (Sandulache et al., 2003; Smith et al., 1969). The semi-synthesis of the di-methyl ether of **1.38** was attempted by refluxing a mixture of sargahydroquinoic acid **(1,38)**, $(CH_3O)_2$ SO_2 , K_2CO_3 and acetone for 16 hours followed by stirring at room temperature for a further 8 hours. This reaction yielded the hydroquinone methyl ester derivative of sargahydroquinoic acid **(5.2)**. Upon standing this compound oxidised smoothly to give the corresponding quinone methyl ester **(5.3)**. Our reaction conditions failed to attain the required selective methylation. This preferential esterification of the carboxylic acid moiety could have resulted from its stronger activity as a Lewis acid compared to the hydroxyl group on the hydroquinone moeity of **1.38**. Further attempts to achieve selectivity were not investigated however, the use of other methylating agents such as CH_3I or the use of protecting groups on this carboxylic group could still be explored in an effort to synthesise the di-methyl ether of **1.38**. Key differences between the NMR spectra of the starting material and those of compounds **5.2** and **5.3** were the appearance of an extra methyl group at δ 51.0 ppm **(5.2)** and at δ 51.2 ppm **(5.3)**. In addition to this, the carboxylic acid signal (C-18') at δ 172 ppm in **1.38** was replaced by a signals at δ

168.4 ppm in both compounds **5.2** and **5.3**. These signals are characteristic of ester carbonyls. The arrangement in the rest of the linear tetraprenyl chain remained intact for both esters.

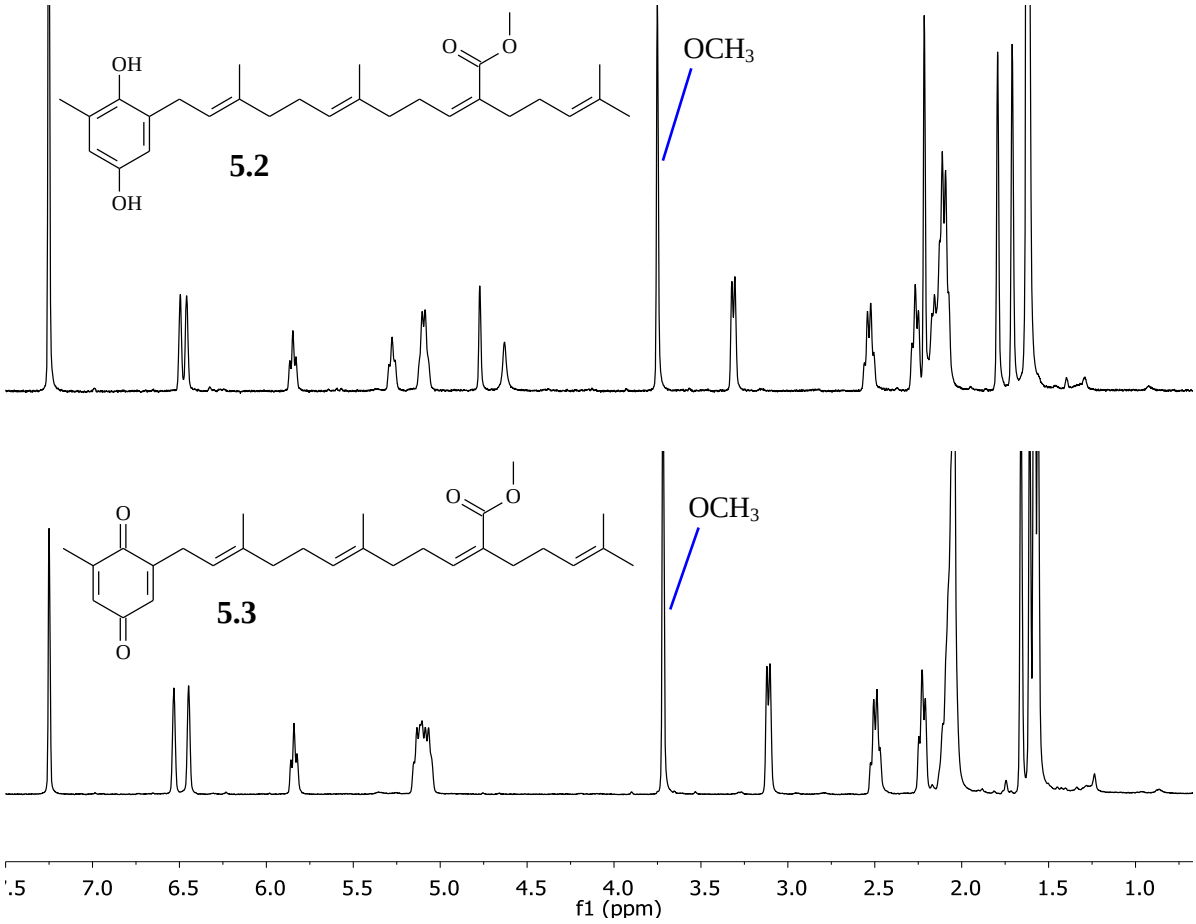


Figure 5.7 ^{1}H NMR spectra of compounds **5.2** and **5.3** (400 MHz, $CDCl_3$)

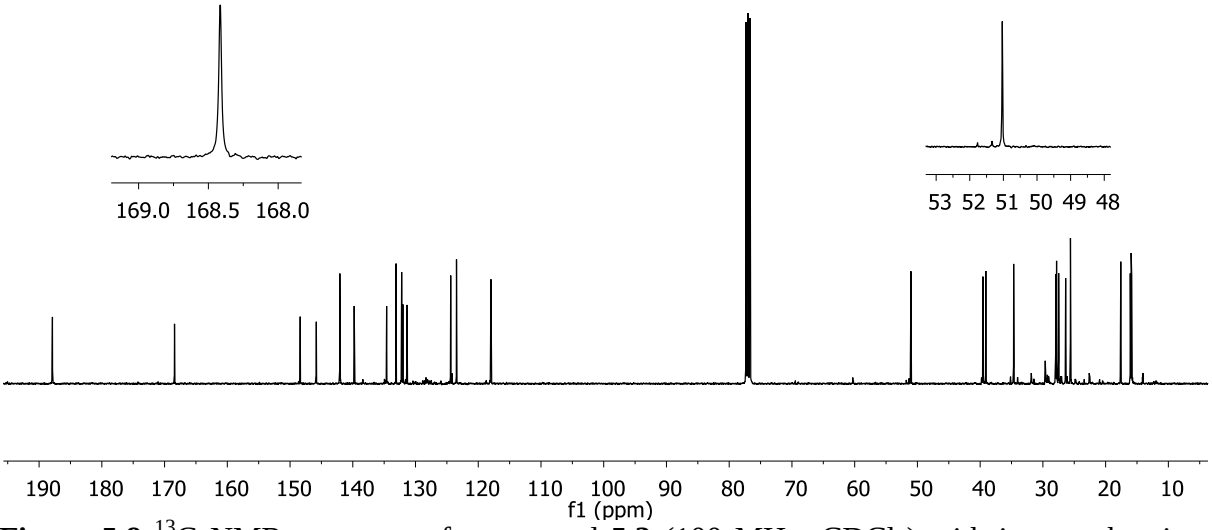


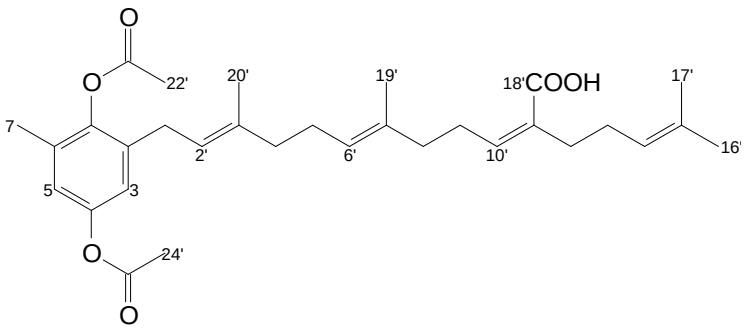
Figure 5.8 ^{13}C NMR spectrum of compound **5.3** (100 MHz, $CDCl_3$) with inserts showing expansions of regions between δ 169.0 and δ 168.0 ppm and δ 53 and δ48 ppm

Table 5.3 Comparison of ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data for compounds **5.2** and **5.3** with 2D NMR data for the more stable **5.3**

Hydroquinone Methyl Ester **(5.2)**			Quinone Methyl Ester **(5.3)**				
Carbon no.	δC	**δ_H, mult, J_{Hz}**	δC	δC mult	**δ_H, mult, J_{Hz}**	**COSY**	**HMBC**
1	146.37		187.92	C			
2	125.5		145.81	C			
3	115.42	6.50, s	133.1	CH	6.52,s		
4	148.88		187.87	C			
5	113.95	6.46, s	132.11	CH	6.44, s		
6	127.51		148.38	C			
7	17.64	1.76, s	16.07	CH_3	1.65, s		
1’	29.99	3.29, s, 7.1	27.48	CH_2	3.11, d, 6.8	H-2',	C-1, C-2', C-3'
2’	121.73	5.27, t, 6.5	117.97	CH	5.10, dd, 7.5		
3’	138.25		139.78	C			
4’	39.5	2.07, m	39.56	CH_2	2.05, d ,8.0		C-6', C-20'
5’	26.12	2.13, m	26.37	CH_2	2.03, d, 8.0	H-6'	
6’	124.2	5.09, m	124.39	CH	5.10, dd, 7.5		C-8'
7’	134.78		134.6	C			
8’	39.1	2.07, m	39.09	CH_2	2.05, d, 8.0		C-7'
9’	28.05	2.56, dd, 7.5	27.95	CH_2	2.49,dd, 6.8	H-10'	C-8', C-10',C-11'
10’	142.44	5.84, t, 7.3	142.06	CH	5.83, t,7.0	H-9'	C-8', C-12', C-18'
11’	131.32		131.36	C			
12’	34.66	2.24, t, 7.7	34.66	CH_2	2.22, t, 7.1	H-13'	C-11',C-18'
13’	27.84	2.50, dd, 7.5	27.82	CH_2	2.04,d	H-14'	C-15'
14’	123.45	5.09, m	123.46	CH	5.10, dd, 7.5		
15’	132.17		133.1	CH			
16’	25.67	2.13, m	25.61	CH_3	1.58, s		
17’	16.17	1.59, s	17.59	CH_3	1.55		C-16'
18’	168.59		168.42	C			
19’	16.07	1.67, s	15.93	CH_3	1.65, s		
20’	15.99	1.76, s	15.84	CH_3	1.61 , s		
21'	51.24	3.73, s	51.03	CH_3	3.71, s		C-18'

