GHANA'S MANGROVE WETLAND ENDOPHYTIC FUNGI: CYTOTOXICITY, ANTI-PLASMODIAL AND APOPTOTIC ACTIVITY OF QUINOLACTACIN A1/A2, CITRINADIN A AND BUTRECITRINADIN

BY

KOFI BAFFOUR-AWUAH OWUSU

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CHEMISTRY

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DECLARATION

I, **Kofi Baffour-Awuah Owusu**, declare that the work contained in this thesis was undertaken solely by me under supervision and has neither wholly nor partially been presented elsewhere for another degree.

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Kofi Baffour-Awuah Owusu (BSc.)

10442006

…………………………………………

Kwaku Kyeremeh (PhD)

(PRINCIPAL SUPERVISOR)

…………………………………………

Mark Ofosuhene (PhD)

(Co-Supervisor)

ABSTRACT

Marine-derived endophytic fungi isolated from mangrove plants are an important and novel resource of natural bioactive compounds with their potential applications in medicine, agriculture and food industry. There are many instances that seem to suggest that, some of the novel metabolites previously isolated from certain species of marine plants and invertebrates are actually metabolites of marine-derived endophytic fungi. In this project, chemical screening of mangrove plants*Conocarpus erectus*, *Laguncularia racemosa* and *Rhizophora racemosa*collected along the banks of the River Butre showed that the compound 14S-bromo-1S-hydroxy-1,2,13,14-tetrahydrosphaerococenol A is not a biosynthetic product of the red alga *Sphaerococcus coronopifolius* but a product of possible endophytic fungi living inside the alga tissues. The compound 3-(phenethylamino)demethyl(oxy)aaptamine and its derivatives like aaptamine are not necessarily the biosynthetic products of the sea sponge *Aaptos aaptos* but originate from endophytic fungi within the tissues of the sponge. The mycospurine amino acid N-methylpalythine-serine originally deemed to be the metabolites of the stony coral *Pocillopora eydouxi*could actually be the biosynthetic products of marine-derived endophytic fungi.

Mangrove plants sampled along the banks of River Butre were investigated for new or novel secondary metabolites. The HRESI/HPLC-DAD-MS^n dereplication technique was employed in identifying and isolating new secondary metabolites from the mangrove plants. Out of twenty-four (24) marine-derived endophytic fungi isolated from various parts of three major mangrove plants growing in and along the banks of the River Butre in the Western Region of Ghana, six (6) different species were selected for chemical profiling, prioritization, identification and subsequent isolation

of compounds. Measurement and analysis of HRESI/HPLC-DAD-MS^n data of the small scale culture of these six (6) species led to the prioritization of *Penicillum* sp. BRS2A-AR2. *Penicillum* sp. BRS2A-AR2 was prioritized due to its ability to make a wide range of interesting secondary metabolites with biosynthesis prowess and ingenuity.

The HRESI/HPLC-DAD-MS^n analysis of *Penicillum* sp. BRS2A-AR2 was prioritised for further investigation because it produced metabolites which did not give any relevant hit when their masses were entered in the marine natural product based commercial database MarinLit, AntiMarin and Antibase database for novelty. The structures of these metabolites were elucidated by 1D and 2D NMR data interpretation.

Quinolactacin A1/A2 was isolated with *m/z* of 270.1362. Citrinadin A and a new compound Butrecitrinadin were also isolated with *m/z* of 625.3962 and 681.4226 respectively. Citrinadin A and Butrecitrinadin were virtually identical with similar mass fragmentation pattern, eluting at the same time, having same retention time and collected together.

Biological activity studies on the compounds and other compounds from the research group showed the anti-proliferative ability of the two steroidal compounds to inhibit human prostate cancer cell line with IC$_{50}$ of 5.58 and 5.28 µM. Butrepyrazinone, Citrinadin Aand Butrecitrinadin showed cytotoxicity against cell lines tested. Quinolactacins isolated also showed anti-plasmodial activity with IC$_{50}$ of 24.80 µM and also, showed apoptotic activity via the loss of mitochondrion membrane potential of the plasmodium parasite.

DEDICATION

This thesis is dedicated to my parents Mr. & Mrs. Owusu-Berko for teaching me the value of hard work and perseverance. Your prayers, motivation, love and support inspired me in the course of the study.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
COSY	Correlation Spectroscopy
CD	Circular dicroism
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Median Effective Concentration
ED_{50}	Median Effective Dose
EtOAc	Ethyl acetate
ESMS	Electrospray Mass Spectrometry
FD	Dichloromethane Fraction
FH	Hexane Fraction
FM	50% aqueous methanol fraction
G8	Group of Eight Nations
GDP	Gross Domestic Product
GI_{50}	Median Growth Inhibition
GPS	Global Positioning System

HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
HTS	High throughput screening
IC_{50}	Median Inhibition Concentration
ICPMS	Inductively Coupled Mass Spectrometry
LC	Liquid Chromatography
LC_{50}	Median Lethal Concentration
LD_{50}	Median Lethal Dose
MeOH	Methanol
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibition Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance Spectroscopy
PDA	Photodiode Array Detector
PTM	Polycyclic Tetramic Acid Macrolactams
RNA	Ribonucleic acid

SASW	Sterile Artificial Sea Water
TCE	Total Crude Extract
TOCSY	Total Correlation Spectroscopy
UV	Ultraviolet
WB	Water/Sec-butanol fraction

CHAPTER ONE

1.0 INTRODUCTION

A review by Newman and Cragg in 2012 provided a solid proof with appropriate statistics of the fact that natural products play an indubitable role in drug discovery at all disease frontiers. Within the period 1981-2010, close to 61% of our approved drugs had some kind of a linkage to natural product sources.[1]

