CHARACTERIZATION, BIOACTIVITY AND QSAR STUDIES OF NATURAL PRODUCTS FROM SELECTED NAMIBIAN RED MARINE ALGAE

A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN PHARMACEUTICAL CHEMISTRY

OF

THE UNIVERSITY OF NAMIBIA

BY

ANTHONY SHANUOLU ISHOLA

200137174

April 2020

MAIN SUPERVISOR: PROF. MICHAEL KNOTT

CO-SUPERVISORS: PROF. EDET ARCHIBONG

DR. JANE MISIHAIRABGWI

ABSTRACT

The objective of this research was to discover new drug leads from Namibian marine algae. *Plocamium* extracts were screened and their phytochemical contents were quantified. Both the antioxidant activity and the antimicrobial activity of the crude extracts were determined, as well as the dose-response relationship for *Plocamium* extracts in *BALB/c* mice. This was done by using acute and sub-acute toxicity parameters. In addition, the structural elucidation of the major metabolite found in the crude extract was determined and the Quantitative Structure Activity Relationships (QSARs) of the compound were determined.

Methods: Frozen *Plocamium* leaves were soaked in dichloromethane (DCM) and methanol (MeOH) in a ratio 1:1 (v/v) for 48 hours. Concentrated *Plocamium* extracts were screened for phytochemical constituents. Total phenolic and flavonoid contents as well as antioxidant activity were quantified. Dried algal extracts were also reconstituted with different solvents and tested *in vitro* for antimicrobial activity against 12 pathogens using the Kirby Bauer disc diffusion method.

Mice of known weights were infected with *Escherichia coli* and *Pseudomonas aeruginosa* by intravenous injection and sub-cutaneous methods respectively. The mice were later treated with gentamycin and ampicillin injections. Other groups of mice were treated with different concentrations of *Plocamium* extract over a period of five days. *E. coli* and *P. aeruginosa* loads in the faeces of the test mice were quantified daily. *Plocamium* extracts were purified using HPLC to fractionate the extracts. Major fractions were collected and identified by means of one and two dimensional NMR spectroscopy data and MS analysis.

In terms of QSAR, the structures of the metabolites were theoretically optimized using the Merck Molecular Force Field. In addition, several physicochemical properties were computed by using the B3LYP variant of Density Functional Theory in conjunction with the 6-31G (d) basis set.

Results: *Plocamium cornutum* and *Plocamium rigidum* extracted using DCM had total phenolic content of 132.85 ± 0.82 mg and 188.65 ± 0.45 mg Gallic acid equivalents per gram respectively. The IC_{50} values for *Plocamium rigidum* and *Plocamium cornutum* were 28.87 ± 0.82 µM and 40.11 ± 0.38 µM respectively. Ethanolic extracts of *Plocamium rigidum* showed a zone of inhibition of 6.35 ± 0.25 mm against *Listeria monocytogenes* while the standard ampicillin had no activity.

From the probit plot, the LD_{50} was calculated to be 3556 mg/kg.

A therapeutic dosage of *P. rigidum* of 355 mg/kg in *BALB/c* mice reduced *E. coli* load to pre-evaluation levels on the fifth day.

The chemical structure of *Plocamium* sample I and II (*Plocamium rigidum* and *Plocamium cornutum* respectively) yielded two known compounds namely, 3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene (from sample I) and 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene (from sample II) respectively.

CPKOvality and HLgap are physicochemical properties that best describe the variation in biological activity of the metabolites. The equation of the best fit was determined as:

pIC_{50} = 9.91CPKovality + 0.270HL-gap-17.149

(R^2 = 0.71, Adj. R^2 = 0.56, R = 0.84, Std error = 0.31, and q^2 = 0.55)

Conclusion: DCM is a better solvent than methanol for the extraction of natural products from *Plocamium* species. *P. rigidum* showed inhibition against *E. coli* and *L. monocytogenes in vitro*. Although *P. rigidum* inhibited the growth of *E. coli*, the possible development of liver lesions (*in vivo*) after chronic exposure is an indication of liver injury which is a sign of the chronic toxicity of *P. rigidum*, even at low concentrations.

The probable compound responsible for this action is sample (II) above. The variables in the equation above are the parameters that best describe the variation in biological activity of the halogenated monoterpenes extracted and identified. The equation also shows that CPKovality (moderate size and shape of ligands) and a small H-L gap with high reactivity are the parameters that best optimised structure to predict improved biological activity of *Plocamium* metabolites.

LIST OF PUBLICATIONS (Related to this dissertation)

1. **Ishola Anthony**[*], Knott Michael, Misihairabgwi Jane. In vitro antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from Namibian coastline. African J Pharm Pharmacol. 2018;12(0):121 – 129.

2. Knott M. G[*], Kapewangolo P, Louw S, Brand J, Kandjengo L, **Ishola A.** The isolation, Structural determination and bioactivity of *1E,3R,4S,5E,7Z-1-bromo 3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene* from a Namibian *Plocamium* species. Int. Sci. Technol. J. Namibia. 2016; 7:59-72.

3. Knott MG* and **Ishola Anthony**. Chemotaxonomy as a Potential Method to Rapidly Identify Various Namibia *Plocamium* Species. Nat Prod Commun. 2018;13 (0): 1-2.

4. **Ishola Anthony*** and Oyedele Opeoluwa. Quantitative Structure Activity Relationships (QSAR) Study of Halogenated Monoterpenes from *Namibian Plocamium* species. Int. Sci. Technol. J. Namibia. 2018; 12:109-116.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2 Literature review

CHAPTER 3 Phytochemical analysis and antioxidant activities of Namibian *Plocamium cornutum* and *Plocamium rigidum*

CHAPTER 4 *In vitro* antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from the Namibian coastline

LIST OF FIGURES

CHAPTER 1 Introduction

CHAPTER 2 Literature review

CHAPTER 3 Phytochemical analysis and antioxidant activities of Namibian *Plocamium cornutum* and *Plocamium rigidum*

LIST OF ABBREVIATIONS

ABTS	2,2-azinobis-3-ethylbenzothizoloine-6-sufonate
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
°C	Degrees Celsius
$CDCl_3$	Deuterated Chloroform
CFU	Colony forming units
CH_2Cl_2	Dichloromethane
CH_3Cl	Chloroform
C_6H_{14}	Hexane
COSY	^1H-^1H Homonuclear Correlation Spectroscopy
COSY-DQF	Cosy-Double Quantum Filter
E. COSY	Exclusive correlation spectroscopy
FTIR	Fourier-transform infrared spectroscopy
d	Doublet
Da	Daltons
dd	Double Doublet
DEPT	Distortionless Enhancement of Polarisation Transfer
DFT	Density functional theory
DPPH	1,1-diphyenyl-2-picrylhydrazyl
dq	Doublet of Quartets
EtOAc	Ethyl Acetate
Eq	Equatorial

FTC	Ferric thiocyanate
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography- Mass Spectrometry
gem	Geminal
HETLOC	Heteronuclear Long-Rang Coupling
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electron Spray Ionisation Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
HSQMBC	Heteronuclear Single Quantum Multiple Bond Correlation
HSQC-TOCSY	HSQC-Total correlation spectroscopy
Hz	Hertz
IC_{50}	Inhibitory Concentration 50%
IKS	Indigenous Knowledge System
IPAP-HSQMBC	In-phase/anti-phase-HSQMBC
IR	Infrared
J	Spin-Spin coupling constant (Hz)
K-C	Potassium clearance
m	Multiplet
m/z	Mass to charge ratio
MIC	Minimum Inhibitory Concentration
MeOH	Methanol
MHz	Megahertz

MMFF	Merck Molecular Force Field
mult	Multiplicity
mw	Molecular Weight
NCRST	National Commission on Research, Science and Technology
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Enhancement Spectroscopy
NPs	Natural Products
PFBHA	Pentafluoro-benzyl-hydroxylamine
q	Quartet
QE	Quercetin equivalent
QSAR	Quantitative structure activity relationships
ROS	Reactive Oxygen Species
s	Singlet
Spp	Species
t	Triplet
TBH	Thiobarbituric acid
TLC	Thin Layer Chromatography
TFC	Total Flavonoid Contents
TPC	Total Phenolic Contents
UV	Ultra Violet
v/v	volume by volume
ZOI	Zone of Inhibition
δ	Chemical shift (ppm)
µL	Microliters

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the University of Namibia who graciously granted me staff development opportunity to undertake these studies. Special thanks to my research supervisors, Prof Michael George Knott, Prof Edet Archibong and Dr Jane Misihairabgwi for their support throughout my study.

A number of people and institutions made valuable contributions that made this study possible. I would like to acknowledge the following:

Mr. Lineekela Kandjengo for his kind assistance with the collection and identification of *Plocamium* species samples.

Namibia Commission on Research Science and Technology (NCRST) for providing me with a research grant.

Prof Jan du Preez of North West University for his assistance with the development of HPLC methods for the purification and collection of fractions for NMR analyses.

Prof. Jacque Petzer and his colleagues at the North West University for their kind assistance with the FTIR and NMR analysis. Profound gratitude is due to Mr. Aloysius Lubega and his colleagues at Makerere University, Kampala, Uganda for the assistance with the laboratory works on dose response.

Prof Timothy Rennie and all my colleagues at the Schools of Pharmacy and Medicine.

Dr Petrina Kapewangolo, Dr Martha Kalili and many others from the Department of Chemistry and Biochemistry for the different ways they supported this initiative.

A very special thanks to my family, especially my children for their patience and moral support during the long and tedious hours in the laboratory.

Above all, a wholehearted and gracious thanks to GOD Almighty who made all this possible.

DEDICATION

This research is dedication to the memory of my dear parents; Mr. Francis Ishola Jemitola and Mrs. Veronica Wuraola Jemitola. They believed in me and always wanted the best for their baby. May their souls rest in eternal peace.

DECLARATION

I, Anthony Shanuolu Ishola, hereby declare that this work on "Structural Characterization, Bioactivity and QSAR studies of Natural Products from selected Namibian red marine algae" is my own work and is a true reflection of my research and that this work, or part thereof has not been submitted for a degree at any other institution.

Anthony Shanuolu Ishola October, 2019

Signed…….

CHAPTER ONE

GENERAL INTRODUCTION

Namibia has a coastline that stretches about 1500 km between the rivers of Kunene ($17^{o}16'S$) and Orange ($28^{o}30'S$). This represents a huge unexplored resource and harbours a tremendous variety of *flora* and *fauna*. The marine environment offers a new frontier for research and attracts scientists from different disciplines.

Namibia has a vast plant biodiversity, which is largely untapped. This fact, complimented by well-established Indigenous Knowledge Systems (IKS) (1,2) puts Namibia in an ideal position to explore and invest in drug discovery from natural resources - known as bio-prospecting (3).

Namibia has over 4,334 different plant taxa (2,4). Although some of these plants are used as herbal medicines, there is limited knowledge about their contents. This is probably due to the fact that screening of plants for pharmacological activity and characterisation of their active chemical compounds is expensive (2). A number of studies into the medicinal properties of Namibian plants have already been undertaken and have demonstrated good pharmacological activity (**1**) on a variety of bioassays including HIV/AIDS (5) and antiplasmodial activity (6).

Seaweeds belong to the division of Thallophyta in plant kingdom. They can be classified into three groups according to their pigments that absorb light of particular wavelengths and give them their characteristic colours of green (Chlorophyta), brown (Phaeophyta) or red (Rhodophyta). For example, the colour observed is complementary to the colour absorbed.

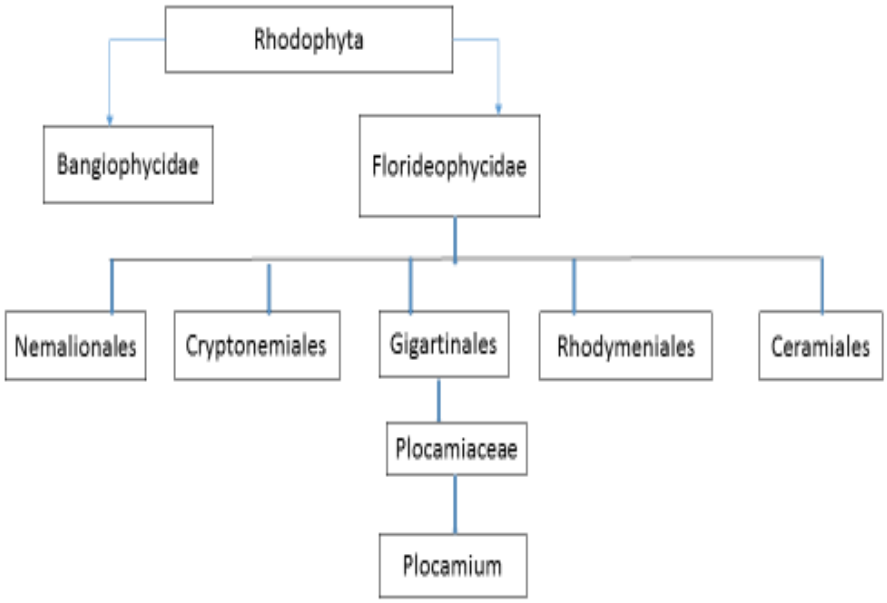
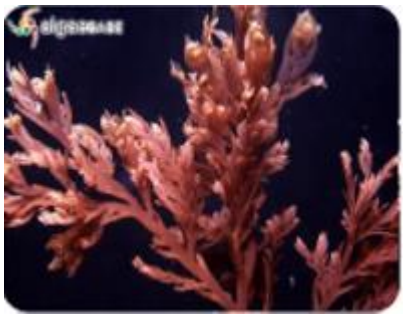


Fig. 1 Classification of red seaweed (Rhodophyta) (7)

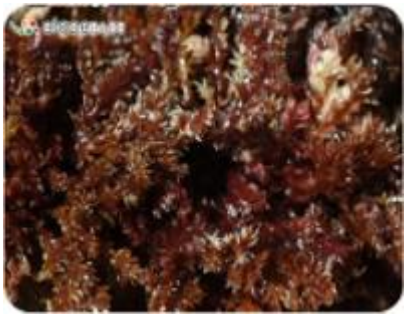
The family *Plocamiaceae* is classified as a red marine alga (Fig. 1) and produces mostly halogenated monoterpenes, which exhibit a range of biological activities including cytotoxic (8), antiherbivory (9), antimicrobial (10) and insecticidal activity (11). Of interest is the impressive selective cytotoxicity that these compounds display, making them potential anticancer leads (12).

Halogenated monoterpenes from South Africa *Plocamium sp.* have shown promising anti-plasmodial (13) as well as cytotoxic (14) activity and this provides motivation to investigate Namibian *Plocamium* species.



A: *Plocamium rigidum*

B: *Plocamium suhrii*



C: *Plocamium cornutum*

Fig. 2. Photographs of selected *Plocamium* species (7)

1.1 Orientation of Study

There has not been any effort to isolate and characterize the secondary metabolites found in red marine algae harvested in Namibia.

1.2 Problem Statement

Marine algae found along the Namibia coast could be holding bioactive compounds of pharmaceutical value. There is a dearth of information on Namibian red marine algae and this study sought to isolate, characterize and perform QSAR studies on potential bioactive natural products. *P. corallorhiza* collected in South Africa was found to demonstrate moderate to good activity towards oesophageal cancer cells (15). The need arose to establish whether the same active compounds could be found in Namibian marine algae.

1.3 Objectives of the Study

The main objective of this research was to determine *in vitro* and *in vivo* biological activities, elucidate the structure of secondary metabolites and use quantitative structure activity relationships (QSAR) to optimise the biological activity of the metabolites.

1.4 Specific Objectives

- To screen red marine algae for phytochemicals and antioxidant activity.
- To determine the biological activity of extracts both *in vitro* and *in vivo.*
- To use HPLC and NMR to determine the molecular structures of the major metabolites.

- To use QSAR technique to optimize the structure of the extract for improve biological activity.

1.5 Significance of the Study

The researcher sought to provide unavailable scientific information on Namibian red marine algae, such as:

Chapter 3 investigated the phytochemical and antioxidant activity of *Plocamium* species using different standard methods.

Chapters 4 and 5 shed light on the *in vitro* and *in vivo* biological activities of *Plocamium* species against an array of pathogens. Biochemical and toxicity information of the major metabolites of *Plocamium* species were investigated in chapter 5.

Chapter 6 investigated the structures of the major metabolites of *Plocamium* species.

Chapter 7 highlights the possible use of QSAR to improve the biological activity of the extract.

1.6 Limitation / Challenges of the Study

After exhaustively combing the coastline of Namibia from Lüderitz to Mowa Bay, in and out of season, only two positively identified *Plocamium* species were found. This had a limiting effect on the scope of the research.

1.7 References

1. Cheikhyoussef A, Shapi M, Matengu K, Ashekele H. Ethnobotanical study of indigenous knowledge on medicinal plant use by traditional healers in Oshikoto region, Namibia. J Ethnobiol Ethnomed. 2011;7(10):1-11.

2. Winschiers-Theophilus H, Bidwell N, Chivuno-Kuria S, Kapuire G. Determining requirements within an indigenous knowledge system of African rural communities. SAICSIT. 2010;332–340.

3. Knott M, Lates J, Ishola A. The Importance of Nuclear Magnetic Resonance in Elucidating the Chemical Structures of Active Components found in Medicinal Plants Indigenous to Namibia. Int J Sci Technol Namibia. 2014;4(2):101–4.

4. Chinsembu K.C, Hedimbi M, Mukaru CW. Putative medicinal properties of plants from the Kavango region, Namibia. J Med. Plants Res. 2011;5(31): 6787-97.

5. Chinsembu KC, Hedimbi M. An ethnobotanical survey of plants used to manage HIV/AIDS opportunistic infections in Katima Mulilo, Caprivi region, Namibia. J Ethnobiol Ethnomed. 2010;6(25):1-9.

6. Nafuka SN. *In Vitro* antiplasmodial activity and phytochemicals screening of ethnomedicinal. plants used to treat malaria associated symptoms. Univ Namibia. 2014;(April).

7. Fakee J. The isolation and characterization of secondary metabolites from selected South African marine red algae (Rhodophyta). Rhodes Univ, South Africa. 2013;1-128.

8. Fuller RW, Cardellina, JH, Jurek J, Scheuer PJ, Alvarado-Lindner B, McGuire M, Gray GN, Steiner JR, Clardy J, Menez E, Shoemaker RH, Newman DJ, Snader KM, Boyd MR. Isolation and structure/activity features of halomon related antitumor monoterpenes from the red alga *Portieria hornemannii*. J Med Chem. 1994; 37:4407–11.

9. Paul VJ, McConnell OJ, Fenical W. Cyclic monoterpenoid feeding deterrents from the red marine alga *Ochtodes crockeri*. J Org Chem. 1980; 45:3401–7.

10. König GM, Wright AD, Linden AD. *Plocamium hamatum* and its monoterpenes: chemical and biological investigations of the tropical marine red alga. Phytochemistry. 1999;52(2):1047–53.

11. Argandona VH, Rovirosa J, Sam-Martin A, Riquelme A, Diaz-marrero AR, Cueto M *et al*. Antifeedant effects of marine halogenated monoterpenes. J Agric Food chem. 2002;50(24):7029-33.

12. Rgandon VH, Rovirosa J, Cueto M, Gonza AC, de IA. Cytotoxic Activity of halogenated monoterpenes from *Plocamium cartilagineum*. Zeitschrift fur naturforsch-Sect A.J. Phys Sci. 2004;59(5-6):339-44.

13. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. Phytochemistry. 2009;70(5):597-600.

14. Antunes EM, Afolayan AF, Chiwakata MT, Fakee J, Knott MG, Whibley CE, Hendricks DT, Bolton JJ, Beukes DR. Identification and in vitro anti-esophageal cancer activity of a series of halogenated monoterpenes isolated from South Africa seaweeds *Plocamium suhrii* and *Plocamium cornutum*. Phytochemistry. 2011;72(8):769-72.

15. Knott MG, Mkwananzi H, Arendse CE, Hendricks DT, Bolton JJ, beukes DR. Ploccoralides A -.C polyhalogenated monoterpenes from the marine alga *Plocamium corallorhiza.* Photochemistry. 2005; 66:1108-12.

CHAPTER TWO

GENERAL LITERATURE REVIEW

The advent of scuba techniques and their utilization by researchers of natural products, led to the identification of several compounds from marine organisms. The search for natural compounds is driven by the exceptional richness of secondary metabolites (including terpenes, steroids, alkaloids) produced by many marine organisms (1). Marine algae are known to produce a wide variety of bioactive secondary metabolites and several compounds have been derived from them for prospective development of novel drugs (2).

Due to the serious side-effects often caused by the use of synthetic drug formulations, the use of plant extracts with medicinal and therapeutic values has gained attention in recent years (3). Presently, interests on immunomodulatory activities of plant based extracts have likewise intensified. The complex compounds found in natural immunomodulators could provide smoother action and produce less allergic reactions compared to synthetic drugs. Furthermore, these compounds do not accumulate to toxic levels and thus, they may be administered for an extended period of time (4).

Marine algae produce a wide variety of chemically active metabolites in their surroundings, potentially as an aid to protect themselves against other settling organisms. These active metabolites, such as halogenated compounds, alcohols, aldehydes and terpenoids are produced by several species of marine macro and micro-algae and have cytotoxic, antibacterial, anti-algal, and antifungal properties which are effective in the prevention of bio-fouling (5,6).

Alkaloids, glycosides, flavonoids, saponins, tannins steroids, related active metabolites, which are of great medicinal value have been extensively used in the drug and pharmaceutical industry (7). Saponins are known to produce inhibitory effects on inflammation. Flavonoids in the human diet may reduce the risk of various cancers, as well as preventing menopausal symptoms. Flavonoids' potent water soluble antioxidant and free radical scavenging activities prevent oxidative cell damage and have strong anti-cancer activity. Flavonoids show anti-allergic, anti-inflammatory, anti- microbial and anti-cancer activity. Furthermore, they show anti-allergic, anti-inflammatory, anti- microbial and anti-cancer activity (7). Phenolic phytochemicals have antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory properties with positive effects on human health (8).

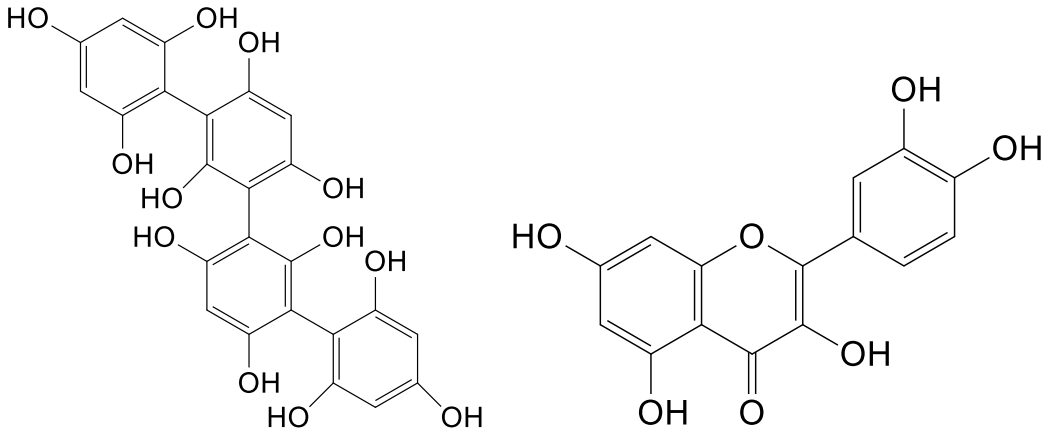
Total phenolic content (TPC) is an important factor in the consideration of antioxidant activity. The higher the value of TPC, the more beneficial the extract is to human health as they can quench reactive free radicals or primary oxidants as reported by Ragan and Glombitza (9). Phenolic compounds have repeatedly been implicated as natural antioxidants due to their redox properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (10). Khairy (11) reported that algal phenolic compounds are effective antioxidants which delay peroxidation. Thus, phenols easily transfer a hydrogen atom to lipid peroxyl cycle and form the aryloxyl, which being incapable of acting as a chain carrier, couples with another radical, thus, quenching the radical process. Phenolic compounds could assist the algae to overcome oxidative stress as well as play a putative adaptive role in the defense against grazers, such as marine herbivores (12). The free radical scavenging activity of seaweeds was

reported by Siriwardhana and Lu (13,14) to be related to their phenolic contents. This observation is in agreement with, Ganesa (15), who reported a high correlation between 2, 2-diphenyl-1 picryl-hydrazyl hydrate (DPPH) scavenging activities (i.e. IC_{50} values) and the total phenolic content. Flavonoids and alkaloids, detected in plant extracts are compounds that have been documented to possess a variety of medicinal properties and health-promoting effects. These classes (such as alkaloids, flavonoids, phenols, saponins, steroids, sugars and tannins) of compounds are known to have curative activity against several pathogens and therefore could suggest their use traditionally for the treatment of various illnesses (16). Flavonoids are crucial antioxidants since they have high redox potential, which allows them to act as reducing agent, hydrogen donors and singlet oxygen quenchers, as well as having metal chelating potential (17).

Packed and refrigerated foods undergo gradual changes during storage, due to auto oxidation which releases reactive oxygen species (ROS) including free radicals like superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), non-free radical species like singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) into the food (18). These ROS induce peroxidation of lipids (polyunsaturated fatty acids) generating secondary oxidants like heptanol and hexanal (19) which contribute to oxidative rancidity and deterioration of the food flavour. These not only cause a loss in food quality but are also believed to be associated with carcinogenesis, mutagenesis, arthritis, diabetes, inflammation and cancer. Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus reduce the level of oxidative stress and slow/prevent the development of complications associated with oxidative stress-related diseases (20). To overcome these problems, a wide range of synthetic

antimicrobial agents (sodium benzoate, calcium benzoate, sorbate) and synthetic antioxidants (butylhydroquinone, propyl gallate, butylated hydroxy toluene (BHT), butylated hydroxyl anisole (BHA)) have been used as food preservatives. Recently, there is a considerable interest in the food as well as pharmaceutical industry for the development of antioxidants from natural sources, such as marine *flora* and *fauna*. Among marine resources, marine algae represent one of the richest sources of natural antioxidants (21). Therefore, the development and utilization of more effective antioxidants of natural origin are desirable.

It has been demonstrated that marine algae have potential antioxidant activity and various classes of Natural Products (NPs) including fucoxanthin, phycoerythrobilin, chlorophyll ∝ and their derivatives have been shown as potent antioxidants. Antioxidant activity of marine algae-derived NPs have been determined by various methods such as ferric thiocyanate (FTC), thiobarbituric acid (TBA),1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2-azinobis-3-ethylbenzothizoloine-6-sulfonate (ABTS) radical scavenging effect, singlet oxygen quenching activity, lipid peroxide inhibition, superoxide radical and hydroxyl radical scavenging assays (22). DPPH has been used extensively as a stable free radical to evaluate a reducing substance and is a useful reagent for investigating free radical scavenging activity of the extracts from *Plocamium* species. The most abundant terrestrial biophenols (flavonoids) have three interconnected rings. Seaweed phlorotannins have up to eight interconnected rings, see Fig. 1 below; making them 10 – 100 times more powerful and more stable as free radical scavengers than other polyphenols. The half-life of phlorotannins which is partially fat soluble, is up to 12 h in the body, compared to 30 – 180 minutes for water soluble, terrestrial polyphenols (23,24).



1 **2**

Fig. 1 Structures of seaweeds phlorotannins (**1**) and terrestrial biophenols (**2**)

Antioxidants are compounds that, when present in low concentration in relation to the oxidant, prevent or delay the oxidation of the substrate (25). Secondary metabolites are produced by plants mainly as products of primary metabolism and as part of the defence mechanisms of plants. Phytochemicals such as alkaloids, tannins and flavonoids are examples of secondary metabolites produced by plants, from which the plants are thought to get their healing properties (26). Antioxidant compounds play an important role against various diseases such as atherosclerosis, chronic inflammation, cardiovascular disorders, cancer and aging processes (27). Phenolic compounds have also been associated with antioxidant activity due to their free radical scavenging activities (28). They can also act as metal chelators which prevent the catalytic function of metal in the process of initiating radicals in the Fenton reaction (20).

Due to emerging resistance of pathogenic microorganisms to existing antibiotics, and the fast spreading of resistant microorganisms, there is an increasing need for new

antibiotics. In marine environments, competition for space and nutrients led to the evolution of antimicrobial defence strategies. This includes the production of chemically active metabolites in their surroundings which act as an aid to protect themselves against other settling organisms, maintenance of unfouled surfaces, deterrence of predation, the ability to successfully reproduce, protection from UV radiation and as allelopathic agents (29,30). Marine algae are one of the largest producers of biomass in the marine environments and are a rich source of structurally novel biologically active metabolites (31). Therefore, they offer a rich source of potentially new drug leads.

Algae metabolites have great industrial potential and accessibility, and thus they have attracted attention for health and cosmetic applications. The use of microalgae and their derivatives in applications to combat skin aging, as well as for depigmentation and antimicrobial applications in the cosmetic industry is wide spread (32). A wide range of metabolites, such as antioxidants, anti-inflammatory agents, alginates, polysaccharides, carotenoids, have been investigated for cosmeceutical preparations. The antimicrobial properties of marine algae have been known since ancient times and well documented in recent years (33). Algae are thus a source of raw materials for one of the most promising and profitable sectors of the biotechnology industry. Phycocolloid substances from marine algae such as alginate, carrageenan and agar have been used globally for decades in medicine and pharmacy. Thus, they are of interest for potential use in cosmetic products (33). Four species of Algerian marine algae were tested for anti-fungi properties and results showed that they had fungi inhibiting effects (33). Algal materials collected from the Red Sea Coast of Jeddah

inhibited the growth of *E. faecalis* (11 mm) but no activity was recorded for the water soluble extracts (2).

Marine algae provide a rich source of structurally diverse secondary metabolites some of which have marked antimicrobial activity against marine pathogens. The bactericidal agents found in algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, cyclic polysulphides and fatty acids. Watson (34) reported that 54 seaweeds were tested *in vitro* for antimicrobial activity and a staggering 95% of the extracts showed activity against different pathogens. Bromophenol compounds have been frequently encountered in various marine algae including red and brown algae. Red algae of family Rhodomaceae are especially known as a rich source of bromophenols (35). Some of these compounds which were previously isolated from the family exhibited a wide spectrum of pharmacological activities such as enzyme inhibition, cytotoxic, antioxidant, feeding deterrent, anti-inflammatory and antimicrobial activities (36). Taskin (29), studied the inhibitory activities of various organic extracts of algae against various fish pathogenic bacteria and their results confirmed the possible use of some marine algae as a source of antimicrobial compounds.

In this study, organic crude extracts from *Plocamium sp.,* a red marine alga from the coastline of Namibia, were studied for their potential inhibitory activities against common pathogens. The twelve pathogens selected for this study are important in our everyday life as they are common causes of a variety of human diseases.

Table 1 Common diseases associated with the pathogens used in this research.

Name	**Type of pathogen**	**Disease**
Escherichia coli	Gram negative	Diarrhoea
Staphylococcus aureus	Gram positive	Sinusitis, skin infection
Staphylococcus saprophyticus	Gram positive	Urinary tract infection and cystitis
Pseudomonas aeruginosa	Gram negative	Sepsis syndromes, hospital acquired infections
Sreptococcus pyogenes	Gram negative	Tonsillitis, sinusitis
Proteus mirabilis	Gram negative	Kidney stones, renal failure
Listeria monocytogenes	Gram positive	Listeriosis
Shigella sonnei	Gram negative	Invade epithelial lining of the colon, diarrhoea
Salmonella typhi	Gram negative	Food poison
Enterococcus faecalis	Gram positive	Urinary tract infection, endocarditis, meningitis
Candida albicans	Fungus (yeast)	Candidiasis (mouth or genital itching)
Epidermidis	Gram positive	Biofilms (catheters)

The ability of marine algae to produce metabolites of potential interest has been extensively documented (37).

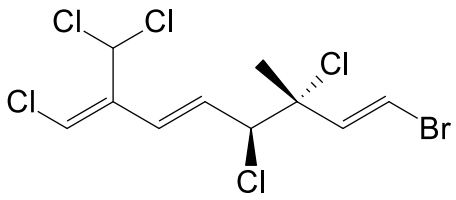
The antimicrobial activity of marine algae may be influenced by some factors such as the habitat, the season of algal collection, different growth stages of plant and experimental methods (2). Although a variety of solvents have been employed in screening marine algae for antimicrobial activity, it is still uncertain what kinds of solvents are most effective and suitable for the extraction of secondary metabolites from various marine algae (38). Mohy El-Din (31), also reported that antimicrobial

activity depends on the solvents used for extraction. It was found that benzene and diethyl ether were suitable solvents for extracting various antibiotic compounds. However, extracts obtained with acetone, ethyl alcohol and ether showed higher antimicrobial activity than extracts from chloroform (38). In a similar study, Sasidharan (39) found that the chloroform extract exhibited the strongest activity, which is in agreement with the findings of Mohy El-Din (31).

The use of plant extracts with medicinal and therapeutic values has gained a lot of attention in recent years due to the serious side-effects often caused by the use of synthetic drug formulations (3). The World Health Organization (WHO) estimated that 80% of African and Asian population use traditional medicine for primary health care. The scenario is similar in developed countries, where 70–80% of the population use some form of complementary or alternative medicine (WHO, 2008). With the global explosion of phythotherapy, the safety of medicinal plants has become a public health problem (40). Medicinal plant species do not receive adequate attention in global discussions related to health (41). The quality, safety and efficacy of many traditionally used herbal formulae are unknown (42). Moreover, there is lack of data for many plants to guarantee their quality and safety (42). Extremely toxic substances like strychnine, the digitoxines, cyanogenic glycosides, amongst others, are extracted from plants. We can only be assured that the use of a particular plant species is safe after a careful investigation (43).

Toxicity from botanical compounds has been underestimated due to the perception that drugs made from plants are absolutely safe. However, severe liver injury has been described after the ingestion of a large variety of different herbal preparations (44).

Determination of the efficacy and safety of herbal remedies is necessary as many people use them for self-medication and little data is available about the pharmacology and toxicology for most of these common herbal remedies (44). It is necessary to determine the *in vivo* efficacy of these extracts and establish the effective dose level in the treatment of pathogens (45). For example, *Galla chinensis* is traditionally used for the treatment of inflammation, dysentery, toxicosis and sores (42). It was reported that *Galla chinensis* could promote mineral ion deposits on the enamel surface layer of dental caries and then modify its remineralization (46). However, herbal formulations are often not subjected to toxicity testing before application to animals or humans. Thus, there are rising concerns about the lack of modern scientific evidence regarding the efficacy and safety of herbal products (47,48). *Plocamium* species are widely distributed throughout the world. The diversity of this species and its ability to produce a wide array of various halogenated monoterpenes with different biological activities has attracted great interest. In recent decades, more attention has been paid to *Plocamium species* as a potential source of pharmaceutical products. *In vitro* studies have demonstrated cytotoxicity, antiproliferative activity, antiplasmodial and antitumor activity (6,49). Six different *Plocamium* species have been identified in Namibia (50). The following compound (**3**) is a major metabolite which was isolated from a *Plocamium* species collected off the Namibian coastline and was characterised using nuclear magnetic resonance (NMR) (Fig. 3).(51,52).



3

Fig. 2 Structure of the major metabolite found in a *Plocamium* sample collected off the Namibian coastline. (51).

The liver has a pivotal role in the regulation of physiological processes and is also involved in detoxification of a variety of drugs and xenobiotics (44). There is growing concern about the toxicity of herbal remedies as many contain substantial amounts of pharmaceutically active ingredients whose mechanisms of action and adverse effects are mostly unknown (53). Severe liver injury, including acute and chronic abnormalities and even cirrhotic transformation and liver failure, have been described after the ingestion of a wide range of herbal products such as mushrooms, germander (*Teucriumchamaedrys*), chaparral (*Larrea tridentate*) (44). Hence, investigations on the biochemical and histopathological alterations associated with the acute oral toxicity of *Plocamium* extract were also conducted.

Due to the possible toxicity of various secondary metabolites that are present in *Plocamium* species (54), it was considered necessary to study the biochemical and histological toxicity of crude *Plocamium* extracts *in vivo*. Much *in vitro* work has been done on halogenated monoterpenes from *Plocamium* species. However, this is the first time the *in vivo* research has been undertaken.

In all these diverse applications of algal metabolites, hundreds of thousands need to be screened or synthesized in the laboratories. This number of compounds as potential algicides is time-consuming and laborious as well as costly. Therefore, theoretical prediction of the activities of algicidal compounds based on quantitative structure–activity relationships (QSAR) is desirable, as it cannot only reduce the heavy experimental burden, but also guide the further design of inhibitory substances (55).