The of acetylation of the hydroxyl groups in the hydroquinone moeity of **1.38** was achieved by stirring a solution of sargahydroquinoic acid **(1.38)**, acetic anhydride and pyridine at room temperature for 30 hours. This reaction resulted in the formation of compound **5.4**. The ^{1}H NMR spectrum of this compound was characterised by a shift in the aromatic signals from δ

6.50 ppm and δ 6.46 ppm in sargahydroquinoic acid to δ 5.21 ppm and δ 5.13 ppm for H-3 and H-5 respectively in compound **5.4**. This was due to the decreased effect of the oxygen's electronegativity on carbons 3 and 5 as its electrons were now influenced by the more electronegative carbonyl oxygens attached to the newly added carbon atoms, C- 21' and C-23'. The ^{13}C NMR spectrum of this compound showed the presence of four additional carbons at δ 169.6, δ 21.1, δ 169.0 and δ 20.5 ppm (C-21', C-22', C-23' and C-24' respectively) (figure 5.9). DEPT-135 NMR was used to confirm that two of these new carbons were quatenary carbons (C-21' and C-23') while the other two signals were confirmed to be due to methyl carbons (figure 5.10). The aromatic nature of the molecule was retained as was shown by the presence of the carbons at δ 145.2 and 148.0 ppm for C-1 and C-4 respectively.



Compound **5.4**

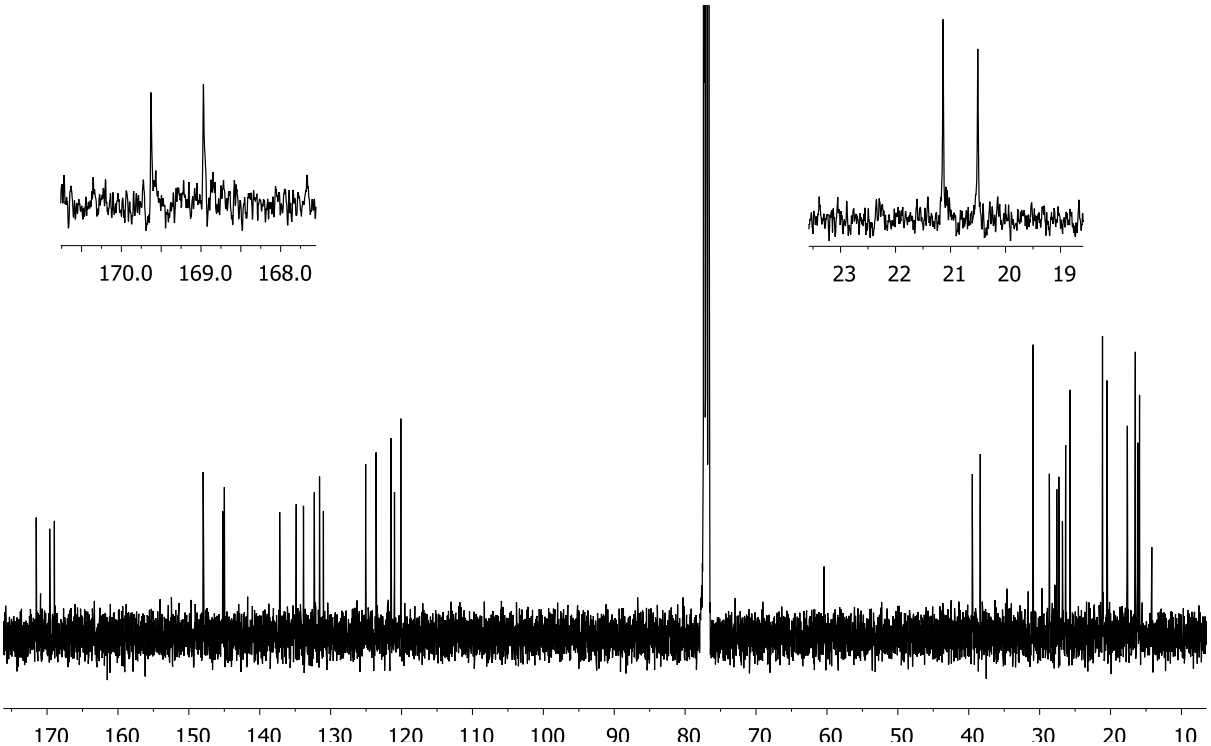


Figure 5.9 ^{13}C NMR spectrum of compound **5.4** (100 MHz, $CDCl_3$) with inserts showing expansions of regions between δ 171.0 and δ 168.0 ppm and δ 23 and δ 19 ppm

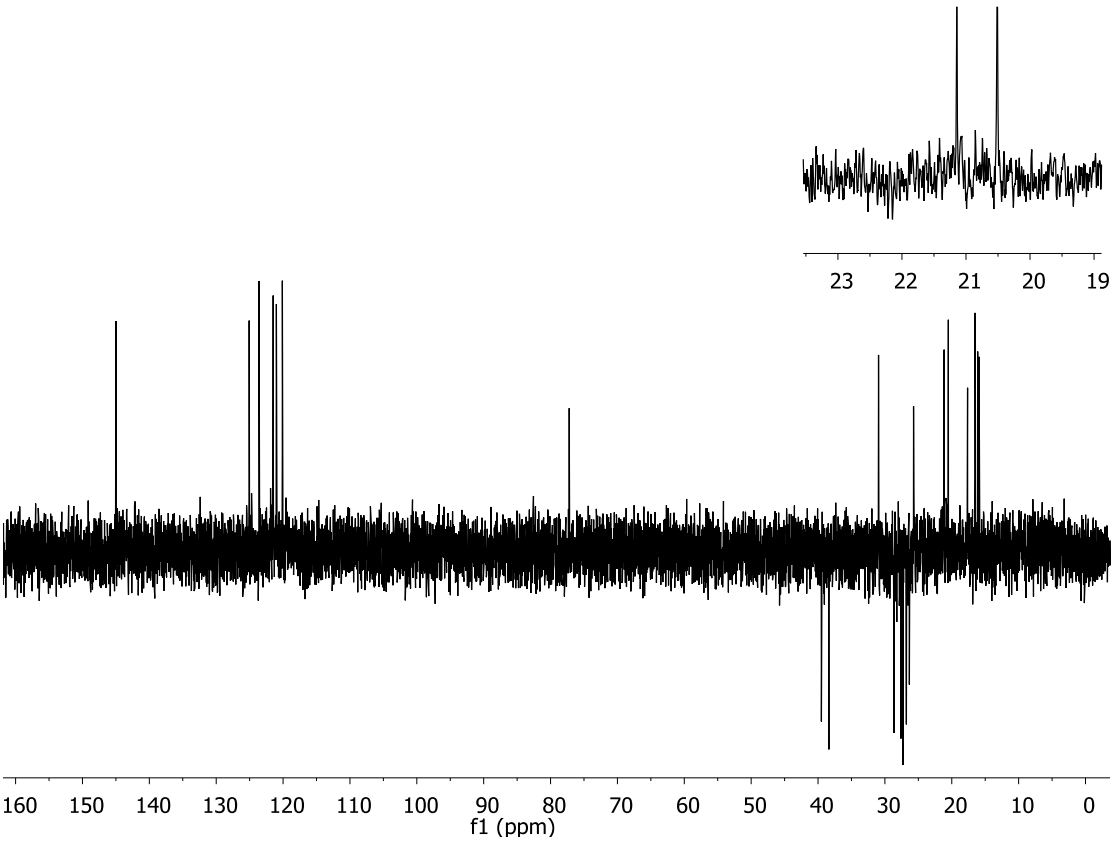


Figure 5.10 DEPT 135 NMR spectrum of compound **5.4** (100 MHz, $CDCl_3$) with the insert showing an expansion of the region between δ 23 and δ 19 ppm

Attempted conversion of (1.38) to sargachromenol (2.10)

During the isolation studies, the slow conversion of sargahydroquinoic acid **(1.38)** to sargaquinoic acid **(1.39)** and finally to sargachromenol **(2.10)** was observed. The *in vitro* transformation of hydroquinones into quinones which are then converted to chromenols has been documented in literatutre (Kusumi et al., 1979; Perez-Castorena et al., 2002). It is also known that an equilibrium exists between hydroquinones, quinones and chromenols. Attempts to mimic the conversion of **1.38** to sargachromenol **(2.10)** by stirring solutions of **1.38** in pyridine, and ethanol proved to be unsuccessful.

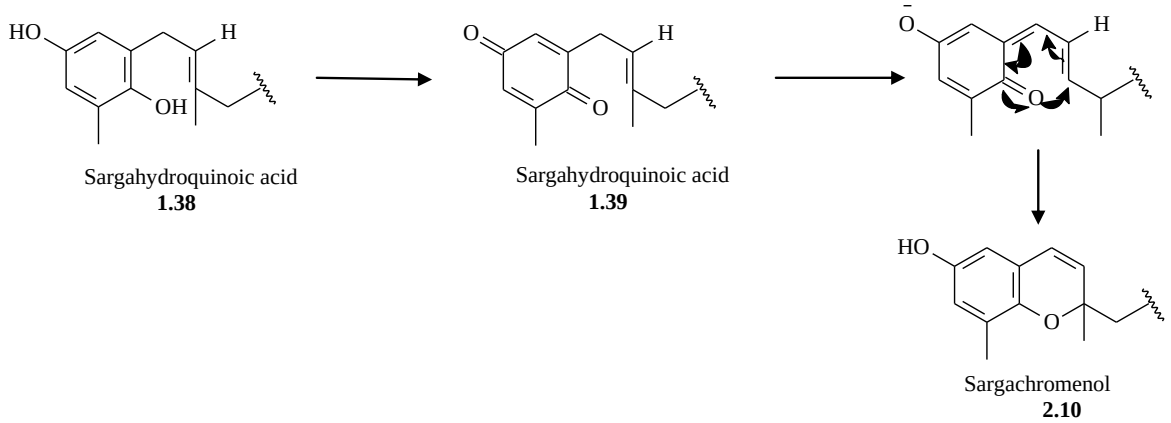


Figure 5.11 The proposed mechanism for the conversion of sargahydroquinoic acid **(1.38)** to sargachromenol **(2.10)**

Formation of sargaquinol (5.5) and sargachromendiol (5.6)

Reduction of the carboxylic acid group in sargahydroquinoic acid was achieved under standard reducing conditions ($LiAlH_4$) to afford two compounds; sargaquinol **(5.5)** and sargachromendiol (**5.6)**. Two products were obtained from this reaction due to the presence of small amounts of sargachromenol in the sargahydroquinoic acid which was used as the starting material. We hypothesise that the carboxylic acid groups in both sargahydroquinoic acid and sargachromenol were reduced simultaneously.