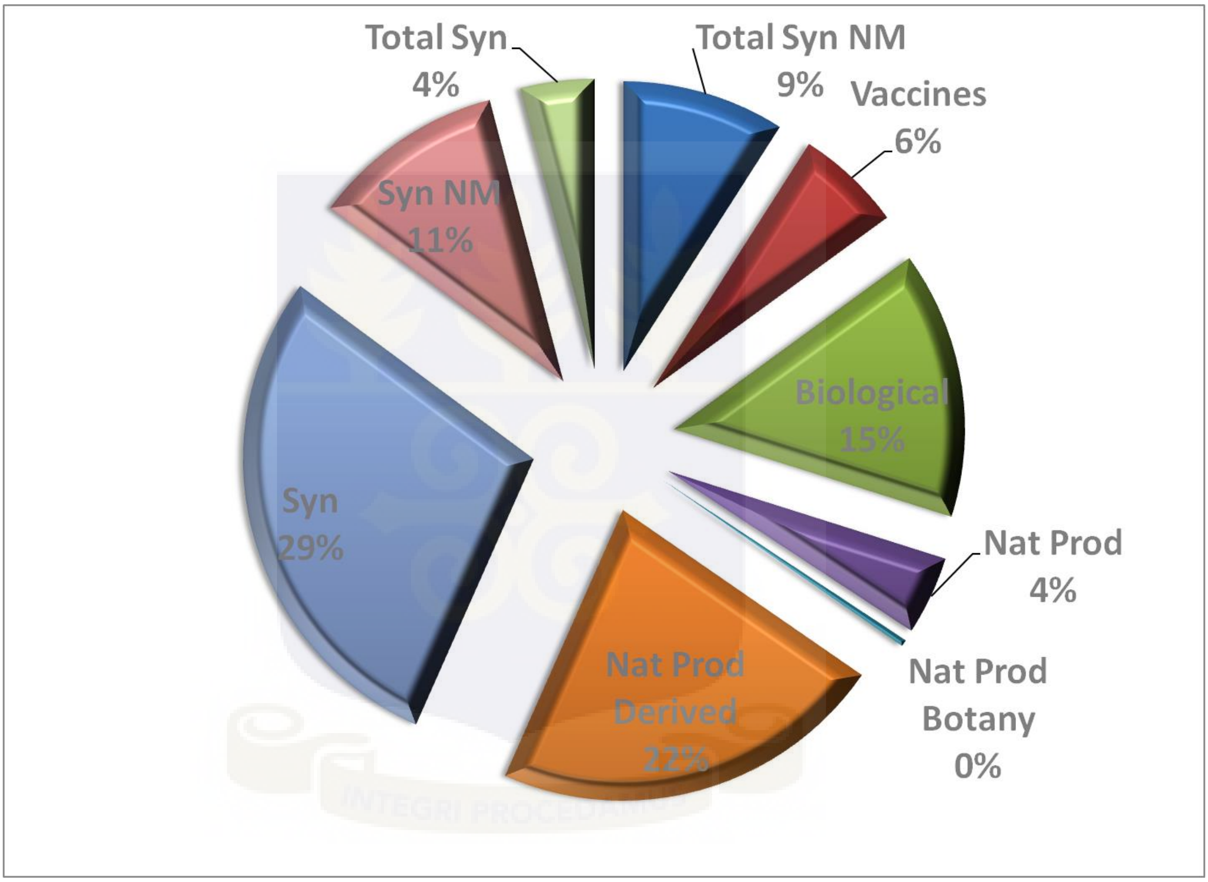
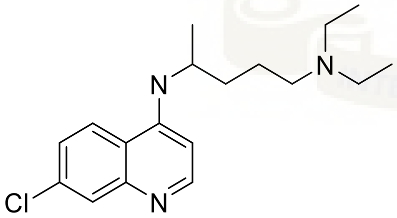


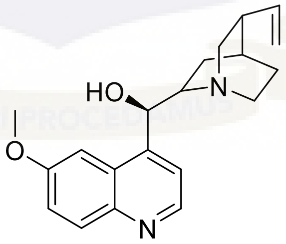
Figure 1.1: All new approved drugs over a period of thirty years 1981-2010.

However, the percentages change when individual disease areas are considered because, while natural product sources provide several leads every year for some diseases, in some disease areas leads from natural sources are particularly rare.[1]For example, natural products have been very successful in the discovery of anticancer drugs with more than 128 new chemical entities approved within 1981-2010 period.[1]In sharp contrast, no natural product drugs were approved as antihistamines, diuretics or hypnotics within the same study period.[1]

Amongst all the parasitic diseases that are endemic to most developing countries malaria is the one that is most researched worldwide.[2-4] Other parasitic diseases like African sleeping sickness, Chagas disease, leishmaniasis, cryptosporidiosis, amoebiasis etc have very low research inputs worldwide.[5] Malaria continues to be a major threat in the world with 500 million infections and 1 million deaths every year. Currently, approximately 40% of the world's population reside in areas of active malaria transmission with the disease and its symptoms most severe in children and pregnant women.[6-13] A licensed vaccine for malaria has not materialised and although chloroquine, the first synthetic drug developed as an antimalarial with inspiration from the naturally occurring quinine provided an almost magical cure for over 30 years, the emergence of the chloroquine-resistant parasites has made it virtually ineffective in most parts of the world.[14-18]

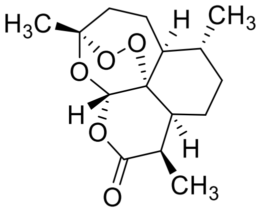


Chloroquine



Quinine

Currently, the plant-derived antimalarial artemisinin is the only available drug that is globally effective against malarial parasites.[19]Although several new drugs have been introduced in the past 30 years, widespread or isolated cases of resistance indicate that their window of effectiveness is most definitely limited.[20, 21]



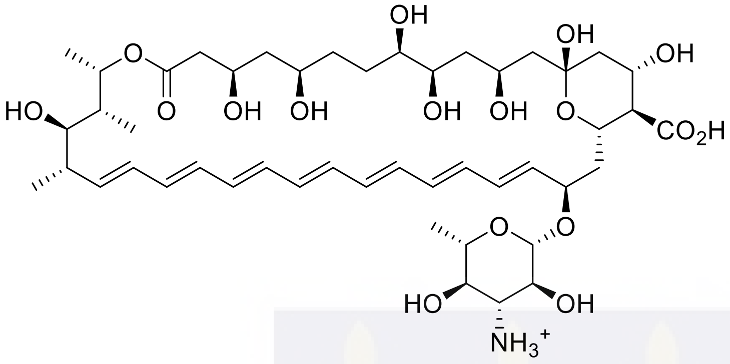
Artemisinin

Hence, there is the need to discover new antimalarials and the previous successes of chloroquine a synthetic derivative of quinine and artemisinin and its synthetic derivatives suggests that nature could prove to be the best source of our future antimalarials.[22-26] Our future antimalarials must be:

- effective and promiscuous by acting through more than one mechanisms of action
- low cost and affordable to the poorest inhabitants on this planet
- novel chemotypes that are entirely different from the currently available structures.

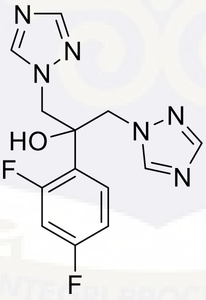
Microbes mainly bacteria and fungi have provided a phenomenal contribution to the health and well-being of people throughout the world. In addition to producing many post-biosynthetically modified primary metabolites such as amino acids, vitamins and nucleotides, they have a tremendous ability to produce secondary metabolites which have become an important constituent of pharmaceuticals on the market and provide industry and agriculture with many essential products.[27-30] However, a comparison of the success rate of bacteria and fungi shows a big skew where discovery and subsequent approval of antifungal drugs have been relatively rare.[31-35]Only three classes of molecules are currently used to treat invasive, life-threatening fungal infections in clinical practice. Out of these three, two of them azolesand polyenes were already in the clinic before 1980. From 1980-to date, only one new class of antifungal have emerged in the clinic and these are known as the echinocandins.[35]

Example of Polyene Class Antifungal
mainly natural products

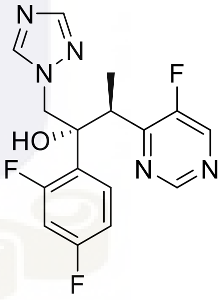


Amphotericin B

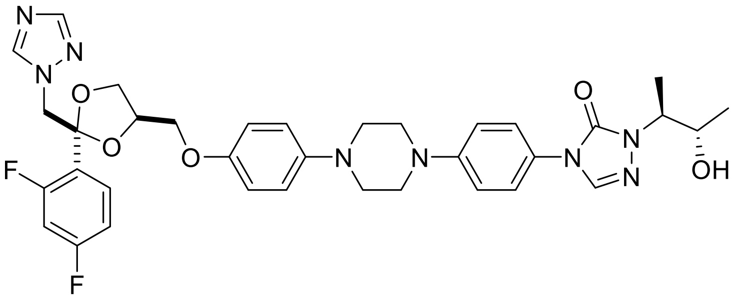
Examples of Azole Class Antifungal
mainly synthetic compounds