QSAR analyses are based on the assumption of linear additive contributions of the different structural properties or features of a compound to its biological activity, provided that there are no nonlinear dependences of transport or binding on certain physicochemical properties. QSAR modelling is a ligand-based drug design method for both exploring and exploiting the relationship between chemical structure and its biological action (56). To predict the activities of marine algae, quantum chemical descriptors like molecular orbitals, dipole moment, charge etc. and molecular property descriptors like hydrophobic, steric coefficient etc. have been applied to develop 2D QSAR models (57).

The main objective of QSAR is development of predictive and robust QSAR equations, with specified chemical domain for prediction of activity of untested molecule. Secondly, QSAR acts as an informative tool by extracting significant pattern in descriptor related to measured biological activity leading to understanding of mechanism of the given biological activity, this could help in suggesting how to design a novel molecule with improved activity profile.

2.2 References

1. Torres FAE, Passalacqua TG, Vel-Squez AMA, de souza RA, Colepicol P, Graminha MAS. New drugs with antiprotozoal activity from marine Algae: A review. Brazilian J Pharmacogn 2014;2493):265-76.

2. Al-Saif SSA. Ilah, Abdel-Raouf N, El-Wazanani HA., Aref, IA. Antibacterial substances from marine algae isolated from Jeddah coast of Red sea, Saudi Arabia. Saudi J Biol Sci. 2014;21(1):57-64.

3. Patil US, Jaydeokar AV, Bandawane DD. Immunomodulators: A pharmacological Review. Int J. Pharm Pharm Sci. 2012;4(1):30–36.

4. Catap ES, Kho MJL, Jimene, MRR. *In vivo* nonspecific immunomodulatory and antispasmodic effects of common purslane (*Portulaca oleracea Linn*.) leaf extracts in ICR mice. J Ethnopharmacol. 2018;215: 191–198.

5. Smith IC, Blandford DE. Nuclear magnetic resonance spectroscopy. Anal Chem.1995;67(12):509-18.

6. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. Phytochemistry. 2009;70(5):597-600.

7. Eluvakkal T, Sivakuamr SR, Arunkumar K. Fucoidan in some Indian brown seaweeds found along the coast of Gulf of Mannar. Int J Bot. 2010;6(2):176-81.

8. Novoa AV, Andrade-Wartha ERS, Linares AF, Silva Amo GMI, González Aeb VP, Costa A, Mancini-Filho J. Antioxidant activity and possible bioactive components in hydrophilic and lipophilic fractions from the seaweed *Halimeda incrassata*. Rev Bras Farm. 2011;21(1):53-7.

9. Ragan MA, Glombitza K. Phlorotannins, brown algal polyphenols. Prog Phycol Res 1986; 4:129-41.

10. Javanmardi J, Stushnoff C, Locke E, Vivanco JM. Antioxidant activity and Total phenolic content of Iranian *Ocimum accessions*. Food Chem. 2003; 83:547-50.

11. Bay A, Khairy HM, El-Sheikh MA. Antioxidant activity and mineral composition of three Mediterranean common seaweeds from Abu-Qir Bay, Egypt. Saudi J Biol Sci. 2015;22(5):623-30.

12. Chew YL, Lim YY, Omar M, Khoo KS. Antioxidant activity of three edible seaweeds from two areas in South East Asia. LWT—Food Sci Technol, 2008;41(6):1067–72.

13. Siriwardhana N, Lee KW, Kim SH, Ha JW, Jeon YJ. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Sci Technol Int 2003;9(5):339– 46.

14. Lu YR, Foo LY. Antioxidant and radical scavenging activities of polyphenols from apple pomace. Food Chem. 2000; 68:81-5.

15. Ganesan K, Kumar CS, Rao PVS. Comparative assessment of antioxidant activity in three edible species of green seaweed, *Enteromorpha* from Okha, Northwest coast of India. Innov Food Sci Emerg.Technol. 2011;12(1):73-8.

16. Rayapu L, Makkar F, Chakraborty K., Valluru L. Phytochemical evaluation and antimicrobial activity of *Gracilaria opuntia*: an important antidiabetic red marine macroalgae. Int J Cur Pharm Res. 2017;9(6):37-41.

17. Mei.Ling Al, Md. Yasir S, Matanjun P, Abu bakar MF. Antioxidant activity, total phenolic and flavonoid contents of selected commercial seaweeds of sabah, Malaysia. Int J Pharm phytopharm Res. 2013;3:234-8

18. Devi K, Suganthy N, Kesika, Pandian S. Bioprotective properties of seaweeds: *In vitro* evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Complement Altern Med. 2008;8(1):38.

19. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production and signaling mechanism of malondialdehyde and 4-hydroxy-2-nonenal. Oxid med cell Longev.2014;1-31.

20. Wu, X.J. and Hansen, C. Antioxidant capacity, phenolic content, and polysaccharide content of *Lentinus edodes* grown in whey permeate-based submerged culture. J Food Sci, 2008;73(1):1-8.

21. Cornish ML, Garbary DJ. Antioxidants from macroalgae: Potential applications in human health and nutrition. Algae, 2010;25(4):155-71.

22. Pangestuti R, Kim S. Biological activities and health benefit effects of natural pigments derived from marine algae. J Functional Foods. 2011;3(4):255–66.

23. Kang K, Park Y, Hwan HJ, Kim SH, Lee JG, Shin HC. Antioxidative properties of brown algae polyphenolics and their perspectives as chemopreventative agents against vascular risk factors. Arch Pharm Res. 2003;26(4):286-93.

24. Mohamed S, Hashim SN, Rahman HA. Seaweeds: A sustainable functional food for complementary and alternative therapy. Trends Food Sci Technol. 2012; 23:83-96.

25. Proesto, C, Lytoud, K, Mavromelanidou OK, Zoumpoulakis P, Sinanoglou VJ. Antioxidant capacity of selected plant extracts and their essential oils. Antioxidant. 2013; 2:11-22.

26. Bhandary SK, Kumari N, Bhat VS, Sharmila K, Bekal MP. Preliminary phytochemical screening of various extracts of *Punica granatum* peel, whole

fruit and seeds. Nitte Univ J Heal Sci. 2012;2(4):34-8.

27. Kohen R, Nyska A. Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for their Quantification. Toxicol Pathol. 2002;30(6):620-50.

28. Rezaie M, Farhoosh R, Iranshahi M, Sharif A, Golmohamadzadeh S. Ultrasonic-assisted extraction of antioxidative compounds from Bene (*Pistacia atlantica subsp. mutica)* hull using various solvents of different physicochemical properties. Food Chem. 2015; 173:577-83.

29. Taskin E, Taskin E, Ozturk M.. Antibacterial activities of some seaweeds from Northern Cyprus against some food -related pathogens. Asian J Biol sci. 2012;5(5):250-56.

30. Chakraborthy K, Lipton AP, Paulraj R, Vijayan KK. Antibacterial. Diterpernoids of *Ulva fasciata Delile* from South-Western coast of Indian peninsula. Food Chem. 2010;119:1399-408.

31. Mohy El-Din SM, El-Ahwany AMD. Bioactivity and phytochemical constitutents of marine red seaweeds. J Taibah Univ Sci. 2015;10(4):471-84.

32. Wang HMD, Chen CC, Huynh P, Chang JS. Exploring the potential of using algae in cosmetics. Bioresour Technol 2015;184: 55–62.

33. Patra JK, Rath SK, Jena K, Rathod VK., Thatoi H. Evaluation of Antioxidant and antimicrobial activity of seaweed (*Sargassum sp*.) Extract: A Study on inhibition of *Glutathione-S-Transferase* activity. Turkish J Biol. 2008; 32:119-25.

34. Watson SB, Cruz-Rivera E. Algal chemical ecology: An introduction to the special issue. Phycologia. 2003;42(4):319-23.

35. Oh KB, Lee JH, Chung SC, Shin HJ, Kim HK, Kee HS. Antimicrobial activity of the bromophenols from the red algae *Odonthalia corymbifera* and some synthetic derivatives. Bioorganic Med Chem Lett. 2008;18**:**104-8.

36. Williamson G, Carughi A. Polyphenol content and health benefits of raisins. Nutr Res. 2010;30(8):511–19.

37. Cabrita M, Vale C, Rauter, AP. Halogenated compounds from marine algae. Mar Drugs; 2010;8:2301-17.

38. Manivannan K, Karthikai devi G, Anantharaman P, Balasubramanian T. Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters, Gulf of Mannar. Asian Pac J Trop Biomed. 2011;1:114-20.

39. Sasidharan D, Darah ., Noordin MKMJ, Kassim M, Jain M. Screening antimicrobial activity of various extracts of *Gracilaria changi.* Pharm Biol. 2009;47(1):72-6.

40. Vidushi S, Neergheen-Bhujun. Understanding the toxicological challenges associated with the use of herbal medicinal products in developing countries. Biomed res Int. 2013;1-9.

41. Tilburt JC, Kaptchuk TJ. Herbal medicine research and global health: an ethical analysis. Bull. WHO. 2008; 86:594-99.

42. Xiang F, Peng L, Yin Z, Jia R, Hu Z, Li Z, Lv C. Acute and subchronic toxicity as well as evaluation of safety pharmacology of *Galla chinensis* solution.J Ethnopharmacol. 2015;162:181–90.

43. Araújo MC, de P M, Barcellos NMS, Vieira PM de A, Gouveia TM, Guerra MO, Peters VM, Saúde-Guimarães DA.. Acute and sub chronic toxicity study of aqueous extract from the leaves and branches of Campomanesia velutina (Cambess) O. Berg. J Ethnopharmacol. 2017;201**:**17–25.

44. Singh T, Sinha N, Singh A.. Biochemical and histopathological effects on liver due to acute oral toxicity of aqueous leaf extract of *Ecliptaalba* on female Swiss albino mice. Indian J Pharmacol. 2013;45(1):61-74.

45. Shanmughapriya S, Manilal A, Sujith S, Selvin J, Kiran GS, Natarajaseenivasan K. Antimicrobial activity of seaweeds extracts against multiresistant pathogens. Ann Microbiol. 2008;58(3):535–41.

46. Cheng L, Li J, Hao Y, Zhou X. Effect of compounds of *Galla chinensis* on remineralization of enamel surface *in vitro*. Arch Oral Biol. 2010; 55(6):435–40.

47. Seef LB. Herbal hepatotoxicity. Clin Liver Dis. 2007;11:577-96.

48. Tang JL, Liu BY, Ma KW. Traditional Chinese medicine. Lancet 2008;372:1938-40.

49. Fakee J. The isolation and characterization of secondary metabolites from selected South African marine red algae (Rhodophyta). Rhodes Univ, South Africa. 2013;1-128.

50. Lluch JR. Marine benthic algae of Namibia**.** Scientia Marina. *2*002; 66(3): 93-118.

51. Knott MG, Kapewangolo P, Louw S, Brand J, Kandjengo L, Ishola A. The isolation, structural determination and bioactivity of 1E, 3R, 4S, 5E, 7Z-1-bromo 3, 4, 8-trichloro-7-dichloromethyl)-3-methylocta-1, 5, 7-triene from Namibian *Plocamium* species. Int Sci Technol J Namibia. 2016; 7:59-72.

52. Antunes EM, Afolayan AF, Chiwakata MT, Fakee J, Knott MG, Whibley CE, Hendricks DT, Bolton JJ, Beukes DR. Identification and *in vitro* anti-esophageal cancer activity of a series of halogenated monoterpenes isolated

from South Africa sea weeds *P. suhrii* and *P. cornutum*. Phytochemistry. 2011; 72(8):769-72.

53. Elvin-Lewis M. "Should we be concerned about herbal remedies" J Ethnopharmacol. 2001*;* 75:141- 64.

54. Ishola A, Knott M, Misihairabgwi J. *In vitro* antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from the Namibian coastline. African J Pharm Pharmacol 2018; 12(10):121-29.

55. Huang H, Xiao X, Shi J, Chen Y. Structure-activity analysis of harmful algae inhibition by congeneric compounds: Case studies of fatty acids and thiazolidinediones. Environ Sci Pollut Res. 2014;21(11):7154–64.

56. Liao C, Sitzmann M, Pugliese A, Nicklaus MC. Software and resources for computational medicinal chemistry. Future Med Chem. 2011;3(8):1057-1085.

57. Chen Jincan, Shen Yong, Liao Siyan, Chen Lanmei Zheng K. DFT-Based QSAR study and molecula design of AHMA derivative as potent anticancer agents. Wiley Intersci. 2007;107(6):1468–78.

CHAPTER THREE

Phytochemical analysis and antioxidant activities of Namibian *Plocamium cornutum* and *Plocamium rigidum*

3.1 Abstract

Objective: The objective of this research was to screen and quantify phytochemical contents and antioxidant activity of Namibian *Plocamium* species.

Methods: Marine algae collected along the Namibian coastline were soaked in DCM and MeOH in a ratio 1:1 (v/v) for 48 hours. Concentrated extracts were screened for phytochemicals. Total phenolic content and flavonoid content as well as antioxidant activity were quantified using appropriate methods.

Results: The phytochemical screening of both DCM and MeOH extracts of *P. cornutum* and *P. rigidum* confirmed the presence of flavonoids, terpenoids, tannins, saponins, resins, cardiac glycosides and alkaloids. The highest total phenolic content recorded for DCM extracts were 188.65 ± 0.45 mg and 132.85 ± 0.82 mg Gallic acid equivalents per gram for *Plocamium rigidum* and *Plocamium cornutum* respectively. Maximum total flavonoid contents were 202.32 ± 0.33 and 126.11 ± 0.58 mg quercetin equivalents per gram respectively for *P. rigidum* and *P. cornutum,* for DCM extracts. The results from MeOH extracts were lower in both *Plocamium* extracts. The free radical scavenging activities of *Plocamium* species were significantly lower than that of ascorbic acid with IC_{50} values for *P. rigidum* and *P. cornutum* of 28.87 ± 0.82 µM and 40.11 ± 0.38 µM respectively, compared to 12.59 ± 0.66 µM for ascorbic acid.

Conclusion: Total phenolic content, total flavonoid content and IC_{50} values were higher for DCM than for MeOH extracts. The high values of phytochemicals are an indication of their antioxidant potential. Although the free antioxidant potential values are lower than that of ascorbic acid, the *Plocamium* extracts present a rich source of antioxidants relative to other algae and plant species.

Keywords: Antioxidant activity, Free radical scavenging activity, *Plocamium* species, Phytochemicals, Flavonoids.

3.2 Literature Review

The advent of scuba techniques and their utilization by researchers of natural products led to the identification of several novel compounds from marine organisms. The search for natural compounds is driven by the exceptional richness of secondary metabolites (including terpenes, steroids, alkaloids) produced by many marine organisms (1). Marine algae are known to produce a wide variety of bioactive secondary metabolites and several compounds have been derived from them for prospective development of novel drugs (2).

Due to the serious side-effects often caused by the use of synthetic drug formulations, the use of plant extracts with medicinal and therapeutic values has gained attention in recent years (3). Presently, interests on immunomodulatory activities of plant based extracts have likewise intensified. The complex compounds found in natural immunomodulators could provide smoother action and produce less allergic reactions compared to synthetic drugs. Furthermore, these compounds do not accumulate to toxic levels and thus, they may be administered for an extended period of time (4).

Marine algae produce a wide variety of chemically active metabolites in their surroundings, potentially as an aid to protect themselves against other settling organisms. These active metabolites, such as halogenated compounds, alcohols, aldehydes and terpenoids are produced by several species of marine macro and micro-algae and some have cytotoxic, antibacterial, anti-algal, and antifungal properties which are effective in the prevention of biofouling (5,6).

Alkaloids, glycosides, flavonoids, saponins, tannins steroids, related active metabolites, which are of great medicinal value have been extensively used in the drug

and pharmaceutical industry (7). Saponins are known to produce inhibitory effects on inflammation. Flavonoids in the human diet may reduce the risk of various cancers, as well as preventing menopausal symptoms. Flavonoids' potent water soluble antioxidants and free radical scavenging activities prevent oxidative cell damage and have strong anti-cancer activity. Flavonoids show anti-allergic, anti-inflammatory, anti- microbial and anti-cancer activity. Furthermore, they also show anti-allergic, anti-inflammatory, anti- microbial and anti-cancer activity (8). Phenolic phytochemicals have antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory properties with positive effects on human health (9–11).

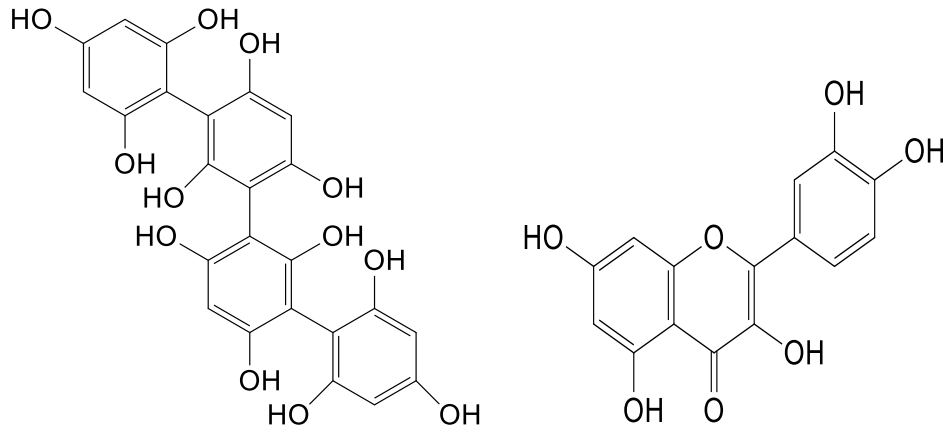
Total phenolic content (TPC) is an important factor in the consideration of antioxidant activity. The higher the value of TPC, the more beneficial the extract is to human health as they can quench reactive free radicals or primary oxidants as reported by Ragan and Glombitza (12). Phenolic compounds have repeatedly been implicated as natural antioxidants due to their redox properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (13). Khairy, (14) reported that algal phenolic compounds are effective antioxidants which delay peroxidation. Thus, phenols easily transfer a hydrogen atom to lipid peroxyl cycle and form the aryloxyl, which being incapable of acting as a chain carrier, couples with another radical, thus, quenching the radical process. Phenolic compounds could assist the algae to overcome oxidative stress as well as play a putative adaptive role in the defense against grazers, such as marine herbivores (15). The free radical scavenging activity of seaweeds was reported to be related to their phenolic contents by Siriwardhana and Lu (16,17). This observation is in agreement with Ganesa (18) who reported a high correlation between

2, 2-diphenyl-1-picryl-hydrazyl hydrate (DPPH) scavenging activities (i.e. IC_{50} values) and the total phenolic content. Flavonoids and alkaloids, detected in plant extracts are compounds that have been documented to possess a variety of medicinal properties and health-promoting effects. These classes of compounds (such as alkaloids, flavonoids, phenols, saponins, steroids, sugars and tannins) are known to have curative activity against several pathogens and therefore could suggest their use traditionally for the treatment of various illnesses (19). Flavonoids are crucial antioxidants since they have high redox potential, which allows them to act as reducing agent, hydrogen donors and singlet oxygen quenchers, as well as having metal chelating potential (20).

Packed and refrigerated food undergo gradual changes during storage, due to auto oxidation which releases reactive oxygen species (ROS) including free radicals like superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), non-free radical species like singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) into the food (21). These ROS induce peroxidation of lipids (polyunsaturated fatty acids) generating secondary oxidants like heptanol and hexanal (22) which contribute to oxidative rancidity and deterioration of the food flavour. These not only cause a loss in food quality but are also believed to be associated with carcinogenesis, mutagenesis, arthritis, diabetes, inflammation and cancer. Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus reduce the level of oxidative stress and slow/prevent the development of complications associated with oxidative stress-related diseases (23). To overcome these problems, a wide range of synthetic antimicrobial agents (sodium benzoate, calcium benzoate, sorbate) and synthetic antioxidants (butylhydroquinone, propyl gallate, butylated hydroxy toluene (BHT),

butylated hydroxyl anisole (BHA) have been used as food preservatives. Recently, there is a considerable interest in the food as well as pharmaceutical industry for the development of antioxidants from natural sources, such as marine *flora* and *fauna*. Among marine resources, marine algae represent one of the richest sources of natural antioxidants (24). Therefore, the development and utilization of more effective antioxidants of natural origin are desirable.

It has been demonstrated that marine algae have potential antioxidant activity and various classes of Natural Products (NPs) including fucoxanthin, phycoerythrobilin, chlorophyll ∝ and their derivatives have been shown as potent antioxidants. Antioxidant activity of marine algae-derived NPs have been determined by various methods such as ferric thiocyanate (FTC), thiobarbituric acid (TBA),1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2-azinobis-3-ethylbenzothizoloine-6-sulfonate (ABTS) radical scavenging effect, singlet oxygen quenching activity, lipid peroxide inhibition, superoxide radical and hydroxyl radical scavenging assays (25). DPPH has been used extensively as a stable free radical to evaluate a reducing substance and is a useful reagent for investigating free radical scavenging activity of the extracts from *Plocamium* species. The most abundant terrestrial biophenols (flavonoids) have three interconnected rings. Seaweed phlorotannins have up to eight interconnected rings, making them 10 – 100 times more powerful and more stable as free radical scavengers than other polyphenols Fig. 1. The half-life of phlorotannins which is partially fat soluble, is up to 12 h in the body, compared to 30 – 180 minutes for water soluble, terrestrial polyphenols (26,27).



1 **2**

Fig. 1 Structures of seaweeds phlorotannins (**1**) and terrestrial biophenols (**2**)

Antioxidants are compounds that, when present in low concentration in relation to the oxidant, prevents or delay the oxidation of the substrate (28). Secondary metabolites are produced by plants mainly as products of primary metabolism and as part of the defence mechanisms of plants. Phytochemicals such as alkaloids, tannins and flavonoids are examples of secondary metabolites produced by plants, from which the plants are thought to get their healing properties (29). Antioxidant compounds play an important role against various diseases such as atherosclerosis, chronic inflammation, cardiovascular disorders, cancer and aging processes (30). Phenolic compounds have also been associated with antioxidant activity due to their free radical scavenging activities (31). They can also act as metal chelators which prevent the catalytic function of metal in the process of initiating radicals in the Fenton reaction (23).

3.3 Chapter Objective

A literature search revealed that *Plocamium* species of marine algae in Namibia have not been investigated for phytochemicals or antioxidant activity. Accounting for the

presence and quantification of important phytochemicals, such as alkaloids, triterpenoids, steroids, tannin, saponin, coumarins, terpenoids, quinine, phenols, phytosterids, phlobatannins and flavonoids found in Namibia *Plocamium* species will enhance our understanding of Namibian *Plocamium* species.

3.4 Materials and Methods

3.4.1 Collection of samples

Plocamium cornutum and *Plocamium rigidum* were collected from the coastline of Namibia in Henties Bay and Luderitz. *Plocamium* species were collected 5 – 10 cm under-water by hand and placed in sealable polythene bags and refrigerated at - 20 ± 2 °C. Collections were done twice during spring and summer of 2015 from the same areas. The coordinates of the collection site at Lüderitz and Henties Bay are 26° 38' 53" S, 15° 9' 34" E and 22° 7' 0"S, 14° 17' 0" E, respectively (see Fig. 2). The samples were washed to remove sand, epiphytes and other necrotic parts before they were subjected to extraction. The samples were identified by a taxonomist based at the Department of Fisheries and Aquatic Sciences of the University of Namibia. A sample voucher was retained at the University of Namibia.

3.4.2 Sample Preparation and Extraction

Following collection and cleaning of *P. cornutum* and *P. rigidum*, 33 g of each sample was soaked in the extraction solvent (MeOH: DCM 1:1 v/v) for 48 h at room temperature (26 ± 2 °C). The extracts were then decanted into a 250 ml beaker. The resulting extracts were filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator (model RE 100; Bibby Sterilin Ltd). The concentrated extracts were separately dissolved in 100 ml of extraction solvent and transferred to a

separating funnel. The MeOH and DCM layers were carefully decanted into pre-weighed glass vials. The extracts were left to dry in a laminar airflow cabinet. The dried extracts were weighed and stored at a temperature of - 80± 2 °C.

Fig. 2 Underlined coastal towns indicate collection sites for *Plocamium* species in Namibia.

3.4.3 Phytochemical Screening Tests

DCM and MeOH extracts of *P. rigidum* and *P. cornutum* were screened for phytochemicals using standardised methods. The method described by Catap (4) was used to test for tannis. Terpenoids, saponins and resins were tested for by using the method described by Evans and Trease (32); the method of Shah and Seth (33) was used to test for alkaloids and steroids. Flavonoids and cardiac glycosides were tested using methods described by Harborne (34).

3.4.4 Total Phenolic Content (TPC)

TPC of the extracts was measured by adopting the procedure of Mia Isabella (35), using a 96-well microplate reader. Gallic acid standard solutions were prepared in 100 % ethanol to give the following final concentrations: 10.0, 20.0, 40.0, 60.0 and 80.0 µg/ml. Plant extracts were prepared to give similar concentrations as those of gallic acid. 20 µL of plant extracts were placed in different wells followed by 100 µL of 0.2 mol/L Folin-Ciocalteu's phenol reagent. 80 µL of saturated sodium carbonate was added to the mixture after 3 minutes. The mixture was left to incubate at room temperature for an hour. The absorbance was then measured at 750 nm using SoftMax Pro M_3 6.3 plate reader and the results are expressed in milligram Gallic acid equivalent per gram of tissue. The following equation was used:

TPC = (C x V)/M

TPC = total phenolic content, C = concentration of Gallic acid established from the calibration curve (µg/ml), V = volume of extract in mL, M = mass of plant extract (g).

3.4.5 Total Flavonoids Content (TFC)

The determination of total flavonoid contents of the different extracts was achieved using the method presented by John (31); using a 96-well microplate reader. 4 mg of quercetin was dissolved in 200 µL of MeOH to yield 20 µg/µl of quercetin standard. The standard solution was diluted to yield different concentrations of quercetin in the range of 10.0, 20.0, 40.0, 60.0, 80.0 µg/ml. Concentrations of the plant extracts in the same range as quercetin were prepared. 100 µL of distilled water was added to the wells followed by the addition of 20 µL of extract to the microplate followed by 180 µL of 90% diethylene glycol and 20 µL of 1 mol /L NaOH. The microplates were left to incubate for 15 minutes after which the absorbance was measured at 515 nm using SoftMax Pro M_3 6.3 plate reader. The flavonoids content was expressed as milligram of quercetin equivalents per gram of tissue.

3.4.6 Antioxidant Activity

The free radical scavenging activity of the crude extracts was evaluated by using the method described by Kuete (36). Working solutions of both ascorbic acid and the extracts which were used as the standard and each of the test samples, were prepared in ethanol to give concentrations of 800 µg/ml and 400 µg/ml respectively. These were placed in 96 plate wells and mixed with 0.3 mM of 2, 2-diphenyl-1- picryl-hydrazyl hydrate (DPPH). The samples in wells were serially diluted to give concentrations of extract ranging from 200 µg/ml to 3.2 µg/ml. After incubating the mixture in the dark for 30 minutes at room temperature, the absorbance values were measured at 520nm. Each assay was repeated four times and the mean results recorded. The percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = \left[1 - \frac{At - Ao}{A}\right] x100$$

Where A_o is the absorbance of the extract without DPPH, A_t is the absorbance of the extracts with DPPH. A is the absorbance of the negative control.

3.5 Statistical Analysis

Means of triplicate analysis were calculated and the data was expressed as mean ± standard deviation (SD). Two-way ANOVA statistical analysis was performed using Graph pad prism 5^{th} edition software for comparison between two or more treatments. A difference was considered statistically significant when $p \leq 0.05$. The same software was used to determine the value of IC_{50} of both the control and plant extracts.

3.6 Results

3.6.1 Phytochemical Screening

The phytochemical screening test results of *P. rigidum* and *P. cornutum* revealed the presence of a range of phytochemicals as shown in Table 1 below.

Table 1 Phytochemical screening of *Plocamium* extracts.

P. Species	Solvent	Tannins	Flavonoids	Terpenoids	Alkaloids	Glycosides	Steriods	Resins	Saponins
P. rigidum	MeOH	+	+	+	+	+	+	+	+
	DCM	+	+	+	+	+	+	+	+
	Water	-	-	-	-	-	-	-	-
P. cornutum	MeOH	+	+	+	+	+	+	+	+
	DCM	+	+	+	+	+	+	+	+
	Water	-	-	-	-	-	-	-	-

Key: + and - indicate the presence or absence of phytochemical respectively.

3.6.2 Total Phenolic Content

The total phenolic contents of *P. rigidum* and *P. cornutum* extracted in DCM and MeOH are as presented in Table 2. They were expressed as Gallic acid equivalent (GAE) per gram of the extract or fraction. Results show that *P. rigidum* has significantly higher total phenolic content of 188.61± 0.45 mg GAE/g compared to *P. cornutum* at 132.15± 0.82 mg GAE/g of DCM extracts ($p < 0.001$) (Fig. 3).

The total phenolic content of the methanolic extracts also showed the same trend where 84.69± 0.18 mg GAE/g for *P. rigidum* and 66.01± 0.38 mg GAE/g for *P. cornutum* were recorded.

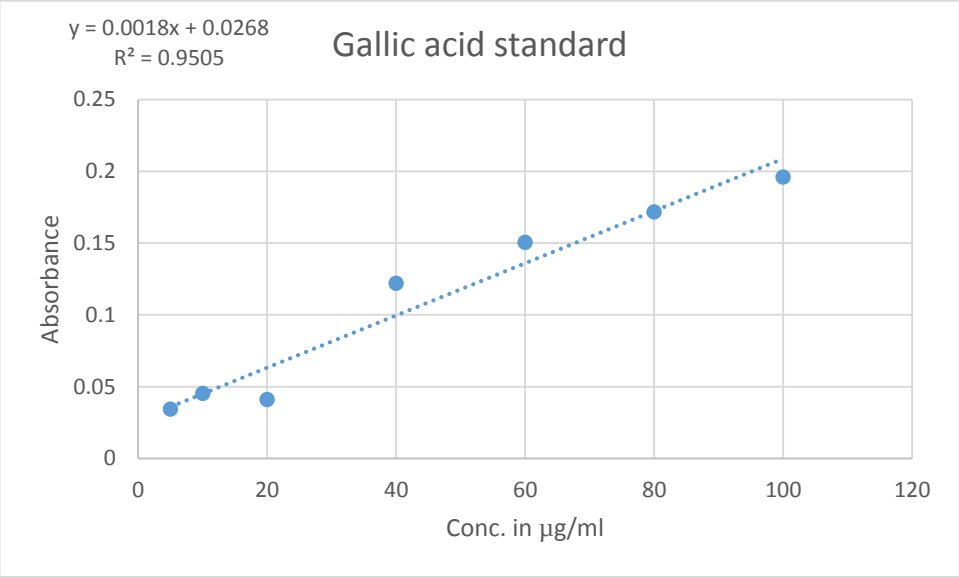


Fig. 3 Gallic acid standard absorption calibration curve.

Table 2 Total phenolic content of *P. rigidum* and *P. cornutum* as GAE in DCM and MeOH

µg/ml	TPC-DCM GAE (µg /g) *P. rigidum*	TPC-MeOH GAE (µg /g) *P. rigidum*	TPC-DCM GAE (µg /g) *P. cornutum*	TPC-MeOH GAE (µg /g) *P. cornutum*
10	43.70 ± 0.02	0	21.27 ± 0.22	0
20	50.83 ± 0.15	7.11 ± 0.15	45.54 ± 0.15	5.31 ± 0.04
40	86.94 ± 0.08	15.85 ± 0.3	57.81 ± 0.06	20.83 ± 0.45
60	105.55 ± 0.03	28.75 ± 0.05	79.91 ± 0.14	40.30 ± 0.22
80	141.69 ± 0.35	51.32 ± 0.41	105.0 ± 0.33	50.62 ± 0.53
100	188.65 ± 0.45	84.69 ± 0.18	132.85 ± 0.82	66.01 ± 0.38

Note: The average of TPC are based on triplicates from a single batch. Results are expressed as means ±SD (n = 3).

The difference in concentrations of TPC of *P. rigidum* in DCM and MeOH are statistically significant ($p < 0.001$). The same trend was observed in the TPC of *P. rigidum, P. cornutum* in the different solvent extracts.

3.6.3 Total Flavonoid Content

The total flavonoid content (TFC) of *P. rigidum* and *P. cornutum* extracted in DCM and MeOH are as presented in Table 4. They are expressed as quercetin equivalent (QE). As in the case of TPC, *P. rigidum* had the highest total flavonoid contents of 202.41±0.33 mg/g compared to 126.48±0.16 mg/g recorded for *P. cornutum* extracted in DCM ($P<0.001$). The TFC of *P. rigidum* and *P. cornutum* extracted in methanol were 164.48±0.58 mg/g and 108.89 ±0.18 mg/g QE respectively. TFC was quantified for extracts of *P. rigidum* and *P. cornutum* based on the quercetin standard curve ($r^2 = 0.9858$).

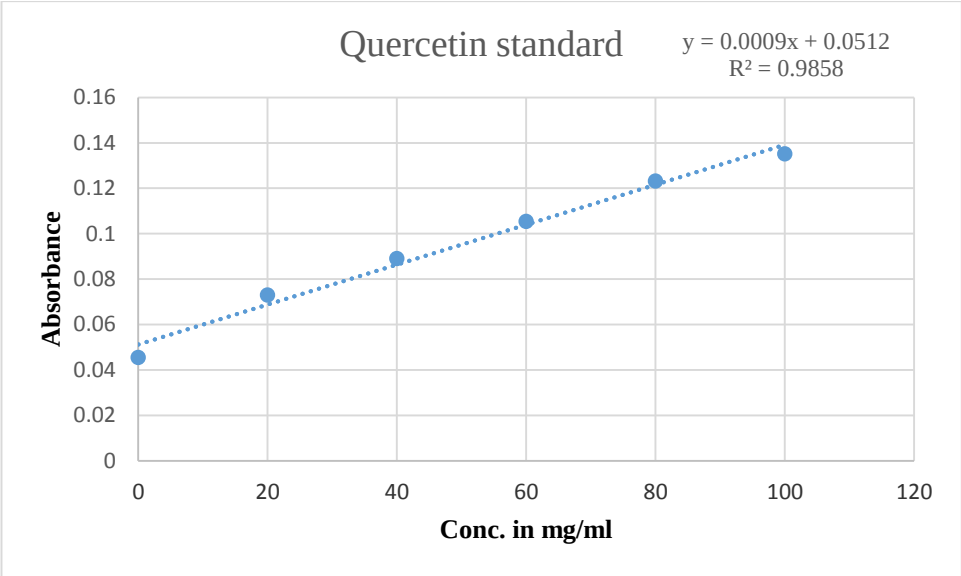


Fig. 4 Quercetin standard absorption calibration curve

Table 3 Total flavonoid content of *P. rigidum* and *P. cornutum* as QE in DCM and MeOH.

Conc µg/ml QE	TFC-DCM *P. rigidum*	TFC-MeOH *P. rigidum*	TFC-DCM *P. cornutum*	TFC-MeOH *P. cornutum*
10	0	0	0	0
20	66.85 ± 0.82	53.33 ± 0.56	18.41 ± 0.59	9.31 ± 0.84
40	104.07 ± 0.15	65.19 ± 0.48	51.54 ± 0.64	37.35 ± 0.05
60	146.67 ± 0.68	94.44 ± 0.18	85.63 ± 0.77	57.44 ± 0.16
80	162.41 ± 0.46	110.94 ± 0.25	107.15 ± 0.13	73.30 ± 0.08
100	202.41 ± 0.33	164.04 ± 0.84	126.11 ± 0.58	102.48 ± 0.16

Note: The averages of TFC are based on triplicates from a single batch. Results are expressed as means ±SD (n = 3). Results are statistically different ($p < 0.001$) between the *P. rigidum, P. cornutum* and the different solvents.

3.6.4 DPPH Free Radical Scavenging Activity

Table 4 indicates the percentage free radical scavenging activity of *P. rigidum* and *P. cornutum* extracted in DCM and MeOH. The DCM extracts showed higher percentage scavenging activity than the MeOH extracts. The free radical scavenging activity increased as the concentration increased in all the extracts. The IC_{50} of the DCM extracts of *P. rigidum* and *P. cornutum* were found to be 28.87 ($R^2 = 0.9705$) and 40.11 µg/ml ($R^2 = 0.9876$) respectively.