These alcohols were identified by the disappearance of the signal due to the carboxylic acid group at δ_C 172 ppm and the appearance of an oxymethylene carbon signal at δ_C 60.3 ppm in both compounds. These changes in the ^{13}C NMR spectra were also accompanied by the emergence of new signals in the ^{1}H NMR spectra at δ 4.1 ppm for both alcohols. In addition to the reduction of the carboxylic acid group in **1.38**, this compound was also oxidised to the quinone. This was shown by the appearance of benzoquinone signals at δ_C 188.0 ppm and δ

187.8 ppm in **5.5** in place of the aromatic signals at δ 146.2 ppm and δ 148.6 ppm in compound **1.38** for C-1 and C-4 respectively. Apart from the changes at the carboxylic group, the rest of the tetraprenyl chain remained intact for both compounds.

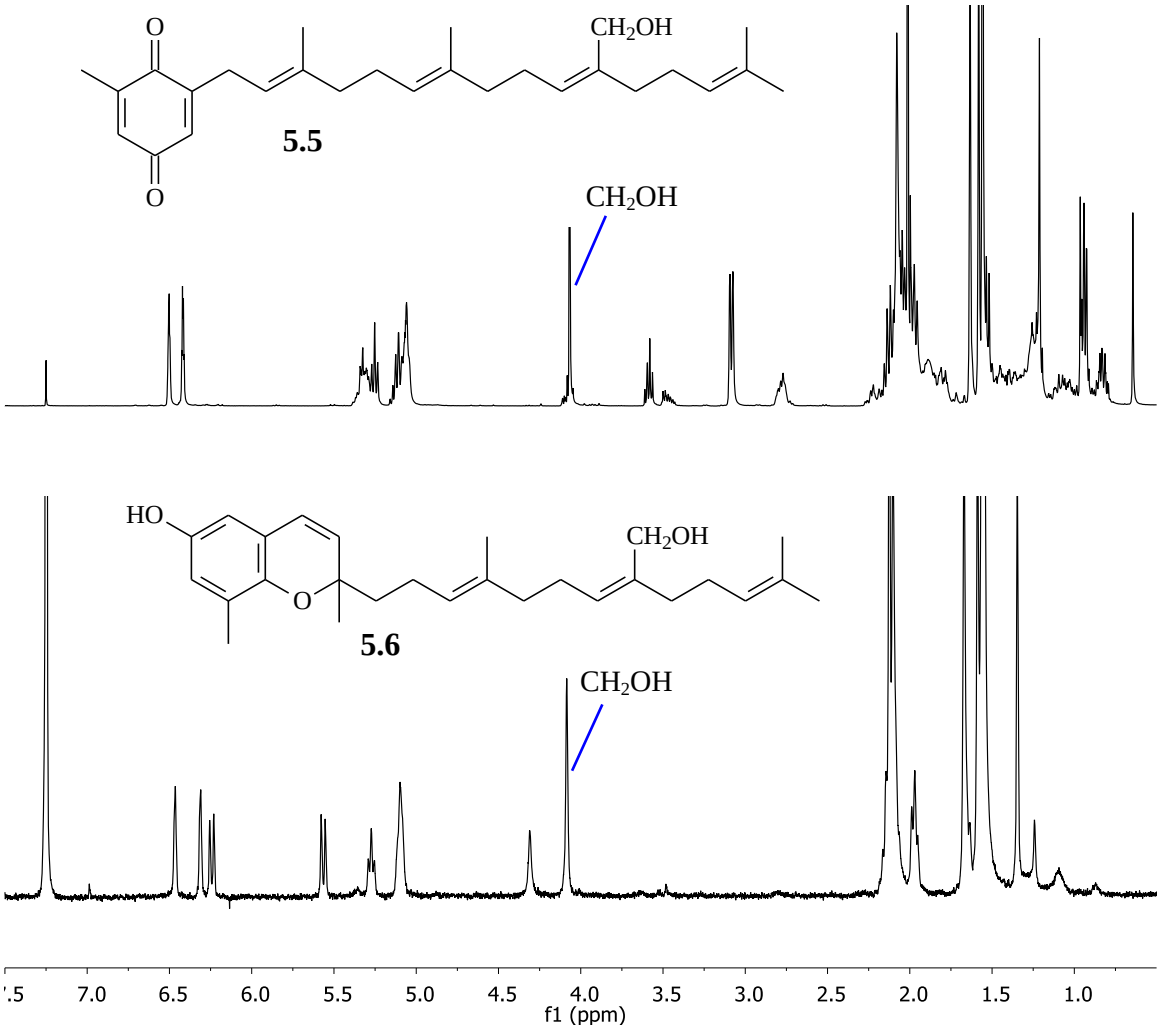
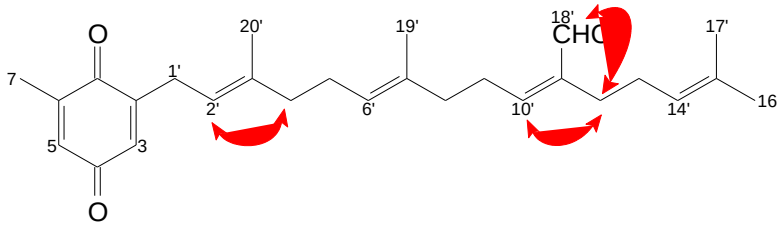


Figure 5.12 ^{1}H-NMR spectra of sargaquinol (**5.5**) and sargachromendiol (**5.6**) (400 MHz, $CDCl_3$)

Conversion of sargaquinol (5.5) to *Z*-sargaquinal (5.7)

2'*E*, 6' E, 10'*E* - sargaquinal **(1.40)** was initially isolated from *S. heterophyllum* in very small quantities (section 2.2.1). The aldehyde derivative of **1.38** was obtained by stirring a solution of sargaquinol (**5.5)** in anhydrous CH_2Cl_2 and Dess Martin's Periodinane. The formation of an aldehyde was identified by the appearance of an aldehyde signal at δ_H 10.1 and δ_C 190.9 ppm in the ^{1}H and ^{13}C NMR spectra of **5.7** respectively. A comparison of ^{1}H NMR spectra of the natural and derived aldehydes revealed differences in chemical shifts of both proton and carbon atoms associated with the aldehyde group. Spectra of the derived aldehyde **(5.7)** showed signals at δ_H 10.1 and δ_C 190.9 ppm compared to δ_H 9.55 and δ_C 205.4 ppm in the natural aldehyde **(1.40)**. This prompted further investigation into compound **5.7** by 2D NMR. ^{1}H-^{1}H NOESY correlations in both compounds revealed a difference in the geometry of the C10'/11' double bond with the derived aldehyde bearing a *Z* conformation at this bond. The derived aldehyde was thus identified as 2'*E*, 6' *E*, 10'*Z* – sargaquinal, the geometrical isomer of the natural form of sargaquinal **(1.40)**. The formation of 2'*E*, 6' *E*, 10'*Z* - sargaquinal **(5.7)** from 2'*E*, 6' *E*, 10'*E* - sargahydroquinoic acid has been reported in literature (Kusumi et al., 1979). However, no ^{13}C or 2D NMR data for this compound has been previously reported for 2'*E*, 6' *E*, 10'*Z* – sargaquinal.