Fluconazole

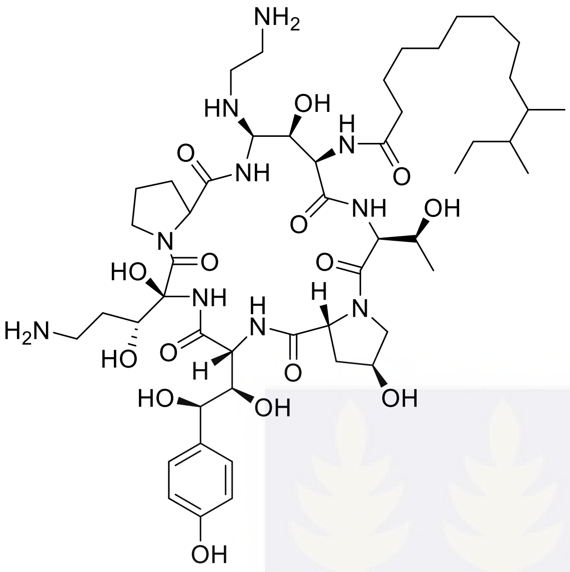


Voriconazole

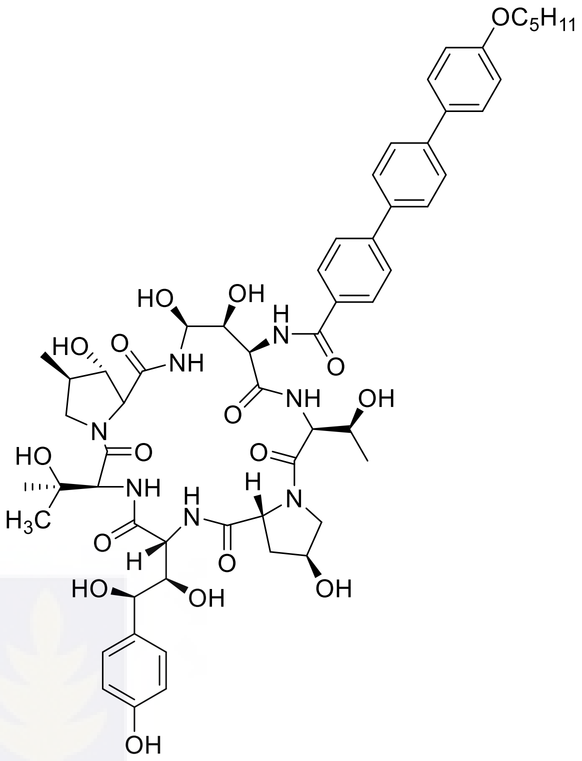


Posaconazole

Examples of Echinocandin Class Antifungals
mainly natural products



Caspofungin



Anidulafungin *(semi-synthetic derivative)*

Three main factors account for this very slow pace in the development and approval of new antifungals and these are as outlined below.

- The first fundamental and philosophical challenge is that, fungal pathogens unlike bacteria are very closely related to their host which in this case represents humans. Many inherent physiological, biochemical and cell biological processes are conserved from fungi to humans. The direct implication is that, many small molecules that are toxic to yeast are likewise toxic to humans through similar mechanisms of action. Prototype antifungals must possess a high level of selectivity and target processes that are only unique to fungi of which there are only a few. Fortunately, such high selectivity also finds application in other drug discovery pipe lines including anticancer and antiparasitic drug development.

- Secondly, the evaluation of new antifungals has challenges directly related to the effective design of the clinical trials stages as a result of the higher risks involved.
- Thirdly, the two challenges mentioned here add to the routine fundamental challenges including well-documented scientific, economic and regulatory factors that are inherent to the discovery of anti-infectives in general.[35-37]

Notwithstanding the problems outlined above, invasive life-threatening fungal infections are an important cause of mobidity and mortality, particularly for patients with heavily compromised immune function like those leaving with HIV-AIDS and primary immune deficiency or those that are on some kind of cancer chemotherapy, immune-modulatory medications or patients of hematologic and solid organ transplantation.[35] Hence, the need to identify new or novel antifungal chemical structures that can also find application as anticancer or antiparasitic drugs is eminent and cannot be overemphasized.

However, as a result of the difficulties already outlined herein, very potent strategies are needed in order to ensure that in the next 5-10 years, new or novel chemical entities will emerge in the drug discovery pipeline for the treatment of yeast infections.

A look at the currently available drugs shows two natural products derived molecular entities polyenes (bacteria derived) and echinocandins (fungi derived) versus one entirely synthetic chemical class, the azoles. Hence, detailed scout of new natural sources (niches or habitats) could yield new or novel antifungal drugs possibly with some anticancer or antiparasitic activity.[38-41]The exact natural source to be investigated must also be chosen in such a way as to maximise the frequency of obtaining hits which could evolve into leads and eventually become drugs. The two main sources where maximised efforts are most likely to yield good results are bacteria and the fungi themselves.

The philosophy behind interests in these two is no different from the current philosophy that drives the discovery of anti-infectives in general. Competition for substrates amongst these microbes forces them to make secondary metabolites that possess great selectivity and target very specific physiological, biochemical and cellular processes of some microbes. These molecules when recruited directly or altered through semi-synthesis can provide novel leads for the development of new antifungals.

This thesis represents an attempt using Ghanaian mangrove plant-derived endophytic fungi to fulfil the need to feed the current drug discovery pipeline with antifungals, antiparasitics and anticancer drug prototypes and ensure that, these molecules will in the next 5-10 years provide new treatments for clinical practice. Several different kinds of endophytic fungi were isolated from a collection of mangrove plants/plant parts that were collected along the banks of the Butre River in the Western Region of Ghana. Small scale culture and subsequent screening of their metabolites using high resolution electrospray ionization liquid chromatography tandem mass spectrometry (HRESI-LCMS^n) led to the prioritization and detailed chemical investigation of one species,*Penicillium* sp. BRS2A-AR2.

1.1 Problem Statement

Current medical practices have been very successful to curtail a majority of the diseases that previously made life on earth very difficult. However, currently there are many problems that seem to suggest a necessary speed-up in the discovery of novel drug entities with entirely different mechanisms of action. There are still diseases that have no cures and this comes amidst the current widespread of new diseases, the spreading geographically of known diseases with increased virulence alongside the development of strong resistance to treatment by known diseases. The current world-wide philosophy is to intensify the search of new un- or underexplored extreme environments or habitats that harbour novel organisms capable of producing novel chemistry. Ghana represents one of the world's biodiversity hotspots with several un- or underexplored extreme habitats where explorations on for example, mangrove plant derived endophytic fungi for possible future drug prototypes is an entirely new subject but has a lot of potential to provide future drug leads.

1.2 Overall Goal

The overall goal of this project is to examine the 'talent profile' of Ghanaian marine endophytic fungi to produce new or novel secondary metabolites that possess the ability to act as future drugs for the treatment of yeast and parasite infections or cancer.

1.3 Hypothesis

The Western region of Ghana is characterized by several mangrove patches that are un- or underexplored with respect to their novel microbial species. Ghanaian mangrove plants harbour a wide variety of new or novel endophytic fungi strains that like their counterparts elsewhere biosynthesise a wide range of structurally intriguing and medicinally important secondary metabolites.