Table 4 Percentage scavenging activity of DCM and MeOH extracts

Conc µg/ml	Ascorbic acid	*P. rigidum* DCM	*P. cornutum* DCM	*P. rigidum* MeOH	*P. cornutum* MeOH
3.1	32.44	26.42	22.08	15.75	14.28
6.5	44.82	30.10	24.94	19.05	18.39
12.5	47.89	34.81	27.97	21.43	19.97
25.0	50.98	38.04	32.21	30.01	25.18
50.0	62.38	46.77	41.96	38.00	33.93
100.0	66.85	53.79	46.41	45.63	36.40
200.0	74.00	59.77	53.22	50.05	42.01

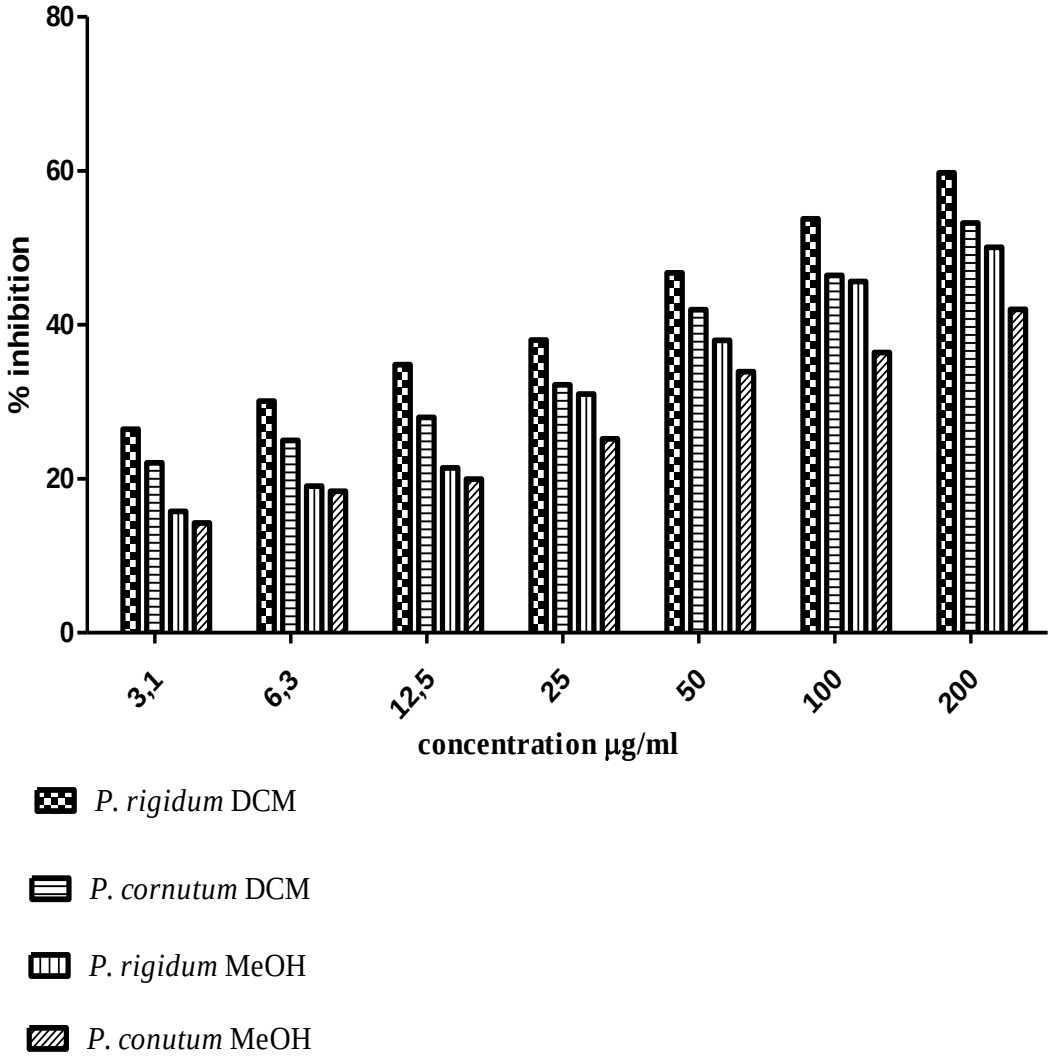


Fig. 5 Percentage inhibition of *Plocamium* extracts against concentration of different solvents.

Graph pad prism 5th edition was used to determine the value of IC_{50} of the extracts. Furthermore, Graph pad prism was used to determine two-way ANOVA of the data. The values were found to be statistically significant with a $p < 0.001$. A two-way ANOVA test revealed that the antioxidant activity varied significantly among algae.

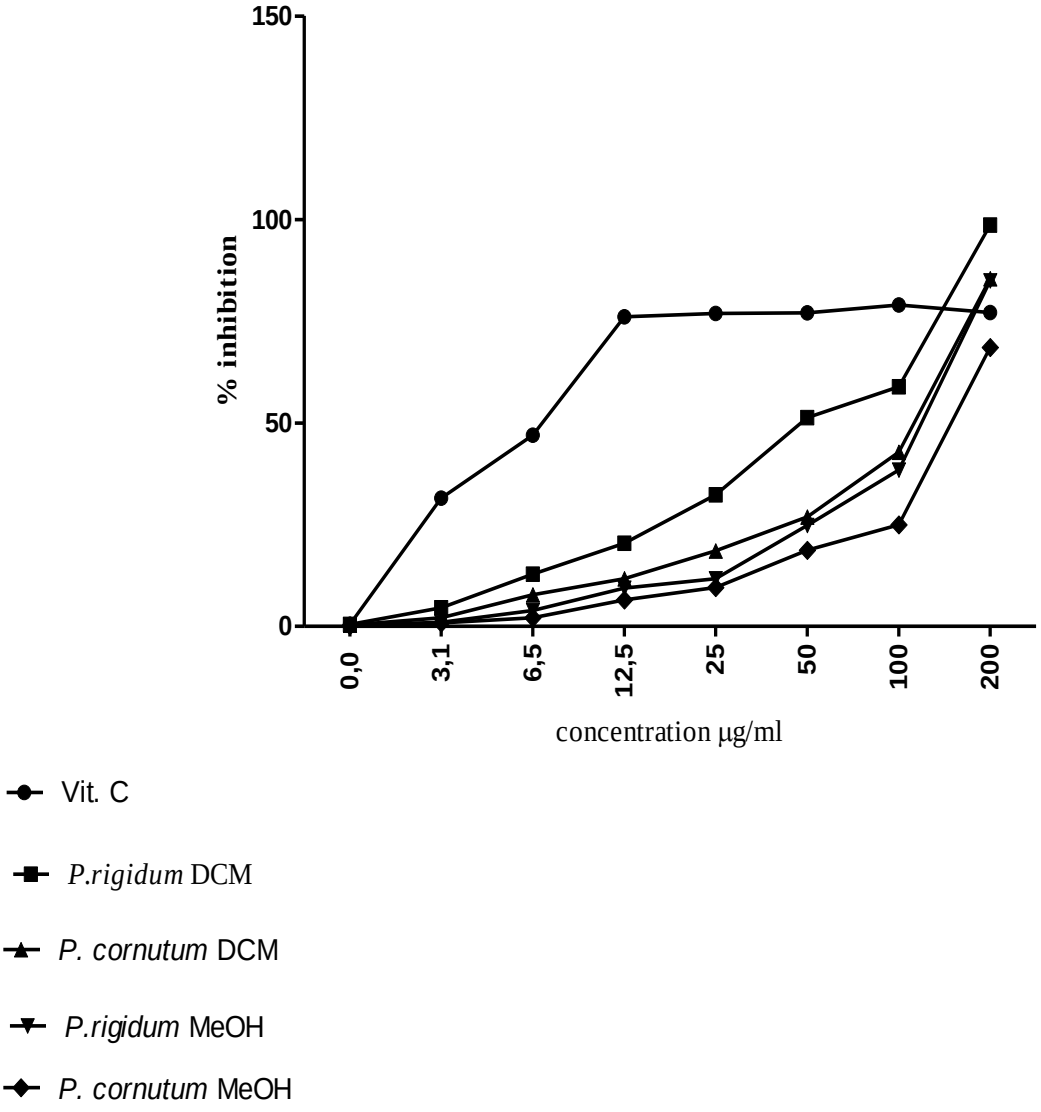


Fig. 6 Antioxidant potentials of extracts in DCM and MeOH against solvent concentration (µg/ml).

3.7 Discussion

In this study, the screening of *P. rigidum* and *P. cornutum* confirmed the presence of important phytochemicals such as alkaloids, flavonoids, steroids, tannins, saponin, terpenoids, resins, and glycosides in all samples tested. The results obtained in this study showed that, TPC in *P. rigidum* and *P. cornutum* extracted in DCM gave 188.65 ± 0.45 and 132.85 ± 0.82 mg GAE/g respectively. The results compare favourably with Chew (15), who reported crude methanolic extracts of red seaweeds to yield results in the range of 144.22 ± 0.21 and 115.35 ± 0.18 mg GAE/g for *C. racemose* and *K. alvarezzi* respectively. The methanolic extracts for *P. rigidum* and *P. cornutum* gave 84.69 ± 0.18 and 66.01 ± 0.38 mg GAE/g respectively which are much lower compared to the findings of Chew (15). It can therefore be inferred that *P. rigidum* has a higher total phenolic content than *P. cornutum* in both extractive solvents. The higher the TPC and TFC the higher the potential of these species being medicinal agents. A two-way ANOVA shows that the difference is statistically significant at $p < 0.001$. Variation in TPC values of marine macro algae could be influenced by extrinsic factors (such as herbivory pressure, irradiance, depth, salinity and nutrients), by intrinsic factors, morphology, age and reproductive stage (18, 37, 38). Thus *P. rigidum* and *P. cornutum* extracted in DCM could be potentially rich sources of natural antioxidants.

TFC in *P. rigidum* and *P. cornutum* extracted in DCM were 202.41 ± 0.33 and 126.11 ± 0.58 mg QE/g respectively. The methanolic extracts for *P. rigidum* and *P. cornutum* were 164.04 ± 0.84 and 102 ± 0.48 mg QE/g respectively.

Mohy El-Din (39) reported that the presence of phytoconstituents such as flavonoids, tannins and polyphenols prevent a number of diseases through their free radical

scavenging activity and these phenolic compounds, which include phenols, tannins and flavonoids, have been found in *P. rigidum* and *P. cornutum.* IC_{50} is the concentration of an antioxidant that is required to reduce the free radical formation by 50% (40); and it gives a better understanding of the free radical scavenging activity of the extracts. Agents with high scavenging activity should have a low IC_{50} value.

Screening for antioxidant activity by means of the free radical scavenging assay revealed that the extracts of *P. rigidum* and *P. cornutum* have antioxidant activity. It was found that *P. rigidum* is a more effective scavenger than *P. cornutum*. This correlated very well with the values of TPC and TFC; the higher values of TPC and TFC in *P. rigidum* is reflected in its more efficient scavenging activity compared to the activity of *P. cornutum* that has lower values of TPC and TFC. The IC_{50} values for scavenging DPPH were found to be 28.87 ± 0.28 µM and 40.11 ± 0.04 µM for *P. rigidum* and *P. cornutum* respectively. The efficacies of antioxidants are often associated with their ability to scavenge stable free radicals (41). Thus, the DPPH radical scavenging activity of DCM and MeOH extracts demonstrate their oxygen radical scavenging capacity indicating potent antioxidant nature. Increased antioxidant activity was observed with increased algal extract concentration. This is consistent with the observation of Moubayed (42), where maximal values were obtained with *Sium latifolium B* at a 150 mg/ml compared to the *Chaetoceros socialis* samples. Results indicated 59% scavenging activity of the *P. rigidum* crude extracts followed by *P. cornutum* (43%) compared with the reference control ascorbic acid having a 77% free radical scavenging activity at a concentration of 100 µg/ml. It can be inferred from the results that, ascorbic acid has a better scavenging activity than *P. rigidum* and *P. cornutum* at lower concentrations. A reversed trend was observed at higher concentrations.

3.8 Conclusion

Plocamium rigidum and *Plocamium cornutum* are both rich in important phytochemicals. Compared to MeOH, DCM proves to be the solvent of choice in the extraction of phytochemicals and in the quantification of both TPC and TFC. The scavenging potential of *Plocamium* species extracted in DCM is about 59 % compared to 77 % scavenging activity of ascorbic acid.

3.9 Acknowledgements

This work is as a result of the support by the University of Namibia and Namibian National Commission on Research, Science and Technology (NCRST).

3.10 References

1. Torres FAE, Passalacqua TG, Vel-Squez AMA, de souza RA, Colepicol P, Graminha MAS. New drugs with antiprotozoal activity from marine Algae: A review. Brazilian J Pharmacogn 2014;2493):265-76.

2. Al-Saif SSA. Ilah, Abdel-Raouf N, El-Wazanani HA., Aref, IA. Antibacterial substances from marine algae isolated from Jeddah coast of Red sea, Saudi Arabia. Saudi J Biol Sci. 2014;21(1):57-64.

3. Patil US, Jaydeokar AV, Bandawane DD. Immunomodulators: A pharmacological Review. Int J. Pharm Pharm Sci. 2012;4(1):30–36.

4. Catap ES, Kho MJL, Jimene, MRR. *In vivo* nonspecific immunomodulatory and antispasmodic effects of common purslane (*Portulaca oleracea Linn*.) leaf extracts in ICR mice. J Ethnopharmacol. 2018;215: 191–8.

5. Smith AJ, Medicinal and pharmaceutical uses of seaweed natural products: A review. J App Phycol. 200; 16:245-62.

6. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. Phytochemistry. 2009;70(5):597-600.

7. Eluvakkal T, Sivakuamr SR, Arunkumar K. Fucoidan in some Indian brown seaweeds found along the coast of Gulf of Mannar. Int J Bot. 2010;6(2):176-81.

8. Azhagu RR, Mala K, Prakasam A. Phytochemical Analysis of marine macroalga Caulerpa racemosa (J Agardh) (*Chlorophyta Caulerpales)* from Tirunelveli District Tamilnadu India. J Glob Biosci. 20164(8):3055-67.

9. Novoa AV, Andrade-Wartha ERS, Linares F, Silva DO, Ines M. *et al.*, Antioxidant activity and possible bioactive components in hydrophilic and lipophilic fractions from the seaweed *Halimeda incrassata*. Rev Bras Farm. 2011;21(1):53-7.

10. Vinay N, Kim S. Potential pharmacological applications of polyphenolic derivatives from marine brown algae. Environ Toxicol Pharmacol. 2011;32(3):325-35.

11. Wijesinghe WAJP, Jeon Y, Enzyme-assistant extraction (EAE) of bioactive compounds: A useful approach for recovery of industrially important metabolites from seaweeds: A review. Fitoterapia. 2012;83(1):6-12.

12. Ragan AM, Glombitza, K. Phlorotannins, brown algal polyphenols. Prog Phycol.Res 1986;4:129-24.

13. Javanmardi J, Stushnoff C, Locke E, Vivanco JM. Antioxidant activity and Total phenolic content of Iranian *Ocimum accessions*. Food Chem. 2003;

83:547-50.

14. Bay A, Khairy HM, El-Sheikh MA. Antioxidant activity and mineral composition of three Mediterranean common seaweeds from Abu-Qir Bay, Egypt. Saudi J Biol Sci. 2015;22(5):623-30.

15. Chew YL, Lim YY, Omar M, Khoo KS. Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT*—Food Sci Technol, 2008;41(6):1067–72.

16. Siriwardhana N, Lee KW, Kim SH, Ha JW, Jeon YJ. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Sci Technol Int 2003;9(5):339–46.

17. Lu YR, Foo LY. Antioxidant and radical scavenging activities of polyphenols from apple pomace. Food Chem. 2000; 68:81-5.

18. Ganesan K, Kumar CS, Rao PVS. Comparative assessment of antioxidant activity in three edible species of green seaweed, *Enteromorpha* from Okha, Northwest coast of India. Innov Food Sci Emerg.Technol. 2011;12(1):73-8.

19. Rayapu L, Makkar F, Chakraborty K., Valluru L. Phytochemical evaluation and antimicrobial activity of *Gracilaria opuntia*: an important antidiabetic red marine macroalgae. Int J Cur Pharm Res. 2017;9(6):37-41.

20. Mei.Ling Al, Md. Yasir S, Matanjun P, Abu bakar MF. Antioxidant activity, total phenolic and flavonoid contents of selected commercial seaweeds of sabah, Malaysia. Int J Pharm phytopharm Res. 2013;3:234-8.

21. Devi K, Suganthy N, Kesika, Pandian S. Bioprotective properties of seaweeds: *In vitro* evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Complement Altern Med. 2008;8(1):1-11.

22. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production and signaling mechanism of malondialdehyde and 4-hydroxy-2-nonenal. Oxid med cell Longev. 2014;1-31.

23. Wu, X.J. and Hansen, C. Antioxidant capacity, phenolic content, and polysaccharide content of *Lentinus edodes* grown in whey permeate-based submerged culture. J Food Sci, 2008;73(1):1-8.

24. Cornish ML, Garbary DJ. Antioxidants from macroalgae: Potential applications in human health and nutrition. Algae, 2010;25(4):155-71.

25. Pangestuti R, Kim S. Biological activities and health benefit effects of natural pigments derived from marine algae. J Functional Foods. 2011;3(4):255–66.

26. Kang K, Park Y, Hwan HJ, Kim SH, Lee JG, Shin HC. Antioxidative properties of brown algae polyphenolics and their perspectives as

chemopreventative agents against vascular risk factors. Arch Pharm Res. 2003;26(4):286-93.

27. Mohamed S, Hashim SN, Rahman HA. Seaweeds: A sustainable functional food for complementary and alternative therapy. Trends Food Sci Technol. 2012; 23:83-96.

28. Proesto, C, Lytoud, K, Mavromelanidou OK, Zoumpoulakis P, Sinanoglou VJ. Antioxidant capacity of selected plant extracts and their essential oils. Antioxidant. 2013; 2:11-22.

29. Bhandary SK, Kumari N, Bhat VS, Sharmila K, Bekal MP. Preliminary phytochemical screening of various extracts of *Punica granatum* peel, whole fruit and seeds. Nitte Univ J Heal Sci. 2012;2(4):34-8.

30. Kohen R, Nyska A. Oxidation of Biological Systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol. 2002;30(6):620-50.

31. John KMM, Ayyanar M, Arumugam T, Enkhtaivan G, Jin K, kim DH. Phytochemical screening and antioxidant activity of different solvent extracts from Strychnos minor Dennst leaves. Asian pacific J Trop Dis. 2015;5(3):204-9.

32. Trease GE, Evans WC. A text book of Pharmacognosy. 4th ed. *Bailliere Tindall Ltd*, London. 832. 2013.

33. Shah BN, Seth AK. Textbook of Pharmacognosy and Phytochemistry. *Elsevier*, India. 2010.

34. Harborne JB.. Phytochemical methods A Guide to modern techniques of plant analysis, Springer. 1998.

35. Isabelle M, Lee BL, Ong CN, Liu X, Huang D. Peroxyl radical scavenging capacity, polyphenolics, and lipophilic antioxidant profiles of mulberry fruits cultivated in Southern China. J Agric Food Chem 2008; 56:9410–6.

36. Kuete V, Metuno R, Keilah PL, Tshikalange ET, Ngadjui BT. Evaluation of the genus *Treculia* for antimycobacterial, anti-reverse transcriptase, radical scavenging and antitumor activities. South African J Bot. 2010; 76(3):530–5.

37. De Alencar DB, Da Silva SR, Pires-Cavalcante KMS, De Lima RL, *et al*., Antioxidant potential and cytotoxic activity of two red seaweeds species, *Amansia multifidi* and *Meristiella echinocarpa*, from the coast of North Eastern Brazil. Ann Brazilian Acad Sci. 2014; 86(1):251-63.

38. Lann KL, Ferret C, Vanmee E, Spagnol C, *et al*., Total phenolic, size-fractionated phenolics and fucoxanthin content of tropical *Sargassaceae* (*Fucales, Phaeophyceae*) from the South Pacific Ocean: Spatial and specific variability. Phycolol Res. 2012; 60:37-50.

38. Mohy El-Din, SM, El-Ahwany AMD. Bioactivity and Phytochemical constituents of marine red seaweeds. *(Janiarubens, coralline mediterranea and Pterocladia capillacea),* J Taibah Univ Sci. 2016; 10(4): 471-84.

40. Zhang J, Ianora A, Wu C, Pellegrini D, Esposito F, Buttino I. How to increase productivity of the copepod *Acartia tonsa* (Dana): effects of population density and food concentration. Aquac Res. 2015; 46(12):2982–90.

41. Bhaigyabati T, Usha K. Preliminary phytochemical screening and antioxidant activity f various extracts of *Sargassum wightii grevillea*. Int J Univers Pharm Bio Sci. 2013; 2(6): 60-69.

42. Moubayed NMS, Al Houri HJ, Al Khulaifi MM, Al Farraj DA. Antimicrobial, antioxidant properties and chemical composition of seaweeds collected from Saudi Arabia (Red Sea and Arabian Gulf). Saudi J Biol Sci. 2016;24(1):162-9.

CHAPTER FOUR

In vitro antimicrobial activities of _Plocamium rigidum_ and _Plocamium cornutum_ from the Namibian coastline

academicJournals

Vol. 12(10), pp. 121-129, 15 March, 2018
DOI: 10.5897/AJPP2018.4642
Article Number: F163A6556266
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

In vitro antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from the Namibian coast line

Anthony Ishola[1*], Michael Knott[1] and Jane Misihairabgwi[2]

[1]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Namibia. Private Bag 13301, Windhoek, Namibia.
[2]Department of Biochemistry and Microbiology, School of Medicine, University of Namibia, Private Bag 13301, Windhoek, Namibia.

Received 12 January, 2018; Accepted 6 February, 2018

4.1 Abstract

Objective: The objective of this research was to determine the antimicrobial activity of two algal species, *P. cornutum* and *P. rigidum* from the coastline of Namibia.

Methods: Dried algae extracts were reconstituted in distilled water, hexane, dichloromethane, ethanol, methanol and chloroform, respectively and tested *in vitro* for antimicrobial activity against 12 pathogens namely; *Escherichia coli, Staphylococcus aureus, Staphylococcus saprophyticus, Pseudomonas aeruginosa, Streptococcus pyogenes, Proteus mirabilis, Listeria monocytogenes, Shigella sonnei, Salmonella sp., Enterococcus faecalis, Candida albicans* and *Staphylococcus epidermidis*.

Results: Screening confirmed that water extracts showed no activity against all the pathogens as the extracts were insoluble in water. The *Plocamium* extracts in the remaining solvents showed varying degrees of antimicrobial activity.

Both DCM and MeOH extracts reconstituted in chloroform showed the greatest activity amongst the five different solvents that were used. Ampicillin (10 µg/disc) showed no antimicrobial activity against *Staphylococcus epidermidis* whilst a zone of inhibition of 6.26 ± 0.07 mm was recorded for 10 µg/disc of *Plocamium cornutum* extract reconstituted in chloroform. An ethanolic extract of *Plocamium rigidum* showed a zone of inhibition of 6.35 ± 0.25 mm against L*isteria monocytogenes* while the standard ampicillin had no activity.

Conclusion: Extracts of *Plocamium rigidum* in ethanol and *Plocamium cornutum* in chloroform are evidently potential lead candidate antibiotics *in vitro* against L*isteria monocytogenes* and *Staphylococcus epidermidis* respectively.

Key words: Antimicrobial activity, *Plocamium cornutum*, *Plocamium rigidum*, *Listeria monocytogenes*.

4.2 Literature review

Due to emerging resistance of pathogenic microorganisms to existing antibiotics, and the fast spreading of resistant microorganisms, there is an increasing need for new antibiotics. In marine environments, competition for space and nutrients led to the evolution of antimicrobial defence strategies. These include the production of chemically active metabolites in their surroundings which act as an aid to protect themselves against other settling organisms, maintenance of unfouled surfaces, deterrence of predation, the ability to successfully reproduce, protection from UV radiation and as allelopathic agents (1, 2). Marine algae are one of the largest producers of biomass in the marine environments and are a rich source of structurally novel biologically active metabolites (3). Therefore, they offer a rich source of potentially new drug leads.

Algae metabolites have great industrial potential and accessibility, and thus they have attracted attention for health and cosmetic applications. The use of microalgae and their derivatives in applications to combat skin aging, as well as for depigmentation and antimicrobial applications in the cosmetic industry is wide spread (4). A wide range of metabolites, such as antioxidants, anti-inflammatory agents, alginates, polysaccharides, carotenoids, have been investigated for cosmeceutical preparations. The antimicrobial properties of marine algae have been known since ancient times and well documented in recent years (5). Algae are thus a source of raw materials for one of the most promising and profitable sectors of the biotechnology industry. Phycocolloid substances from marine algae such as alginate, carrageenan and agar have been used globally for decades in medicine and pharmacy. Thus, they are of interest for potential use in cosmetic products (5). Four species of Algerian marine algae were tested for anti-fungi properties and results showed that they had fungi

inhibiting effects (5). Algal materials collected from the Red Sea Coast of Jeddah inhibited the growth of *E. faecalis* (11 mm) but no activity was recorded for the water extracts (6).

Marine algae provide a rich source of structurally diverse secondary metabolites some of which have marked antimicrobial activity against marine pathogens. The bactericidal agents found in algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, cyclic polysulphides and fatty acids. Watson, (7) reported that 54 seaweeds were tested *in vitro* for antimicrobial activity and a staggering 95% of the extracts showed activity against different pathogens. Bromophenol compounds have been frequently encountered in various marine algae including red and brown algae. Red algae of family Rhodomaceae are especially known as a rich source of bromophenols (8). Some of these compounds which were previously isolated from the family exhibited a wide spectrum of pharmacological activities such as enzyme inhibition, cytotoxic, antioxidant, feeding deterrent, anti-inflammatory and antimicrobial activities (9). Taskin (1), studied the inhibitory activities of various organic extracts of algae against various fish pathogenic bacteria and their results confirmed the possible use of some marine algae as a source of antimicrobial compounds.

In this study, organic crude extracts from *Plocamium sp.*, a red marine alga from the coastline of Namibian, were studied for their potential inhibitory activities against common pathogens. Twelve pathogens selected for this study are important in our everyday life as they are common causes of a variety of human diseases (Table 1).

Table 1 Common diseases associated with the pathogens used in this research.

Name	**Type of pathogen**	**Disease**
Escherichia coli	Gram negative	Diarrhoea
Staphylococcus aureus	Gram positive	Sinusitis, skin infection
Staphylococcus saprophyticus	Gram positive	Urinary tract infection and cystitis
Pseudomonas aeruginosa	Gram negative	Sepsis syndromes, hospital acquired infections
Sreptococcus pyogenes	Gram positive	Tonsillitis, sinusitis
Proteus mirabilis	Gram negative	Kidney stones, renal failure
Listeria monocytogenes	Gram positive	Listeriosis
Shigella sonnei	Gram negative	Invade epithelial lining of the colon, diarrhoea
Salmonella typhi	Gram negative	Food poison
Enterococcus faecalis	Gram positive	Urinary tract infection, endocarditis, meningitis
Candida albicans	Fungus (yeast)	Candidiasis (mouth or genital itching)
Staphylococcus epidermidis	Gram positive	Biofilms (catheters)

The ability of marine algae to produce metabolites of potential interest has been extensively documented (11).

The antimicrobial activity of marine algae may be influenced by some factors such as the habitat, the season of algal collection, different growth stages of plant and experimental methods (6). Although a variety of solvents have been employed in screening marine algae for antimicrobial activity, it is still uncertain what kinds of solvents are most effective and suitable for the extraction of secondary metabolites from various marine algae (12). Mohy El-Din (3), also reported that antimicrobial activity depends on the solvents used for extraction. It was found that benzene and diethyl ether were suitable solvents for extracting various antibiotic compounds. However, extracts obtained with acetone, ethyl alcohol and ether showed higher antimicrobial activity than extracts from chloroform (12). In a similar study, Sasidharan (13) found that the chloroform extract exhibited the strongest activity, which is in agreement with the findings of Mohy El-Din (3).

4.3 Chapter Objective

There is a dearth of information concerning the antimicrobial activities of Namibian marine algae. Antimicrobial potential of Namibian marine algae is unexplored despite the availability of marine algae in the coastal areas of the country. This study therefore aimed to screen for potentially bioactive metabolites from Namibian marine algae and test them for antimicrobial activity. This would be beneficial to the development of Namibian medicine if a novel solution was found to combat microbial infections.

4.4 Materials and Methods

4.4.1 Sample Collection

Plocamium species were collected during low tide along the coastline of Namibia from Henties Bay and Lüderitz, where they are abundant in intertidal, shallow and coastal

estuaries. As the samples co-exist with other settling organisms, they were first washed in seawater and then in fresh water to remove sand, epiphytes and other necrotic parts. The identification of *P. cornutum* and *P. rigidum* was done on the basis of their morphological characteristics, including colour and the arrangement of the branchlets. The final identification was done at the Marine and Fisheries Department of the University of Namibia by visual appearance and standard collection guides (14). The samples were transported to the laboratory in polythene bags under ice and were frozen at -87 °C for future analyses.

4.4.2 Test Microorganisms

Extracts of *Plocamium rigidum* and *Plocamium cornutum* were investigated to evaluate their antimicrobial activity against 6 Gram positive human pathogens (*S. aureus, S. saprophyticus, L. monocytogenes S. pyogenes S. epidermidis* and *E. faecalis*), 5 Gram negative human pathogens (*E. coli, P. aeruginosa, P. mirabilis, S. sonnei* and *Salmonella*) and a fungus, *C. albicans* using a disc diffusion method. The 12 pathogens used in this study were obtained from the Department of Biochemistry and Microbiology, School of Medicine, University of Namibia.

4.4.3 Preparation of Marine Algae Extracts

Frozen samples of marine algae (33 g) were soaked in extraction solvent (MeOH:DCM 1:1 v/v) for 48 hours at room temperature (26 ± 2^{o} C). The extracts were then decanted into a 250 ml beaker. The resulting extracts were filtered through Whatman No 1 filter paper and concentrated using a rotary evaporator (model RE 100; Bibby Sterilin Ltd). The concentrated extracts were then dissolved in 100 ml of extraction solvent and transferred to a separating funnel. Two layers of immiscible liquids were

formed in the separating funnel, of which the upper layer consisted of MeOH extract which form the polar fraction and the lower layer was composed of DCM extracts which is the non-polar fraction. The MeOH and DCM layers were carefully decanted into pre-weighed glass vials. The extracts were left to dry in a laminar airflow cabinet. The dried extracts were weighed and placed in a freezer at a temperature of about -80 ^{o}C. DCM and MeOH extracts of *Plocamium* species were reconstituted in six different solvents to yield solutions of 1 mg/ml in water, hexane, DCM, EtOH, MeOH and $CHCl_3$ (H_2O, C_6H_{14}, DCM, EtOH, MeOH and $CHCl_3$) to evaluate the antimicrobial potential of the different solvent extracts.

4.4.4 Microbial Inoculum Preparation

All media used in the study were supplied from Hi Media laboratories, India. Standard ampicillin antibiotic susceptibility discs were obtained from SRL chemicals Ltd, India. All media were prepared in deionised water and autoclaved at 121°C for 15 min prior to use according to the manufacturers' instructions. Twelve selected pathogens were cultured on nutrient agar plates. The plates were incubated at 35-37 ^{o}C in an incubator (Scientific series 2000, L. digital incubator; model 286) for 24 hours. Colonies of each of the selected twelve bacteria were inoculated into Tryptone Soya Broth in 2 ml culture tubes. The culture tubes were left to incubate at 35-37 ^{o}C for 24 h.

4.4.5 Determination of Antimicrobial Activity of *Plocamium* Species

The antimicrobial activity of the *Plocamium* extract was carried out using the Kirby Bauer disc diffusion method (15). The DCM and MeOH extracts were tested separately against 12 pathogens and the tests were run in triplicate and average inhibition zones were recorded using Vernier callipers. The negative controls consisted

of six different sterile solvents (H_2O, C_6H_{14}, DCM, EtOH, MeOH and $CHCl_3$) used to dissolve the extracts. The culture solutions were used to impregnate the diffusion disc used for antimicrobial screening.

Twelve petri dishes with Mueller-Hinton agar were impregnated with a different bacteria and each dish was divided into three sectors. Discs were soaked in different extracts in their respective solvents were placed in different sectors of the petri dish along with both negative and positive controls. Three petri dishes with the same extract and controls (triplicate) were incubated at 37 ^{0}C for 24 hours. The zones of inhibition were measured after 24 hours.

4.4.6 Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined for all extracts that showed antimicrobial activities in the screening phase. This was carried out using a modified procedure from (16). Each algal extract was serially diluted to yield 800 µg/ml, 600 µg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml, 20 µg/ml and 10 µg/ml. Filter paper discs were placed in each of the diluted extract to absorb their respective solution for fifteen minutes and allowed to dry. The discs were respectively placed onto the plates containing each of the twelve selected bacteria. Standard ampicillin antimicrobial susceptibility test discs of 10 µg/disc and blank solvent discs served as the positive and negative controls respectively. Different discs with different loading along with positive and negative controls were placed in their respective divisions and labelled on the petri dishes. The petri dishes were prepared in triplicates. The plates were incubated at 37 ^{o} C for 24 hrs. MIC was defined as the lowest loading at which no visible growth was observed; this indicates the presence of antimicrobial activity.

In Fig. 1 below, the petri dish with Mueller-Hinton agar (MHA) was impregnated with *E. coli*. Discs soaked in different concentrations (10 µg/ml, 20 µg/ml and 100 µg/ml) of *P. cornutum* reconstituted in EtOH and 10 µg/ml ampicillin were placed in different section of the disc. The disc was incubated at 37 ^{0}C for 24 hours.

4.4.7 Effect of *Plocamium* species on Vero Cells.

Different concentrations of the compound were incubated with Vero cells for 72 h at 37°C (humidified, 5% CO_2). Viability was measured using MTT dye and absorbance was recorded at 550 nm using a spectramax M3 plate reader.

4.5 Statistical analysis

Means of triplicate analysis were calculated and the data was expressed as mean ± standard deviation (SD). *Post hoc* ANOVA statistical analysis was performed using SPSS 22 software for comparison between two or more treatments. A difference was considered statistically significant when $p \leq 0.05$.

4.6 Results

4.6.1 Antimicrobial Screening

Water extracts showed no antimicrobial activity against all the pathogens. The antimicrobially active algae extracts were probably not soluble in water. The remaining solvents had varying degrees of antimicrobial activity as shown in Tables 2 and 3 for DCM and MeOH extracts respectively.

Table 2 highlights that DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform and ethanol showed high antimicrobial activity (+++) against, *E. coli,* and *S. sonnei*. Similar extracts only demonstrated moderate antimicrobial activity (++) against *P. aeruginosa, E. faecalis, L. monocytogenes* and *S. aureus* when reconstituted in EtOH, DCM and hexane. The other pathogens only demonstrated weak or mild antimicrobial activity (+) in DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform, ethanol and other solvents.

Table 3 shows that the antimicrobial activity of MeOH extracts of algae reconstituted in chloroform, hexane, DCM and EtOH showed less activity against the selected pathogens, compared to DCM extracts. MeOH extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform, hexane and ethanol showed high antimicrobial activity (+++) against *E. coli* and *S. sonnei*. Similar extracts only demonstrated moderate antimicrobial activity (++) against, *E. faecalis, L. monocytogenes, Salmonella* species and *S. saprophyticus* when reconstituted in ethanol, chloroform and hexane.

Table 2. Screening DCM extracts reconstituted in different solvents for antimicrobial activity against pathogen.

Solvents	Algal species	*E. coli*	*P. aeruginosa*	*S. sonnei*	*S. pyogenes*	*P. mirabilis*	*E. faecalis*	*L. monocytogenes*	*Salmonella sp.*	*S. epidermidis*	*S. saprophyticus*	*C. albicans*	*S. aureus*
$CHCl_3$	*P. rigidum*	+++	-	+	-	-	+	-	-	+	-	-	-
	P. cornutum	-	-	-	+	+	+	-	-	+	-	-	-
C_6H_{14}	*P. rigidum*	+	-	+++	-	-	-	++	-	-	-	-	-
	P. cornutum	+	-	++	-	-	-	+	-	-	-	-	-
DCM	*P. rigidum*	+++	++	+	+	+	+	++	-	-	-	-	-
	P. cornutum	+++	++	+	+	+	+	+	-	+	-	-	-
EtOH	*P. rigidum*	-	-	+++	+	-	++	-	+	-	-	-	++
	P. cornutum	-	++	+	+	-	++	-	-	+	-	-	+
Water	*P. rigidum*	-	-	-	-	-	-	-	-	-	-	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-

Inhibition zone diameters: -, < 6 mm; +, 6 – 10 mm; ++, 10 – 15 mm; +++, 15 – 20 mm. Chloroform = $CHCl_3$, Hexane=C_6H_{14}, Ethanol = EtOH, Dichloromethane = DCM.

Table 3 Screening MeOH extracts reconstituted in different solvents for antimicrobial activity against pathogens.