2'E, 6' E, 10'Z – sargaquinal **(5.7)**

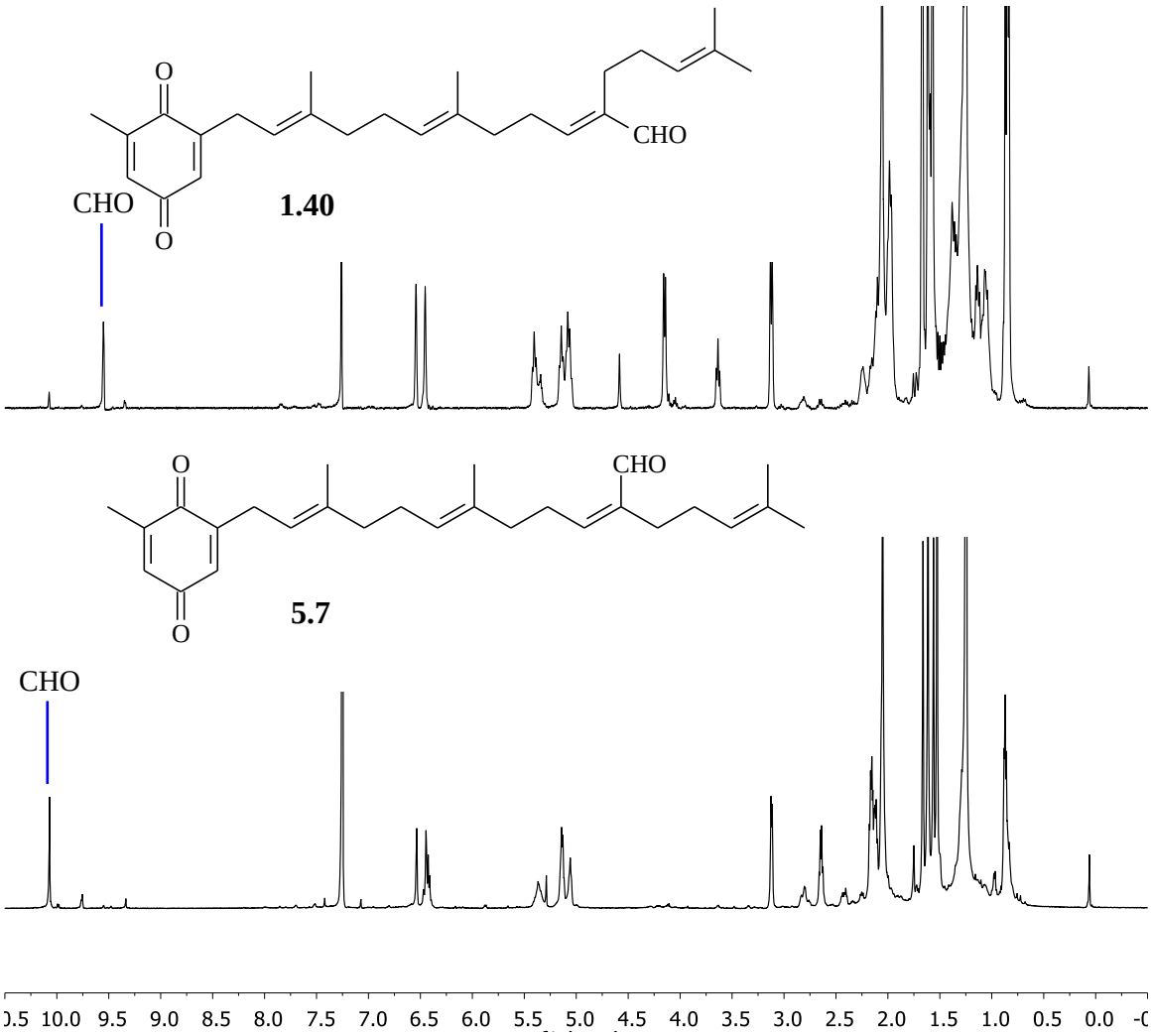


Figure 5.13 ^{1}H-NMR spectra of the natural (**1.40**) and derived (**5.7**) sargaquinal (400 MHz, $CDCl_3$)

Table 5.4 Comparison of NMR spectral data for the natural (**1.40**) and derived aldehydes **(5.7)** ($CDCl_3$) in addition to 2D NMR data for **5.7**

	Natural aldehyde **(1.40)**				Derived aldehyde **(5.7)**			
Carbon No	**δ_C**	**δ_H, mult, J_{Hz}**	**δ_C**	**δ_C, mult**	**δ_H, mult, J_{Hz}**	**HMBC**	**COSY**	**NOESY**
1	187.96		187.96	C				
2	145.9		145.9	C				
3	132.3	6.54, s	132.27	CH	6.54, s	C-1'		
4	187.91		187.91	C				
5	132.1	6.43, m	132.12	CH	6.43,m			
6	148.4		148.4	C				
7	16.0	2.06, m	15.99	CH_3	2.05, d, 6.3			H-5
1'	27.3	3.12, d, 7.2	27.33	CH_2	3.13, d, 6.9	C-2', C-3'	H-2'	H-2'
2'	118.2	5.10, dt, 6.8	118.2	CH	5.14, d, 5.8		H-1'	H-4'
3'	139.9		139.9	C				
4'	39.5	2.06, m	39.5	CH_2	2.05, d, 6.3			
5'	26.4	2.06, m	26.4	CH_2	2.14, dd, 15.0, 6.8			H-6'
6'	125.5	5.10, dt, 6.8	125.5	CH	5.06, s		H-5'	
7'	133.6		133.6	C				
8'	39.4	2.24, m	39.38	CH_2	2.05, d, 6.3	C-6'	H-6'	H-10'
9'	27.5	2.06, m	27.54	CH_2	2.14, dd, 15.0, 6.8		H-10'	H-10'
10'	154.9	6.41, m	145.9	CH	6.43 m	C-8', C-9'		
11'	132.1		132.1	C				
12'	27.3	2.24, m	27.33	CH_2	2.64, m		H-13'	H-10'
13'	25.7	1.67, s	27.45	CH_2	2.14, dd, 15.0, 6.8		H-12'	
14'	123.6	5.10, t, 6.8	123.6	CH	5.14,d ,5.8		H-13'	
15'	133.2		133.2	CH				
16'	25.2	1.67, s	25.15	CH_3	1.67,s			
17'	17.7	1.57, s	17.72	CH_3	1.57, s			
18'	205.4	9.55, s	190.9	C	10.08, s		H-10'	H-12'
19'	16.0	1.62, s	16.04	CH_3	1.62			
20'	16.1	1.62, s	16.1	CH_3	1.62			

Conversion of sargahydroquinoic acid (1.38) to sargalactone (5.8)

Several macrocyclic lactones have been shown to exhibit potent antiplasmodial activities. These include the bromophycolides recently discovered by the Kubanek group from the seaweed, *Callophycus serratus* (Stout et al., 2011). This was the motivation behind the attempted cyclization of sargahydroquinoic acid to sargalactone **(5.8)**. The conversion of **1.38** to a lactone was achieved by stirring a solution of sargahydroquinoic acid, acetone, cyanuric chloride ($C_3Cl_3N_3$) and triethylamine ($C_6H_{15}N$) at 30°C for 5 hours to give compound **5.8**. The key differences between ^{1}H NMR data for this compound and that of the starting material were an increased de-shielding of the aromatic protons from δ 6.5 ppm (H-3) and δ 6.47 ppm (H-5) to δ 6.96 ppm and δ 6.81 ppm for H-3 and H-5 respectively in **5.8** (figure 5.14). This was expected in the intended product as the movement of the carbonyl oxygen to the aromatic region would contribute to an increased de-shielding of protons in this region. The ^{13}C NMR spectrum of **5.8** also showed a key change in the chemical shift of the C-18' signal from δ 172.4 ppm in **5.8** to δ 168.4 ppm in sargalactone **(5.8)**, which is characteristic of a change from a carboxylic acid to an ester. This information was supported by the mass spectral data of compound **5.8** which showed the expected molecular ion at m/z 407.26 $[M-H]^+$. All 2D data supported the basic structure of this compound but no unambiguous correlations were observed connecting the carboxylic ester carbonyl and the aromatic ring system. This is not unexpected due the long range correlations (5-bond) which are required. Ultimately, an X-ray structure of this novel compound is required in order to unambiguously assign its structure, however, this reaction yielded insufficient material for x-ray structure determination.

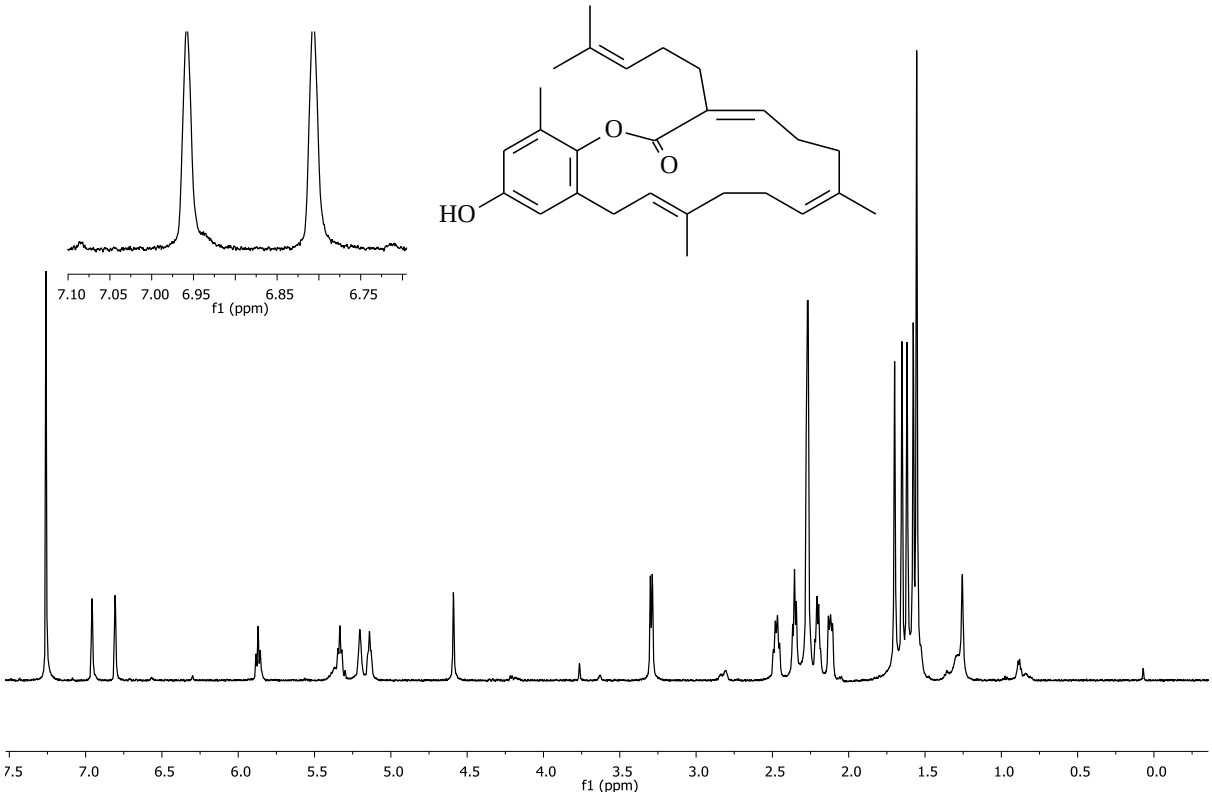


Figure 5.14 ^{1}H NMR spectrum of compound **5.8** (600 MHz, $CDCl_3$) with the insert showing an expansion f the region between δ 7.1 and δ 6.6 ppm

Table 5.5 NMR spectroscopic data for compound **5.8** ($CDCl_3$)

Carbon no.	**δ_C**	**δ_C, mult**	**δ_H, mult J_{Hz}**	COSY	HMBC	NOESY
1	143.6	C				
2	123.6	C				
3	121.4	CH	6.96, s		C-1	H-5
4	148.9	C				
5	118.8	CH	6.81, s			
6	127.1	C				
7	16.7	CH_3	2.27, s		C-1	
1’	27.3	CH_2	3.29, d, 37.5	H-2'	C-2, C-2'	H-20' lr
2’	121.5	CH	5.33, dd, 17.8, 10.1	H-1'	C-4'	H-4',
3’	132	C				
4’	39.4	CH_2	2.12, m		C-3', C-5'	H-6'
5’	26.7	CH_2	1.70,s	H-6'	C-3',	
6’	123.6	CH	5.17, d, 37.5			H-19'
7’	135.3	C				
8’	39.1	CH_2	2.12, m			H-6'
9’	29.0	CH_2	2.27, s			
10’	136.1	CH	5.87, t, 8.3		C-12'	H-8', H9’
11’	133.5	C				
12’	34.5	CH_2	2.36, t, 7.1	H-13'	C-11', C-14'	
13’	27.4	CH_2	2.12, m	H-12'		
14’	123.3	CH	5.33, dd, 17.8, 10.1			
15’	132.5	CH				
16’	25.7	CH_3	2.27, s			H-14'
17’	17.7	CH_3	1.65, s		C-15, C-16'	H-16'
18’	168.4	C				
19’	14.4	CH_3	1.57, d, 13.4		C-8'	H-9'
20’	16.1	CH_3	2.27, s			

5.2.2 Bioactivity of sargahydroquinoic derivatives and naturally occurring prenylated toluquinones

The nine derivatives of sargahydroquinoic acid **(1.38)** together with the naturally occurring compounds; 2'*E*, 6'*E*-10'*E* -sargaquinal **(1.40)**, sargachromenol **(2.10)** and sargahydroquinoic acid itself, were assessed for both antiplasmodial and cytotoxic activity. The 12 compounds were tested against the chloroquine resistant strain of *P. falciparum*, FCR-3 for antiplasmodial activity and against the MDA-MB-231 breast carcinoma cell line for cytotoxic activity.