1.4 Objectives

1) To collect indigenous mangrove plant samples from several well chosen sites on the Butre River, starting from the source to about 1000 m up-river where there is little or no human activity.
2) To mark the positions of all the plants collected in a GPS and store data on the Google Earth GPS satellite.
3) To identify all the plants sampled and store voucher specimens at the University of Ghana in Legon.
4) To choose different mangrove plant parts and isolate the endophytic fungi that can be found within the tissues of these parts using a standard protocol and malt extract media.
5) To purify isolated endophytic fungi by repeated sub-cultures until very pure strains are obtained on malt extract agar plates.
6) To obtain the chemistry profile of the isolated species of mangrove endophytic fungi through small scale fermentation, subsequent extraction of metabolites and acquisition of high resolution HPLC-Mass spectrometry data.
7) To single out high priority endophytic fungi that are capable of making new or novel secondary metabolites by entering UV and accurate mass data into two marine natural product databases; Marin-Lit and Antimarin.
8) To do a large scale fermentation of high priority species of endophytic fungi and obtain enough extracts that allows the subsequent isolation of compounds of interest in reasonable quantities.
9) To extract secondary metabolites from the large scale fermentation media using HP-20 resin.

10) To extract secondary metabolites from the HP-20 resin and fungal mycelia using an alternating sequence of methanol and dichloromethane extractions (Total Crude Extract or TCE).

11) To subject TCE to a modified Kupchan solvent partitioning process that eventually results into four fractions FH, FD, WB and FM.

12) To obtain high resolution HPLC-Mass spectrometry data for Kupchan solvent partitioning fractions and determine which fractions contain compounds of interests.

13) To isolate compounds of interest from high priority Kupchan fractions using size-exclusion, gravity column and HPLC chromatography.

14) To obtain spectroscopic and spectrometric data of the isolated compounds for the elucidation of their structures.

15) To test for anticancer, antimalarial and anti buruli ulcer activity of the pure compounds and determine the mechanisms of action in the case of any positive tests.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mangrove wetlands

Collectively, mangroves are a group of trees and shrubs that live in the coastal inter-tidal zone. In order to survive in this habitat, these plants are normally very tolerant to salt, high humidity, low oxygen content of sediments, and constantly varying conditions of temperature, pressure and nutrients.[42-44] Typically, there are about eighty (80) different kinds of trees that inhabit mangroves worldwide but, each mangrove habitat has its own collection of trees and shrubs that can be found to recur throughout the area occupied by the mangrove. Some of the floras in mangroves are true mangrove plants while others are basically normal plant species that are associated with the mangroves.[45,46]

Mangrove forests only grow at tropical and sub-tropical latitudes near the equator because; they are not able to withstand freezing temperatures. Many mangroves can easily be recognized by their dense tangle of prop and buttress breathing roots that make the trees appear to be standing on stilts above the water. This tangle of roots allows the trees to take up oxygen and handle the daily rise and fall of tides, which means that most mangroves get flooded at least twice per day. The roots also slow the movement of tidal waters, causing sediments to settle out of the water and build up a very muddy bottom. Mangrove forest generally stabilizes the coastline, reducing erosion from storm surges, currents, waves and tides.[42-44]

Mangroves have become an important extreme habitat in the continued research to discover new drug entities from marine derived organisms as a result of the following reasons:

These wetlands are a great assembly point of novel marine microbes including bacteria and fungi. As a result of the rather harsh environmental conditions in these wetlands, microbes that have been isolated and studied in these habitats have proven to exhibit a lot of biosynthetic prowess.

Compared to the deep oceans, mangrove habitats are much easier to access and sample. Small boats, canoes, rafts and other small vessels can be used to access the most remote of mangrove wetlands and in addition sample grabs, shovels or much simpler equipment's can be used for sampling. The deep oceans require ships, larger boats, paid crew and divers with expensive remotely operated submarines to effectively access invertebrates and sediments.

Large sections of mangroves are under constant threat of extinction worldwide and their novel species if not studied might be lost forever.[40,47-51]

In Ghana, mangrove wetlands cover close to 140 km^{2}but are largely limited to a very narrow, non-continuous coastal area that surrounds lagoons on the western and eastern sections of the country and the lower fringes of the Volta River. Mangrove wetlands are most extensive to the west in the stretch between Cape Three Points and Côte d'Ivoire, especially in areas around Half Assini, Sekondi Tarkoradi, Amanzure lagoon, Axim, Princes Town and Shama. To the east of the country, they are best developed at Apam, Muni lagoon, Winneba, Sakumono lagoon, Bortiano, Korle lagoon, Teshie, Ada, Sroegbe and Keta lagoon.[52-54] Six species of mangroves that are typical of West-Central African mangroves are found in Ghana and these include: *Acrostichum aureum*, *Avicennia germinans*, *Conocarpus erectus*, *Laguncularia racemosa*, *Rhizophora harrisonii*, and *Rhizophora racemosa*. The open lagoons tend to be dominated by *Rhizophora racemosa*, whilst closed lagoons with an elevated salinity contain *Avicennia germinans*, *Conocarpus erectus*, *Laguncularia racemosa* and *Acrostichum aureum*.[52-55]

Ghanaian mangrove wetlands have not been previously explored for the possible isolation of new endophytic fungi and subsequent chemical screening thereof. This project represents the first ever attempt to screen these wetlands for possible isolation of new or novel species that have the ability to make biologically active compounds.

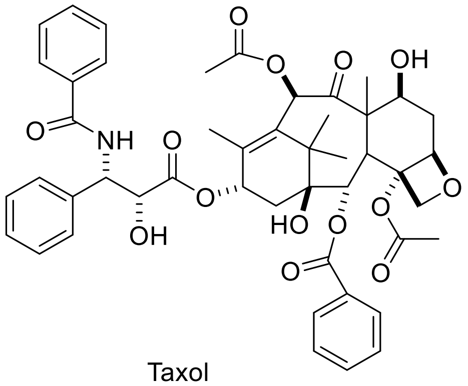
2.2 Brief introduction to mangrove plant endophytic fungi

Endophytic fungi are indeed eukaryotic fungal microorganisms which spend all or part of their lifecycle inter- and/or intra-cellular colonizing healthy tissues of their hosts plants.[56-63]They constitute a polyphyletic group of highly diverse, primarily ascomycetous fungi defined functionally by their occurrence within asymptomatic tissues of plants. Typically, they cause no disease symptoms and their relationships with the respective plant hosts have been mostly described as symbiotic although this idea has in some cases proved contentious.[56-63]Endophytic fungi have been found in each plant species examined, and it is estimated that, there are over 1.5 million endophytic fungi existing in nature out of which only about 7% are known.[64] They are found in both the roots and aerial plant parts or for those types of marine flora which are entirely submerged by sea water in the cells and within the intercellular spaces of the plant. They are found in all plant types from the arctic to the tropics and from agricultural fields to the most biotic and diverse tropical forests.[56-63]

Their ecological significance is particularly complex and has most of the times been simplified to the scenario where the host plant provides good shelter, water, wide array of ready available nutrients and biosynthetic raw materials while in return the fungi protects its host from predators both micro and macro. As endophytes, they have an inherent ability to adapt to several sometimes rapidly changing environmental factors. For example, leaves and roots represent the most dynamic interfaces between plants and their environments. Endophytic fungi that inhabit these biologically active and fast growing tissues may share characteristics that allow them to grow and persist in an ever-changing biochemical milieu or in the context of rapidly changing gene expression as host tissues grow and age.[65,66] In contrast to this, endophytic fungi that colonize the submerged roots of mangrove plants, constantly find themselves in a matrix that in many cases contains lots of moisture, organic and inorganic materials, high salt content, low nutrient, temperatures or pressures and low to no light conditions. As a result of these harsh environmental stresses and changes, endophytic fungi have developed the most biosynthetically versatile and bio-diverse genomes allowing them to produce a very wide range of secondary metabolites that exhibit very important bioactivities.[65,66]