Solvents	Algal species	*E. coli*	*P. aeruginosa*	*S. sonnei*	*S. pyogenes*	*P. mirabilis*	*E. faecalis*	*L. monocytogenes*	*Salmonella sp.*	*S. epidermidis*	*S. saprophyticus*	*C. albicans*	*S. aureus*
$CHCl_3$	*P. rigidum*	+++	-	+	-	-	+	-	-	+	-	-	-
	P. cornutum	-	-	-	-	-	-	-	++	-	+	-	+
C_6H_{14}	*P. rigidum*	+	-	+++	-	-	-	+	-	-	++	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-
DCM	*P. rigidum*	-	-	-	-	-	-	-	-	-	-	-	-
	P. rigidum	-	-	-	-	-	-	-	-	-	-	-	-
EtOH	*P. rigidum*	-	+	+++	+	+	++	++	+	-	+	+	+
	P. cornutum	-	++	-	-	-	-	+	-	-	-	-	-
MeOH	*P. rigidum*	++	+	-	+	+	+	+	-	+	+	-	+
	P. cornutum	++	+	-	-	-	-	-	-	-	+	-	+

Water	*P. rigidum*	-	-	-	-	-	-	-	-	-	-	-	-
	P. cornutum	-	-	-	-	-	-	-	-	-	-	-	-

Inhibition zone diameters: -, < 6 mm; +, 6 – 10 mm; ++, 10 – 15 mm; +++, 15 – 20 mm. Chloroform = $CHCl_3$, Hexane = C_6H_{14}, Ethanol = EtOH, Dichloromethane = DCM

4.6.2 Minimum inhibitory concentration of algal extracts

Evaluation of antimicrobial activity of the algal extracts was recorded in Tables 4, 5 and 6. Of the six solvents used to reconstitute extracts from DCM and MeOH for antimicrobial activity, water extracts showed no activity against any of the twelve pathogens used in the test. This is in agreement with the results obtained by Al-Saif (6,17). The other algal extracts reconstituted in C_6H_{14}, DCM, EtOH and $CHCl_3$ demonstrated varying degrees of inhibitory activity against the test pathogens. Chloroform proved to the solvent of choice, as it was observed to have significantly higher inhibitory activity against tested pathogens ($p < 0.05$). Ampicillin at a loading concentration of 10 µg/disc showed no activity against *S. epidermidis* in DCM extract of *P. rigidum* reconstituted in chloroform.

4.6.3 Antimicrobial activity of DCM extracts of algae reconstituted in chloroform

The DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform showed prominent antimicrobial activity (15–20 mm) against *E. coli* but only weak or mild antimicrobial activity (6-10 mm) against *E. faecalis, S. epidermidis* and *S. sonnei* species.

S. saprophyticus and *C. albicans* proved resistant to all the extracts of DCM reconstituted in chloroform and ethanol. *P. mirabilis, S. pyogenes* and *S. sonnei* also showed weak/mild of activity (6-10 mm) in all DCM extracts. DCM extracts of sample *P. rigidum* demonstrated a ZOI of 10-15 mm against *L. monocytogenes*.

DCM extracts of *P. rigidum* reconstituted in hexane showed activity of (10–15 mm) against *L. monocytogenes* and *S. sonnei* but only weak or mild antimicrobial activity

against *E. coli*. While *P. rigidum* reconstituted in EtOH showed high antimicrobial activity (15-20 mm) against *S. sonnei* and *S. aureus* but moderate antimicrobial activity (10-15 mm) against *E. faecalis*. The extract of *P. rigidum*, showed only weak or mild inhibition against *S. pyogenes* and *S. epidermidis.*

The results of MeOH extracts of *Plocamium species* reconstituted in EtOH (Table 6) revealed that the algal extracts were potentially active in suppressing microbial growth of *S. saprophyticus* and *L. monocytogenes*. It was found that the ZOI of 10 µg/disc ampicillin against *L. monocytogenes* was 10.05 mm compared to 10.26 mm recorded for the MeOH extract of *P. rigidum* reconstituted in EtOH.

Table 4 MIC of DCM extracts of *Plocamium* species reconstituted in chloroform against pathogens.

Extracts	Loading µg/disc	**Inhibition zone (mm)**				
		Gram (+ve) pathogens			**Gram (-ve) pathogens**	
P. rigidum		*E. faecalis*	*S. epidermidis*	*S. pyogenes*	*E. coli*	*S. sonnei*
	5	-	-	-	-	-
	10	7.41 ± 0.20	6.21 ± 0.08	-	7.42 ± 0.31	-
	20	7.55 ± 0.61	6.41 ± 0.44	-	8.06 ± 1.21	6.88 ± 0.06
	100	8.15 ± 0.32	6.55 ± 0.51	-	8.62 ± 0.18	7.38 ± 1.05
	200	8.33 ± 0.17	6.71 ± 0.04	-	9.86 ± 0.41	7.45 ± 0.32
	400	8.42 ± 0.60	6.84 ± 0.11	-	9.67± 0.34	7.86 ± 0.13
	600	9.05 ± 0.15	6.92 ± 0.06	-	10.49 ± 0.62	9.27 ± 0.05
	800	9.64 ± 1.07	7.81 ± 0.62	-	13.35 ± 1.06	11.26 ± 0.16

P. cornutum	5	-	-	-	-	-
	10	-	6.26 ± 0.07	-	-	-
	20	-	6.43 ± 0.11	-	-	-
	100	-	6.88 ± 0.13	-	-	-
	200	-	7.16 ± 0.08	-	-	-
	400	-	7.55 ± 0.18	6.88 ± 0.17	-	-
	600	-	7.75 ± 0.15	7.75 ± 0.12	-	-
	800	8.95 ± 0.52	8.17 ± 0.31	8.49 ± 0.13	-	-

Key: – No inhibition

Table 5 MIC of DCM extracts of *Plocamium* species reconstituted in ethanol against pathogens.

Extracts	Loading µg/disc	**Inhibition zone (mm)**					
		Gram (+ve) pathogens				**Gram (-ve) pathogens**	
P. rigidum		*E. faecalis*	*S. epidermidis*	*L. monocytogenes*	*S. pyogenes*	*E. coli*	*S. sonnei*
	5	-	-	-	-	-	-
	10	-	-	6.35 ± 0.25	-	-	-
	20	-	-	6.37 ± 0.04	-	-	-
	100	-	-	6.44 ± 0.13	-	-	-
	200	-	-	6.51 ± 0.04	-	-	-
	400	6.38 ± 0.16	-	6.83 ± 0.12	-	-	-
	600	6.43 ± 0.13	-	7.55 ± 0.81	-	-	9.38 ± 0.42
	800	8.77 ± 0.12	-	10.94 ± 0.15	-	-	12.45 ± 0.33

Key: – No inhibition

The lowest extract loading that prevents visible bacterial growth was determined for all three extracts. *Plocamium rigidum* showed activity at a loading dose of 10 µg/disc. This could be an indication of better antibacterial activity of this extract in comparison to other extracts.

DCM extracts of *P. rigidum* reconstituted in chloroform showed activity at a minimum loading of 10 μg/disc and a ZOI of 7.41 ± 0.2 mm against E *faecalis.* The same extract with minimum loading of 10 μg/disc showed a ZOI of 6.21 ± 0.08 mm against *S. epidermidis* which indicates a better growth inhibition of *E. faecalis* than *S. epidermidis*. Algal extract of minimum loading of 10 μg/disc have a ZOI of 7.42 ± 0.31 growth inhibition against *E. coli*. However, as the algal loading increases, there is a marked growth of inhibition demonstrated *in E. coli* and *S. sonnei* than the Gram positive *E. faecalis* and *S. epidermidis*. DCM extracts of *P. rigidum* reconstituted in chloroform thus has the characteristics of a broad spectrum antibiotic *in vitro*. DCM extracts of *P. cornutum* reconstituted in chloroform showed no activity at lower extract loading against *E. faecalis* and *S. pyogenes*. There was no activity against *E. coli* and *S. sonnei*.

DCM extracts of *P. rigidum* reconstituted in ethanol showed pronounced activity against *L. monocytogenes* at low loading (minimum loading dose of 10 μg/disc and ZOI of 6.35 ± 0.25 mm). This result was better than the observed activity of standard ampicillin antimicrobial susceptibility test disc against the same pathogen.

4.6.4 Antimicrobial activity of MeOH extracts of algae reconstituted in ethanol

MeOH extracts of *P. rigidum* reconstituted in ethanol showed no activity against *E. faecalis, E. coli, S. sonnei* and *S. pyogenes.* The extracts however demonstrated strong

activity against *S. saprophyticus* and *L. monocytogenes*. While the standard ampicillin antimicrobial susceptibility test disc of 10 µg/disc did not inhibit the growth of *L. monocytogenes*, 10 µg/disc of *P. rigidum* reconstituted in ethanol inhibited the growth of *L. monocytogenes* by 10.26 ± 0.01 mm. MeOH extracts of *P. cornutum* reconstituted in ethanol only inhibited the growth of *E. coli* and *P. aeruginosa*. The zone of inhibition of 10 µg/disc of *P. cornutum* against *P. aeruginosa* was 9.76 ± 0.05; this is a significant result as this pathogen is resistant to standard ampicillin.

Table 6 MIC *Plocamium* species of MeOH extract reconstituted in ethanol against pathogens.

Extracts	Loading µg/disc	**Inhibition zone (mm)**					
		Gram (+ve) pathogens				**Gram (-ve) pathogens**	
P. rigidum		*E. faecalis*	*S. saprophyticus*	*L. monocytogenes*	*S. pyogenes*	*E. coli*	*S. sonnei*
	5	-	0.0 ± 0.00	0.0 ± 0.00	-	-	-
	10	-	9.28 ± 0.10	10.26 ± 0.01	-	-	-
	20	-	9.89 ± 0.01	11.05 ± 0.02	-	-	-
	100	-	10.88 ± 0.03	11.23 ± 0.05	-	-	-
	200	-	11.43 ± 0.02	11.90 ± 0.05	-	-	-
	400	-	11.15 ± 0.06	12.60 ± 0.03	-	-	-
	600	-	13.57 ± 0.02	13.25 ± 0.02	-	-	-
	800	-	14.37 ± 0.04	13.91 ± 0.05	-	-	-

Key: – No inhibition

Table 7 MIC *Plocamium* species of MeOH extract reconstituted in ethanol against pathogens.

Extracts	loading µg/disc	**Inhibition zone (mm)**			
		Gram (+ve) pathogens		**Gram (-ve) pathogens**	
P. cornutum		*E. faecalis*	*L. monocytogenes*	*E. coli*	*P. aeruginosas*
	5	-	-	-	-
	10	-	-	-	9.76 ± 0.44
	20	-	-	-	10.11± 0.13
	100	-	-	-	10.38 ± 0.06
	200	-	-	7.26 ± 0.33	10.75 ± 0.06
	400	-	-	8.22 ± 0.04	11.34 ± 0.22
	600	-	-	12.94 ± 0.60	10.80 ± 0.41
	800	-	-	13.00 ± 0.11	11.52 ± 0.51

Key: – No inhibition

Fig. 1 Effect of *Plocamium* extracts on growth of *E. coli.*

Plocamium species demonstrated some level of cytotoxicity with a CC_{50} value of 6.3 µg/mL Each bar represents the mean of at least 3 different independent experiments. Auranofin (Aurano) was used as a standard control for cytotoxicity (2.5 µg/mL). CC_{50} value = 6.30 ± 0.02 µg/mL.

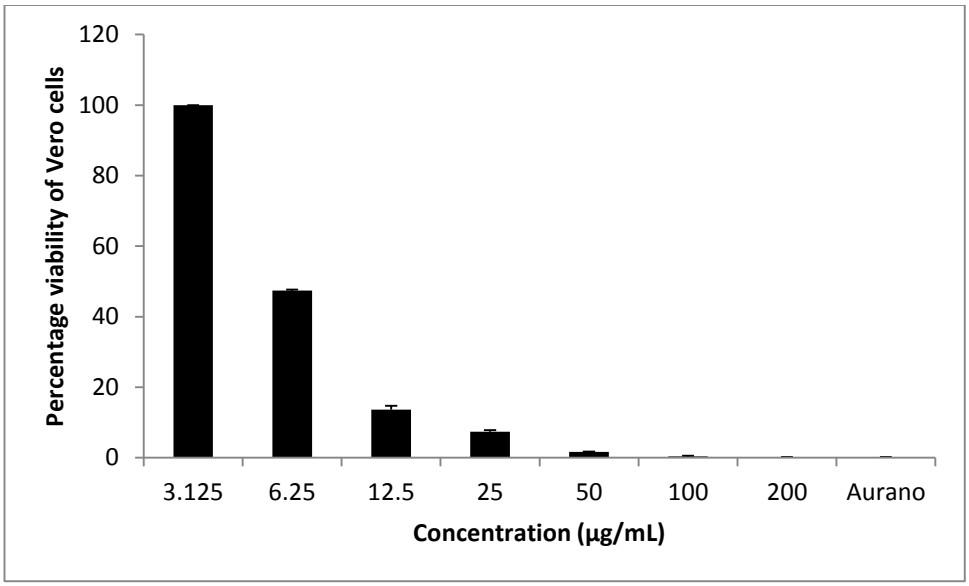


Fig. 2 Effect of *Plocamium* extracts on Vero cells.

4.7 Discussion

The data obtained in the present study indicated that chloroform was the most effective solvent for the extraction of bioactive compounds, followed by ethanol. Organic solvents always have a higher efficiency in extracting antimicrobial compounds than water as the solvent for extraction (18). This result is consistent with literature that extracts from organic solvents give more consistent antimicrobial activity than water extracts (18,19).

Antimicrobial activity of marine algae could be attributed to combined effects of the high percentage of phenolic content, due to the presence of various phytochemicals (20) and the presence of halogenated monoterpenes which are regularly present in marine algae (11). The type and amount of halogens present in the algal molecule also have a role in the overall defense against pathogenic Gram-positive and Gram-negative bacteria (21). The fats and fatty acids from marine algae also play an important role in the formation of many other bioactive secondary metabolites since some fatty acids have been shown to possess antibacterial activities (8, 22).

The DCM extract of *P. rigidum* reconstituted in chloroform demonstrated antimicrobial activity against *E. faecalis*, *E. coli* and *S. epidermidis*. The ZOI for the extracts are 7.41 ± 0.2 mm, 7.42 ± 0 31 mm and 6.21 ± 0.08 mm respectively. Although these extracts were not very active *in vitro* compared to 10 µg/disc loading of ampicillin that showed activity with ZOI in the range of 16.84 ± 0.22 mm (*E. coli*) and 8.94 ± 0.44 mm (*E. epidermidis*), they offer potential leads in the search for alternative antibiotics that could be active against *E. faecalis* and *S. sonnei.*

The DCM extract of *P. rigidum* reconstituted in ethanol showed a noteworthy result. 10 µg/disc of this extract showed weak or mild antimicrobial activity of 6.18 ± 0.56 mm against *S. epidermidis*, while the standard ampicillin of same loading showed no activity at all against *S. epidermidis*. The DCM extract of *P. cornutum* reconstituted in chloroform showed ZOI of 6.26 ± 0.07 mm against *S. epidermidis* while the standard ampicillin showed no inhibitory activity against *S. epidermidis*. Loadings higher than 10 µg/disc of extract showed varying degrees of inhibition against all pathogens tested.

In addition, DCM extracts of *P. rigidum* and *P. cornutum* reconstituted in chloroform showed antimicrobial activity against both Gram positive (*E. faecalis* and *S. epidermidis*) and Gram negative (*E. coli* and *S. sonnei*) pathogens, which is an indication of a broad spectrum of activity. The DCM extract of *P. rigidum* reconstituted in chloroform, was the most effective marine algae extract against the various pathogens. The ZOI and minimum loading dose (MLD) obtained in this research are comparable to the results presented in a similar work reported by Ashraf (17).

4.8 Conclusion

In this study presenting the first report of antimicrobial activity of Namibian marine algae, chloroform was the most suitable extraction solvent for antimicrobial compound extraction among the solvents used. It can be inferred from this study that the antimicrobial potential of *P. cornutum* and *P. rigidum* depend on the solvent medium used for extraction and the type of organism tested. *P. cornutum* and *P. rigidum* collected along the coastline of Namibia can be used as agents for the development of new drug leads for bacterial infections. DCM extracts of *P. rigidum* (in ethanol) and

P. cornutum (in chloroform) are evidently potent antibiotics lead candidates *in vitro* against *L. monocytogenes* and *S. epidermidis*.

Conflict of interest

The authors declare that there is no conflict of interest.

4.9 Acknowledgements

This study was partly funded by the Namibian Commission for Research Science and Technology (NCRST) and the University of Namibia Staff Development Fund.

4 10 References

1. Taskin E, Taskin E, Ozturk M. Antimicrobial activities of some seaweeds from northern Cyprus against some food- related pathogens. Asian J Biol Sci. 2012;5(5):250-6.

2. Chakraborthy K, Lipton AP, Paulraj R, Vijayan KK. Antibacterial diterpernoids of *Ulva fasciata Delile* from South-Western coast of Indian peninsula. Food Chem. 2010;119(4):1399-408.

3. Mohy El-Din SM, El-Ahwany AMD. Bioactivity and phytochemical constitutents of marine red seaweeds. J Taibah Univ Sci. 2015;10(4):471-84.

4. Wang HMD, Chen CC, Huynh P, Chang JS. Exploring the potential of using algae in cosmetics. Bioresour Technol 2015;184: 55–62.

5. Patra JK, Rath SK, Jena K, Rathod VK, Thatoi H. Evaluation of antioxidant and antimicrobial activity of seaweed (*Sargassum sp*.) extract: A study on inhibition of *Glutathione-S-transferase* activity. Turkish J Biol. 2008; 32 119-25.

6. Al-Saif SSA. Ilah, Abdel-Raouf N, El-Wazanani HA., Aref, IA. Antibacterial substances from marine algae isolated from Jeddah coast of Red sea, Saudi Arabia. Saudi J Biol Sci. 2014;21(1):57-64.

7. Watson SB, Cruz-Rivera E. Algal chemical ecology: An introduction to the special issue. Phycologia. 2003;42(4):319-23.

8. Oh KB, Lee JH, Chung SC, Shin HJ, Kim HK, Kee HS. Antimicrobial activity of the bromophenols from the red algae *Odonthalia corymbifera* and some synthetic derivatives. Bioorganic Med Chem Lett. 2008;18**:**104-8.

9. Williamson G, Carughi A. Polyphenol content and health benefits of raisins. Nutr Res. 2010; 30(8):511–19.

10. Cabrita M, Vale C, Rauter, AP. Halogenated compounds from marine algae. Mar Drugs; 2010;8:2301-17.

11. Manivannan K, Karthikai devi G, Anantharaman P, Balasubramanian T. Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters, Gulf of Mannar. Asian Pac J Trop Biomed. 2011;1:114-20.

12. Sasidharan D, Darah ., Noordin MKMJ, Kassim M, Jain M. Screening antimicrobial activity of various extracts of *Gracilaria changi.* Pharm Biol. 2009;47(1):72-6.

13. Lluch JR.. Marine benthic algae of Namibia. Scientia Mar. 2002;66(3):245-56.

14. Arullappan S, Zakaria Z, Basri DF. Preliminary screening of antibacterial activity using crude extracts of *Hibiscus rosa sinensis*. Trop Life Sci Res.

2009;20(2):109-18.

15. Peng Q, Huang B, Hou D, Hua F, Qian Y. Green tea extracts weakens the antimicrobial effects of amoxicillin in methicillin-resistant *Staphylococcus aureus* infected mice. Phytother Res. 2010;24:141-5.

16. Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. Antimicrobial activity of some plants extracts against bacterial strain causing food poisoning diseases. Saudi J Biol Sci. 2018;25:361-6.

17. Kamra A, Bhatt AB. Evaluation of antimicrobial and antioxidant activity of *Ganoderma luciduma* extracts against human pathogenic bacteria. Int J Pharm Pharm. Sci. 2012;4(2):359-62.

18. Prashant T, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: A review. Int. Pharm. Sci. 2011;1(1):98-106.

19. Govindasamy C, Narayani S, Arulpriya M, Ruban P, Anantharaj K, Srinivasan R.. J Pharm Res. 2011;4(7):2076-77.

20. Andrianasolo EH, France D, Cornell-Kennon S, Gerwick WH. DNA methyl transferase inhibiting halogenated monoterpenes from Madagascar red marine algae *Portieria honemannii*. J Nat.Prod. 2006;69: 576-9.

21. Barbosa JP, Fleury BG, da-Gama BAP, Teixeira VL, Pereria RC. Natural products as antifoulants in the Brazilian brown alga *Dictyota pfaffii*

(Phaeophyta, Dictyotales). Biochem Syst Ecol. 2007;35:549-53.

CHAPTER FIVE

In vivo antimicrobial activity and toxicity of an extract from a Namibian *Plocamium* species of marine algae.

5.1 Abstract

Objective: The purpose of this research was to determine the acute toxicity parameters of *Plocamium* species and investigate the dose-response relationship in infected male *Balb/ c* mice using *in vivo* techniques.

Methods: Organisation for Economic Co-operation and Development (OECD) guidelines and Miller-Tainter method were used to determine the acute oral toxicity (LD_{50}) of *Plocamium* extract.

Eighteen mice of weights between 19.2 - 21.9 g were infected with *E. coli* and *P. aeruginosa* by intravenous injection and sub cutaneous methods respectively. After incubation and disease development, different groups of mice were subjected to various treatments which included gentamycin, ampicillin and different dosages of *Plocamium* extract over a period of five days by oral gavage. One group was not treated and was used as a negative control. The *E. coli O157:H7* and *P. aeruginosa* loads in faeces of test mice was quantified daily in each mouse for the duration of treatment.

Results: The LD_{50} was calculated to be 3556 mg/kg.

Plocamium extract of 355 mg/kg reduced *E. coli* in feces to pre-evaluation level on the fifth day. *P. aeruginosa* was not inhibited by any concentration of the marine algal extract.

Conclusion: It can be inferred that 355 mg/kg of *Plocamium rigidum* could be a potential drug lead in the treatment of *E. coli* infection *in Balb/c* mice.

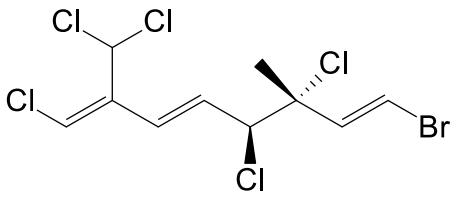
Key words: LD_{50}, dose response.

5.2 Literature Review

The use of plant extracts with medicinal and therapeutic values has gained attention in recent years due to the serious side-effects often caused by the use of synthetic drug formulations (1). The World Health Organization (WHO) estimated that 80% of African and Asian population use traditional medicine for primary health care. The scenario is similar in developed countries, where 70–80% of the population use some form of complementary or alternative medicine (WHO, 2008). With the global explosion of phythotherapy, the safety of medicinal plants has become a public health problem (2). Medicinal plant species do not receive adequate attention in global discussions related to health (3). The quality, safety and efficacy of many traditionally used herbal formulae are unknown (4). Moreover, there is a lack of data for many plants to guarantee their quality and safety (4). Extremely toxic substances like strychnine, the digitoxines, cyanogenic glycosides, amongst others, are extracted from plants. We can only be assured that the use of a particular plant species is safe after a careful investigation (5).

Toxicity from botanical compounds has been underestimated due to the perception that drugs made from plants are absolutely safe. However, severe liver injury has been described after the ingestion of a large variety of different herbal preparations (6). Determination of the efficacy and safety of herbal remedies is necessary as many people use them for self-medication and little data is available about the pharmacology and toxicology for most of these common herbal remedies (6). It is necessary to determine the *in vivo* efficacy of these extracts and establish the effective dose level in the treatment of pathogens (7). For example, *Galla chinensis* is traditionally used for the treatment of inflammation, dysentery, toxicosis and sores (4). It was reported that

Galla chinensis could promote mineral ion deposits on the enamel surface layer of dental caries and then modify its remineralization (8). However, herbal formulations are often not subjected to toxicity testing before application to animals or humans. Thus, there are rising concerns about the lack of modern scientific evidence regarding the efficacy and safety of herbal products (9,10). *Plocamium* species are widely distributed throughout the world. The diversity of this species and its ability to produce a wide array of various halogenated monoterpenes with different biological activities has attracted great interest as a potential source of pharmaceutical products. *In vitro* studies have demonstrated cytotoxicity, antiproliferative activity, antiplasmodial and antitumor activity (11,12). *Plocamium rigidum* is one of the six different *Plocamium* species identified in Namibia (13). The major metabolite of *P. rigidum* was isolated and characterised using nuclear magnetic resonance (NMR). The structure of this compound is shown in Fig. 1 below.



1

Fig. 1 Structure of the major metabolite found in *Plocamium* (14).

The liver has a pivotal role in the regulation of physiological processes and is also involved in detoxification of a variety of drugs and xenobiotic (6). There is growing concern about the toxicity of herbal remedies as many contain substantial amounts of pharmacologically active ingredients whose mechanisms of action and adverse effects are mostly unknown (15). Severe liver injury, including acute and chronic

abnormalities and even cirrhotic transformation and liver failure, have been described after the ingestion of a wide range of herbal products such as mushrooms, germander (*Teucriumchamaedrys*), chaparral (*Larrea tridentate*) (6).

Due to the possible toxicity of various secondary metabolites that are present in *Plocamium* species (16), it was considered necessary to study the toxicity of crude *Plocamium* extracts *in vivo*. Many *in vitro* evaluation has been done on halogenated monoterpenes from *Plocamium* species. However, this is the first time the *in vivo* research has been undertaken.

5.3 Chapter Objective

This study aimed to determine the acute oral toxicity (LD_{50}) of the extract of *Plocamium* species in *Balb/c* mic and to observe dose response of infected *Balb/c* mice.

5.4 Methods

5.4.1 Determination of LD_{50} /Acute Dose

Male *Balb/c* mice were obtained from the College of Veterinary Studies at the University of Makerere. 26 mice of weights between 19.2 - 21.9 g were randomly selected and divided into two groups labelled as Red Head (RH) and Green Head (GH). They were fasted overnight in a disinfected cage.

A toxicity range-finding test was done according to the guideline of OECD (17). There is no information on the lethality of *Plocamium* extract. Therefore, according to OECD guidelines, in the absence of an estimate of the substance's lethality, dosing of *Plocamium* extract prepared in vegetable oil was initiated in the mice at 1750 mg/kg.

One RH mouse was dosed at the test dose (1750 mg/kg) and observed for morbidity or mortality. The first mouse survived, therefore three additional RH mice were sequentially dosed so that a total of four mice were tested (17). In the absence of mortality or signs of morbidity, dosing was increased to 2000 mg/kg, 2250, mg/kg, 2500mg/kg and 2750 mg/kg.

For upper limit test, 4500 mg/kg of *Plocamium* extracts were prepared in vegetable oil and administered to the test mice (GH) by oral gavage.

One mouse (GH) was dosed at the test dose and observed for morbidity or mortality. Two additional mice were dosed one at a time and observed for morbidity or mortality. The procedure was repeated with a lower dose of 4250 mg/kg which was determined as the upper range dose.

The mice were observed hourly for the first twelve hours and later every four hours for the last twelve hours for any behavioural changes and signs of discomfort and mortality. Animals were kept in a well ventilated environment (23 ± 2 ºC) with a 12 h light-dark cycle.

The Miller-Tainter method (18) was used to determine the LD_{50} value. The amount of toxic agent that is sufficient to kill 50 % of a population of animals usually within 24 hours is known as the Lethal Dose (LD_{50}) (18). This is expressed as milligrams of substance per kilogram of body mass (mg/kg). This gives a measure of the acute toxicity of the substance.

Two doses of plant extracts (2500 mg/kg and 4250 mg/kg) obtained from range finding tests were selected as the lower and upper limit doses for the determination of LD_{50}. 54 mice with weights between 19.2 - 21.9 g were randomly selected and divided into

nine groups and labelled as Group 1 to 9 (n = 6 per group). They were fasted overnight in disinfected cages. Various dosages of *Plocamium* extract were prepared starting from 2500 mg/kg and an incremental dose of 250 mg/kg up to 4250 mg/kg. The first group of mice were administered by oral gavage with only vegetable oil which is the vehicle in which *Plocamium* extract was dissolved. Groups 2 to 9 test mice were administered with different concentrations of *Plocamium* extracts. Group 2 mice received the lowest dose (2500 mg/kg) and group 9 received the highest dose (4250 mg/kg). They were housed in a stainless steel cages and provided with a standard pellet diet and water *ad libitium*. They were observed hourly for the first twelve hours and later every four hours for the last twelve hours for any behavioural changes and signs of discomfort. The log dose values were plotted against the probit values. From the regression of the probit-log dose line, the log dose was extrapolated corresponding to probit units of 5. The extrapolated dose corresponds to the median lethal log dose, and the antilog of this log dose value would be the LD_{50} value. The dose corresponding to 50% or probit 5 was taken as LD_{50}.

5.4.2 Dose Response

Inoculation of *E. coli O157:H7, P. aeruginosa* and the administration of *Plocamium* extracts and antibiotic into *BALB/c* mice was carried out on 24 mice of average weights between (19.2 - 21.9 g). The animals were randomly selected and divided into 6 groups of 4 replicates (n = 4 per group). According to Hussein, Al-shokair & Ashry, (19) a safe upper limit for the evaluation of dose-response in animals is taken as 10% of LD_{50}. Three different concentrations of *Plocamium* extracts were prepared in the range of 119, 178 and 355 mg/kg. 355 mg/kg represented 10% of the LD50. 355 mg/kg of the standard antibiotics gentamycin and ampicillin were also prepared.

The presence of *E. coli O157:H7* was pre-determined in each mouse before commencement of experiment. Mice were infected with 10^7 colony forming units of *E. coli O157:H7*, in 0.2 ml of sterilized de-ionised water by intravenous injection according to the prescription of Venkatesan (20). The volume of the inoculum introduced into each mouse was as prescribed by Eman and Hoda (21). After the incubation period three days, the mice were observed for any signs of disease development. Treatment of the animals commenced with Groups I and II treated with gentamycin and ampicillin respectively and they also served as the positive control. Groups III, IV and V were treated with 355, 178 and 119 mg/kg by oral gavage of *Plocamium* extracts respectively. Group VI was not treated and was used as the negative control. The mice were treated with 1.0 ml of their respective doses every twelve hours for five days. Faeces were collected from each test mice daily for the duration of treatment and quantified the *E. coli O157:H7* present in the faeces. Test animals treated with *Plocamium* extracts and the negative control groups were treated with gentamycin at the end of the experiment.

The procedure above was repeated to determine the response of *P. aeruginosa* to *Plocamium* extracts as well as standard antibiotics such as gentamycin and ampicillin. Mice were infected with *P. aeruginosa* via sub-cutaneous method.

5.4.3 Enumeration and /or Detection of *E. coli O157:H7*

The faeces of the test animals were collected four times daily from transparent plastic dishes placed beneath the individual rat cages. This was done for five 5 days after inoculation to determine the number of mice shedding the pathogen and the faecal counts shed (22). *E. coli O157:H7* in each faecal sample was quantified as follows; *E.*

coli was isolated by a conventional process which employs a tenfold dilution method. 10 mg of the faecal sample was weighed, suspended and homogenized in 9 mL of peptone water in falcon tubes and serially diluted up to the ninth dilution. 100 μL of the sample from each dilution was plated on MacConkey agar by surface spreading, using sterile glass spreaders. The plates were then incubated at 45° C for 24 hours. This was followed by counting the colonies and hence converting the counts into colony forming units (CFU). The colonies were preliminarily identified by their phenotypic appearance namely; size, shape, color and the nature of margins. This was followed by Gram staining with smears prepared from the colonies that were pink (*E. coli* presents with gram negative short rods). All colonies that appeared pink, medium sized, elevated with entire margins on MacConkey as described by Cheesbrough (2009) were tentatively considered as *E. coli*. A confirmatory test was performed using a biochemical test. The *E. coli* suspect colony was inoculated in peptone water in a tube and incubated at 37° C for 24 hours after Kovac's reagent was added to the broth. A pink ring was formed at the surface of the broth indicating presence of *E. coli*.

A similar test was carried out to determine the presence of *Pseudomonas aeruginosa* in the collected faeces of mice.

5.5 Ethical Consideration.

Toxicity studies was carried out according to the guidelines of the School of Biomedical Sciences Higher Degree Research Ethics Committee (SBSHREC), Standard Operating Procedures and Guidelines of Makerere University adopted in September 2014.

5.6 Results

5.6.1 Determination of LD_{50}

The results of range finding test carried out according to OECD guideline (17) yielded the following observations. There were no noticeable signs of discomfort or mortality in the group of RH mice dosed with 1750 mg/kg up to 2500 mg/kg. A mortality was recorded at a dose of 2750 mg/kg within 24 hours. 2500 mg/kg was taken as the lowest dose at which none of the test mice died and no signs of morbidity was observed. The LD_{50} is more than the test dose (2500 mg/kg) when three or more animals survived. GH mice dosed with 4250 mg/kg up to 4500 mg/kg showed reduced motor activity, tremors, arching, muscle spasm, writhing and death. Test mice died within 24 hours. The two additional mice that were dosed sequentially also died within 24 hours. The LD_{50} is lower than the test dose (4250 mg/kg) when three or more animals died.

Lower range finding 1750 mg/kg	0		
	0	0	0
Lower range finding 2000 mg/kg	0		
	0	0	0
Lower range finding 2250 mg/kg	0		
	0	0	0
Lower range finding 2500 mg/kg	0		
	0	0	0
Upper range finding 4500 mg/kg	x	0	
	x	x	
Upper range finding 4250 mg/kg	x	0	
	x	x	

Fig. **2** Range finding results. (O=survival, X=death).

There were no noticeable signs of discomfort or mortality in the group of RH mice (see Fig 3 (a) and (b) below). All the GH mice died under 24 hours (between 02h00 and 06h00).

(a) Healthy mouse

(b) Unhealthy mouse

Fig. 3 (a) Healthy mouse and (b) Unhealthy mouse

Table 1 Results of lethal doses of *Plocamium* extract for the determination of the LD_{50}.

Groups	**mg/kg**	**Log dose**	**Dead/6**	**Dead %**	**Corrected probit %**	**Probit**
1	2500	3.3979	0	0	2.5	3.04
2	2750	3.4393	1	17	17.0	4.05
3	3000	3.4771	2	33	33.0	4.56
4	3250	3.5119	3	50	50.0	5.00
5	3500	3.5441	4	67	67.0	5.44
6	3750	3.5740	5	83	83.0	5.95
7	4000	3.6021	6	100	97.5	6.96
8	4250	3.6284	6	100	97.5	6.96

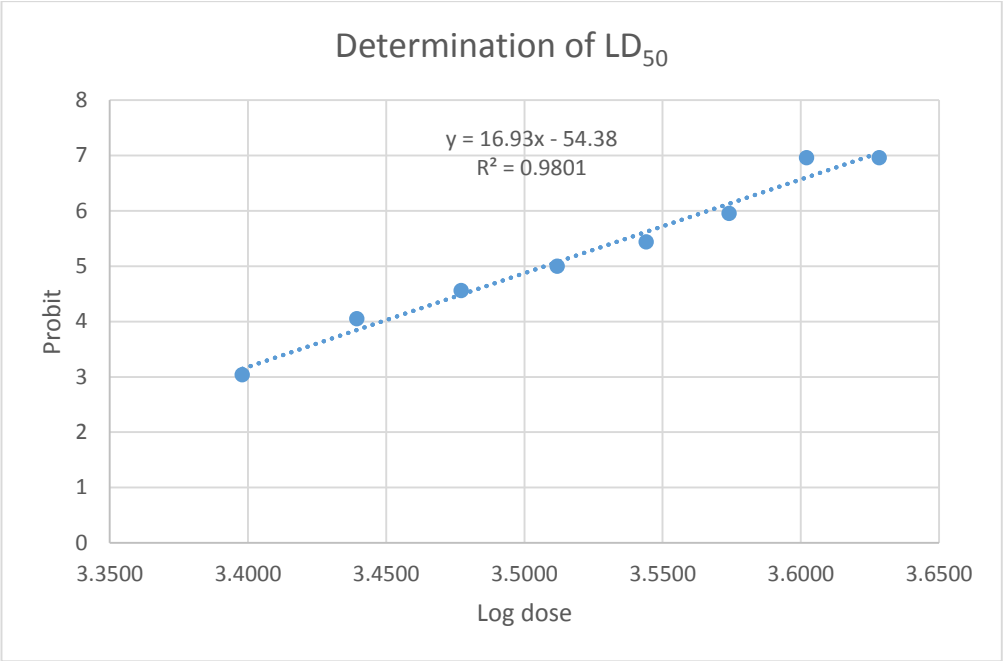


Fig. 4 Plot of log-doses versus probits from Table 1 for the calculation of oral LD_{50} of *Plocamium* extract.