Antiplasmodial assay

All compounds were tested in triplicate against the chloroquine-resistant Gambian FCR-3 strain of *P. falciparum*.

Cytotoxicity assay

The cytotoxicity of the compounds was determined using the WST-1 assay method (Roche). Compounds were tested against MDA-MB-231 breast carcinoma cells.

The selectivity index of these compounds was calculated by dividing the IC_{50} value (µM) obtained from the cytotoxicity assay with the same parameter obtained from the antiplasmodial assay. The results for both assays as well as the selectivity indices of all compounds are shown in table 5.6.

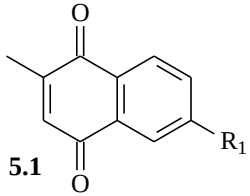
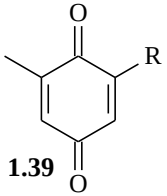
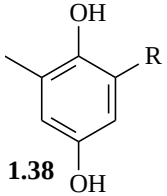
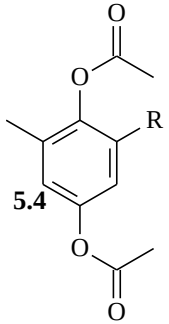
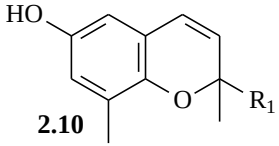
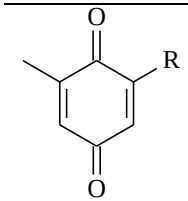
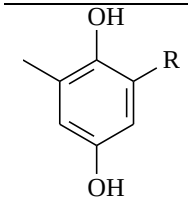
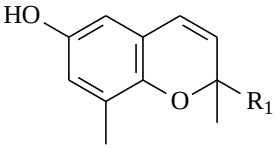
From the results, it was difficult to definitively discuss the structure activity relationships of the tested compounds. However, the following was observed: of the assayed compounds, four compounds were more active against the antiplasmodial parasite compared to sargahydroquinoic acid **(1.38)**. These included sarganaphthoquinone (**5.1**), both the hydroquinone and quinone methyl esters (**5.2** and **5.3**) and sargalactone (**5.8**). These compounds also had good selectivity (> 10) for the *plasmodium* parasite except for the quinone methyl ester which had a selectivity index of 8.55. Most compounds had higher cytotoxic activity compared to the weakly cytotoxic sargahydroquinoic acid **(1.38)**. The hydroquinone methyl ester **(5.2)** had the highest cytotoxic activity (44 µM).

Table 5.6 Bioassay results for compounds **1.38 – 1.40** and **5.1 – 5.8**

Compound	**Antiplasmodial activity(FCR-3) IC_{50} µM**	**Cytotoxicity (MDA-MB-231) IC_{50} µM**	**Selectivity Index (SI)**
Sargahydroquinoic acid **(1.38)**	38.58	70	1.81
Sargaquinoic acid **(1.39)**	10.81	658	60.87
Sarganaphthoquinone **(5.1)**	5.44	2410	443.01
Hydroquinone Methyl ester **(5.2)**	1.94	44	22.68
Quinone Methyl ester **(5.3)**	8.19	70	8.55
Hydroquinone di-acetate **(5.4)**	84.3	286	3.39
Sargachromenol **(2.10)**	114.82	56	0.49
Sargaquinol **(5.5)**	93.12	99	1.06
Sargachromendiol **(5.6)**	34.21	187	5.47
Natural Sargaquinal **(1.40)**	104.43	69	0.66
Derived Sargaquinal **(5.7)**	72.58	211	2.91
Sargalactone **(5.8)**	5.22	107.9	20.67
Quinine	0.17	-	-

In order to discuss the effects of functional groups on antiplasmodial activity, the tested compounds were separated into different groups based on the functional group at C-18' in sargahydroquinoic acid or the moiety group at the head of the compound i.e quinone, hydroquinone or chromenol (table 5.7).

Table 5.7 Antiplasmodial activity of compounds **1.38 -1.40** and **5.1 -5.8**

Common Moiety	**Compound and IC_{50} value**				
COOH	 **5.1**	 **1.39**	 **1.38**	 **5.4**	 **2.10**
	5.44	10.81	38.58	84.3	114.82
	COOMe **(5.3)**	COOH **(1.39)**	CHO **(5.7)** (Derived)	CH_2OH **(5.5)**	CHO **(1.40)** (Natural)
	8.19	10.81	72.58	93.12	104.43
	COOMe **(5.2)**	COOH **(1.38)**			
	1.94	38.58			
	CH_2OH **(5.6)**	COOH **(2.10)**			
	34.21	114.82			

For molecules with a carboxylic acid functional group at C-18', the naphthoquinone **(5.1)** had the highest antiplasmodial activity followed by the quinone bearing molecule, sargaquinoic acid **(1.39)**. Amongst the molecules bearing a quinone moeity, the quinone methyl ester **(5.3)** was the most active. An interesting difference between the activity of the synthetic and natural aldehydes was also observed amongst molecules in this group. While both aldehydes did not show significant antiplasmodial activity, a change in the geometry at the C10'/11' double bond seemed to affect both antiplasmodial and cytotoxic activity. This was seen by the higher antiplasmodial activity of the derived aldehyde **(5.7)** compared to its natural isomer **(1.40)** which is more cytotoxic than compound **(5.7)**. The conformation at this bond may be further investigated where the cytotoxicity of these compounds is concerned. Another interesting difference in antiplasmodial activity is observed with the hydroquinone and quinone methyl esters with the former being the most active of all 12 compounds. Results for this compound **(5.2)** and the lactone **(5.8)** suggest that the hydroquinone moeity is important for antiplasmodial activity. If this is the case, increasing the lipophilicity of this compound further e.g. by methylating the hydroquinone hydroxyl groups of **(5.2)** or by increasing the ester's chain length at C-18' may provide more information on how the antiplasmodial activity of **(5.2)** may be improved. Sargalactone **(5.8)** was expected to show more cytotoxic activity based on deductions from other large macroesters such as geldanamycin. However, this compound's antiplasmodial activity is in line with trends observed with the esterification of the carboxylic acid. Despite the fact that none of the derivatives had significant antiplasmodial activity, this study was successful in providing preliminary information on the functional groups that influence the antiplasmodial activity of sargahydroquinoic acid.

5.3 Experimental

5.3.1 *General Experimental*

All solvents were of chromatographic grade (Merck, Germany) and were used without further purification. Ethanol (95%) was redistilled before use. Column chromatography was performed with silica gel (40-63 μm particle size) from Merck, Germany. Normal Phase HPLC was carried out on a Phenomenex Luna C_{18} column (10mm × 250 mm, 10 μm), while a Whatman Partisil 10 semi-preparative column (10 mm × 500 mm, 10 μm) was used for reverse phase HPLC. NMR spectra were recorded on Bruker Avance 400 MHz and 600 MHz spectrometers and referenced to $CDCl_3$ solvent signals δ_H 7.26 ppm and δ_C 77.0 ppm. UV spectra were obtained from a Perkin Elmer Lambda 25 uv/vis spectrometer while FT-IR data was measured using a Perkin Elmer Spectrum 100 FT-IR Spectrometer. High resolution mass spectroscopy (ESI) was determined on a Waters Synapt G2 mass spectrometer at 20 V.

5.3.2 Plant material

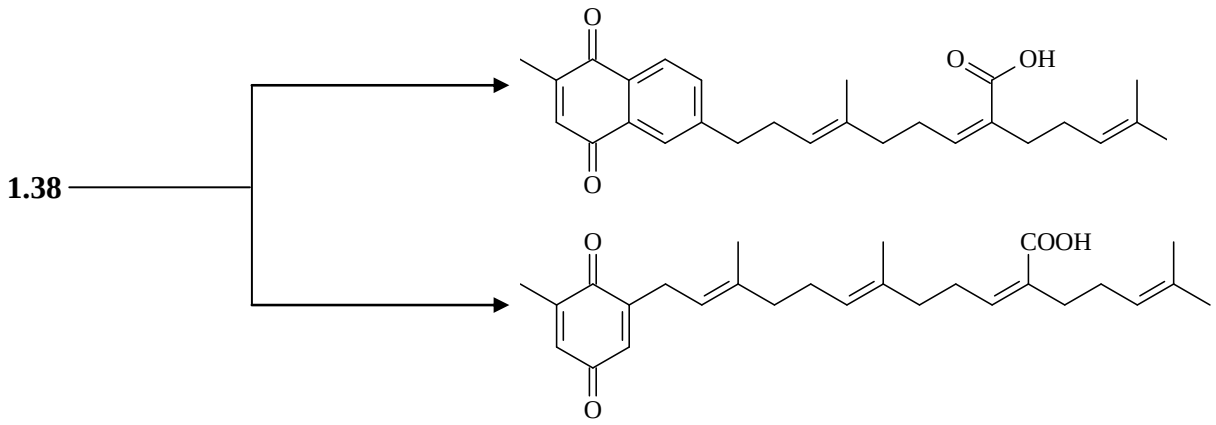
Specimens of *S. heterophyllum* were collected from Noordhoek on the south east coast of South Africa. Algae was transported and stored as outlined in chapter 2, section 2.3.2.