Since the first endophytic fungus was identified, a lot of attention has been given to the potential of exploitation of these fungi for the production of novel antibacterial, antifungal, anti-parasitic and anticancer agents. In some cases, in addition to producing a great number of diverse bioactive compounds which have been implicated in the protection of their host against pathogens and herbivores, some endophytic fungi have evolved and possess the ability to produce the same or similar compounds characteristic of their host plants.[56,67,68]

An important example is the anticancer agent taxol, which is currently used to treat different types of cancer including ovarian, breast, lung and pancreatic cancer amongst others. Taxol acts primarily by interfering with the normal breakdown of microtubules during cell division. This compound was originally discovered in 1962 as a result of a U.S. National Cancer Institute-funded screening program. It was isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. Thirty-one years later, the taxol producing endophytic fungus *Taxomyces andreanae* was identified.[62]



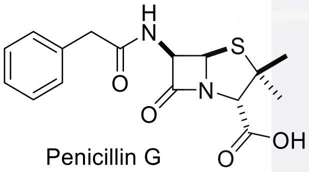
Taxol

The ability of some endophytes to produce the same rare and important bioactive compounds originally deemed characteristic of their host plant is a very important revelation. This phenomenon is of great importance and offers a prudent solution to the drug resupply issues. It effectively reduces the need to harvest plant or various plant parts which are rather slow-growing and possibly rare in occurrence and therefore, helps to preserve the world's ever diminishing biodiversity. Moreover, the production of a high value phytochemical by exploiting a microbial source is easier and more economical and it leads to increased availability and the reduced market price of the drug products.[69]

In conclusion, mangrove plant endophytic fungi represent a niche that should be meticulously investigated and used as a base for sustainable research and development of new antibacterial, antifungal, anti-parasitic and anticancer agents that can respond to current pathogenic, parasitic or cancer resistance and anticipate evolving resistance.

2.3 Bioactive compounds from marine-derived endophytic fungi

Since the discovery of penicillin in the last century, the diverse and bioactive fungal metabolites have played an important role in drug development. By the year 2005, nearly 35 drugs that originated from fungi were identified as antibacterials, immune suppressives and other therapeutics. Some were also found in clinical trials like the diketopiperazine compound phenylahistine which was projected as a future anticancer drug.[70,71]Furthermore, it is worth mentioning the antibacterial drug cephalosporin C which was originally discovered in 1945 from the marine fungus *Cephalosporium acremonium*.[72]

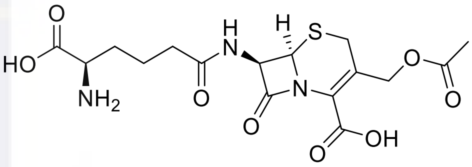


Penicillin G

Penicillium chrysogenum

Phenylahistine

Aspergillus ustus



Cephalosporin C

Cephalosporium acremonium

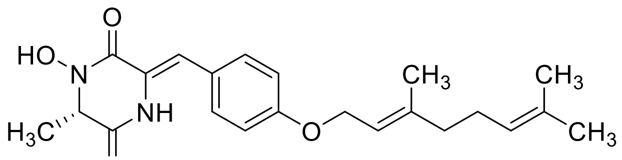
Despite the discovery of such important drugs from fungi, the number of bioactive natural products originated from marine fungi increased extremely slowly for a very long time. It is only during the last decade that researchers have returned focus on marine-derived fungi to search for novel bioactive secondary metabolites that could potentially be used as drugs.[73,74] Between the years 2006-2011, intensified research on marine derived fungi yielded many compounds of different functionalities and bioactivity.

2.3.1 Alkaloids isolated from marine-derived fungi

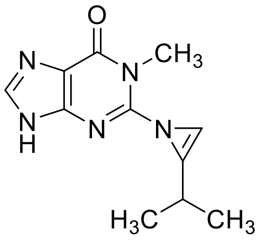
Marine-derived endophytic fungi have produced quite a number of alkaloids with fascinating structures and excellent biological activities. A detailed description of some of the structures is given below to provide a good comparison with the compounds presented in the results of this project.

The compound acremolin is a novel alkaloid obtained from the culture broths of *Acremonium strictum*. Structurally, the molecule constitutes a methyl guanine base containing an isoprene unit and a ^{1}H-azirine moiety, which is entirely unique in natural product chemistry. Acremolin exhibited weak cytotoxicity against A549 cell line with IC_{50} value of 45.90 µg/ml. It is however inactive against several strains of Gram-positive and Gram-negative bacteria.[75,76]

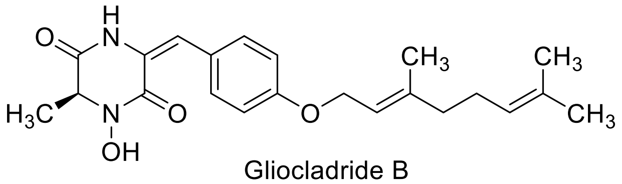
Generally, diketopiperazines are widespread in the chemistry of microbes especially fungi. Apart from the already mentioned phenylahistine, some important examples include the compounds gliocladride A and B and deoxymycelianamide which were isolated from the mycelia of marine fungus *Gliocladium* sp. Gliocladride A and B were found to possess moderate activity against HL-60, U937 and T47D with IC_{50} values from 11.6 µg/ml to 52.83 µg/ml. Deoxymycelianamide on the other hand, showed strong cytotoxic activity against U937 cell line with IC_{50} value 0.785 µg/ml.[77]



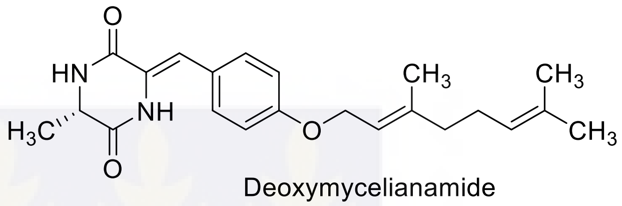
Gliocladride A



Acremoline

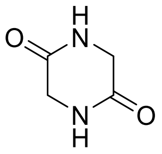


Gliocladride B

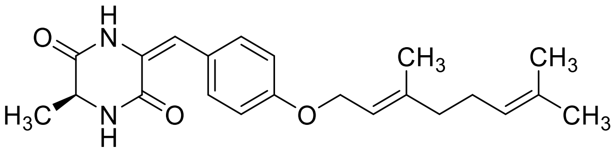


Deoxymycelianamide

The same fungus *Gliocladium* sp. produced the compounds PJ157 and glioclaride which appears to be an artefact. However, glioclaride exhibited strong cytotoxicity with an IC_{50} value of 3.86 µg/ml against human A375-S2 melanoma cell line. Looking at their structures therefore, it is easy to realise that the glioclaride moiety present in the structures of these compounds are responsible for the cytotoxicity activity generally seen in these compounds.[78,79] Also, it appears that the N-OH moiety in gliocladride A and B is likely to be responsible for the decreased cytotoxicity seen in these two compounds.[77]