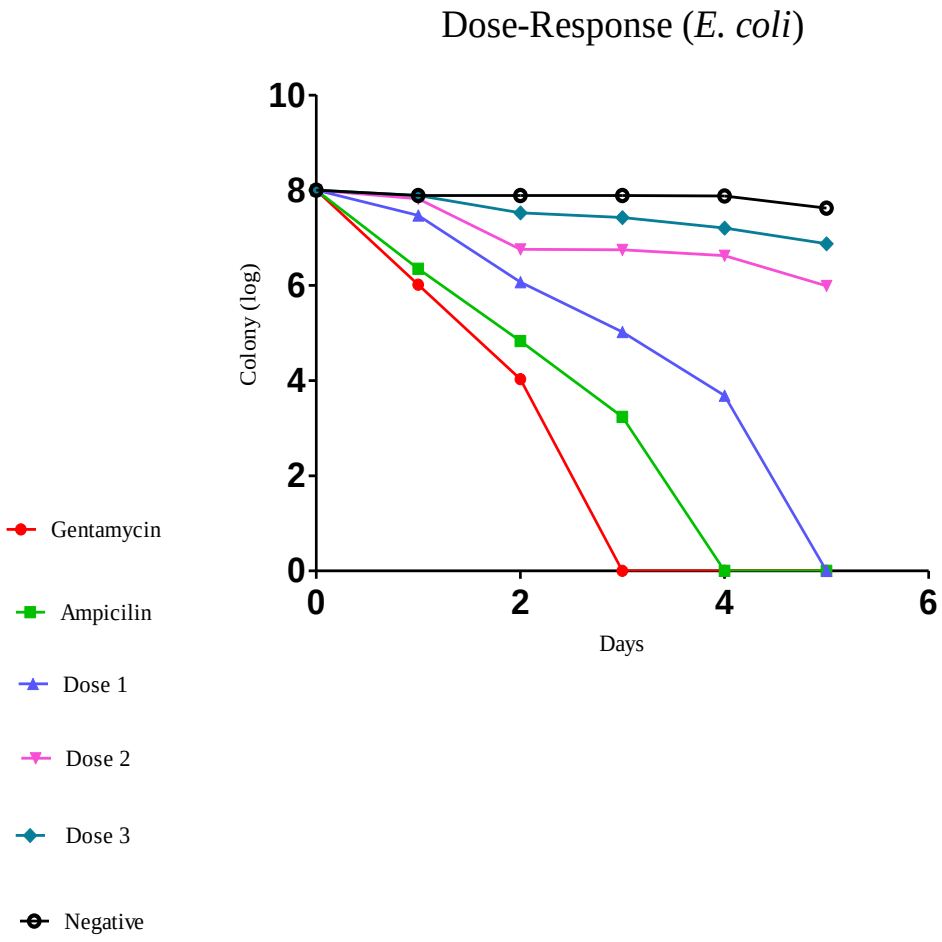
From Table 1 and Fig. 4 above, the value of LD_{50} was found to be 3556 mg/kg. The LD_{50} gives the measure of the immediate or acute toxicity of *Plocamium* extract. The log-dose corresponding to 50% or probit 5 was 3.551.

The antilog of 3.551 =3556

Therefore, the LD_{50} of *Plocamium* extract = 3556 mg/kg.

5.6.2 Dose-Response

From dose-response experiment, it was found that only dose 1, corresponding to 355 mg/Kg of *Plocamium* extract was able to reduce the *E. coli* load to zero on the fifth day. The lower concentrations of the extract had no impact on the bacterial load within the duration of the experiment. Gentamycin and Ampicillin reduced the bacterial load to zero on the third and fourth days respectively. *P. aeruginosa* was found to be resistant to all concentrations of the extract.



(a)

Key

Gentamycin

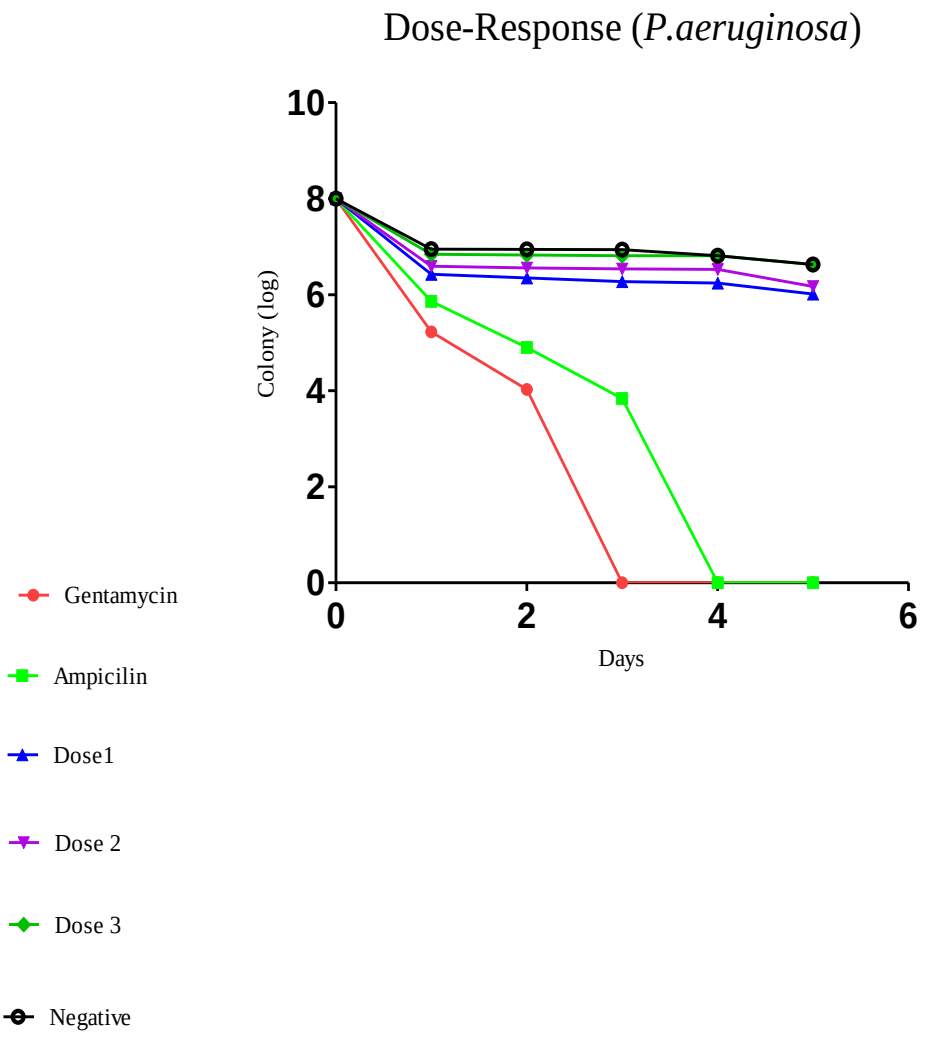
Ampicillin

D1 = 355 mg/kg

D2 = 178 mg/kg

D3 = 119 mg/kg body weight

Negative = is the negative control



(b)

Fig. 5. Dose-response: (a) response of *E. coli* to treatment of control antibiotics and *Plocamium* extract. (b) response of *P. aeruginosa* to treatment of control antibiotics and *Plocamium* extract.

D1 = 355 mg/kg, D2 = 178 mg/kg and D3 = 119 mg/kg

5.7 Discussion

In the acute toxicity experiment, it was found that the acute toxicity (LD_{50}) of *Plocamium* extract was 3556 mg/kg. From the value of LD_{50}, *Plocamium* extract can be classified as a category 5 poison according to OECD 423; LD_{50} estimated between 2000-5000 mg/kg are classified as category 5 poison (23). Toxicity of category 5 substances are slightly toxic. The signal word is caution, possibly followed by, "Harmful if swallowed" or "May be harmful if absorbed through the skin" or "May be harmful if inhaled", or "May irritate eyes, nose, throat, and skin." They are low toxicity substances and can be considered only harmful to vulnerable individuals. However, category 5 materials are estimated to be fatal to an adult human at some dose in excess of 30 grams (24).

E. coli responded positively to the concentration of *Plocamium* extracts. 355 mg/kg (10% of LD_{50}) of the extract inhibited the growth of *E. coli* on the fifth day. However, *P. aeruginosa* were resistant to the presence of *Plocamium* extract.

5.8 Conclusion

In the present study, the acute toxicity (LD_{50}) of the *Plocamium* extract was determined for the first time and found to be 3556 mg/kg. *E. coli* responded positively to the concentration of *Plocamium* extracts. 355 mg/kg (10% of LD_{50}) of the extract inhibited the growth of *E. coli* on the fifth day. However, *P. aeruginosa* were resistant to the presence of *Plocamium* extract.

Recommendations.

Sub-acute test should be conducted to investigate the effects of prolonged use of *Plocamium* extract on other major organs.

5.9 Acknowledgement

The author wishes to thank the University of Namibia through the directorate of staff development for sponsoring this study. Profound gratitude is due to Mr. Aloysius Lubega and his colleagues at Makerere University, Kampala, Uganda for the assistance with the laboratory works. Special thanks to Prof P. Nyarango and Prof C. Hunter for supporting my trip to Uganda.

5.10 References

1. Patil US, Jaydeokar AV, Bandawane DD. Immunomodulators: A pharmacological Review. Int J. Pharm Pharm Sci. 2012; 4(1):30–36.

2. Neergheen-Bhujun VS. Underestimating the toxicological challenges associated with the use of herbal medicinal products in developing countries. BioMed Research Int. 2013; 1-9.

3. Tilburt JC, Kaptchuk TJ. Herbal medicine research and global health: an ethical analysis. Bull. WHO. 2008; 86:594-99.

4. Xiang F, Peng L, Yin Z, Jia R, Hu Z, Li Z, Lv C. Acute and subchronic toxicity as well as evaluation of safety pharmacology of *Galla chinensis* solution.J Ethnopharmacol. 2015;162:181–90.

5. Araújo MC, de P M, Barcellos NMS, Vieira PM de A, Gouveia TM, Guerra MO, Peters VM, Saúde-Guimarães DA. Acute and sub chronic toxicity study of aqueous extract from the leaves and branches of Campomanesia velutina (Cambess) O. Berg. J Ethnopharmacol. 2017;201**:**17–25.

6. Singh T, Sinha N, Singh A.. Biochemical and histopathological effects on liver due to acute oral toxicity of aqueous leaf extract of *Ecliptaalba* on female Swiss albino mice. Indian J Pharmacol. 2013;45(1):61-74.

7. Shanmughapriya S, Manilal A, Sujith S, Selvin J, Kiran GS, Natarajaseenivasan K. Antimicrobial activity of seaweeds extracts against

multiresistant pathogens.Ann Microbiol. 2008;58(3):535–41.

8. Cheng L, Li J, Hao Y, Zhou X. Effect of compounds of *Galla chinensis* on remineralization of enamel surface *in vitro*. Arch Oral Biol. 2010; 55(6):435–40.

9. Seef LB. Herbal hepatotoxicity. Clin Liver Dis. 2007;11:577-96.

10. Tang JL, Liu BY, Ma KW. Traditional Chinese medicine. *Lancet* 2008;372:1938-40.

11. Fakee J. The isolation and characterization of secondary metabolites from selected South African marine red algae (Rhodophyta). Rhodes University, South Africa. 2013; 1-128.

12. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. Phytochemistry, 2009; 70(5):597-600.

13. Lluch JR. Marine benthic algae of Namibia**.** Scientia Marina. *2*002; 66(3): 93-118.

14. Antunes EM, Afolayan AF, Chiwakata MT, Fakee J, Knott MG, Whibley CE, Hendricks DT, Bolton JJ, Beukes DR. Identification and *in vitro* anti-esophageal cancer activity of a series of halogenated monoterpenes isolated

from South Africa sea weeds *P. suhrii* and *P. cornutum*. Phytochemistry. 2011; 72(8):769-72.

15. Elvin-Lewis M. "Should we be concerned about herbal remedies" J Ethnopharmacol. 2001; 75:141- 64.

16. Ishola A, Knott M, Misihairabgwi J. *In vitro* antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from the Namibian coastline. African J Pharm Pharmacol. 2018; 12(10):121-9.

17. Organisation for Economic Co-operation and Development (OECD) Guideline for testing of chemicals 425. Adopted: December 2001.

18. Raj J, Chandra M, Dogra TD, Pahuja M, Raina A.. Determination of median lethal dose of combination of endosulfan and cypermethrin in Wistar Rat, Toxicol Int. 2013;20(1):1–6.

19. Hussein YA, Al-shokair SS, Ashry KM.. Acute and sub-chronic toxicological potential of *Withania somnifera* extract on rats. Alexandria J Vet Sci. 2017;55(2):10–8.

20. Venkatesan N, Vadivu T, Sathiya N, Arokya A, Sundararajan R, Sengodan G, Thandavarayan JB. Anti-diarrhoea potential of Asparagus racemosus wild root extracts in laboratory animals. J Pharm Pharm Sci. 2005; 8(1):39-46.

21. Eman MA, Hoda MZ. Studies on the effect of garlic preparation on *Escherichia coli* O157:H7 causing enteritis in lambs. Egyptian J Clin Pathol. 2008; 21(4):102-29.

22. Itelima JU, Agina SE. *In vivo* antimicrobial activity of plant species on *Escherichia coli* O157:H7 inoculated into albino rats. World J Microbiol. 2014; 1(1): 2-9.

23. Brondani CJ, Reginato FZ, da Silva Brum E, de Souza Vencato M, Lima Lhamas C, Viana C, Manfron MP. Evaluation of acute and subacute toxicity of hydroethanolic extract of Dolichandra unguis-cati L. leaves in rats. J Ethnopharmacol. 2017; 202:147–53.

23. WHO. The *WHO* Recommended Classification of Pesticides by Hazard. 2010:9-12.

24. Hasan KMM, Tamanna N, Haque MA. Biochemical and histopathological profiling of Wistar rat treated with Brassica napus as a supplementary feed. Food Sci Hum Wellness. 2018; 7(1):77–82.

CHAPTER SIX

Structural Elucidation of the Major Metabolites found in two Namibian *Plocamium* species

NPC Natural Product Communications

2018

Vol. 13

No. 0

1 - 2

Chemotaxonomy as a Potential Method to Rapidly Identify Various Namibian *Plocamium* Species

Michael G. Knott* and Anthony Ishola

School of Pharmacy, University of Namibia, Private Bag 13301, Windhoek, Namibia

mknott@unam.na

6.1 Abstract

Objective: The objective of this research was to elucidate the structures of the major metabolites found in *Plocamium* samples I and II by means of Nuclear Magnetic Resonance.

Methods: Marine algae collected along the Namibian coastline were soaked in DCM and MeOH in a ratio 1:1 (v/v) for 48 hours. Concentrated extracts were purified using HPLC to fractionate the extracts and collect the major fractions. The isolated compounds were identified by means of one and two dimensional NMR spectroscopy data and MS analysis.

Results: The major metabolite of *Plocamium* sample I yielded a known compound (**11)** namely, 3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene. The major metabolite of the *Plocamium* sample II is also a known compound namely, 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene (**17)**.

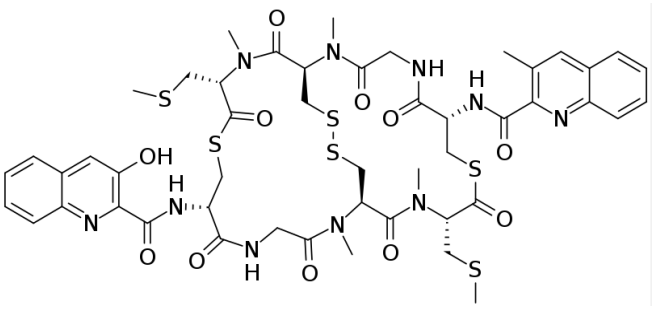
Conclusion: The structures obtained from samples I and II collected along the Namibian coastline correlate with the major metabolites from *Plocamium cornutum* and *Plocamium rigidum* respectively, which had previously been isolated and identified in South Africa.

Key words*:* NMR, marine algae, structural elucidation, *Plocamium* species, chemotaxonomy

6.2 Literature Review

Over the years' marine algae has gained prominence in drug prospecting in different parts of the globe. The rise in prominence of marine algae is not unconnected with the bioactivity demonstrated by different species of marine algae against different bacteria and cancer cell-lines *in vitro* (1,2).

In marine environments, competition for space and nutrients has led to the evolution of antimicrobial defence strategies. This includes the production of chemically active metabolites in their surroundings which act as an aid to protect themselves against other settling organisms, maintenance of unfouled surfaces, deterrence of predation, the ability to successfully reproduce, protection from UV radiation and as allelopathic agents (3,4). A number of promising compounds have been isolated and identified from marine sources that are at advanced stages of clinical trials, or have been selected as promising candidates for extended preclinical evaluation (5). For example, Thiocoraline (**1**) from marine *Micromonospora* species (Fig 1), is a molecule that inhibits DNA α-polymerase and is currently in late preclinical development for the treatment of cancer (6).

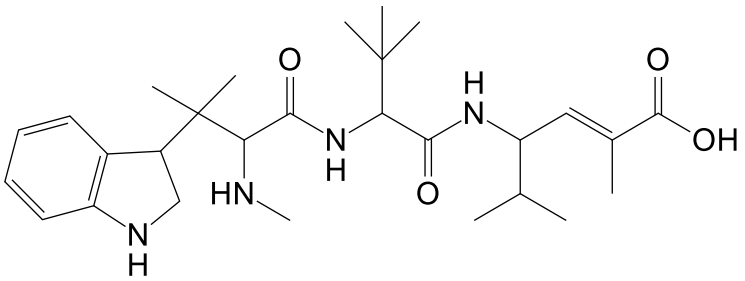


1

Fig. 1 Anticancer drug candidate, Thiocoraline (**1**).

Other examples include several antitumor marine natural products, Fig. 2, derived

mainly from marine sponges or molluscs but also bryozoans and cyanobacteria, exhibit potent antimitotic properties. Several have advanced to Phase I and II clinical trials (7). For example, Hemiasterlin (**2**) below.



2

Fig 2 Antitumour marine drug candidate, Hemiasterlin (**2**).

Since the search for the structures of novel metabolites started, different methods were used ranging from various chemical methods, UV and IR, MS, ^{1}H NMR (60-100 MHz) to the high resolution ^{1}H NMR (300-600 MHz) and 2D-NMR. Today a major tool to assist in achieving the structural elucidation of these secondary metabolites is to use high resolution Nuclear Magnetic Resonance (NMR) technology. Once the structures of these lead compounds or secondary metabolites have been determined, they can then be artificially synthesised in a laboratory for further drug development. NMR spectroscopy is a powerful, non-destructive technique which is capable of complete structural and conformational analysis of complex molecules (8).

The general morphology of the *Plocamium* species is described as being upright, oval shaped and grows up to 50 cm in length. A red, distinctive tinge is common amongst the species in addition to fronds which further separate into smaller branchlets. Since the morphological characteristics of *Plocamium* species are so similar, various methods of species differentiation need to be employed. For example, *P. vulgare* was

harvested in South Africa and incorrectly identified as *P. cartilagineum* in Namibia according to Lluch J. R. (9). Two of the compounds (**3** and **4**) isolated from *P. cartilagineum* are shown below (Figure 3) (10). Only DNA analysis will help to overcome the confusion associated with various closely related *Plocamium* species.

3 **4**

Fig. 3 Halogenated monoterpenes (**3-4**) isolated from *P. Cartilagineum.*

Nature comprises of an untapped pool of unique compounds with high structural uniqueness which is often accompanied by exceptional pharmacological properties. At the core of NP drug discovery is identification and NMR remains the most efficient method (11) for structural elucidation. NMR spectroscopy in combination with high-resolution mass-spectrometry (HRMS) make up a basic set of methods to solve the problem of structural elucidation of most unknown metabolites. Several advances have taken place concerning the inherent capabilities of the NMR apparatus, for example, they are able to reduce experiment times and increase sensitivity toward more efficient analyses of natural materials available in microgram quantities (7).

The determination of the relative configuration of NPs is still a major concern, especially among those of marine origin. In NPs, configurational analysis (Murata's method) through NMR is typically performed with the measurement of both H–H and C–H coupling constants through a combination of experiments such as COSY-DQF,

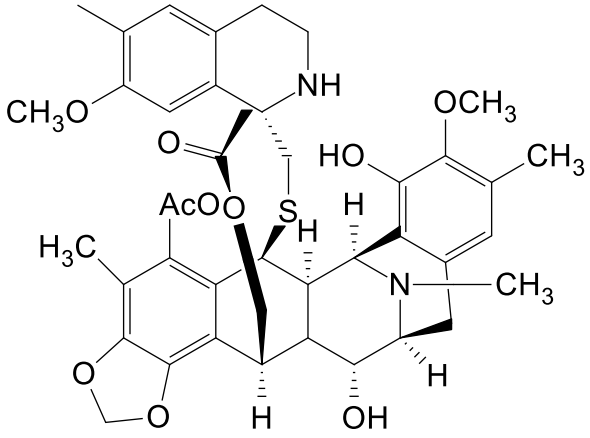
E.COSY, HETLOC, HSQC-HECADE, HSQMBC, IPAP-HSQMBC and *J*-resolved methodologies (8,12). Recently, selective pulse sequences like *J*-selHSQMBC-IPAP allow the simultaneous measurement of H–H and long-range C–H coupling constants ($^{n}J_{CH}$, n > 1) (13).

Relative stereochemistry is inferred not only from NOE correlations between protons and proton–proton coupling constants ($^{n}J_{H,H}$), but also proton–carbon coupling constants ($^{n}J_{C,H}$). NOE-type correlations can now be effectively obtained for a broad range of molecular weights by using newer experiments, whereas many techniques are now available for extracting values of $^{n}J_{H,H}$ even when multiplet structures are obscured by spectral overlap. Measurements of $^{n}J_{C,H}$, are now routinely available and provide an alternative line of evidence with which to support a proposed structure. Finally, the proposed structure is verified in light of all of the observed data.

The first step in the structural determination of an unknown compound is a spectral search against the relevant or available databases using MS and NMR spectra. If the spectrum of the unknown compound fully coincides with a reference spectrum, it means that the structure and formula of the unknown is identical to that of the reference. This is termed structural identification. Otherwise, the problem of structural elucidation arises. Given that the structure has not been elucidated, it is necessary to establish if the compound is new or a known molecule. The structural search against corresponding databases to answer this question is called dereplication. This procedure is also interpreted in literature as structural identification of a known chemical entity based on previously reported analytical and spectroscopic information (14). First, skeletal connectivity is deduced by combining data from both homonuclear (COSY)

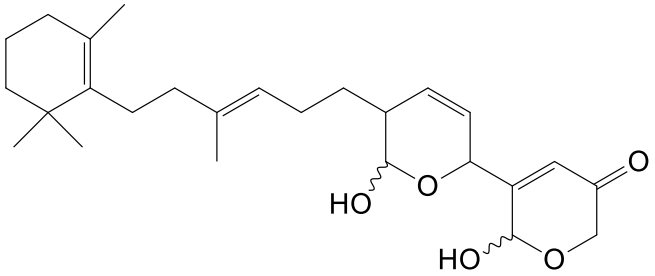
and heteronuclear (HSQC and HMBC) correlation spectroscopy. These experiments give good results even when significant overlap exists in the 1D ^{1}H NMR spectrum and do not require the time-consuming acquisition of a 1D ^{13}C NMR spectrum. Small structural fragments inferred from this can, in turn, be iteratively combined to establish the overall connectivity of the unknown compound. Nowadays, NMR probably is the most important technique for structure elucidation, material characterization and studying molecular motion. Continuing efforts have been made to develop different NMR methods so as to obtain more information from NMR measurements, a number of experiments such as 1D-NMR (^{1}H DEPT, ^{13}C, ^{15}N, ^{19}F, ^{31}P, etc.), 2D-NMR (COSY, DQFCOSY, MQFCOSY, HETCOR, HSQC, HMQC, HMBC, TOCSY, NOESY, EXSY, etc.) and Multidimensional NMR (Homonuclear and Heteronuclear) have been developed (15).

Today, all but the most complex organic molecules are amenable to routine analysis, even with sub-milligram sample quantities. Recent work has uncovered the structural details of not only NPs of every class but also of many of the synthetic creations. A prominent example of a successful compound isolated from the marine environment is Trabectedine (**5**) (Figure 4**)** (16). This compound was the first marine anticancer drug to be approved by the European Union (17). Figure 5 contains examples of other clinically significant marine compounds derived from NPs (18). Other well-characterized examples include halogenated monoterpenes isolated from *Plocamium cornutum* (19) shown in Figure 6. Figure 7 shows the structures of halogenated monoterpenes isolated from *Plocamium rigidum* (2). Figure 8 shows halogenated monoterpenes previously isolated from *Plocamium corallorhiza* (20).

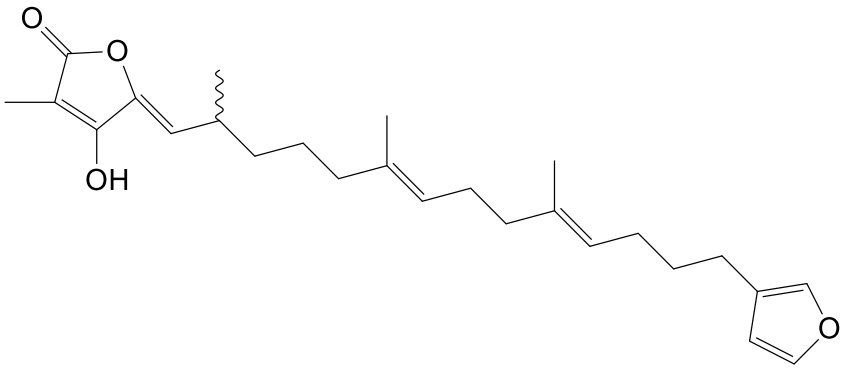


5

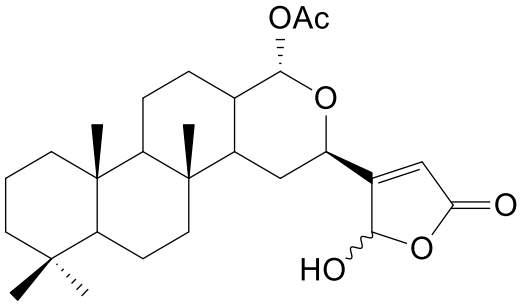
Fig. 4 The anticancer drug Trabectedine (**5**).



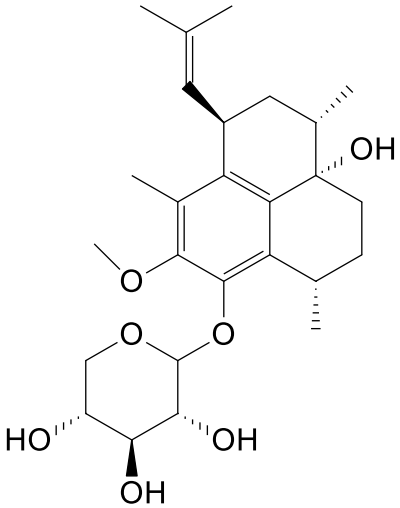
Manoalide **(6)**



Variablin **(7)**

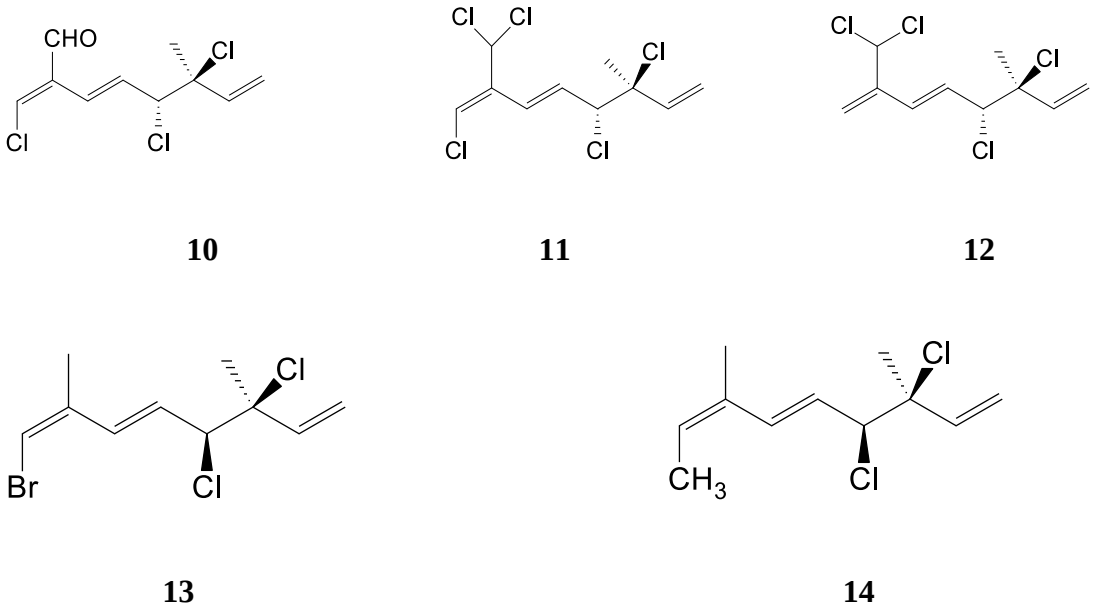


Petrosaapongiolide **(8)**



Methopterosin **(9)**

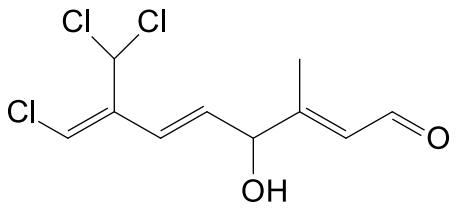
Fig. 5 Examples of clinically significant marine compounds derived NPs (**6 –9**).



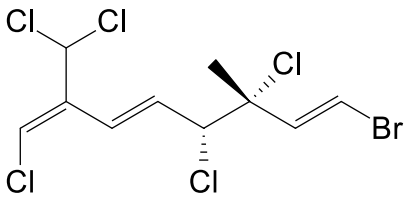
10 **11** **12**

13 **14**

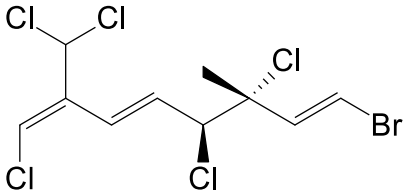
Fig. 6 Structures of halogenated monoterpene (**10**-**14**) isolated from *Plocamium cornutum* (19).



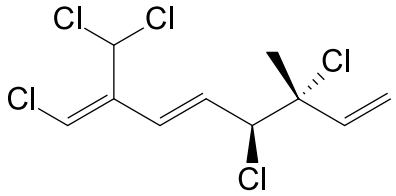
15



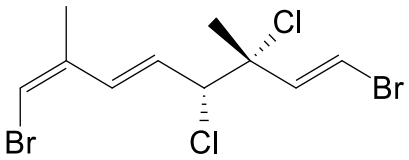
16



17

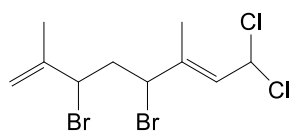


18

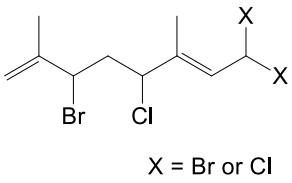


19

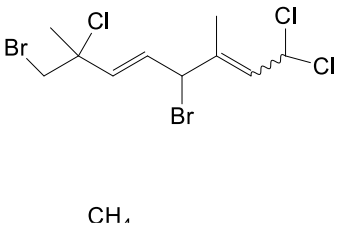
Fig. 7 Structures of halogenated monoterpene (**15-19**) isolated from *Plocamium rigidum.*(2).



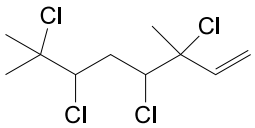
20



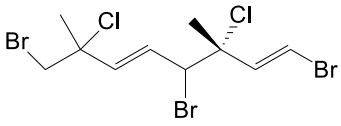
21



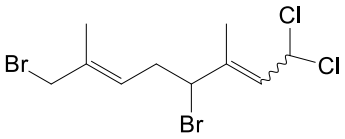
22



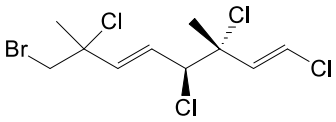
23



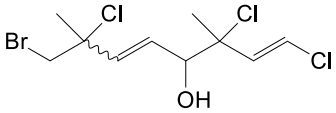
24



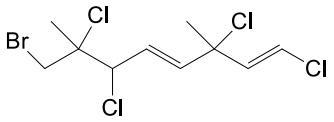
25



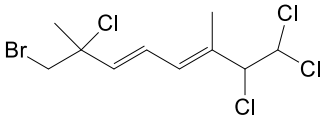
26



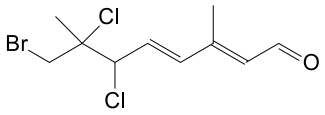
27



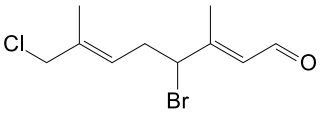
28



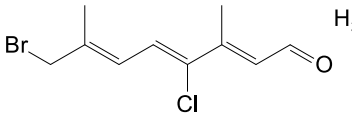
29



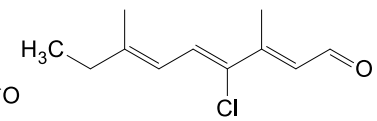
30



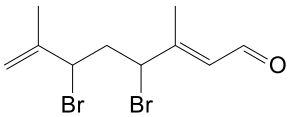
31



32

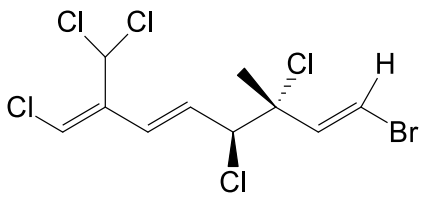


33

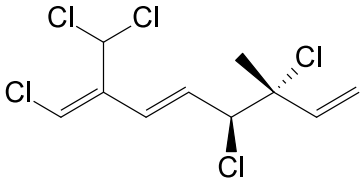


34

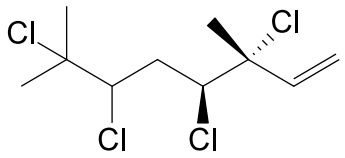
Fig. 8a Halogenated monoterpenes (**20**-**34**) previously isolated from *Plocamium corallorhiza* (20).



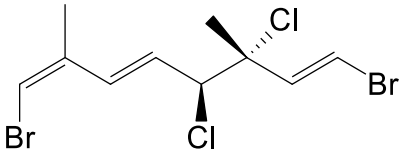
36



37



38



39

Fig. 8b Halogenated monoterpenes (**36 -39**) previously isolated from *Plocamium suhrii.*

6.3 Chapter objectives

- To isolate and employ NMR technology to characterize the halogenated metabolites produced by Namibian *Plocamium* species.
- To identify potential chemotaxonomic markers from these species.

6.4 Materials and Methods

6.4.1 Plant Material

Two different *Plocamium* species were collected by hand at the intertidal zone in Lüderitz and Swakopmund in 2015. The samples were collected about 5 to 10 cm under water by hand and placed in sealable polythene bags and refrigerated at -20 ^{0}C until extraction. A voucher specimen for each sample has been stowed away at the Department of Fisheries and Aquatic Sciences, University of Namibia. They were

labeled as *Plocamium* samples I and II and tentatively identified as *P. cornutum* and *P. rigidum* respectively.

6.4.2 Extraction

Partially thawed samples of *Plocamium* species I and II (~25 g dry mass each) were respectively steeped in 1:1 DCM/MeOH (150 ml) and left for 48 hours at room temperature. The partially concentrated organic extracts were filtered through cotton wool and partitioned three times with hexane (3 x 50 ml). The hexane extracts of samples I and II were concentrated and weighted 0.356 g and 0.218 g respectively.

6.4.3 High Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis was performed on Agilent technologies HPLC 1200 series with a quaternary Gradient Elution Module, Agilent series 1200 Auto-sampler, Agilent series 1200 Fraction Collector and Agilent series 1200 normal UV Detector was used for the separation of the components of the extracts. Prior to HPLC separation, samples were filtered using pudf 0.22 μm prefilters. Isocratic elution method for the separation of the components from the plant extract was developed. The method consists of two mobile phases: Mobile phase A; 90 %v/v acetonitrile (ACN), in deionised in water. The detector was set to run from 210 to 800 nm and the total run time was 15 minutes. The isocratic elution programme was then adapted to the preparative mode. The chromatographic system consisted of semi preparative column, 250 x 10 mm synergi fusion RP 4μm. The chemicals used in the chemistry laboratory were purchased from Merck KGaA, Germany. The separated components were concentrated in a rotary evaporator (model RE 100; Bibby Sterilin Ltd) and dried under nitrogen gas. The crude organic extract was fractioned into six separate fractions by the HPLC system. Only

one major fraction was obtained and it was prepared for NMR analysis. The retention times for the major compounds are 7.759 and 9.977 minutes respectively. See Appendices 10a and 10b.