5.3.3 Extraction and isolation of sargahydroquinoic acid (1.38)

The extraction and isolation of sargahydroquinoic acid **(1.38)** was carried out as outlined in section 2.3.3.

5.3.4 The Chemical Modification of Sargahydroquinoic acid (1.38)

5.3.4.1 Oxidation of sargahydroquinoic acid (1.38) to sargaquinoic acid (1.39)



Procedure A

To a solution of **1.38** (154.0 mg, 0.36 mmol) dissolved in a mixture of $CHCl_3$ (8 mL) and MeOH (7 mL) was added Ag_2O (100 mg, 0.43 mmol). The reaction mixture was stirred at room temperature for 24 hours after which the resulting suspension was filtered through diatomaceous earth and concentrated under reduced pressure. The crude product was passed over a plug of charcoal in a solvent system of Hexane: EtOAc (40:60) to give a yellow mixture of compounds which was separated by silica gel chromatography Hexane: EtOAc (70:30). Separation of this crude mixture gave two yellow oils; sargaquinoic acid **(1.38)** (80 mg, 70%) and compound **5.1** (9.8 mg, 6%).

*Sargaquinoic acid **(1.39)***: ^{1}H and ^{13}C NMR data - see table 2.1.

*Sarganaphtoquinone **(5.1)**:* ^{1}H and ^{13}C NMR data see table 5.2

UV (EtOH) λ_{max} 205 nm; IR ν_{max} (film) 1049, 1377, 1454, 1600, 1663, 2850, 2924 cm^{-1}; EIMS m/z 419.22 $[M-H]^+$

Procedure B

To a solution of sargahydroquinoic acid **(1.38)** (94.1 mg, 0.22 mmol) dissolved in a mixture of $CHCl_3$ (7 mL) and MeOH (8 mL) was added MnO_2 (30.3 mg, 0.348 mmol). The reaction mixture was stirred for 24 hours at room temperature. The reaction mixture was then filtered through diatomaceous earth and concentrated under reduced pressure. The ^{1}H NMR spectrum of the crude product showed 100% conversion of **1.38** to sargaquinoic acid **(1.39)**.

Procedure C

A solution of sargahydroquinoic acid **(1.38)** (25.1 mg, 0.06 mmol) dissolved in 5 mL of ACN was stirred for 14 hours at room temperature in an open round bottomed flask. The reaction mixture was concentrated under reduced pressure. The ^{1}H NMR spectrum of the crude product showed 25% conversion of **1.38** to **1.39**.

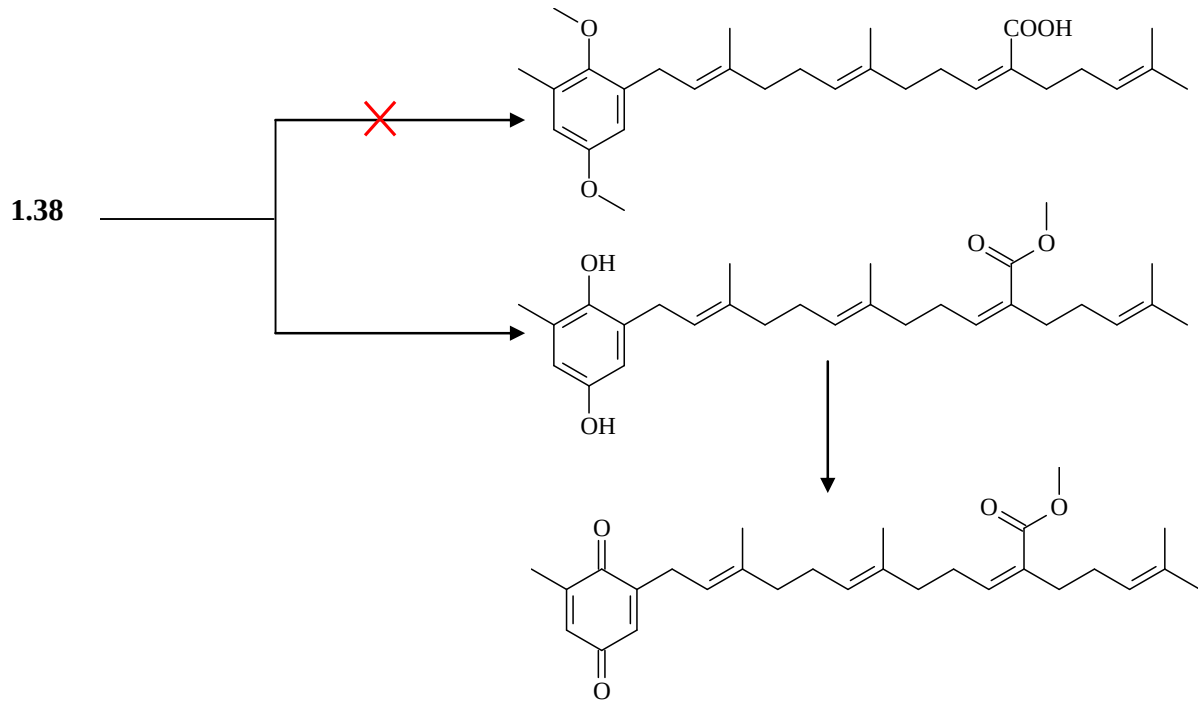
Procedure D

To a solution of sargahydroquinoic acid **(1.38)** (26 mg, 0.06mmol) dissolved in 5 mL ACN was added oxone (228.9 mg, 0.40 mmol). The reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was filtered and concentrated under reduced pressure. The ^{1}H NMR spectrum of the crude product showed that most of the starting material had not been converted to sargaquinoic acid. To a solution of the crude product dissolved in 5 mL ACN was added another portion of oxone (228.9 mg, 0.40 mmol). The reaction mixture was stirred at room temperature for 4 hours, filtered and concentrated under reduced pressure. The ^{1}H NMR of the second crude product showed 20% conversion of **1.38** to **1.39**.

Procedure E

To a solution of sargahydroquinoic acid **(1.38)** (6 mg, 0.01 mmol) dissolved in 3 mL CH_2Cl_2, triethylamine (0.3 mL, 2.15 mmol) and Ag_2CO_3 (80 mg, 0.29 mmol) was added. The reaction mixture was stirred at room temperature for 5 hours, filtered and concentrated in vacuo. The ^{1}H NMR spectrum of the crude product showed no conversion of **1.38** to **1.39**.

5.3.4.2 Methylation of sargahydroquinoic acid



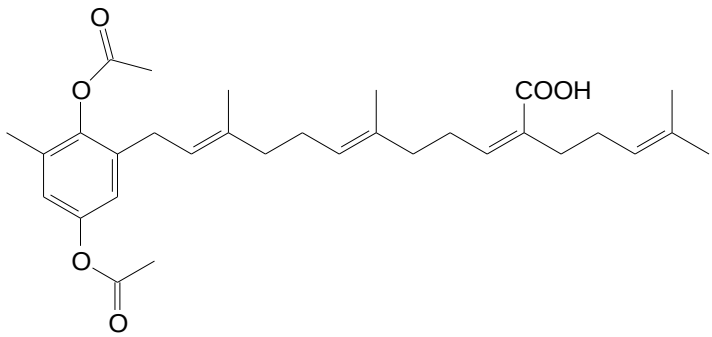
To a solution of **1.38** (122.4 mg, 0.29 mmol) dissolved in 2 mL acetone, K_2CO_3 (207.4 mg, 1.50 mmol) in 5mL acetone and dimethylsulphate (250 µl, 2.63 mmol) was added. The mixture was heated at 40°C for 8 hours followed by stirring at room temperature for 16 hours. The reaction mixture was filtered and concentrated under reduced pressure. The crude reaction mixture was separated by silica gel column chromatography (Hexane: EtOAc, 80:20) to give compound **5.2** (50 mg, 44%). Upon standing the hydroquinone methyl ester **(5.2)** oxidised to the quinone methyl ester **(5.3)**. The mixture of the two esters was separated by HPLC (Hexane: EtOAc, 80:20) to give two yellow oils; **5.2** (10 mg) and **5.3** (20 mg).

*Hydroquinone methyl ester **(5.2)***: ^{1}H and ^{13}C NMR data- see table 5.3

*Quinone methyl ester **(5.3)***: ^{1}H and ^{13}C NMR data- see table 5.3

5.3.4.3 Acetylation of sargahydroquinoic acid

1.38 →

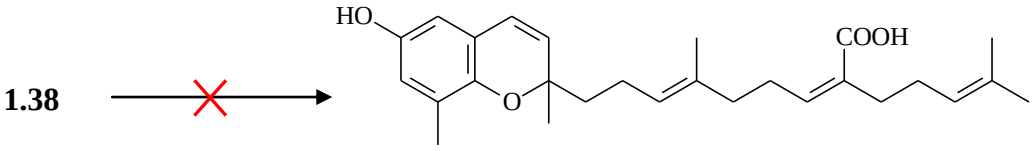


To sargahydroquinoic acid **(1.38)** (110.0 mg, 0.26 mmol), acetic anhydride (3 mL, 31.8 mmol) and pyridine (2 mL, 24.8 mmol) was added. The reaction mixture was stirred at room temperature for 30 hours. The crude product was acidified with 1 M HCl (10 mL) and extracted with EtOAc (5 mL x 3). The organic layer was collected and concentrated under reduced pressure to give a crude product. The crude product was passed over a plug of charcoal in a solution of EtOAc: Hexane (60:40), concentrated under reduced pressure and purified by silica gel column chromatography (Hexane: EtOAc, 70:30) to give compound **5.4** (12.8 mg, 12 %) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (d, *J* = 7.5 Hz) (H-3), 6.76 (d, *J* = 2.4 Hz) (H-5), 5.21 (t, *J* = 6.5 Hz) (H-2’), 5.13 (d, *J* = 7.1 Hz) (H-6’, 14’), 3.18 (d, *J* = 7.0 Hz) (H-1’), 2.34 – 2.28 (m) (H-23’, 24’)), 2.27 (d, *J* = 3.7 Hz) (H-12’), 2.17 (s) (H-7), 2.11 (d, *J* = 14.4 Hz) (H-5’, 13’), 2.05 (s) (H-4’,8’,22’, 24’), 1.66 (d, *J* = 6.0 Hz) (H-16’, 19’,20’), 1.60 (d, *J* = 7.5 Hz) (H-17’).