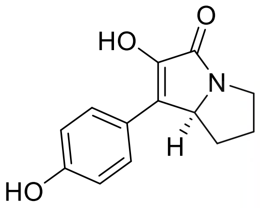


Glioclaride

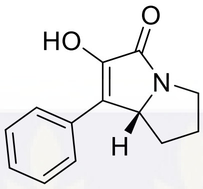


PJ157

The compound p-hydroxyphenopyrrozin isolated from the culture broths of the marine-derived fungi *Chromocleista* sp. exhibited potent antifungal properties by killing the yeast cells *Candida albicans* with MIC of 25 µg/ml. Interestingly, its dehydroxy derivative, phenopyrrozin did not produce any relevant activity against yeast cells.[80]

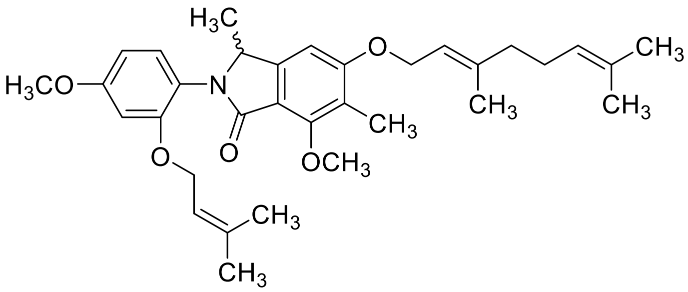


p-Hydroxyphenopyrrozin

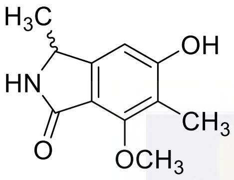


Phenopyrrozin

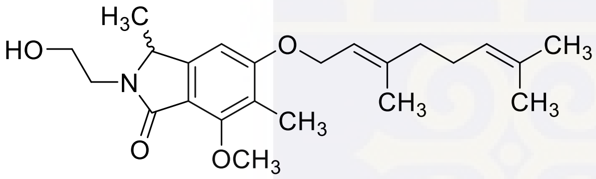
The culture extracts of a marine-derived fungus *Stachylidium* sp. isolated from the sponge *Callyspongia flammea*, produced four phthalimidine derivatives that were named marilines A1 and A2, marilines B and C. Marilines A1 and A2 are enantiomers which were identified by a combination of circular dichroism (CD) analysis and quantum chemical CD calculations. Marilines afford an unusual skeleton, which is most definitely derived from some uncommon biochemical reactions characteristic only of fungal secondary metabolism. The two enantiomers marilines A1 and A2, inhibited human leukocyte elastase (HLE) with an IC_{50} value of 0.86 µM. Furthermore, mariline A1 also moderately inhibited the growth of pathogens of the tropical infectious disease, African sleeping sickness and leishmaniasis.[81]



Marilines A1 and A2



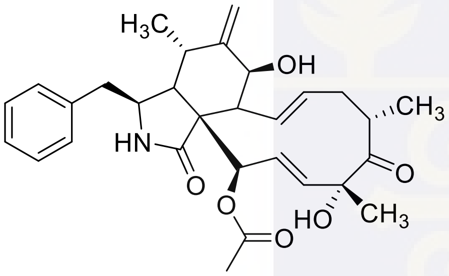
Mariline B



Mariline C

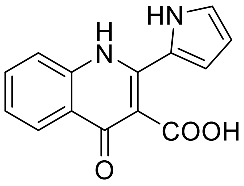
Cytochalasins are a group of fungal metabolites that possess the ability to bind to actin and thereby alter its ability to polymerize. This class of fungal metabolites have been widely used to study the role of actin in biological processes and as models for actin-binding proteins. By virtue of their mechanisms of action, the cytochalasins display a wide array of biological effects which include the inhibition of cytoplasm division, reversible inhibition of cell movement, nuclear extrusion, platelet aggregation and clot retraction, glucose transport and thyroid secretion, antimicrobial and anticancer properties, HIV-1 integrase inhibition and various other activities. Due to their commercial availability during the last four decades, several cytochalasin derivatives have been synthesized and sold for various cytological research programs.[82-98]

The compound cytochalasin D represents a typical example of the cytochalasins. It was originally obtained from the mold *Xylaria* sp. SCSIO156 collected from South China Sea Marine sediments. It is a cell permeable potent inhibitor of actin polymerization and disrupts actin filaments through activation of the p53-dependent pathways causing arrest of the cell cycle at the G1-S transition. It is also believed to bind F-actin polymer and prevent polymerization of actin monomers. [99,100]

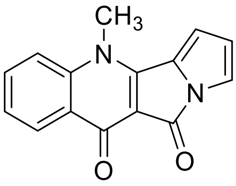


Cytochalasin D

The pyrrolyl-4-quinolinone alkaloid possesses an unprecedented ring system and was named penicinoline. This compound was obtained from a mangrove endophytic fungus and its complete structure was determined by spectroscopic methods and in comparison to its penicinotam, an unexpected lactam that was obtained from penicinoline by intramolecular dehydration. Eventually, the structure ofpenicinoline was confirmed by X-ray analysis and it was shown to possess potent *in vitro* cytotoxicity towards 95-D and HepG2 cell lines with IC_{50} values of 0.57 and 6.5 µg/ml respectively.[101]



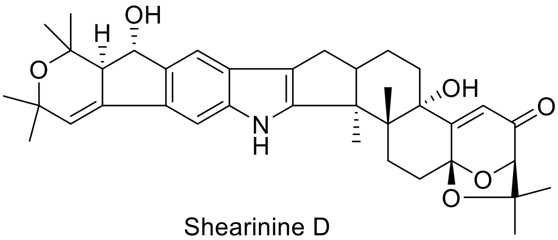
Penicinoline



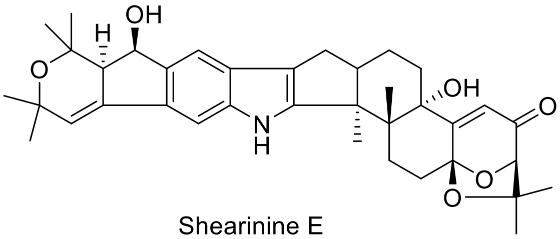
Penicinotam

Penicinoline and its derivative penicinotam where further evaluated to check their potential activity against the insects *Aphis gossypii*, *Plutella xylostella*, *Heliothis virescens*, *Septoria tritici* and *Uromyces fabae*. Penicinoline was found to possess an impressively strong activity against *Aphis gossypii* with 100% mortality at 1000 ppm whereas penicinotam not only killed completely all pests tested but, it also showed total control of the larvae of the pests *Plutella xylostella* at 500 ppm concentration. Penicinotam also moderately controlled the pests *Heliothis virescens*.[101]

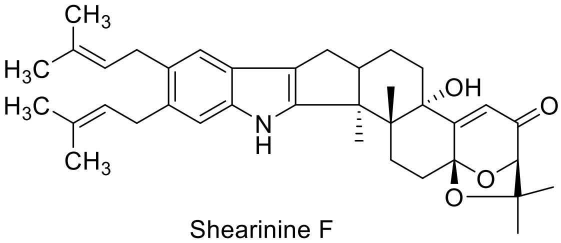
Marine-derived fungus *Penicillium janthinellum* is the source of the three alkaloids shearinines D-F. Shearinine E inhibited EGF-induced malignant transformation of JB6 P + Cl41 cells in a soft agar with INCC50 (inhibition of number of colonies) of 13 µM. Shearinines D and F induced apoptosis in human leukemia HL-60 cells at 100 µM concentration by 10%, 39% and 34% of the apoptotic cells respectively.[102]