6.4.4 NMR Analysis

General experimental procedures for *Plocamium* sample I

The major fraction of sample I from the HPLC procedure was subjected to NMR analysis. The ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were all recorded on a Bruker 600 NMR spectrometer using standard pulse sequences. Spectra were referenced to residual protonated solvent resonances ($CHCl_3$ δ_H 7.25, δ_C 77.0). Chemical shifts were reported in parts per million (ppm), while coupling constants were reported in Hertz (Hz). (Table 1). The major fraction of sample II from the HPLC procedure was subjected to NMR analysis. The ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were all recorded on a Bruker 600 NMR spectrometer using standard pulse sequences. Spectra were referenced to residual protonated solvent resonances ($CHCl_3$ δ_H 7.25, δ_C 77.0). Chemical shifts were reported in parts per million (ppm), while coupling constants were reported in Hertz (Hz).

6.4.5 GC-MS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analyses were performed on a Thermo Scientific Focus GC coupled to an ITQ 700 MS using Xcalibur Software, version 2.1, for data acquisition. A SGE BP5MS capillary GC column (30 m × 0.25 mm i.d., film thickness of 0.25 μm) was used with helium as carrier gas at a flow rate of 1.0 mL/min (constant flow) and a split ratio of 10. The GC injector was maintained at a temperature of 220 ^{0}C. Samples were injected in the split mode using a split ratio

of 1:10. The oven temperature was programmed at 5 ^{0}C/min from 40 ^{0}C to 300 ^{0}C. Electron Ionization-Mass Spectrometry (EI-MS) data were acquired at 70 eV and a mass range of *m/z* 25 to 625 was scanned. Ion source and interface temperatures of 200 and 250 ^{0}C, respectively, were used for the analysis. (See Fig. 16).

6.5 Results and Discussion

6.5.1 Identification of the Major Metabolite of *Plocamium* Sample I

The major metabolite presents in *Plocamium* sample I was confirmed by NMR and identified based on a comparison of both experimental and literature values. This compound had previously been isolated from *Plocamium cartilagineum* (21) and *Plocamium cornutum* (2).

The ^{1}H NMR spectrum (Figures 9 and 10) shifts and multiplicity demonstrated a doublet of doublets for the methine (CH) at δ 6.09 (J = 10.8, 16.8 Hz) (H-2) which is coupled to a terminal CH_2 group which is presented by a doublet at δ 5.30 (J = 10.8 Hz) (H-1a) and a doublet at δ 5.42 (J = 16.8 Hz) (H-1b). This suggests the presence of a –CH=CH_2 fragment. The shift at δ 1.77 (H-10) indicates the presence of one methyl group, while two deshielded methine (CH) proton singlets at δ 6.76 (H-9) and 6.39 (H-8) can also be observed. A methine proton doublet at δ 6.62 (J = 16.2 Hz) (H-6) is coupled to a methine proton double doublet at δ 6.46 (J = 16.2, 8.4 Hz) (H-5). In addition, the methine doublet at δ 4.59 (J = 8.4 Hz) (H-4) is also coupled to the double doublet at δ 6.46 (J = 16.2, 8.4 Hz) (H-5). This suggests the presence of a –CH=CH=CHX fragment. (See Figures 9 and 10).(22)

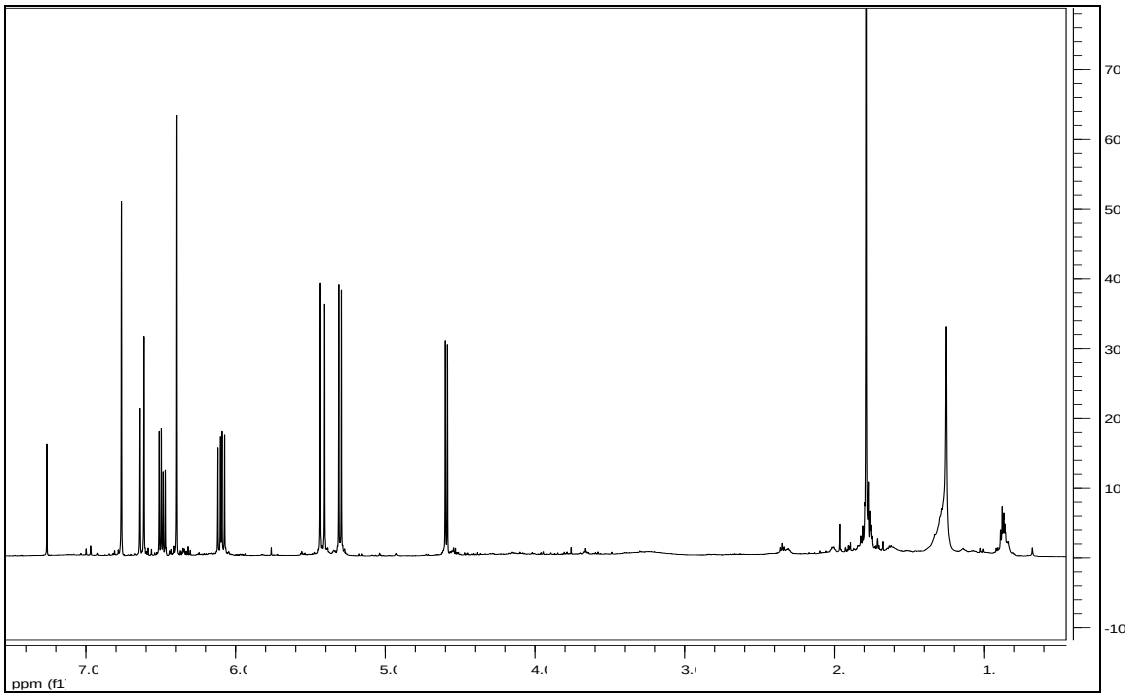


Fig. 9 *Plocamium* sample I ^{1}H NMR spectrum ($CDCl_3$, 400 MHz).

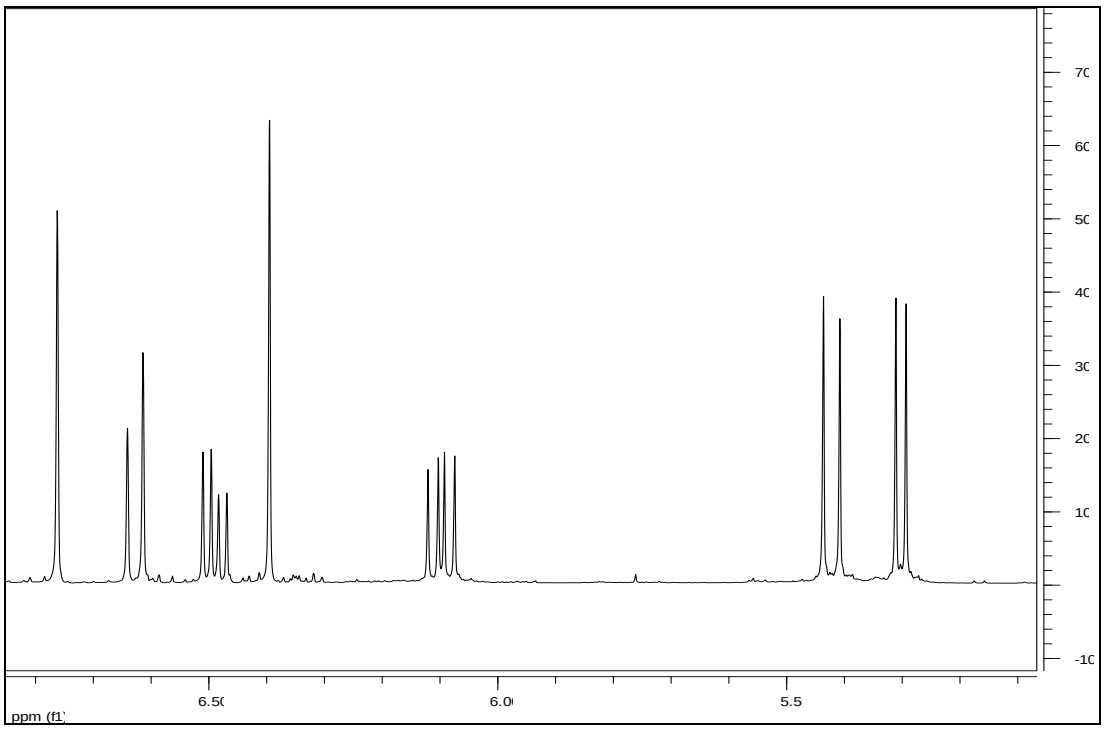


Fig. 10 *Plocamium* sample I ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) (expanded view).

^{13}C NMR data indicated the presence of ten carbons (Figure 11). The six resonances at δ 116.5, 123.8, 124.2, 131.9, 136.0, and 139.4 suggested the presence of three double

bonds. While at C-4 and C-9 two halomethine carbons overlapped at δ 69.4. A quaternary carbon was noted at δ 71.7 while the shift at δ 25.1 suggests a methyl group with a 3,4-*erythro* configuration (22).

The major metabolite isolated from *Plocamium* sample I has been found in South African species of *Plocamium cornutum*. Although the chemistry of the major secondary metabolite isolated and characterised from this Namibian *Plocamium* sample has the characteristic of *Plocamium cornutum*, further taxonomical investigations still need to confirm the full identity of this Namibian *Plocamium* sample using DNA analysis.

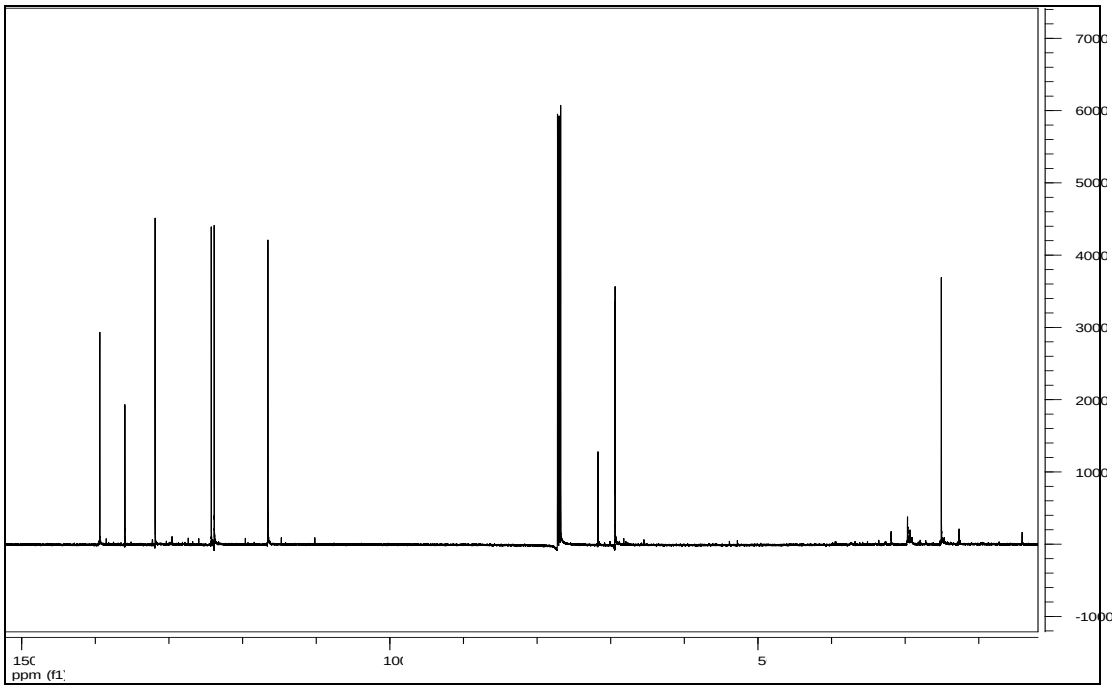


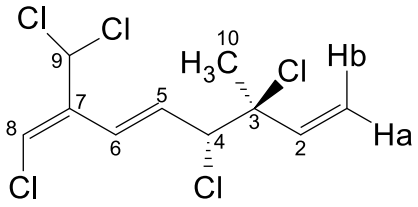
Fig. 11 *Plocamium* sample I ^{13}C NMR spectrum ($CDCl_3$, 100 MHz).

The ^{13}C and ^{1}H NMR data below for *Plocamium* sample I is consistent with that reported by Afolayan (2) (Table 1).

Table 1 ^{13}C NMR ($CDCl_3$, 100 MHz) data and ^{1}H NMR ($CDCl_3$, 400 MHz) data for *Plocamium* sample I **(23).

Carbon no	δC	δ_C**	δ_H	δ_H**
1a	116.5	116.6(CH_2)	5.30, d, 10.8	5.29, d, 10.7
1b			5.42, d, 16.8	5.42, d, 17.1
2	139.4	139.4 (CH)	6.09, dd, 10.8, 16.8	6.09, dd, 10.7, 17.1
3	71.7	71.7 (C)	--	--
4	69.4	69.4 (CH)	4.59, d, 8.4	4.58, d, 8.5
5	124.2	124.2 (CH)	6.46, dd, 8.4, 16.2	6.48, dd, 8.5, 16.2
6	123.8	123.9 (CH)	6.62, d, 16.2	6.62, d, 16.2
7	136.0	136.0 (C)	--	--
8	131.9	131.9 (CH)	6.39, s	6.38, s
9	69.4	69.4 (CH)	6.76, s	6.75, s
10	25.1	25.1 (CH_3)	1.77, s	1.78, s

It is therefore safe to infer that the structure of sample I is consistent with that of *Plocamium cornutum* which was already isolated, characterized and identified as 3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene (**11**) as shown in Figure 12 below.



11

Fig. 12 Structure of Namibian *Plocamium* sample I.

6.5.2 Identification of the Major Metabolite of *Plocamium* Sample II

The major metabolite present in this *Plocamium* sample II was confirmed by NMR and MS and identified based on a comparison of both experimental and literature values. The data obtained for this compound are consistent with a previously isolated compound from *Plocamium cartilagineum* (21), *Plocamium suhrii* (23) and *Plocamium rigidum* (24). The ^{13}C NMR data and ^{1}H NMR data below for *Plocamium* sample II are consistent with that reported by Fakee (24) (Table 2). These correlations were also confirmed by means of a ^{1}H-^{1}H Homonuclear Correlation Spectroscopy (COSY) experiment (See Figure 13-18 supporting evidence).

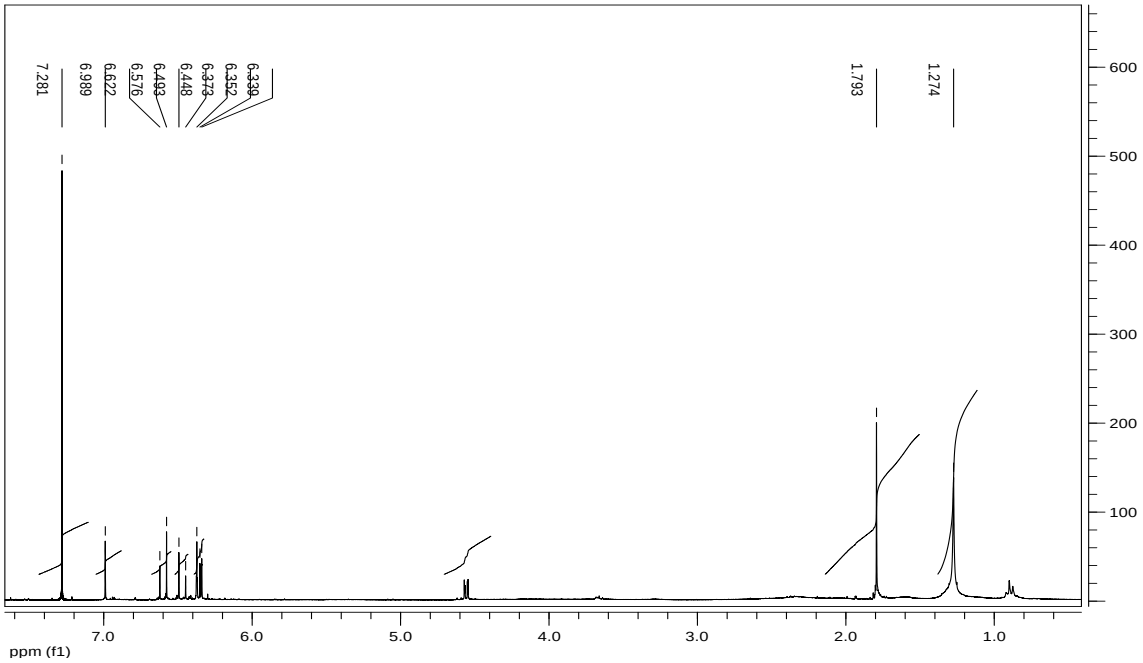


Fig. 13 *Plocamium* sample II ^{1}H NMR spectrum ($CDCl_3$, 400 MHz).

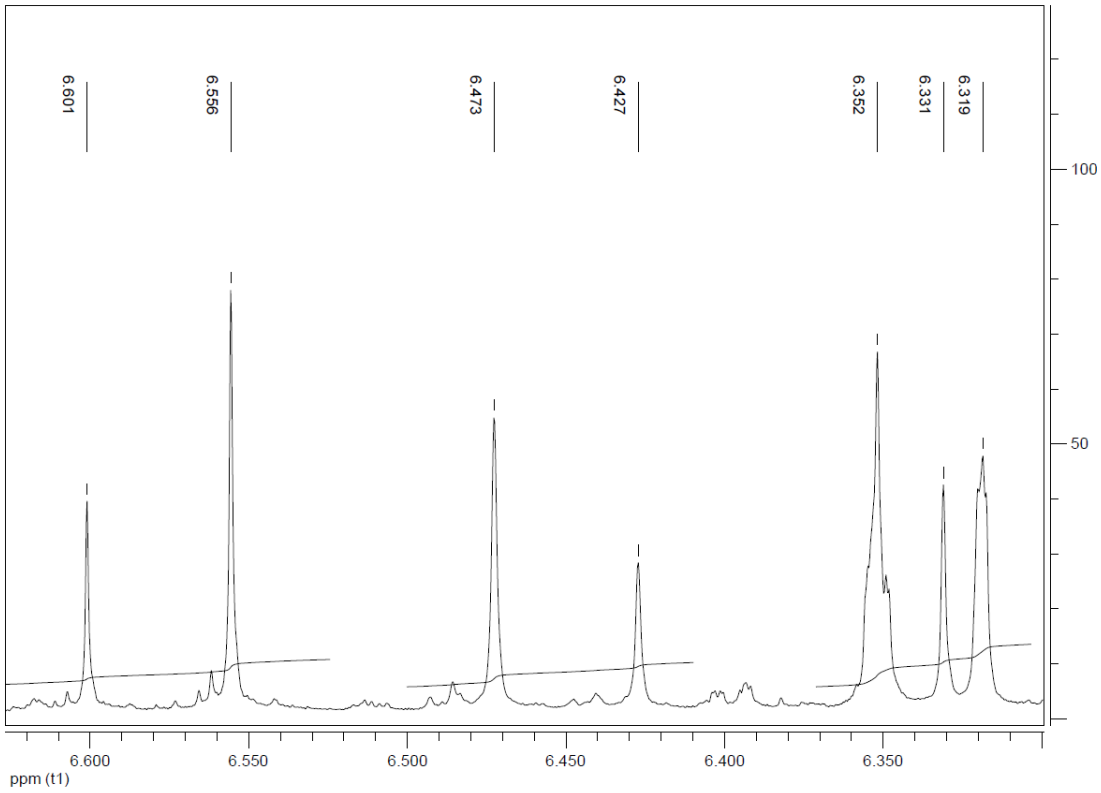


Fig. 14 *Plocamium* sample II ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) (expanded view).

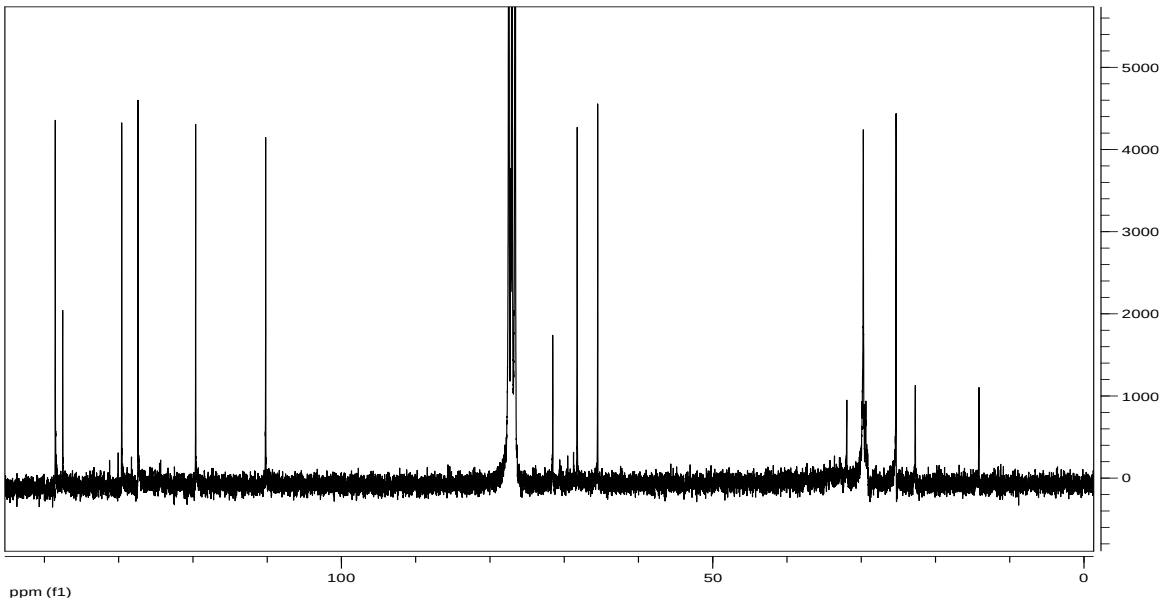


Fig. 15 *Plocamium* sample II ^{13}C NMR spectrum ($CDCl_3$, 100 MHz).

Table 2 ^{13}C NMR and ^{1}H NMR ($CDCl_3$, 400 MHz) data for *Plocamium* sample II **(24).

Carbon no	δC	δ_C**	δ_H	δ_H**
1	110.2	110.2	6.58, d, 16.0	6.57, d, 13.6
2	137.5	137.5	6.44, d, 16.0	6.45, d, 13.6
3	71.5	71.5		
4	68.2	68.2	4.53, d, 12.0	4.54, d, 6.8
5	127.4	127.4	6.33, m	6.33, dd, 15.5, 5.3
6	129.6	129.6	6.35, m	6.34, d, 15.6
7	138.5	138.5		
8	119.6	119.7	6.32, s	6.32, s
9	65.5	65.5	6.97, s	6.96, s
10	25.3	25.3	1.77, s	1.77, s

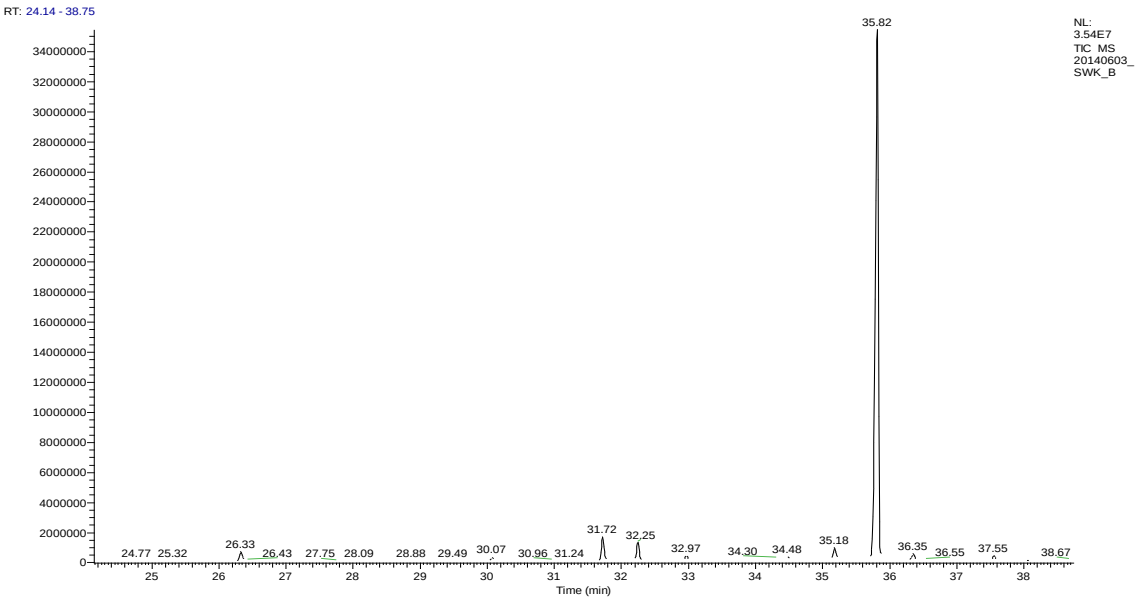


Fig. 16 Total ion chromatogram of the hexane partition of the *Plocamium* sample II.

The molecular ion for this compound could not be observed. (Figure 16). The mass spectrum exhibited an abundant ion at mass to charge ratio (*m/z*) 167,169 (base peak), 171 (relative abundance: 3:4:1) which corresponds to an ion with a formula $C_4H_5BrCl^+$, based on the isotope pattern and MS data reported by Mynderse and Faulkner (21). This fragment ion is formed by the homolytic cleavage of the 3,4-bond of the compound according to Mynderse and Faulkner (21)**.**

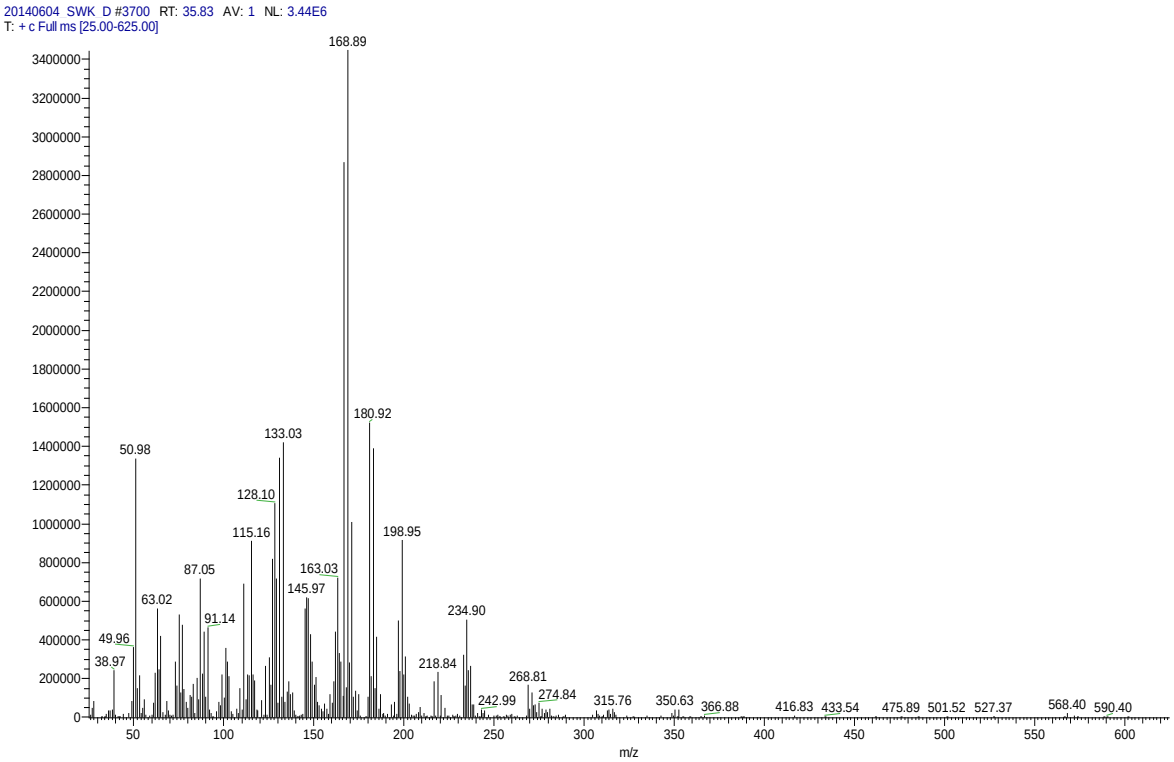


Fig. 17 Electron ionisation mass spectrum of the major metabolite, *Plocamium* sample II.

Of concern was the stereochemistry at position 4. However, the up field shift of the methyl signal at position 10 from δ 1.82 to δ 1.77 suggests *S* stereochemistry at position 4 (21,24).

Another point of contention was the nature of *E/Z* geometry at the $\Delta^{7,8}$ double bond. Literature values supported an *E* geometry, for example a proton shift of δ 6.33 at position 8 is characteristic of *E* geometry at the $\Delta^{7,8}$ double bond, while *Z* geometry at the $\Delta^{7,8}$ double bond has a proton shift of δ 6.28 (21). Fakee (24) indicated that the carbon at position 8 has a shift of δ 119.7 for *E* geometry at the $\Delta^{7,8}$ double bond, and a shift at δ 119.3 for *Z* geometry at the $\Delta^{7,8}$ double bond. Based on both these literature values, an *E* geometry might have been assigned to the $\Delta^{7,8}$ double bond.

However, a Nuclear Overhauser Enhancement Spectroscopy (NOESY) experiment showed strong correlations between H-8 and both H-4 and the methyl group at position 10 which strongly suggested *Z* geometry at the $\Delta^{7,8}$ double bond (Figure 18 and 19).

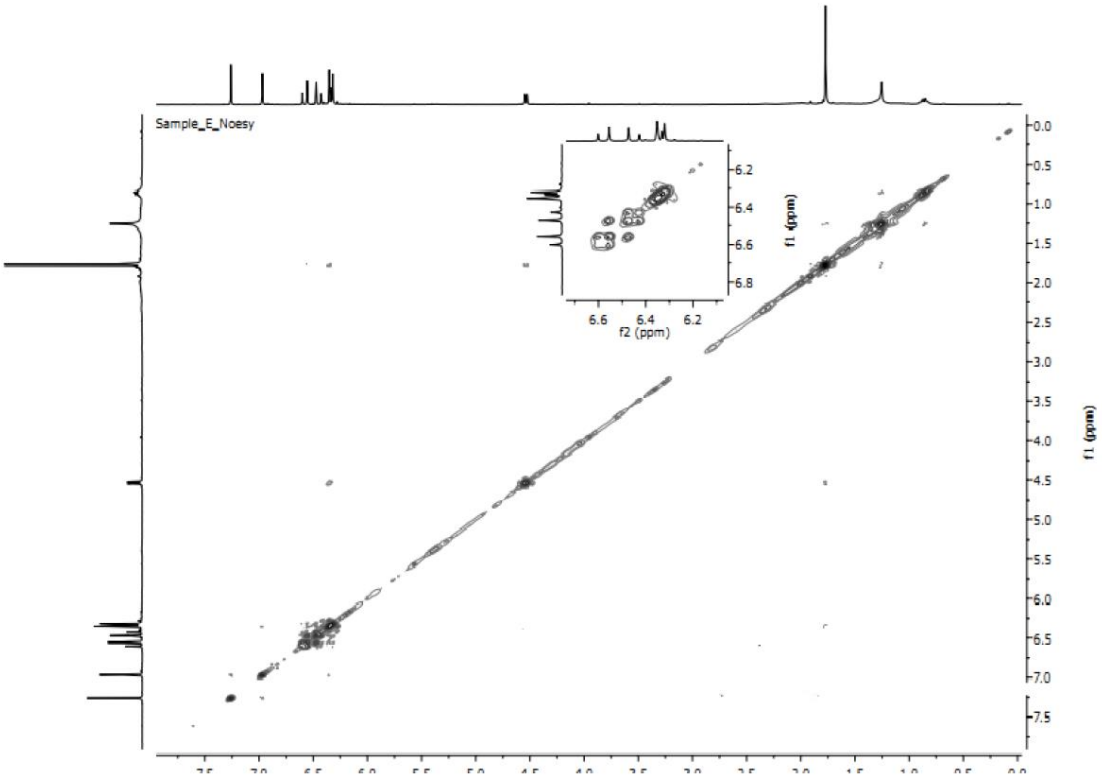


Fig. 18 *Plocamium* sample II H-8 NOESY correlations.

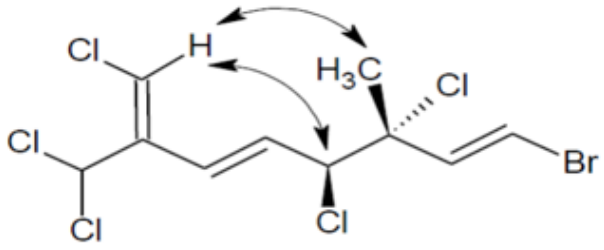
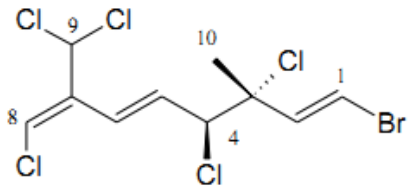


Fig. 19 *Z* geometry at the $\Delta^{7,8}$ double bond based on NOESY correlations.

As a result, structure from *Plocamium* sample II was tentatively named, 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo 3,4,8-trichloro-7-(dichloromethyl)- 3-methylocta-1,5,7-triene (**17**).



17

Fig. 20 Structure of *Plocamium* sample II.

6.6 Chemotaxonomy

Chemotaxonomy is the systematic study of chemical variation between plant taxa. Evidence of chemical variation has essentially been used for classification purposes ever since 'folk taxonomies', based on certain obvious plant characteristics were instinctively employed by mankind centuries ago according to Mannheimer (25). For example, species separation in the genus Laurencia (Rhodomelaceae, Rhodophyta) is complicated by the high degree of morphological variation within the species. Chemical investigations on a worldwide basis of over 15 species indicate that one or more of the halogenated natural products synthesized by Laurencia are unique to each species (26). Observations under a light microscope are often insufficient to determine species identity. Confusion may persist even after examination by transmission electron microscopy as quality fixations of the fragile raphidophyte cells are difficult to achieve (27).

In different *Plocamium* species, it is common to find the same metabolites present in one or more species. However, Fakee (24) identified species specific metabolites even though these metabolites might not be the major products from those species.

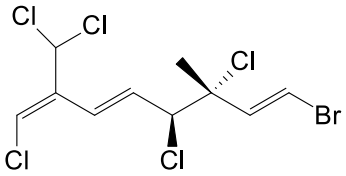
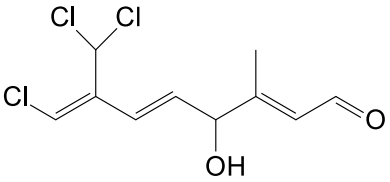
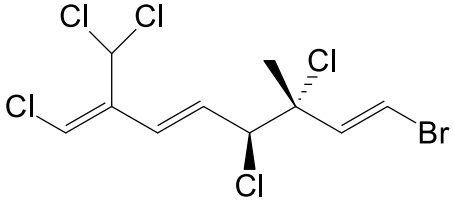
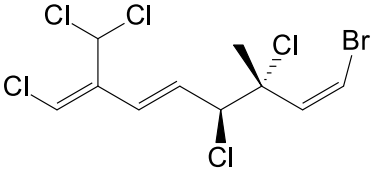
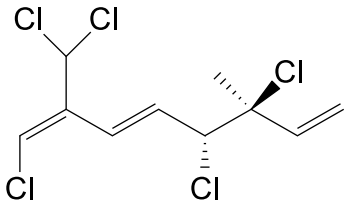
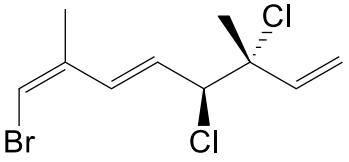
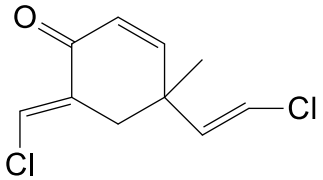
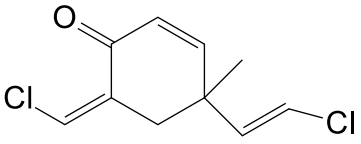
An alternative method is to rely on the major product obtained from the species as the flag bearer of that species. Investigations into the six common species of *Plocamium* confirmed that the major product of each species are unique to that species (28). Therefore, the major products of each species could be used as a tool in the identification of the different *Plocamium* species.