^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5 (C-18’), 169.6 (C-21’), 168.97 (C-23’), 148.0 (C-4), 145.23 (C-1,C-10’) , 144.99 (C-9’), 137.2 (C-3’), 134.9 (C-7’), 133.8 (C-6), 132.3 (C-14’), 131.0 (C-11’), 125.1 (C-2), 123.56 (C-6’), 123.59 (C-5), 121.5 (C-3), 121.0 (C-2’), 120.1, 39.5 (C-4’), 38.4 (C-8’), 30.9 (C-12’), 28.6 (C-8’), 28.3 (C-5’), 27.6 (C-1’), 27.3 (C-13’),25.7 (C-15’),20.50 (C-22’), 21.1 (C-22’), 17.6 (C-16’), 16.5 (C-7), 16.1 (C-19’), 15.9 (C-20’).

5.3.4.4 Attempted synthesis of sargachromenol (2.10)



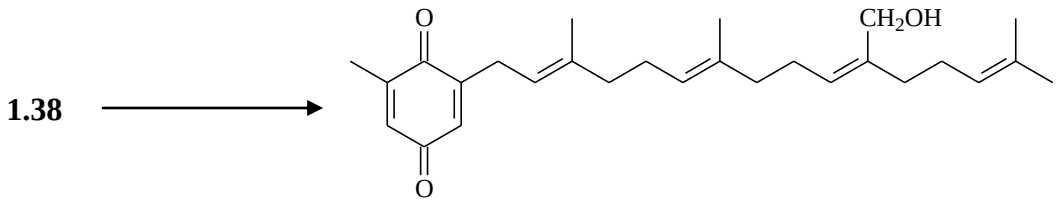
Procedure A (Mori et al., 2006)

To a solution of sargaquinoic acid **(1.39)** (1.2 mg, 0.003 mmol) pyridine, (1.5 mL, 18.6 mmol) was added. The reaction was stirred at room temperature over night. The reaction mixture was acidified with 1 M HCl (10 mL) and extracted with CH_2Cl_2 (3 mL).The organic phase was concentrated under reduced pressure. The ^{1}H NMR spectrum of the crude showed no formation of sargachromenol.

Procedure B (Kusumi et al., 1979)

A solution of sargaquinoic acid **(1.39)** (85 mg, 0.2 mmol) dissolved in EtOH (2 mL) and water (48 mL) was stirred at room temperature for five days. 5 mL of the reaction mixture was extracted with CH_2Cl_2, concentrated under reduced pressure and analysed by NMR during the course of the reaction. All ^{1}H NMR spectra showed unreacted starting material.

5.3.4.5 Reduction of sargahydroquinoic acid to sargaquinol (5.5)



To a solution of sargahydroquinoic acid **(1.38)** (140.7 mg, 0.33 mmol) dissolved in anhydrous THF (5 mL), $LiAlH_4$ (0.104 g, 2.74 mmol) was added. The reaction mixture was stirred at room temperature, under a nitrogen line for 1.25 hours. The crude product was extracted with EtOAc (10 mL x 2) and H_2O (5 mL). The organic layer was concentrated

under reduced pressure to give a crude product which was purified by silica gel chromatography (Hexane: EtOAc, 80:20) to give sargaquinol (**5.5)** (12.2 mg, 30%) and the alcohol derivative of sargachromenol **(5.6)** (2.8 mg, 3.5%).

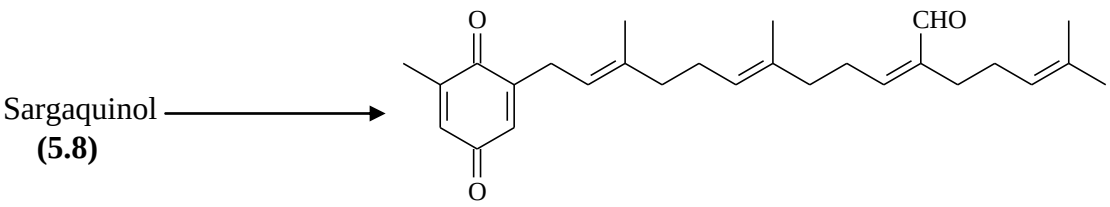
*Sargaquinol **(5.5)*** yellow oil (30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.54 (s, 1H) (H-3), 6.46 (s, 1H) (H-5), , 5.15 (dd, *J* = 21.0, 13.2 Hz) (H- 2’, 6’, 14’), 4.11 (s) (H-18’), 3.63 (t, *J* = 6.5 Hz) (H-10’), 3.12 (d, *J* = 7.1 Hz) (H-1’), 2.12 (s) (H-5’, 9’, 13’), 2.05 (s) (H-4’, 8’), 1.67 (s) (H- 7, 16’), 1.60 (s) (H-19’, 20’) , 1.57 (s) (H-17’).

^{13}C NMR (100 MHz, $CDCl_3$) δ 188.0 (C-1), 187.97 (C-4), 148.5 (C-6), 145.9 (C-2), 139.7 (C-3’), 135.0 (C-7’), 133.1 (C-3), 131,2 (C-11’), 132.24 (C-5), 133.7 (C-15’), 124.7 (C-6’) , 124.2 (C-14’), 118.1 (C-2’), 71.8 (C-18’), 62.8 (C-10’), 39.8 (C-4’), 39.5 (C-8’), 35.2 (C-12’), 27.1 (C-13’),27.5 (C-1’), 26.2 (C-5’) , 26.3 (C-9’), 25.6 (C-16’), 17.7 (C-7), 16.11 (C-17’), 16.07 (C-19’), 15.96 (C-20’).

*Sargachromenol alcohol derivative **(5.6)*** yellow oil (3.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.47 (d, *J* = 2.4 Hz) (H-5), 6.32 (d, *J* = 2.5 Hz) (H-2), 6.26 (s) (H-2’), 5.98 (t, *J* = 7.2 Hz) (H-9’), 5.57 (d, *J* = 9.8 Hz) (H-3’), 5.12 (dt, *J* = 19.0, 6.4 Hz) (H-5’, 14’), 2.59 (q, *J* = 7.3 Hz (H-8’), 2.27 (t, *J* = 7.4 Hz), 2.13 (s) (H-12’), 2.09 – 2.04 (m) (H-4’, 7’), 1.68 (s) (H-8), 1.58 (d, *J* = 3.5 Hz) (H-17’, 19’), 1.36 (s) (20’).

^{13}C NMR (100 MHz, $CDCl_3$) δ 148.6 (C-5), 144.95 (C-10’), 144.82 (C-8), 134.7 (C-7’), 131.8 (C-15’), 130.6 (C-11’), 126.3 (C-3), 124.7 (C-6’), 124.1 (C-1’), 122.9 (C-14’), 121.3 (C-2), 117.0 (C-4), 110.3 (C-6), 77.8 (C-3’), 60.3 (C-18’), 40.7 (C-4’), 39.8 (C-8’), 35.1 (C-12’), 35.3 (C-12’), 27.0 (C-9’), 26.1 (C-13’), 25.88 (C-20’), 25.67 (C-16’), 22.56 (C-5’), 17.7 (17’), 15.9 (C-7), 15.5 (C-19’).

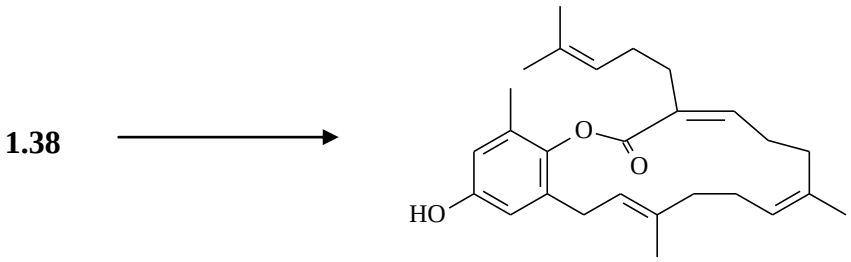
5.3.4.6 Conversion of sargaquinol (5.5) to Z-sargaquinal (5.7)



To a solution of sargaquinol **(5.5)** (37.2 mg, 0.09 mmol) dissolved in anhydrous CH_2Cl_2 (8 mL), Dess-Martins Periodinane (107mg, 0.26 mmol) was added. The reaction mixture was stirred at room temperature for 2 hours. The reaction was quenched with CH_2Cl_2 (10 mL) and de-ionised water (10 mL). The organic phase was washed with saturated solutions of $NaHCO_3$ (10 mL x 3) and $Na_2S_2O_3$ (10 mL x 3), dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (Hexane: EtOAc, 80:20) to give compound **5.7** (42%, 14.9 mg), as a yellow oil.

*Sargaquinal **(5.7)***: ^{1}H, ^{13}C and 2D NMR data - see table 5.4

5.3.4.7 Conversion of sargahydroquinoic acid (1.38) to sargalactone (5.8)



To a solution of sargahydroquinoic acid **(1.38)** (89.8mg, 0.21 mmol) dissolved in acetone (2 mL), cyanuric chloride (59.7mg, 0.32 mmol) in acetone (8 mL) was added. To this solution triethylamine (0.5 mL,1.98 mmol) was added. The reaction mixture was stirred at 30°C for 5hours, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (Hexane: EtOAc, 80:20) followed by HPLC (Hexane: EtOAc, 85:15) to yield compound **(5.8)** (4.4 mg, 5%) as a yellow oil.

*Sargalactone **(5.8)***: ^{1}H, ^{13}C and 2D NMR data - see table 5.5

UV (EtOH) λ_{max} 202 nm; IR ν_{max} (film) 1366, 1746, 2910cm^{-1}; EIMS m/z 407.26 $[M-H]^+$.