Shearinine D



Shearinine E

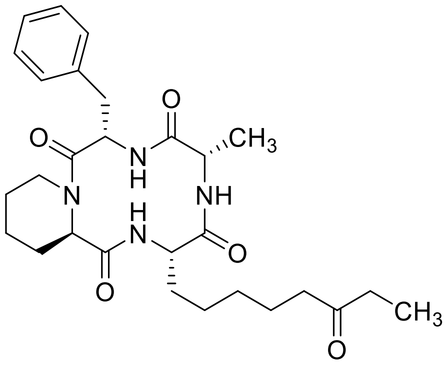


Shearinine F

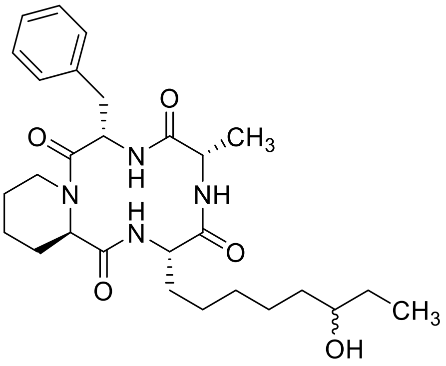
2.3.2 Peptides isolated from marine-derived fungi

Marine-derived fungi produce a lot of peptides that includes cyclic peptides, cyclic depsipeptides, and straight chain peptides. Some of these have interesting biological activity and a few examples are mentioned here.

Microsporin A and B were isolated from the marine fungus *Microsporum gypseum* and found to possess cytotoxicity activity. The unique structural feature of these two peptides is the presence ofα-amino acids in their structure resulting in a 12-membered cyclic peptide ring. Three of the amino acids have L-configurations, while the pipecolic acid has a D-configuration. Both microsporin A and B have been reported as potent inhibitors of histone deacetylase and demonstrate activity against human colon adenocarcinoma (HCT-116) with IC_{50} 0.6 µg/ml. They also proved potent in the NCI 60 cancer cell panel with average IC_{50} value of 8.5 µg/ml. In all these assays, microsporin A was more active than microsporin B and this was attributed to the importance of the ketone carbonyl group in the bioactivity observed.[103]

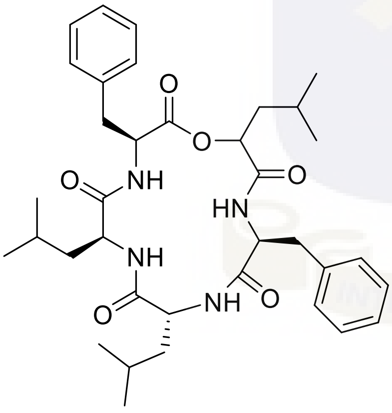


Microsporin A

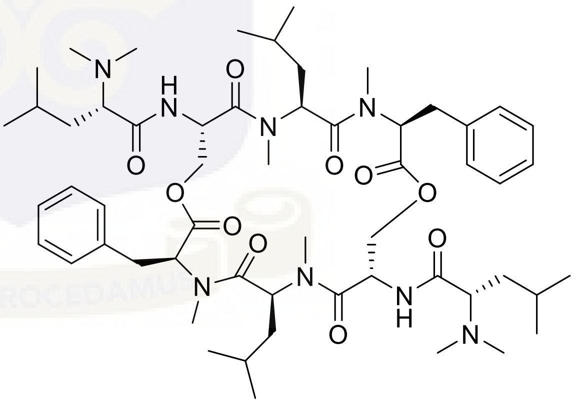


Microsporin B

Zygosporamide is a cyclic pentadepsipeptide that was discovered in the fermentation broth of the marine fungus *Zygosporium masonii*. Despite a rather simple structure compared to other cyclic peptides, this compound showed significant cytotoxicity GI_{50} of 9.1 µM against NCI 60 cell line panel. Zygosporamide showed highly enhanced selectivity with GI_{50} of 6.5 nM against the CNS cancer cell line SF-268 and the renal cancer cell line RXF 393, GI_{50} 6.0 nM.[104]



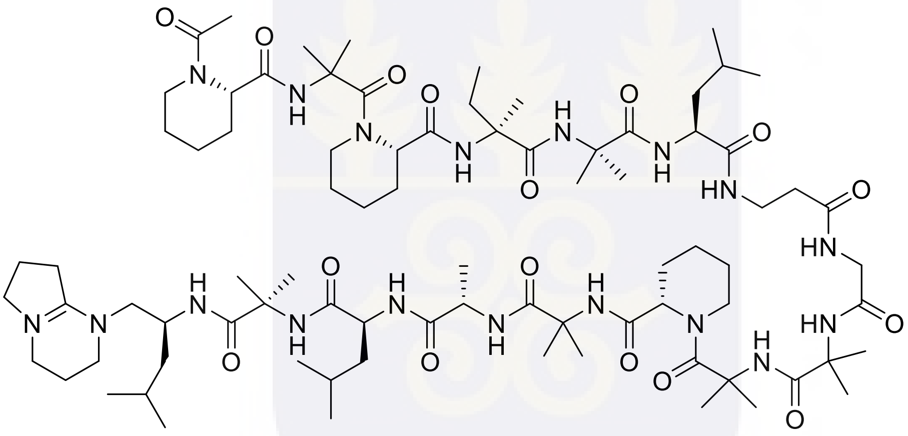
Zygosporamide



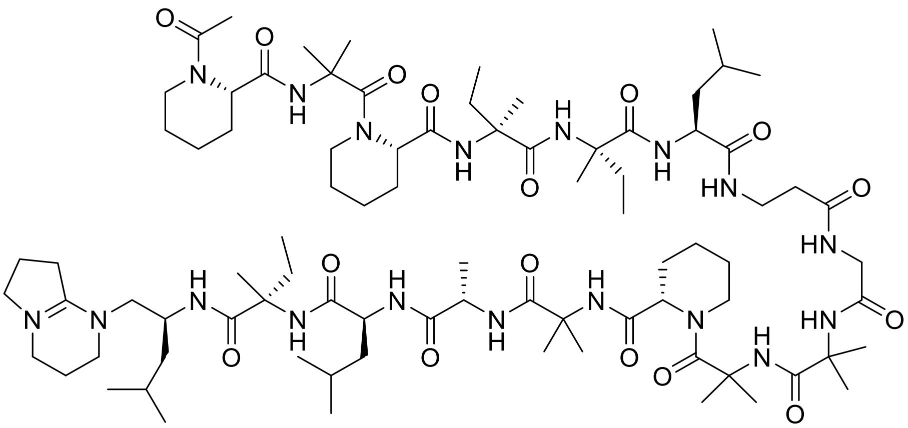
cyclodepsipeptide IB-01.212

The cyclodepsipeptide IB-01.212 was obtained from the mycelium of *Clonostachys* sp. and found to exhibit leishmanicidal activity on promastigote and amastigote forms of the parasite with LC_{50} of 10.5 and 7.5 µM respectively. Other derivatives of this compound isolated from the same organism were also tested but, cyclodepsipeptide IB-01.212 showed double potential compared to the other analogues.[105]