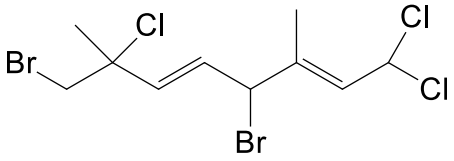
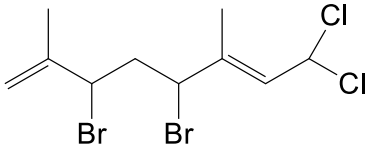
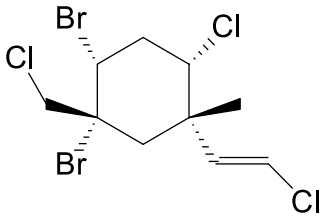
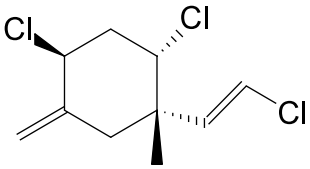
From a chemotaxonomic perspective, it is interesting to observe that South African *Plocamium* species can be classified on the basis of their major metabolites which are

unique to each species (29). Table 3 shows the mass and structures of the major metabolites alongside the mass and structures of the minor metabolites of common *Plocamium* species found in Southern Africa.

Tentative extrapolation can therefore be made on the basis of different major metabolites, namely, 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo 3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene and 3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene that were isolated from the two different Namibian *Plocamium* species. 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo 3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene and 3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene are known major metabolites of *Plocamium rigidum* and *Plocamium cornutum* (in terms of quantity in milligrams per sample extracted) (2,24) respectively.

Table 3 Major and minor metabolites unique to common *Plocamium* species.

Plocamium species	Structure and quantity of the unique major metabolite	Structure and quantity of the unique minor metabolite	Reference
P. rigidum	 (**16**) 10.7 mg	 (**15**) 8.0 mg	(24)
P. suhrii	 (**35**) 44.4 mg	 (**36**) 12.6 mg	(23)
P. cornutum	 (**11**) 185.5 mg	 (**37**) 64.4 mg	(24) (2)
P. maxillosum	 (**38**) 2.3 mg	 (**39**) 0.9 mg	(30).

P. corallorhiza	 (**29**) 15.8 mg	 (**20**) 5.5 mg	(20)
P. robertiae	 (**40**) 34.5 mg	 (**41**) 10.9 mg	(28).

The significance of the same metabolite occurring in more than one species, for example structure **37** was found as a minor metabolite in *P. cornutum* (2) which was isolated in *P. suhrii* (23) is unclear. According to Knott (29), "The taxonomy of *Plocamium* in southern Africa is not properly sorted out". Until the taxonomy of *Plocamium* in southern Africa is well established, tentative identification of *Plocamium* species in Namibia will remain an uncertain element of this research.

6.7 Conclusion

The results obtained in this research showed that the structures of the major metabolites of *Plocamium* species collected along the coastline of Namibia are known and do not correlate well with the proposed identification by the taxonomist at the University of Namibia.

6.8 Acknowledgements

Identification of the algae was done by Mr. Lineekela Kandjengo from the Department of Fisheries and Aquatic Sciences, University of Namibia. A special thank you goes to Prof Klaus R. Koch at the University of Stellenbosch and Prof Jan du Preez at North West University for enabling us to perform the NMR and HPLC purification experiments respectively. This work is as a result of the support by the Namibian National Commission on Research, Science and Technology (NCRST) and the National Research Foundation (NRF) of South Africa for the project Capacity development in NMR spectroscopy for the molecular structure determination of indigenous plant extracts.

6.9 References

1. Ishola A, Knott M, Misihairabgwi J. *In vitro* antimicrobial activities of *Plocamium rigidum* and *Plocamium cornutum* from the Namibian coastline. African J Pharm Pharmacol 2018; 12(10):121-29.

2. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. Phytochemistry. 2009;70(5):597-600.

3. Taskin E, Taskin E, Ozturk M.. Antibacterial activities of some seaweeds from Northern Cyprus against some food -related pathogens. Asian J Biol sci. 2012;5(5):250-56.

4. Chakraborthy K, Lipton AP, Paulraj R, Vijayan KK. Antibacterial. Diterpernoids of *Ulva fasciata Delile* from South-Western coast of Indian peninsula. Food Chem. 2010;119:1399-408.

5. Faulker DJ. Marine Natural Products. Nat Prod Rep. 2001; 17(1):1-6.

6. Fenical W, Jensen PR. Developing a new resource for drug discovery: marine actinomycete bacteria. Nat Chem Biol. 2006;2(12):666-73.

7. Molinski TF. NMR of natural products at the ‘nanomole-scale. Nat Prod Rep. 2010; 27:321–29.

8. Smith IC, Blandford DE. Nuclear magnetic resonance spectroscopy. Anal Chem.1995; 67(12):509-18.

9. Lluch JR. Marine benthic algae of Namibia**.** Scientia Marina. *2*002; 66(3): 93-118.

10. de Ines C, Argandoña VH, Rovirosa J, San-Martin A, Diaz-Marrero AR, Cueto M, Gonzalez-Coloma A. Cytotoxic activity of halogenated monoterpenes from *Plocamium artilagineum*. J Biol Sci. 2004; 59:339-44.

11. Halabalaki M, Vougogiannopoulou K, Mikros E, Skaltsounis AL. Recent advances and new strategies in the NMR-base identification of natural products. Curr Opin Biotechnol. 2014; 25:1–7.

12. Molinski TF, Morinaka BI. Integrated approaches to the configurational assignment of marine natural products. Tetrahedron. 2012; 68:9307-43.

13. Sauri J, Parella T. Simultaneous measurements of J(HH) and two different nJ(CH) coupling constants from a single multiply edited 2D cross-peak. Magn Reson Chem. 2013; 51:397–402.

14. Elyashberg M. Identification and structural elucidation by NMR spectroscopy, TrAC-Trends Anal Chem. 2015; 69:88-97.

15. Fuloria NK, Shivkanya F. Structural elucidation of small organic molecules by 1D, 2D and Multi-dimensional-solution NMR Spectroscopy. *J Anal Bioanal Tech.* 2013; 11:1-9.

16. Cuevas C, Francesch A. Development of Yondelis (trabectdin, ET-743). A semi-synthetic process solves the supply problem. Nat Prod Rep. 2009; 26:322-37.

17. Mayer AMS, Glaser KB, Cuevas C, jacobs RS, Kem W, Little RD, *et al*., The odyssey of marine pharmaceuticals: a current pipeline perspective. Trends Pharmcol Sci. 2010;31:255-65.

18. Haefner B. Drugs from the deep. Marine natural products as drug candidates. Drug discov Today. 2003;8(12):536-44.

19. Cabrita MT, Vale C, Rauter AP. Halogenated compounds from marine algae. *Mar Drugs*, 2010;8:2301-17.

20. Mann MGA, Mkwananzi H, Antunes EM, Whibley CE, Hendricks DT, Bolton JJ, Beukes DR. Halogenated monoterpenes aldehydes from South African marine algae. *Plocamium corallorhiza*. J Nat Prod. 2007; 70:596-9.

21. Mynderse JS, Faulker DJ. Polyhalogenated monoterpenes from the red algae *Plocamium cartilagineum*. Tetrahedron. 1975; 31:1963-7.

22. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. *Phytochemistry*, 2009;70(5):597-600.

23. Antunes EM, Afolayan AF, Chiwakata MT, Fakee J, Knott MG, Whibley CE, Hendricks DT, Bolton JJ, Beukes DR. Identification and *in vitro* anti-esophageal cancer activity of a series of halogenated monoterpenes isolated from South Africa sea weeds *P. suhrii* and *P. cornutum*. Phytochemistry. 2011; 72(8):769-72.

24. Fakee J. The isolation and characterization of secondary metabolites from selected South African marine red algae (Rhodophyta). Rhodes University, Grahamstown, South Africa. 2013;1-128.

25. Mannheimer CA. An overview of chemotaxonomy and its role in creating a phylogenetic classification system. Agricola. 1998;99(10):87-90.

26. Fenical W, Norris J. Chemotaxonomy in marine Algae: Chemical separation of some *Laurencia* species (Rhodophyta) from the Gulf of California. *J Phycol*. 1975;11(1):104-8.

27. Marshall J-A, Nichols DP, Hallegraeff MG. Chemotaxonomic survey of sterols and fatty acids in six marine raphidophyte algae. J Appl Phycol. 2002; 14:255–65.

28. Knott MG. Structural characterisation and evaluation of cytotoxic activity of natural products from selected south African marine red algae. Rhodes Univ, South Africa. 2012.

29. Knott M, Mkwananzi H, Arendse C. *Plocoralides* A – C, polyhalogenated monoterpenes from the marine alga *Plocamium corallorhiza*. Phytochemistry. 2005; 66:1108-1112.

30. Knott MG, mare JD, Edkins AL, Zhang A, Stillman MJ, Bolton JJ, *et al*., Plaxenone A and B: Cytotoxic halogenated monoterpenes from South Africa red seaweed *Plocamium maxillosum*. Phytochem Lett. 2019; 29:182-5.

CHAPTER SEVEN

Quantitative structure activity relationships (QSAR) study of halogenated monoterpenes from *Plocamium* rigidum

Int. Sci. Technol. J. Namibia Ishola & Oyedele/ISTJN 2018, 12:107-115.

Quantitative Structure Activity Relationships (QSAR) Study of Halogenated Monoterpenes from *Plocamium* species collected from the Namibian coast line

Anthony Ishola[1]*, and Opeoluwa Oyedele[2]

[1]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Namibia.

[2]Department of Statistics and Population Studies, Faculty of Science, University of Namibia

Received: 23rd April, 2018. Accepted: 14th October, 2018. Published: 23rd October, 2018

7.1 Abstract

Objective: The objective of this study was to optimize the structures of monoterpenes isolated from *Plocamium rigidum*, towards improvement of their anti-esophageal cancer activities through the use of Quantitative Structure Activity Relationships (QSAR) study.

Method: The structures of the metabolites were optimized with Merck Molecular Force Field (MMFF). The geometry of the stable conformers from the MMFF calculations were employed to compute several physicochemical properties using the B3LYP variant of Density Functional Theory (DFT) in conjunction with the 6-31G (d) basis set. The relationship between the pIC_{50} and the descriptors were determined using the statistical package, R, and the analysis toolpak in excel. Validation of the models were made via statistical parameters like R-squared, adjusted R-squared, Root means square (RMSE) and cross validation (CV) coefficient (q^2).

Results: Two descriptor models were developed. Multi-Linear regression analysis indicated that CPKOvality and HOMO-LUMO (H-L) gap are physicochemical descriptors within the two-descriptor models, that best describe the variation in biological activity of the metabolites studied in this work. The best predictive two parameter model is characterized by the following Hansch equation:

pIC50 = 9.91CPKovality + 0.270HL-gap-17.149

(R^2 = 0.71, Adj. R^2 = 0.56, R = 0.84, Std. error = 0.31, RMSE = 0.229 and q^2 = 0.55)

Conclusion: The Hansch equation above is the best that can be developed for the number of compounds (seven) considered in this study. It also satisfied the rigorous requirements that q2 > 0.5 and R^2.> 0.6 and RMSE < 0.3.

Key words: QSAR, *Plocamium* species, quantum chemical descriptors, molecular property descriptors, squared correlation coefficient ($\mathbf{r^2}$) value, cross-validation coefficient (q^2), Bayesian Information Criterion (BIC).

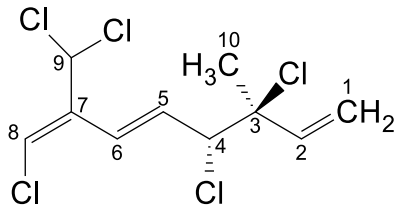
7.2 Literature Review

The marine environment represents a vast proportion of the Earth's biodiversity and it has been estimated that biological diversity in marine ecosystems may be higher than that in tropical rain forests (1,2). Thus, the marine environment potentially offers an abundance of highly potent secondary metabolites for exploitation as bioactive compounds. Marine natural products provide unusual and unique chemical structures from which molecular modeling and chemical synthesis of new drugs can be based, and these natural products are known to act with greater efficiency and specificity for the treatment of many human diseases (1,3,4). Unique chemical compounds of marine origin with various biological activities have been isolated, and some of them are under investigation and are being used to develop new pharmaceuticals (5).

Algae have attracted considerable research interest in the biomedical areas and recent trends in macro-algae natural drug research have revealed the biomedical potential of these organisms in human diseases treatment. They are producers of bioactive substances which exhibit antioxidant, antimicrobial, antiviral, anthelmintic, anti-inflammatory, anticoagulant, antituberculosis, antiviral and antitumor properties (5–8). The use of marine algae and their derivatives in applications to combat skin aging, as well as for depigmentation and antimicrobial applications in the cosmetic industry is wide spread as reported by Wang (9).

The genus *Plocamium* contains more than 40 species that are widely distributed throughout the world's oceans (10). These algae typically produce acyclic and cyclic halogenated monoterpenes of which more than 100 have been reported. They have a distinct evolution on their biosynthetic pathways that frequently yield complex molecules with no counterparts in the terrestrial environment.

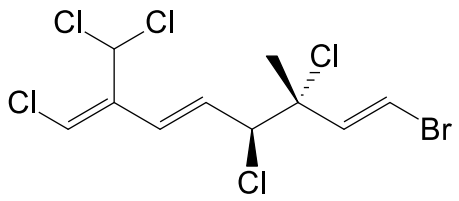
The major secondary metabolite from a species of red marine algae, *P. cornutum* (3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene), shown in Fig 1 was evaluated for antiplasmodial activity against the chloroquine sensitive strain of *Plasmodium falciparum* (11).



1

Fig. 1 Structure of the major metabolite found in *Plocamium cornutum.*

While the major metabolite from *P. rigidum,* (1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene) shown in Fig 2 was found to be active against different cancer cell lines (11).



2

Fig. 2 The structure of the major metabolite found in *Plocamium rigidum.*

QSAR Theory

Quantitative structure-activity relationship (QSAR) modeling pertains to the construction of predictive models of biological activities as a function of structural and molecular information of a compound library. The concept of QSAR has typically been used for drug discovery and development and has gained wide applicability for correlating molecular information with not only biological activities but also with other physicochemical properties, which has therefore been termed quantitative structure-property relationship (12). All QSAR analyses are based on the assumption of linear additive contributions of the different structural properties or features of a compound to its biological activity, provided that there are no nonlinear dependences of transport or binding on certain physicochemical properties.

Traditional QSAR modelling is a ligand-based drug design method for both exploring and exploiting the relationship between chemical structure and its biological action (13). To predict the activities of chemical compounds extracted from marine algae, quantum chemical descriptors like molecular orbitals, dipole moment, charge etc. and molecular property descriptors like hydrophobic, steric coefficient etc. have been applied to develop 2D QSAR models (14).

In all these diverse applications of algal metabolites, hundreds of thousands need to be screened or synthesized in the laboratories. This number of compounds as potential algicides is time-consuming and laborious as well as costly. Therefore, theoretical prediction of the activities of algicidal compounds based on quantitative structure–

activity relationships (QSAR) is desirable, as it cannot only reduce the heavy experimental burden, but also guide the further design of inhibitory substances (15).

The main objective of QSAR study is the development of predictive and robust QSAR equations, with specified chemical domain for prediction of activity of untested molecule. Secondly, QSAR acts as an informative tool by extracting significant pattern in descriptor related to measured biological activity leading to understanding of mechanism of the given biological activity. This could help in suggesting how to design a novel molecule with improved activity profile.

Physicochemical descriptors.

These are physical and chemical properties of a molecule, for example, solubility, partition coefficient, ionization, redox potential hydrogen bonding etc that can affect directly the biological activity of the molecule. For example, the lipophilicity of a drug molecule refers to its affinity for a lipophilic environment. It is a key property in the transport of drugs in the body. Lipophilicity is the ability of a drug to dissolve or interact with a non-polar environment, organic layer. Lipophilicity is a key physicochemical property that plays a crucial role in determining ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties and the overall suitability of drug candidates. A calculated log P is used as an assessment of lipophilicity *in vivo.* (16).

Molecular descriptors are thus essential part of methodological toolbox widely used to optimize the characteristics of molecules.

Partition-coefficient (*P*) is the ratio of concentrations of a compound in a mixture of two immiscible phases , organic phase and aqueous phase at equilibrium. Log P is a simple way to express Partition coefficient. Lipophilicity is useful in estimating the distribution of drugs within the body.

Lipophilic substituent constant (π) relates to the lipophilicity of the substituents. Log P described above is related to the lipophilicity of the whole molecule.

Polar surface area (PSA) is the surface sum over all polar atoms, primarily oxygen and nitrogen, also including their attached hydrogen atoms. PSA is a very useful parameter for prediction of drug transport properties it is a commonly used in medicinal chemistry metric for the optimization of a drug's ability to permeate cells.

Polarizability (α) characterizes how readily the atomic or molecular charge distribution is distorted by external static or oscillating electromagnetic fields. Indirectly polarizability can be used to measure the volume of a molecule.

The HOMO-LUMO gap, i.e. the difference in energy between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) is an important stability index. It is also an indicator of chemical reactivity of a molecule. Molecules with large HOMO-LUMO gaps are kinetically stable and less reactive while those with small gaps are generally more reactive. The HOMO-LUMO gap is used as an approximation to the lowest excitation energy of the molecule (17)

Hammett (σ) is a measure of electronic effects. It takes into account both resonance and inductive effects. Electron donating groups (EDG) have negative Hammet constant and electron withdrawing groups (EWG) have positive Hammet constant. The value of Hammet constant depends on whether the substituent is para or meta

substituted in aromatic system. Ortho substituents are not considered due to correlation with steric effects.

Ovality is a measure of how the shape of a molecule approaches a sphere (at one extreme) or a cigar shape (at the other). Ovality is described by a ratio of volume to area. Ovality O quantifies the deviation of a molecule from the spherical shape (O = 1.0) (18).

Ovality is important because the shape and size of a drug will influence how easily it can approach and interact with a binding site. A bulky substituent may act like a shield and hinder the ideal interaction between drug and its binding site (19).

Electrochemical Pot (Min & Max): Electrochemical potential is a thermodynamic measure of chemical potential that does not omit the energy contribution of electrostatics.

Dipole moment: The dipole moment is a measure of the polarity of the molecule and it can occur between two ions in an ionic bond or between atoms in a covalent bond. It is the overall descriptor of the electronic interaction among the solvent and solute molecules.

7.3 Chapter Objective

In this work, a quantitative structure activity relationship (QSAR) study using computational approach is reported for different monoterpenes isolated from the algae *Plocamium rigidum* and *Plocamium cornutum*. An attempt is made to correlate reported IC_{50} of the monoterpenes with their physicochemical properties in order to shed light on the mode of action of these important compounds. To our knowledge,

there are no reports of computational QSAR studies of these *Plocamium* species in the literature.

7.4 Computational Methods

Starting with the structures in Fig. 3 obtained from Antunes (20), the equilibrium conformers of the monoterpenes reported in this study were computed using Merck Molecular Force Field (MMFF). Physicochemical properties employed in the QSAR studies were subsequently obtained via single point energy calculations at the B3LYP/6-31G(d)//MMFF level. In order to develop the QSAR model, the pIC_{50} calculated from the experimental IC_{50} was employed as the dependent variable. The eleven descriptors used as independent variables were ClogP, polarizability, minimum electrostatic potential (MinElpot), maximum electrostatic potential (MaxElpot), CPKOvality, CPKArea, Dipole moment, Hammet constants (σ_I Inductive electronic effect), hydrophobic constituent constants (π), molar refractivity (MR) and the HOMO-LUMO gap (H-L). Spartan 14 Version 1.1.8 programme was used to build the structures and to compute the geometric and electronic properties of the molecules.

A stepwise regression approach was used to fit the regression model. This approach fits the model in a step-by-step forward elimination process (21). In each step, a descriptor was considered for addition or subtraction into the final model based on its significance power or some pre-specified criterion. More precisely, the first descriptor to enter the regression model was the variable that had the strongest association with the activity. Then, this initial descriptor was paired with each of the remaining descriptors one at a time. Afterwards, the descriptor that offered the best improvement

to the explained variation in the activity was chosen next. This continued until there were no more descriptors left to consider or until the next improvement was not significant. The best improvement was determined using the R-squared value of the fitted model. However, since R-squared increases as more descriptors are added to a model, to avoid over-fitting, the adjusted R-squared (a modified version of the R-squared that has been adjusted for the number of descriptors in the model) was used as a better measure. Additionally, the Bayesian Information Criterion (BIC) was employed whereby the model with the lowest BIC value was chosen as the final fitted regression model. When fitting models, it is possible to increase the likelihood by adding descriptors, but doing so may result in overfitting. BIC attempts to resolve this problem by introducing a penalty term for the number of descriptors in the model. The statistical analysis was done with the *R* programme. This model is better suited when there is a sizable number of structures.

The most important part of QSAR model development is the model validation. In situations where the number of samples in the data set is limited, the most popular validation criteria, is the leave-one-out- cross validated R^2 (LOO q^2) (22). The number of folds in this model is equal to the number of samples (7) present in the data set. One sample is left out as the testing set while the rest is used as the training set for the model construction. Finally, validation is performed on the data set that was left out initially. This is iteratively performed until all data samples are given the chance to be left out as the testing set (12). Often a high value of this statistical characteristic ($q^2 > 0.5$) is considered as a proof of a high predictive ability of the model.

Predictive QSAR model

In evaluating the performance of the constructed QSAR model, a commonly used approach in the field of QSAR follows the recommendation of Tropsha (23) that a predictive QSAR model should possess the following statistical characteristics.

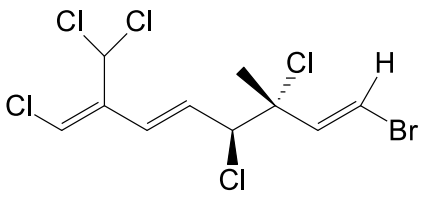
$$q2 > 0.5$$

$$R^2 .> 0.6$$

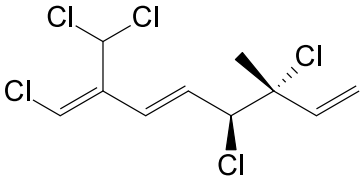
$$RMSE < 0.3$$

Where q2 represents cross-validated explained variance, R^2 represents coefficient of determination (12).

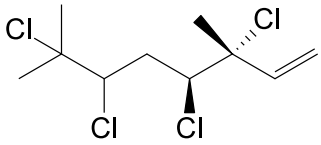
When the difference between the R^2 and R^2 adj value is less than 0.3 it indicates that the number of descriptors involved in the QSAR model is acceptable. The number of descriptors is not acceptable if the difference is more than 0.3.



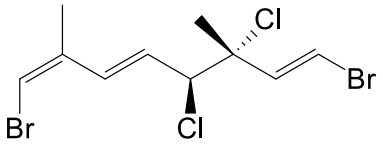
a (IC_{50} = 6.6 µM)



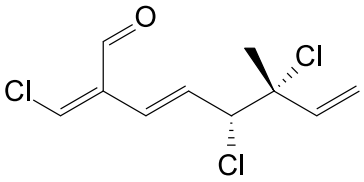
b (IC_{50} = 8.8 µM)



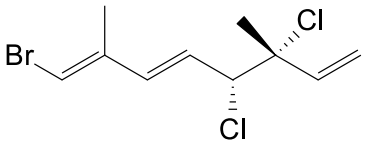
c (IC_{50} = 7.9 µM)



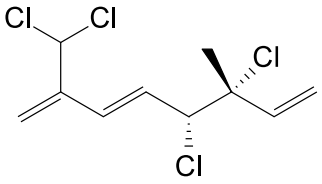
d (IC_{50} = 8.4 µM)



e (IC_{50} = 47.3 µM)



f (IC_{50} = 87.6 µM)



g (IC_{50} = 40.2 µM)

3(a-g)

Fig. 3 Structurally diverse cytotoxic compounds of *Plocamium* species from the Seaweed Metabolite Database (SWMD).(20).

7.5 Results and Discussion

Seven unique monoterpenes from the *Plocamium* species were considered in this study and they are depicted in Fig. 3 above. These metabolites were isolated from *Plocamium* rigidum. Their cytotoxic effects on oesophageal cancer cell line have been investigated and the results (20), suggest their potential as leads in the development of new anticancer drugs. Table 1 lists the seven metabolites, their pIC_{50} (calculated from experimental IC_{50}), and several physicochemical properties computed at the B3LYP/6-31G(d) level.

Table 2 shows a fit of the activity (pIC_{50}) against each property, the individual model fit, yields poor results. The best result in this category is found for pIC_{50} vs polarizability with R^2 of 0.657 and adjusted R^2 of 0.589. A stepwise procedure in which each descriptor is included in the fitting apparently yielded improved results. Fitting of two, three and four descriptors yielded R^2 of 0.667, 0.752, 0.752 and 0.863, respectively. Higher order combinations give spurious results due to overfitting. However, it is known that increasing the number of independent variables in the fitting process often and erroneously results in improved R^2. To guide against this pitfall, the values of adjusted R^2 are of importance and as indicated in Table 3, the adjusted R^2 clearly indicates that the models obtained for the stepwise fitting for the first four combinations are also invalid. Other combinations including five or more descriptors are definitely in error due to overfitting. The R^2 values of 1 are unrealistic based on corresponding undefined adjusted R^2. This is a common challenge in situations where the number of samples in the data set is limited.

Table 1 The activity and the physicochemical properties of structures [Fig 3 (a-g)] computed at the B3LYP/6-31G(D) level

	pIC_{50}	LogP	Polarizability	MinElPot	MaxElPot	CPKOvality	CPKArea	Dipole	Sigma	Pi	MR	HLgap
a	-0.82	5.38	62.24	-94.63	143.43	1.51	307.95	3.69	2.85	4.41	39.03	5.42
b	-0.93	4.71	60.79	-107.81	136.69	1.48	288.09	3.81	2.35	3.55	30.15	5.51
c	-0.90	4.75	60.00	-112.86	112.64	1.44	276.67	1.76	1.88	2.84	24.12	6.97
d	-0.92	5.26	60.44	-63.11	110.52	1.46	280.40	1.82	1.94	3.14	29.82	4.97
e	-1.67	3.12	59.00	-154.67	141.48	1.42	258.73	2.54	1.66	1.48	24.97	4.48
f	-1.94	4.59	58.99	-85.75	99.54	1.43	260.96	1.14	1.44	2.28	20.94	5.03
g	-1.60	4.31	59.80	-85.86	131.52	1.45	272.98	2.11	1.88	2.84	24.12	5.18

Table 2. A fit of activity (pIC50) against each descriptor

Fitted Model	LogP	Polarizability	MinElPot	MaxElPot	CPKOvality	CPKArea	Dipole	Sigma	Pi	MR	HLgap	R^2	Adj R^2
1	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.433	0.320
2	OUT	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.657	0.589
3	OUT	OUT	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.038	0.000
4	OUT	OUT	OUT	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.051	0.000
5	OUT	OUT	OUT	OUT	IN	OUT	OUT	OUT	OUT	OUT	OUT	0.511	0.413
6	OUT	OUT	OUT	OUT	OUT	IN	OUT	OUT	OUT	OUT	OUT	0.661	0.593
7	OUT	OUT	OUT	OUT	OUT	OUT	IN	OUT	OUT	OUT	OUT	0.287	0.144
8	OUT	OUT	OUT	OUT	OUT	OUT	OUT	IN	OUT	OUT	OUT	0.576	0.492
9	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	IN	OUT	OUT	0.577	0.492
10	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	IN	OUT	0.526	0.431
11	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	IN	0.287	0.144

Table 3 Stepwise fitting of activity against descriptors. One descriptor is included one at a time.

ClogP	Polarizability	MinElPot[a]	MaxElPot[b]	CPKOvality	CPKArea	DM[c]	σ[d]	π[e]	MR[f]	HLgap[g]	R^2	Adj R^2
IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.433	0.320
IN	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.667	0.501
IN	IN	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.752	0.504
IN	IN	IN	IN	OUT	OUT	OUT	OUT	OUT	OUT	OUT	0.752	0.256
IN	IN	IN	IN	IN	OUT	OUT	OUT	OUT	OUT	OUT	0.863	0.175
IN	IN	IN	IN	IN	IN	OUT	OUT	OUT	OUT	OUT	1	NaN
IN	IN	IN	IN	IN	IN	IN	OUT	OUT	OUT	OUT	1	NaN
IN	IN	IN	IN	IN	IN	IN	IN	OUT	OUT	OUT	1	NaN
IN	IN	IN	IN	IN	IN	IN	IN	IN	OUT	OUT	1	NaN
IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	OUT	1	NaN
IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN	1	NaN

* The shaded region (IN) indicates descriptors included in stepwise fitting and the unshaded (OUT) are left out; [a]Minimum electrostatic potential; [b]Maximum electrostatic potential; [c]Dipole moment; [d]Hammet constant; [e]Lipophilic substituent constant; [f]Molar refractivity; [g]Homo-Lumo gap;

Table 4. Correlation values of 2 descriptors

	LogP	Polarizability	MinElPot	MaxElPot	CPKOvality	CPKArea	Dipole	Sigma	Pi	MR	HLgap
pIC_{50}	0.6586	0.8108	0.1933	0.2257	0.7150	0.8132	0.5356	0.7593	0.7599	0.7242	0.5386
	LogP	0.7272	0.7903	-0.2801	0.7209	0.7435	0.1268	0.5674	0.8479	0.5594	0.3999
		Polar	0.3082	0.4419	**0.9768**	**0.9973**	0.7209	**0.9742**	**0.9565**	**0.9464**	0.2489
			MinElP	-0.5125	0.3661	0.3185	-0.2687	0.1245	0.5026	0.1608	0.0364
				MaxElP	0.4483	0.4210	0.8504	0.6180	0.2558	0.5669	-0.2292
					CPKOv	**0.9780**	0.7516	**0.9616**	**0.9662**	**0.9207**	0.1431
						CPKAr	0.7145	**0.9716**	**0.9701**	**0.9221**	0.3002
							Dipole	0.8489	0.5996	0.7779	-0.0361
								Sigma	**0.9016**	**0.9450**	0.2002
									Pi	0.8277	0.3351
										MR	-0.0092
											Hlgap

For a small number of compounds (**Fig. 3, a-g)** as exemplified in this study, the Leave-one-out (LOO) cross validation (CV) procedure is more appropriate for the treatment of QSAR data. Further, in addition to R^2 and adjusted R^2, the BIC can also be used to assess the validity of the models. The model with the lowest BIC value provides the best combination of descriptors that could properly describe the variation in biological activity of the compounds under study. Table 5 summarizes the two models with lowest BIC, when all the descriptors were considered.

Table 5 Two models obtained from all the descriptors using the lowest BIC criterion.

pIC_{50} = 0.482LogP + 0.0140MinElpot – 78.466CPKOvality + 0.101CPKArea + 0.922 Dipole moment + 81.86833. **Equation 1** (R^2 = 0.975, Adj. R^2 = 0.85, p-value = 0.265, BIC = -14.147)
pIC50 = 0.0173MinElpot + 0.013MaxElpot – 0.0117CPKOvality – 79.555 + 0.951Dipole moment + 81.8414 **Equation 2** (R^2 = 0.9735, Adj. R^2 = 0.8409, p-value = 0.2728, BIC = -13.737)

The major drawback of the above models is the small ratio of compounds to descriptors used in their derivation; this small ratio of compounds to descriptors will likely invalidate the reliability of the models. A rule of thumb in QSAR specifies five compounds for every descriptor in order to minimize chance correlation and overfitting. In the current study, the number of samples in the data set is limited and the best that can be done is to construct a 2-descriptor model validated by the LOO approach. In this case the predictive power is a direct function of the cross-validation parameter, the q2 value.

7.6 A 2-Descriptor model

The models obtained in the equations above cannot be used as a predictive tool due to the high numbers of descriptors relative to the experimental data and possible overfitting.

In the construction of 2–descriptor models, the value of pIC_{50} were plotted against a combination of two descriptors. In Table 4, the correlation between pair of descriptors are listed. To avoid collinearity a variable that gives a correlation value of 0.9 and above with two or more variables was removed. The remaining descriptors with correlations of < 0.9 were used to generate the 2-descriptor models which are validated statistically.

From Table 4 above, CPKovality and HOMO-LUMO gap shows a correlation of a mere 0.1431, indicating they are independent from each other as descriptors. When the CPKovality and HOMO-LUMO gap (as independent variables) were subjected to multilinear regression analysis (MLR) with experimental pIC_{50} (as dependent variable), a satisfactory Hansch equation with predictive parameters were generated. See equation 3 below.

Equation 3 was the best model involving CPKovality and HL-gap that has been found to give the best prediction of biological activity for the group of compounds considered in this study. All other 2-descriptors models have less predictive power compared with equation 3. Appendix 11 shows the predictive biological activity of other 2-descriptor models.

.

The best predictive model was characterized by the following parameters:

pIC_{50} = 9.91 ± 4.12CPKovality + 0.270 ± 0.16HL-gap -17.149. **Equation 3**

(R^2 = 0.71, Adj. R^2 = 0.56, R = 0.84, Std. error = 0.31, RSME 0.229 and q^2 = 0.55)

A moderate value of CPKovality as found in the model is favourable for binding. Lucas (25) reported that higher values of Ovality reduces the biological activity of pyridazine amide as cell specific spleen tyrosine kinase inhibitors. A bulky substituent may act like a shield and hinder the ideal interaction between drug and its binding site (19).

Large HOMO-LUMO gap is related to high kinetic stability and low chemical reactivity. A small HOMO-LUMO gap as shown in the model above implies low kinetic stability and high reactivity (22).

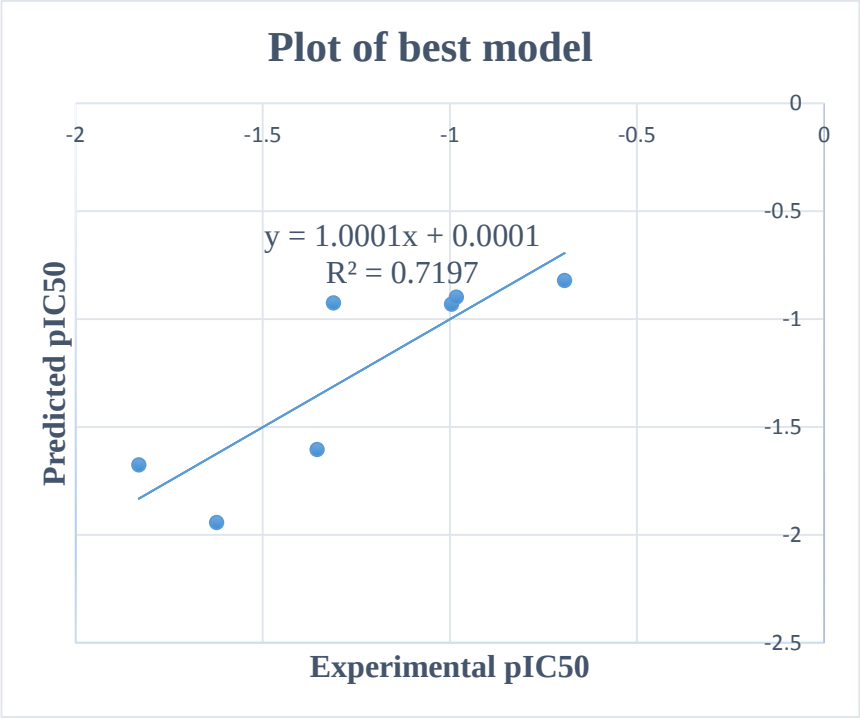


Fig. 4 Plot of predicted pIC_{50} of monoterpenes of *Plocamium rigidum* (n=7) activity against experimental pIC_{50}

7.7 Conclusion

QSAR-based on a 2-descriptors model involving CPKovality and HL-gap was found to generate the best prediction of biological activity of monoterpenes from *Plocamium rigidum*

(R^2 = 0.71, Adj. R^2 = 0.56, R = 0.84, Std error = 0.31, RMSE = 0.229 and q^2 = 0.55). The results suggest that the design of potential drug molecules that would increase the activity should consider substituents that will lead to compounds having moderate size, shape and H-L gap.

7.7 Recommendations.

- To synthesize and test for biological activity of the optimized structures.
- Optimized structures which takes into consideration the CPKOvality which is related to the size and shape of the molecule and having moderate HOMO-LUMO gaps.
- Carry out additional *in vivo* trials with the optimized structures of pure *Plocamium* metabolites to determine LD_{50}, sub-chronic toxicity and measure biochemical parameters.

7.8 Acknowledgement

The author wish to thank the University of Namibia through the directorate of staff development for sponsoring this study.

7.9 References

1. Alves C, Pinteus S, Horta A, Pedrosa R. High cytotoxicity and anti-proliferative activity of algae extracts on an in vitro model of human hepatocellular carcinoma Springer Plus. 2016; 5(1339):1-13.