5.3.5 Antiplasmodial Assays

Screening for antiplasmodial activity was conducted by Professor Robyn Van Zyl at the Department of Pharmacy and Pharmacology, University of the Witwatersrand. All compounds were tested in triplicate against the chloroquine-resistant Gambian FCR-3 strain of *P. falciparum*. The *in vitro* erythrocytic stage of the parasite was maintained using the method outlined by Trager and Jensen (1976). The antimalarial activity of the compounds was determined using the tritiated hypoxanthine incorporation assay (Desjardins et al. 1979). All assays were carried out using untreated parasites and uninfected red blood cells as controls. The concentration that inhibited 50% parasite growth (IC_{50} value) was determined from the log sigmoid dose response curve. Quinine was used as the reference antiplasmodial agent.

5.3.6 Cytotoxicity Assay

Screening for cytotoxicity was conducted by Dr. Adrienne Edkins at the Biomedical and Biotechnology Research Unit at Rhodes University. All compounds were tested in triplicate against MDA-MB-231 breast carcinoma cells. The cytotoxicity of the compounds was determined using the WST-1 assay method (Roche). The cells were treated with a range of concentrations of the test compounds or vehicle control (DMSO). Cells treated with DMSO were considered to represent 100% viability and the viability of cells at each dose was represented relative to this value. The concentration resulting in a decrease of cell viability to 50% was calculated from the linear portion of the dose response curve.

Supplementary information

See attached CD (supplementary information) for 2D NMR data for sarganaphthoquinone **(5.1)**, sargahydroquinoic acid methyl ester **(5.2),** sargaquinoic acid methyl ester **(5.3),** sargaquinol **(5.5)**, 2’E, 6’E, 10’Z – sargaquinal **(5.7)** and sargalactone **(5.8)**.

References

Afolayan, A.F., Bolton, J.J., Lategan,C.A., Smith, P.J., and Beukes, D.R. (2008). Fucoxanthin, Tetraprenylated Toluquinone and Toluhydroquinone Metabolites from *Sargassum heterophyllum* Inhibit the *in vitro* Growth of the Malaria Parasite *Plasmodium falciparum*. Z Naturforsch 63c:848-852.

Alonso, M.A., Lopez-Alvarado, P., Avendano, C., and Menendez, J.C. (2004). Regioselective Diels-Alder Reactions of 3-Vinylindoles with Quinones. Letters in Organic Chemistry 1:20-22.

Bahl, A., Brunk, B., Crabtree, J., Fraunholz, M.J., Gajria, B., Grant, G.R., Ginsburg, H., Gupta, D., Kissinger, J.C., Labo, P., Li, L., Mailman, M.D., Milgram, A.J., Pearson, D.S., Roos, D.S., Schug, J., Stoeckert, C.J., and Whetzel, P. (2003). PlasmoDB: the *Plasmodium* genome resource. A database integrating experimental and computational data. Nucleic Acids Research 31:212-215.

Basco, L.K., Marquet, F., Makler, M.M., and Lebras, J. (1995). *Plasmodium falciparum* and *Plasmodium vivax*: Lactate-Dehydrogenase Activity and Its Application for *in vitro* Drug Susceptibility Assay. Experimental Parasitology 80:260-271.

Couladouros, E.A., Plyta, Z.F., and Papageorgiou, V.P. (1996). A General Procedure for the Efficient Synthesis of (Alkylamino) naphthoquinones. Journal of Organic Chemistry 61:3031-3033.

de Koning, C.B., Rousseau, A.L., and van Otterlo, W.A.L. (2003). Modern methods for the synthesis of substituted naphthalenes. Tetrahedron 59:7-36.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., and Chulay, J.D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrobial agents and Chemotherapy 16:710-718.

Fidock, D.A., Rosenthal, P.J., Croft, S.L., Brun, R., and Nwaka, S. (2004). Antimalarial Drug Discovery: Efficacy Models For Compound Screening. Nature Reviews - Drug Discovery 3:509-520.

Figueiredo, J.N., Raz, B., and Sequin, U. (1998). Novel Quinone Methides from *Salacia kraussii* with *in vitro* Antimalarial Activity. Journal of Natural Products 61:718-723.

Francis, S.E. and Sullivan, D.J. (1997). Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. Annual Review of Microbiology 51:97.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M.A., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R.W., Fraser, C.M., and Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498-511.

Janse, C.J. and Waters, A.P. (1995). *Plasmodium berghei*: The application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitology Today 11:138-143.

Kayser, O., Kiderlen, A.F., and Croft, S.L. (2003). Natural products as antiparasitic drugs. Parasitology Research 90:S55-S62.

Kusumi, T., Ishitsuka, M., Kinoshita, T., Kakisawa, H., and Shibata, Y. (1979). Structures of new plastoquinones from the brown alga *Sargassum serratifolium*. Chemistry Letters277-278.

Laurent, D. and Pietra, F. (2006). Antiplasmodial Marine Natural Products in the Perspective of Current Chemotherapy and Prevention of Malaria. A Review. Marine Biotechnology 8:433-447.

Lin, T.S., Zhu, L.Y., Xu, S.P., Divo, A.A., and Sartorelli, A.C. (1991). Synthesis and antimalarial activity of 2-aziridinyl- and 2,3-bis(aziridinyl)-1,4-naphthoquinonyl sulfonate and acylate derivatives. Journal of Medicinal Chemistry 34:1634-1639.

Mancini, I., Guella, G., and Defant, A. (2009). Synthesis of Marine Natural Products with Antimalarial Activity. Mini- reviews in Medicinal Chemistry 8:1265-1284.

Mori, J., Iwashima, M., Takeuchi, M., and Saito, H. (2006). A Synthetic Study on Antiviral and Antioxidative Chromene Derivative. Chemical & Pharmaceutical Bulletin 54:391-396.

Olliaro, P. (2001). Mode of action and mechanisms of resistance for antimalarial drugs. Phamacology & Therapeutics 89:207-219.

Perez-Castorena, A., Arciniegas, A., Apan, M.T.R., Villasenor, J.L., and de Vivar, A. (2002). Evaluation of the anti-inflammatory and antioxidant activities of the plastoquinones derivatives isolated from *Roldana barba-johanis*. Planta Medica 68:645-647.

Pink, R., Hudson, A., Mouries, M.A., and Bendig, M. (2005). Opportunities and Challenges in Antiparasitic Drug Discovery. Nature Reviews - Drug Discovery 4:727-740.

Sandulache, A., Silva, A.M.S., and Cavaleiro, J.A.S. (2003). New Benzo[b]xanthones from Diels-Alder Reactions of Chromone-3-carboxaldehydes with ortho-Benzoquinodimethanes. Monatshefte Fur Chemie 34:551-563.

Sheu, J-H., Su, J-H., Sung, P-J., Wang, G-H., and Dai, H. (2004). Novel Meroditerpenoid-Related Metabolites from the Formosan Soft Coral *Nephthea chabrolii*. Journal of Natural Products 67:2048-2052.

Smith, R.G., Vanterpool, A., and Kulak, H.J. (1969). Dimethyl sulfoxide as a solvent in the Williamson ether synthesis. Canadian Journal of Chemistry 47:2015-2019.

Stout, E.P., Cervantes, S., Prudhomme, J., France, S., La Clair, J.J., Le Roch, K., and Kubanek, J. (2011). Bromophycolide A Targets Heme Crystallization in the Human Malaria Parasite *Plasmodium falciparum*. ChemMedChem 6:1572-1577.

Vennerstrom, J.L. and Eaton, J.W. (1988). Oxidants, oxidant drugs, and malaria. Journal of Medicinal Chemistry 31:1269-1277.

Wright, A.D., Wang, H., Gurrath, M., Konig, G.M., Kocak, G., Neumann, G., Loria, P., Foley, M., and Tilley, L. (2001). Inhibition of Heme Detoxification Processes

Underlies the Antimalarial Activity of Terpene Isonitrile Compounds from Marine Sponges. Journal of Medicinal Chemistry 44:873-885.

Ziegler, H.L., Staerk, D., Christensen, J., Hviid, L., Hagerstrand, H., and Jaroszewski, J.W. (2002). In Vitro *Plasmodium falciparum* Drug Sensitivity Assay: Inhibition of Parasite Growth by Incorporation of Stomatocytogenic Amphiphiles into the Erythrocyte Membrane. Antimicrobial agents and Chemotherapy 46:1441-1446.

Conclusion

The investigation of the chemical differences between *S. heterophyllum* and *S. elegans* raised interesting questions with respect to the chemical composition of the two species. While this project provided preliminary information on the metabolite profiles of the two species, further studies involving a higher number of sampled specimens and the separation of crude extract components are required to conclusively prove that these species are chemically in-distinct.

Despite the relatively high standard deviations obtained in the comparison of four different techniques for the extraction of metabolites from *S. heterophyllum*, qNMR allowed for simple and efficient quantification of analytes extracted by the investigated techniques. However, the method presented in this project may be improved by validation in addition to the determination of certain parameters such as the upper and lower limits of quantification. It would have been useful to compare results obtained from the qNMR study with those obtained from gravimetric experiments; however, the rapid oxidation of pure sargahydroquinoic acid **(1.38)** would have presented significant challenges for the accurate gravimetric quantification of this metabolite.

Despite the fact that none of the derivatives had significant antiplasmodial activity, this study was successful in providing preliminary information on the functional groups that influence the antiplasmodial activity of sargahydroquinoic acid **(1.38)**. From the information obtained here, several other modifications of **1.38** may be made in an effort to increase the antiplasmodial activity of the derivatives synthesised in this project. Such alterations may include combining portions of the most active compounds such as methylating the carboxylic acid group of sarganaphthoquinone **(5.1)**. In addition to this, the antiplasmodial activity of sargahydroquinoic acid derivatives obtained by other alterations to this compound still remain to be investigated. Examples of these alterations include the substitution of the carboxylic acid moeity with various functional groups such as amides, halides or heterocycles or esterifying the hydroxyl functional groups on the hydroquinone moeity of **1.38**. The use of a natural product **(1.38)** as the starting material in chemical reactions required extra caution in the selection of reaction conditions. Hence, exposure to harsh conditions required to cause certain reactions in typical chemical syntheses were generally avoided. The optimisation of

reaction conditions as was accomplished for the oxidation of **1.38** to **1.39** may be required to successfully complete the reactions which were not successful in the current study.