2. Allsopp M, Page R, Johnston P, Santillo D. State of the World's Oceans. Springer,Heidelberg. 2009; 3(4):255- 66.

3. Ebada S.S, Lin W, Proksch P. Bioactive sesterterpenes and triterpenes from marine sponges: occurrence and pharmacological significance. Mar Drugs. 2010; 8:313–46.

4. Stonik VA Marine natural products: a way to new drugs. Acta Naturae. 2009; 1(2):15–25.

5. Kijjoa A, Sawangwong P. Drugs and cosmetics from the sea. Mar Drugs. 2004; 2:73–82.

6. Murray PM, Moane S, Collins C, Beletskaya T, Thomas OP, Duarte AWF, Walsh DJ. Sustainable production of biologically active molecules of marine based origin. N Biotechnol. 2013; 30(6):839–50.

7. Pinteus S, Alves C, Monteiro H *et al*., *Asparagopsis armata* and *Sphaerococcus coronopifolius* as a natural source of antimicrobial compounds. World J Microbiol Biotechnol. 2015; 31(3):445–51.

8. Ye J, Li Y, Teruya K *et al*., Enzyme-digested fucoidan extracts derived from seaweed Mozuku of Cladosiphon novaecaledoniae kylin inhibit invasion and angiogenesis of tumor cells. Cytotechnology. 2005; 47:117–26.

9. Wang HMD, Chen CC, Huynh P, Chang JS. Exploring thepotential of using algae in cosmetics. Bioresour Technol. 2015;184: 355–62.

10. Saunders GW, Lehmkuhl KV. Molecular divergence and morphological diversity among four cryptic species of *Plocamium (Plocamiales, Florideophyceae*) in northern Europe. Eur J Phycol. 2005; 40(3): 293–12.

11. Afolayan AF, Mann MGA, Lategan CA, Smith PJ, Bolton JJ, Beukes DR. Antiplasmodial halogenated monoterpenes from the marine red algae *Plocamium cornutum*. Phytochemistry. 2009; 70(5):597-600.

12. Nantasenamat C, Isarankura-Na-Ayudhya C, Naenna T, Prachayasittikul V. A practical overview of quantitative structure-activity relationship. EXCLI J. 2009; 8:74-88.

13. Liao C, Sitzmann M, Pugliese A, Nicklaus MC. Software and resources for computational medicinal chemistry. Future Med Chem. 2011;3(8):1057–85.

14. Chen J, Shen Y, Liao S, Chen L, Zheng K.. DFT-Based QSAR study and molecular design of AHMA derivatives as potent anticancer agent. Int J Quantum Chem. 2007;*107*:1468–78.

15. Huang H, Xiao X, Shi J, Chen Y. Structure-activity analysis of harmful algae inhibition by congeneric compounds: Case studies of fatty acids and thiazolidinediones. Environ Sci Pollut Res. 2014;21(11):7154–64.

16. Danishuddin and Asad, U. K (2016). Descriptors and their selection methods in QSAR analysis: paradigm for drug design. *Drug Discovery Today*. **21**(8) 1291-1302.

17. Karelson M, Lobanov VS, Katritzky AR. Quantum-Chemical Descriptors in QSAR / QSPR Studies. Chemical Reviews. 1996;96(3):1027-1043.

18. Loschen, C, Klamt A. Computational Screening of Drug Solvates. Pharmaceutical Research. 2016;33(11):2794-2804.

19. Graham LP. An introduction to medicinal chemistry. United Kingdom. Oxford university Press, 2013.

20. Antunes EM, Afolayan AF, Chiwakata MT, Fakee J, Knott MG, Whibley CE, Beukes DR. Identification and in vitro anti-esophageal cancer activity of a series of halogenated monoterpenes isolated from the South African seaweeds *Plocamium suhrii* and *Plocamium cornutum*. Phytochemistry. 201172(8):769–72.

21. Aczel AD, Sounderpandian J. Complete Business Staistics, 7th Ed McGraw-Hill Primis. 2008.

22. Moniruzzaman, Mohammed JH, Amrin A, Md Belayet H. Molecular Docking, Pharmacokinetic, and DFT Calculation of Naproxen and its Degradants. Biomed J Sci Tech Res 2018;9(5):7360-7365.

23. Tropsha A, Gramatica P, Gombar VK. The importance of being earnest: validation is the absolute essential for successful application and interpretation of QSAR models. QSAR Comb Sci. 2003; 22:69-77.

24. Veerasamy R, Rajak H, Jain A, et al. Validation of QSAR models- strategies and importance. Int J drug des discovery. 2011; 2(3):511-519.

25. Lucas M, Bhagirath N, Chiao E, et al. Using Ovality to Predict Nonmutagenic, Orally Efficacious Pyridazine Amides as Cell Specific Spleen Tyrosine Kinase Inhibitors. J Med Chem. 2014; 57(6):2683-2691.

CHAPTER EIGHT

8.1 Concluding Remarks

This comprehensive research was undertaken to explore the potential of *Plocamium* species as possible drug leads. The extracts of *Plocamium cornutum* and *Plocamium rigidum* were screened. Phytochemical contents and antioxidant activity of the *P. cornutum* and *P. rigidum* were quantified.

It was found that both DCM and MeOH extracts confirmed the presence of flavonoids, terpenoids, tannins, saponins, resins, cardiac glycosides and alkaloids. The highest total phenolic content recorded for DCM extracts was 188.65 ± 0.45 mg and 132.85 ± 0.82 mg GAE per gram for *Plocamium rigidum* and *Plocamium cornutum* respectively. Maximum total flavonoid contents were 202.32 ± 0.33 and 126.11± 0.58 mg QE per gram respectively for *Plocamium rigidum* and *Plocamium cornutum* DCM extracts. The TPC and TFC results from MeOH extracts were lower in both *Plocamium* extracts compared to DCM extracts.

The free radical scavenging activities of *Plocamium* species were significantly lower than that of ascorbic acid. IC_{50} values for *Plocamium rigidum* and *Plocamium cornutum* were 28.87 ± 0.82 and 40.11 ± 0.38 respectively, compared to 12.59 ± 0.66 for ascorbic acid.

The high values of phytochemicals are an indication of their antioxidant potential.

The rich presence of phytochemicals and antioxidant potential serves as motivation to screen for the antimicrobial activity of *Plocamium rigidum* and *Plocamium cornutum.* Extracts were reconstituted in distilled H_2O, C_6H_{14}, DCM, EtOH, MeOH and $CHCl_3$, respectively and tested *in vitro* for antimicrobial activity against 12 pathogens (*Escherichia coli, Staphylococcus aureus, Staphylococcus saprophyticus,*

Pseudomonas aeruginosa, Streptococcus pyogenes, Proteus mirabilis, Listeria monocytogenes, Shigella sonnei, Salmonella sp., Enterococcus faecalis, Candida albicans and *Staphylococcus epidermidis*).

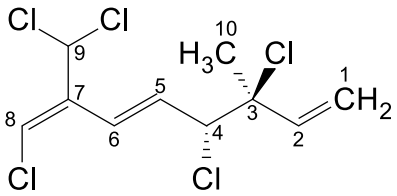
Plocamium rigidum extracted in DCM demonstrated prominent activity against *E. coli in vitro*. The positive response of *Plocamium rigidum* against *E. coli* (*in vitro*) which is the vector pathogen for diarrhoea provided the necessary motivation to investigate the effects of the major metabolite on the dose-response relationship. The acute (LD_{50}) and sub-acute toxicity parameters of *Plocamium rigidum* were determined in *Balb/C* mice using *in vivo* techniques.

The Miller-Tainter method was used to determine the acute (LD_{50}) toxicity of the extract. The LD_{50} was calculated to be 3556 mg/kg. 355 mg/kg (10% of LD_{50}) of these crude extracts of *Plocamium rigidum* reduced *E. coli* to zero on the fifth day. *P. aeruginosa* was not inhibited by any concentrations of the marine algal extract.

Despite the wide applications and antimicrobial activity potential of *Plocamium* marine algae, there is no information on the toxicity levels of the different extracts. This is the first time research of this nature has been conducted on crude *Plocamium* extracts.

Having determined the phytochemicals, antioxidant activity, *in vitro* and *in vivo* parameters of the crude *Plocamium* extract; it became pertinent to explore the chemical nature of the metabolites responsible for the activity. The structures of the major metabolites found in two different Namibian *Plocamium* species were determined by means of Nuclear Magnetic Resonance (NMR) and mass spectroscopy (MS).

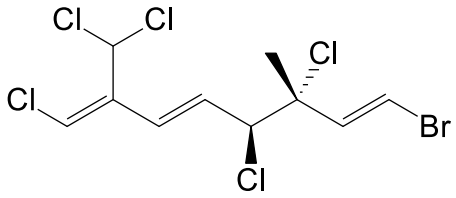
The major metabolite found in *Plocamium cornutum* was found to be 3,4-*erythro*-7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5*E*,7*E*-octatriene (**1**). (See Fig. 1).



1

Fig. 1 Structure of the major metabolite found in Namibian *Plocamium cornutum.*

In sample II, the major metabolite was found to be 1*E*,3*R*,4*S*,5*E*,7*Z*-1-bromo-3,4,8-trichloro-7-(dichloromethyl)-3-methylocta-1,5,7-triene (**2**). (See Fig. 2) This sample was tentatively identified as *P. rigidum*, however there is a possibility that it might also be *P. suhrii*. For the sake of simplicity, *P. rigidum* is the name used throughout the thesis. Only DNA analysis is able to truly identify the sample used.



2

Fig. 2 Structure of the major metabolite found in Namibian *Plocamium rigidum.*

Quantitative Structure Activity Relationships (QSAR) study was undertaken to theoretically determine the physicochemical properties that would enhance the anti-oesophageal cancer activity of various metabolites previously isolated from *Plocamium* species, based on published IC_{50} values and their corresponding structures.

The structures of the metabolites were optimized with Merck Molecular Force Field and several physicochemical properties computed using the B3LYP variant of Density Functional Theory (DFT) in conjunction with the 6-31G(d) basis set.

Statistical analysis indicated that CPKOvality, and HOMO-LUMO gap are physicochemical properties that best describe the variation in biological activity of the metabolites studied in this work.

The best model developed in this work is given by the Hanch equation:

pIC_{50} = 9.91CPKovality + 0.270HL-gap -17.149.

(R^2 = 0.71, Adj. R^2 = 0.56, R = 0.84, Std error = 0.31, and q^2 = 0.55).

The equation above was found to be the best that could be developed for the number of compounds (seven) considered in this study. The parameters in the equation are the combination that best describe the variation in biological activity of secondary metabolites extracted and identified. The equation shows that substitution with CPKovality (moderate size and shape of ligands) and a small H-L gap with high reactivity are the parameters that best optimised structure to predict improved biological activity of *Plocamium* extract.

8.2 Summary of Major Findings

- The favourable values of phytochemicals are an indication of their antioxidant potential.
- *Plocamium rigidum* extracts in DCM demonstrated prominent activity against *E. coli in vitro*.

- The LD_{50} was calculated to be 3556 mg/kg for crude extract of *Plocamium rigidum.*
- *Plocamium rigidum* of 355 mg/Kg (10% of LD_{50}) reduced *E. coli* to zero on the fifth day *in vivo.*
- The two structures of the major metabolites found in Namibian *Plocamium* species are **1** and **2** as shown above.
- The equation of the best fit is as shown below.

  pIC_{50} = 9.91CPKovality + 0.270HL-gap -17.149.

  (R^2 = 0.71, Adj. R^2 = 0.56, R = 0.84, Std error = 0.31, and q^2 = 0.55).
- Moderate size CPKovality and a small H-L gap with high reactivity are expected to increase the biological activity

8.3 Recommendations

More studies are recommended to be done on the effects of prolonged use of the extract on major organs of the human body.

Further *in vivo* trials to investigate whether the use of pro-drugs and new analogues (based on QSAR results), may ameliorate the toxicity of the *Plocamium* compounds.

APPENDICES

Appendix 1 Ethical clearance

UNAM
UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SOM/93/2016 **Date: 29 April, 2016**

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

Title of Project: STRUCTURAL CHARACTERIZATION, BIOACTIVITY AND COMPUTATIONAL STUDIES OF NATURAL PRODUCTS FROM SELECTED NAMIBIAN RED MARINE ALGAE

Nature/Level of Project: Doctorate

Researcher: Anthony S. Ishola

Student Number : 200137174

Faculty: School of Medicine

Supervisor : Dr. M. Knott (Main) Prof. E. Archibong (Co) Dr. J. Misihairabgwi (Co)

Take note of the following:

(a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.
(b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.
(c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.
(d) The UREC retains the right to:
  (i). withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
  (ii). request for an ethical compliance report at any point during the course of the research.

UREC wishes you the best in your research.

PP Kapenda

Dr. H. Kapenda
Director –Centre for Research and Publications
ON BEHALF OF UREC

Appendix 2 Sample collection permit

REPUBLIC OF NAMIBIA

MINISTRY OF FISHERIES AND MARINE RESOURCES
(Aquaculture Licensing Regulation 3, Annexure B)

Aquaculture Licence issued under the Aquaculture Act, 2002 (Act No 18 of 2002)

Name of Licensee: Dr Sam Nuyoma Marine and Coastal Research Center.
Licence Number: HENTIES BAY 0052-06
Date of Issue: 1st FEBRUARY 2006

The person or entity described in this licence is licensed in accordance with the Aquaculture Act, to engage in the type of aquaculture in such parts of Namibia or Namibian waters as described below, for the period described in this licence and in accordance with the terms and conditions set out in the Aquaculture Act and the conditions set out in this licence.

1. The name and business address of the licence holder;
   Prof O Mwandemele, Dr Sam Nuyoma Marine and Coastal Research Center, P O Box 462, Henties Bay.

2. Location, size and description of the site at which the aquaculture is authorized;
   South of Henties Bay. The area designated by the Town Council for aquaculture purposes. An area of 15ha.
   North of Henties Bay at the Dr Sam Nuyoma Marine and Coastal Research Center.

3. The aquatic organism to be cultivated and type of grow out system to be used to which the authorization applies;
   All marine species to be farmed with for research purposes.
   Ponds, tanks and aquaria.

4. The maximum annual production authorized (number of weight);
   For research purposes.

5. The source of water supply at the aquaculture facility;
   Ocean water.

5. The source of water supply at the aquaculture facility;

Ocean water pumped onshore.

6. The composition and annual, quantity of any effluent to be discharged from the aquaculture facility.

No effluent containing toxins or environmental pollutants should be discharged back to the ocean. Discharge sea water should be filtered through and gravel/sand filter prior to be pumped back to the ocean.

CONDITIONS OF LICENCE

A. This licence is to be utilised within the Aquaculture Act and Regulations.

B. To abide by conditions laid down by Henties Bay Town Council pertaining to the abstraction and discharge of sea water.

7. Notifications required.

Any changes to the company and ownership of this licence, the site and effluent discharges, should be communicated to the office of the Permanent Secretary, Ministry of Fisheries and Marine Resources.

PERIOD OF VALIDITY

Subject to the Aquaculture Act, and the Regulations made there under, this licence is valid from 1st day of February 2006, to the 1st February day of February 2021.

Dr Abraham Iyambo
MINISTER

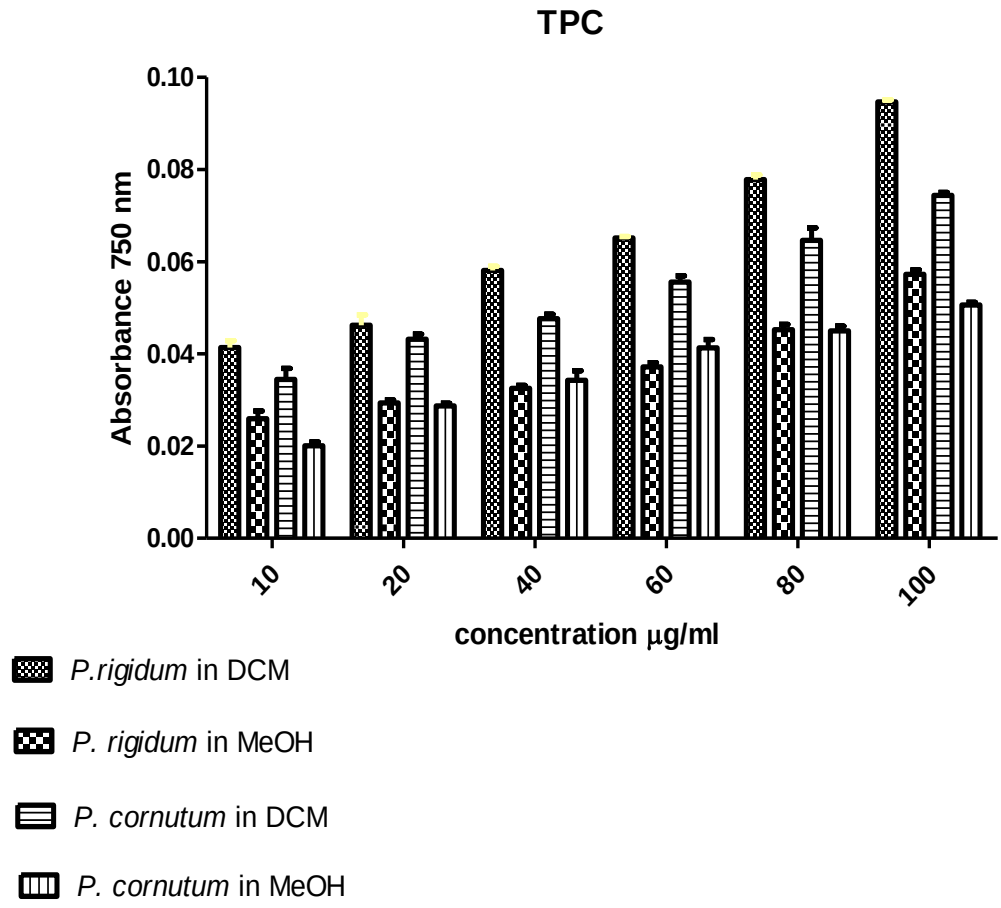
DATE

MINISTRY OF FISHERIES+MARINE RESOURCES
2006 -02- 01
PRIVATE BAG 13355
WINDHOEK

Appendix 3 Gallic acid standard absorption calibration data

Conc. mg/ml	Triplicate readings			Average values
	1	2	3	
5	0.0340	0.0338	0.0348	0.0342
10	0.0450	0.0447	0.0460	0.0452
20	0.0407	0.0428	0.0394	0.0497
40	0.1212	0.1235	0.1212	0.1220
60	0.1568	0.1420	0.153	0.1506
80	0.1697	0.1732	0.1723	0.1717
100	0.1971	0.1949	0.1963	0.1961

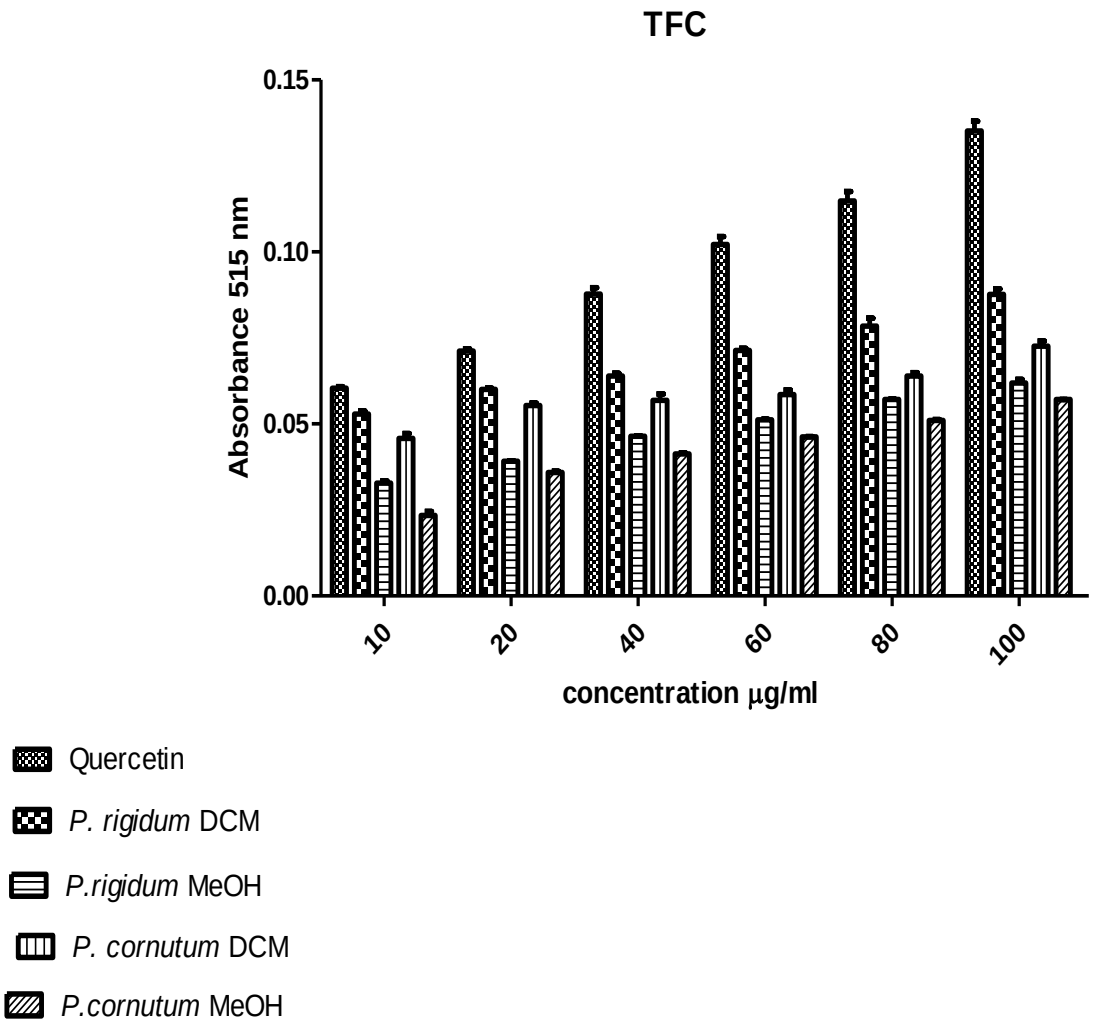
Appendix 4. Bar charts of phenolic absorption of extracts against concentration



Appendix 5. Quercetin standard absorption calibration data

Conc mg/ml	1	2	3	Average
0	0.0454	0.0465	0.0445	0.0455
20	0.0691	0.0748	0.0749	0.0729
40	0.0853	0.0855	0.0964	0.0891
60	0.0987	0.1012	0.1165	0.1055
80	0.1159	0.1237	0.1297	0.1231
100	0.1194	0.1386	0.1474	0.1351

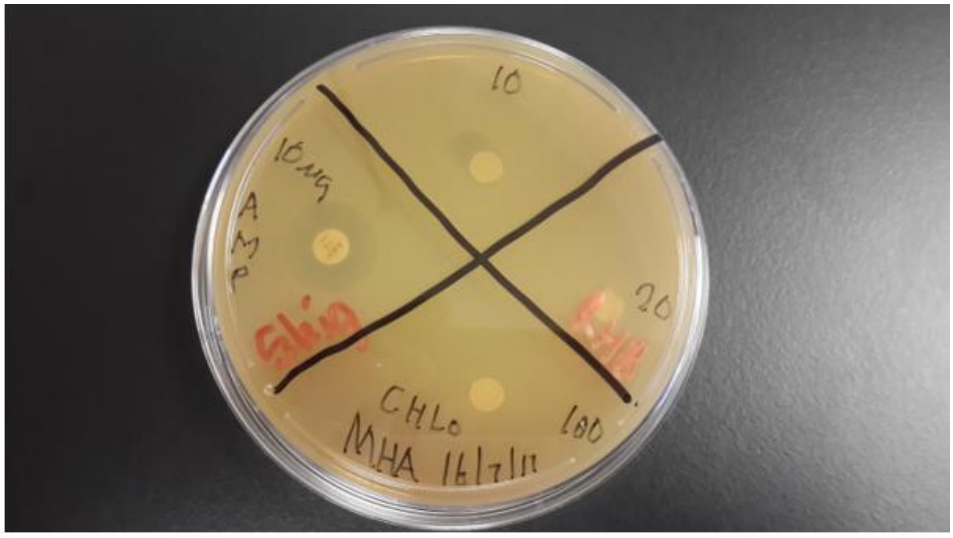
Appendix 6. Bar charts of flavonoid absorption of extracts against concentration.



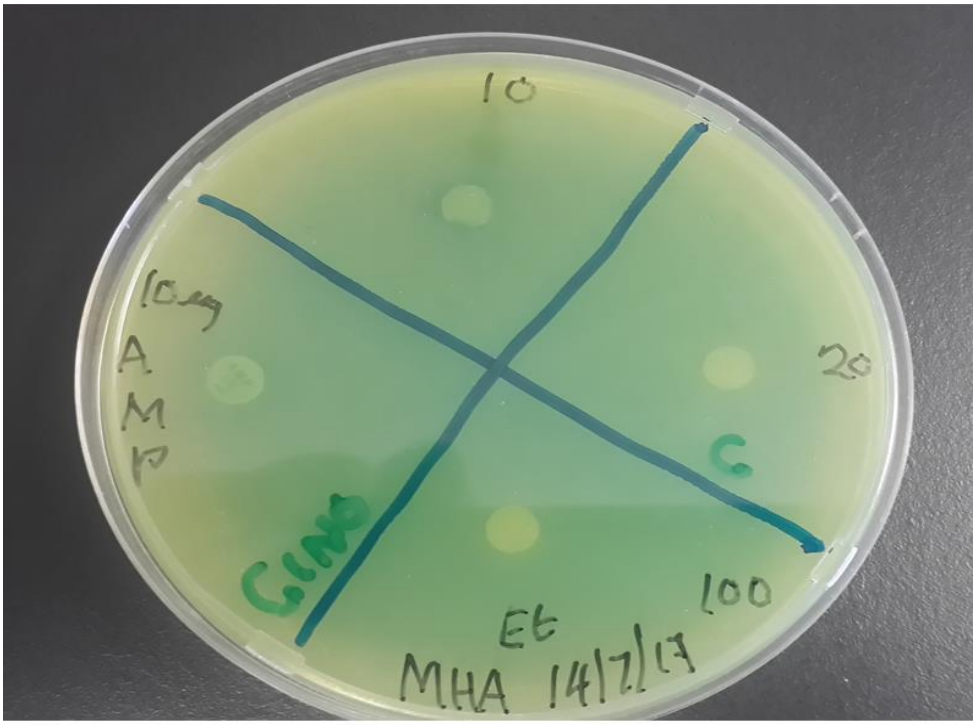
Appendix 7. Percentage scavenging activity of extracts in DCM and Methanol

Conc µg/ml	Ascorbic acid	*P. rigidum* DCM	*P. cornutum* DCM	*P. rigidum* MeOH	*P.cornutum* MeOH
3.1	32.44	26.42	22.08	15.75	14.28
6.5	44.82	30.10	24.94	19.05	18.39
12.5	47.89	34.81	27.97	21.43	19.97
25	50.98	38.04	32.21	30.01	25.18
50	62.38	46.77	41.96	38.00	33.93
100	66.85	53.79	46.41	45.63	36.4
200	74.00	59.77	53.22	50.05	42.01

Appendix 8. Antimicrobial activity



Inhibition of ampicillin and *Plocamium rigidum* against *Shigela sonnei*



Inhibition of ampicillin and *Plocamium rigidum* against *P. aeruginosa*

Inhibition of ampicillin and *Plocamium rigidum* against *S. epidermidis*

Appendix 9. Dose-response measurement (a) = *E. coli* and
(b) = *P. aeruginosa*

a. Log of average colony value for *E. coli*

	T_0	T_1	T_2	T_3	T_4	T_5
Gentamycin	8	6.323018	4.029523	0	0	0
Ampicillin	8	6.349999	3.829412	3.236196	0	0
D-1	8	7.470191	6.070954	5.022149	3.685084	0
D-2	8	7.821632	6.756441	6.748767	6.624745	5.988862
D-3	8	7.886965	7.524614	7.425244	7.205192	6.877271
Negative	8	7.888443	7.889637	7.889848	7.878127	7.623839

b. Log of average colony value for *P. aeruginosa*

	T_0	T_1	T_2	T_3	T_4	T_5
Gentamycin	8	5.223931	4.569035	0	0	0
Ampicillin	8	5.441643	4.901362	3.83694	0	0
D-1	8	6.423453	6.351017	6.271597	6.242502	6.012892
D-2	8	6.59428	6.558481	6.535872	6.524514	6.172859
D-3	8	6.840803	6.82827	6.813503	6.813503	6.627745
Negative	8	6.948407	6.945124	6.93944	6.810971	6.627745

Key: T_0 = Before treatment started, T_1 = first day after treatment, T_5 = five days after treatment

Appendix 10a. HPLC purification of *Plocamium* sample I

Injection date: 5/29/2017 10:15:30 AM

Sample name: corn_sw Location: vial 1

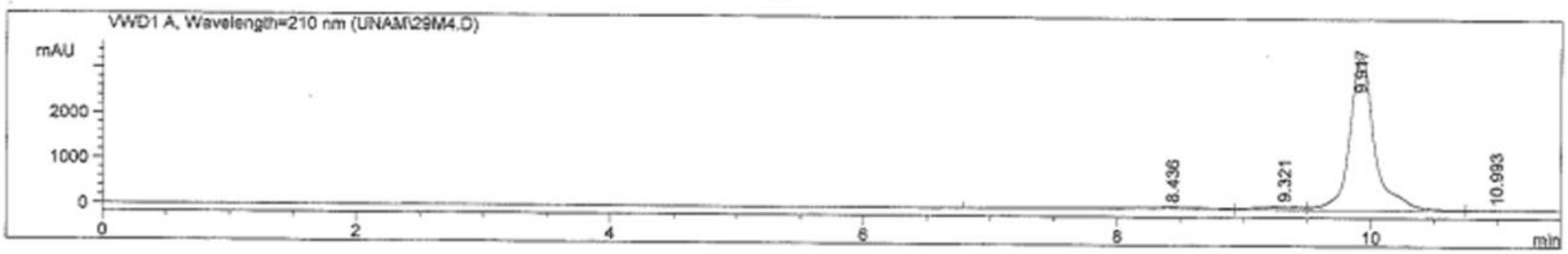
Acq Operator: J du Preez / A. Ishola

Acq. Instrument: H8 Inj. volume: 50 µl

Plant extract on 250 x 10 mm Synergi Fusion RP 4 µm

90% acn stop 15 min 210 nm.

3 ml/min, 50.0 µl inj.



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Appendix 10b. HPLC purification of *Plocamium* sample II

Injection date: 5/29/2017 11:29:49 AM

Sample name: Rigid. SW Location: vial 101

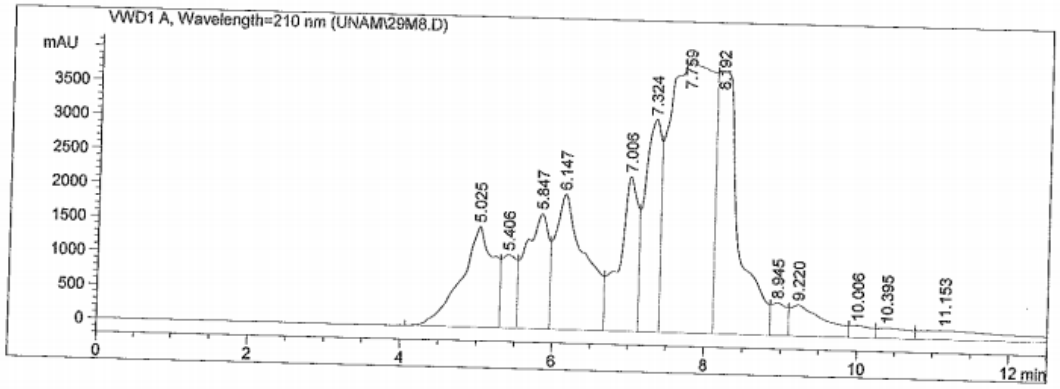
Acq Operator: J du Preez / A. Ishola

Acq. Instrument: H8 Inj. volume: 50 µl

Plant extract on 250 x 10 mm Synergi Fusion RP 4 µm

90% acn stop 15 min 210 nm

3 ml/min, 50.0 µl inj.



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=210 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	5.025	BV	0.3694	4.36679e4	1471.85938	8.6412
2	5.406	VV	0.1947	1.40912e4	1068.32336	2.7884
3	5.847	VV	0.2861	3.52996e4	1678.85498	6.9853
4	6.147	VV	0.3623	5.48984e4	1974.21667	10.8636
5	7.006	VV	0.2318	3.81173e4	2259.42310	7.5429
6	7.324	VV	0.2202	4.46415e4	3104.33447	8.8339
7	7.759	VV	0.4847	1.56464e5	3955.79321	30.9619
8	8.192	VV	0.2959	7.89685e4	3828.94678	15.6267
9	8.945	VV	0.1955	6507.49951	472.90726	1.2877
10	9.220	VV	0.4059	1.41780e4	452.99054	2.8056
11	10.006	VV	0.2750	3352.42236	171.24673	0.6634
12	10.395	VV	0.4209	4222.62646	146.40662	0.8356
13	11.153	VBA	1.0816	1.09345e4	129.06062	2.1638
Totals :				5.05343e5	2.07144e4	

Results obtained with enhanced integrator!

Appendix 11 Parameters for pIC50 against 2-molecular descriptors.

a. pIC_{50} against $LogIC_{50}$ and MinElPot

pIC_{50} = 0.54logP -0.01MinElP – 6.525

(R^2 =0.719, R^2 (adj) = 0.578, Std. error = 0.303, RMSE = 0.228, q2 = 0.134)

b. pIC_{50} against $LogIC_{50}$ and MaxElPot

pIC_{50} = 0.49LogP + 0.10maxElPot – 4.992

R^2, = 0.616, R^2 (adj) 0.425, Std error, 0.354, RMSE 0.267, q2 = 0.252

c. pIC_{50} against $LogIC_{50}$ and CPKOvality

pIC_{50} = 0.70LogP + 2.57CPKovality – 13.646

R^2, = 0.554, R^2 (adj) 0.331, Std error, 0.381, RMSE 0.223, q2 = 0.256

d. pIC_{50} against $LogIC_{50}$ and Dipole moment

R^2, = 0.642, R^2 (adj) 0.462, Std error, 0.342, RMSE 0.259, q2 = 0.194

pIC_{50} = 0.38LogP + 0.2Dipole – 3.328

e. pIC_{50} against $LogIC_{50}$ and HOMO-LUMO gap

pIC50 = 0.33LogP + 0.195H-L gap – 3.418

R^2, = 0.524, R^2 (adj) 0.286, Std error, 0.394, RMSE 0.297, q2 = 0.352

f. pIC_{50} against Polarizability and MinElPot

pIC_{50} = 0.61Polar – 0.00MinElPot – 38.281

R^2, = 0.661, R^2 (adj) 0.491, Std error, 0.332, RMSE 0.180, q2 = 0.253

g. pIC50 against Polarizability and MaxElPot

pIC_{50} = 0.76Polar +0.00MaxElPot – 46.225

R^2, = 0.679, R^2 (adj) 0.519, Std error, 0.323, RMSE 0.194, q2 = 0.252

h. pIC_{50} against Polarizability and CPKOvality

pIC_{50} = 1.38Polar – 32.92ovality – 36.780

R^2, = 0.787, R^2 (adj) 0.680, Std error, 0.264, RMSE 0.042, q2 = 0.208

i. pIC_{50} against Polarizability and Dipole moment

pIC_{50} = 0.60Polar – 0.06Dipole – 37.093

R^2, = 0.662, R^2 (adj) 0.494, Std error, 0.332, RMSE 0.193, q2 = 0.225

j. pIC_{50} against Polarizability and HOMO-LUMO gap

pIC_{50} = 0.49Polar + 0.153H-L gap – 31.565

R^2, = 0.778, R^2 (adj) 0.668, Std error, 0.269, RMSE 0.164, q2 = 0.241

k. pIC_{50} against MinElPot and MaxElPot

pIC_{50} = 0.01MinElPot + 0.01MaxElPot – 2.058

R^2, = 0.180, R^2 (adj) -0.230, Std error, 0.517, RMSE 0.391, q2 = 0.518

l. pIC_{50} against MinElPot and CPKOvality

pIC_{50} = -0.00 MinElPot + 11.16ovality – 17.639

R^2, = 0.517, R^2 (adj) 0.275, Std error, 0.397, RMSE 0.298, q2 = 0.242

"*The real voyage of discovery consists not in seeking new lands but seeing with new eyes*" Marcel Proust

TO GOD BE THE GLORY FOR EVER