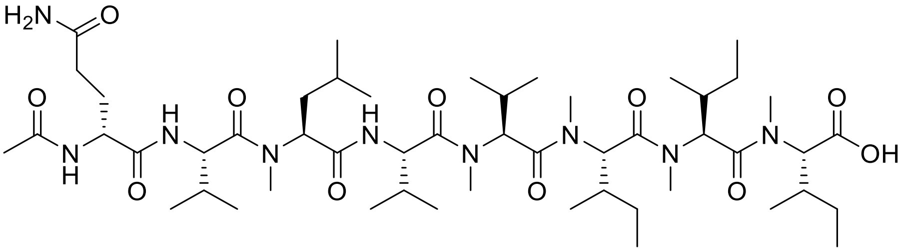
Apart from the cyclic peptides mentioned above, marine-derived fungi also produce a very wide variety of linear peptides that possess very interesting biological activity. Examples of such peptides are efrapeptin E, efrapeptin H, N-methylated octapeptide RHM3 and RHM4 which were separated from a marine-derived fungus *Acremonium* sp. Efrapeptin E displayed IC_{50} of 1.3 nM against H125 cells. The other analogues of this peptide separated from the same species have been reported to possess the same activity with different potential.[106]



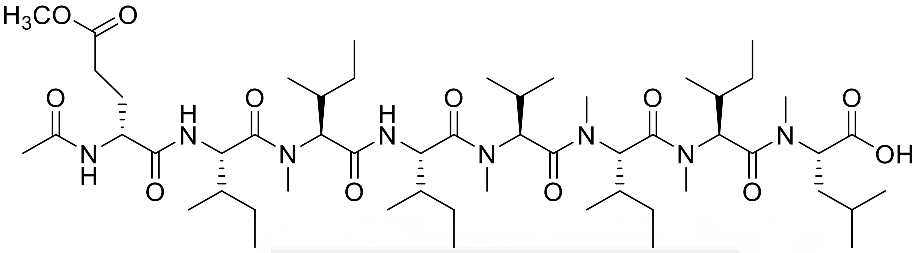
Efrapeptin E



Efrapeptin H



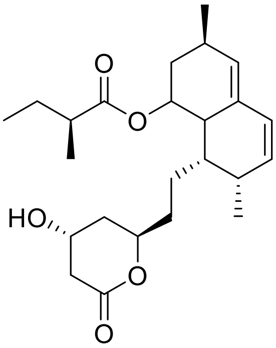
RHM3



RHM4

2.3.3 Polyketides isolated from marine-derived fungi

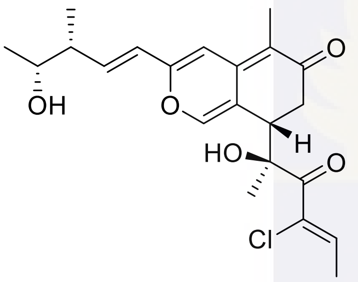
Polyketides are a highly diverse group of natural products with structurally intriguing carbon skeletons which are assembled from a very simple building block which is acetate. Many polyketides have become important therapeutics in clinical use for example; the fungal polyketide lovastatin is a very well-known cholesterol-lowering agent on the pharmaceutical market and represents one of the success stories of natural products in drug discovery.[107]



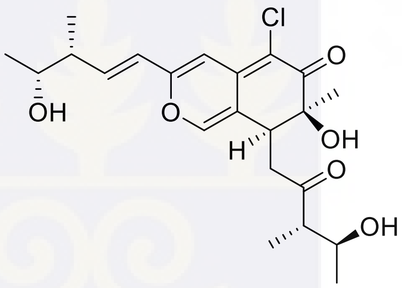
Lovastatin

In addition to this, several marine-derived fungal species also make a large number of polyketides and their derivatives. The medicinal importance of this capability is very important and only further buttress the importance of marine fungi as a source of our future drug prototypes. A few examples are mentioned below to emphasise the importance of this resource.

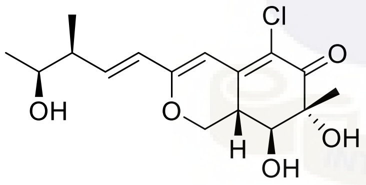
The azaphilone polyketides, chaetomugilins P-R and 11-epi-chaetomugilin I were isolated from the broth culture of *Chaetomium globosum* which was retrieved from the marine fish *Mugil cephalus*.[108]



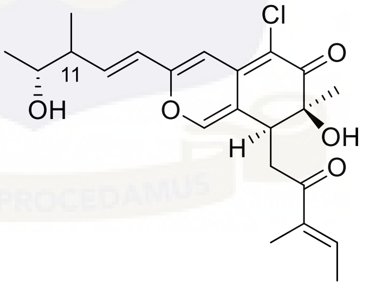
Chaetomugilin P



Chaetomugilin Q



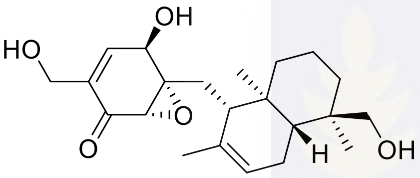
Chaetomugilin R



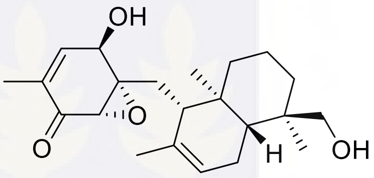
11-Epi-chaetomugilin I

Chaetomugilins P and 11-epi-chaetomugilin I significantly inhibited the P388, HL-60, L1210 and KB cancer cell lines with highest activity in the range of IC_{50} 0.7-1.8 pM. The reported results suggested the importance of the enone functionality within the acyclic side chain for strong cytotoxicity.[108]

Marine derived fungus *Phoma* sp. produced the two compounds epoxyphomalin A and B. The cytotoxicity of the two compounds was evaluated with 36 different cancer cell lines. Epoxyphomalin A showed significant *in vitro* tumour cell selectivity. IC_{50} values in the tested above-average sensitive cell lines ranged from 0.010 µg/ml for breast cancer MAXF 401NL to 0.038 µg/ml in adeno lung cancer LXFA 629L. Epoxyphomalin B exhibited 22% of selectivity with IC_{50} values in the eight above-average sensitive cell lines ranging from 0.251 µg/ml for pleuramesothelioma PXF 1752 L to 0.402 µg/ml for bladder cancer BXF T24. However, summary of the results indicated that epoxyphomalin A showed superior cytotoxicity at nanomolar concentrations for pleuramesothelioma PXF 1752 L and bladder cancer BXF T24.[109]

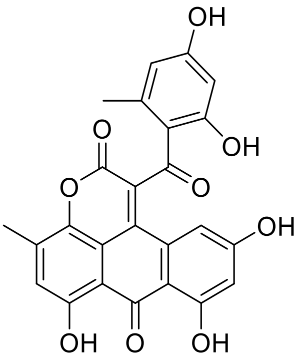


Epoxyphomalin A



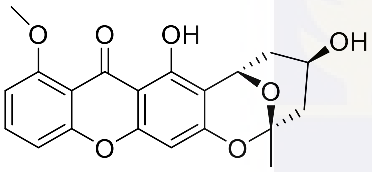
Epoxyphomalin B

The marine fungus *Aspergillus glaucus* produced the cytotoxic anthraquinone-derived polyketide aspergiolide A. Aspergiolide A exhibited selective cytotoxicities against A-549, HL-60, BEL-7402 and P388 cell lines with IC_{50} values of 0.13, 0.28, 7.5, and 35.0 µM respectively.[110]

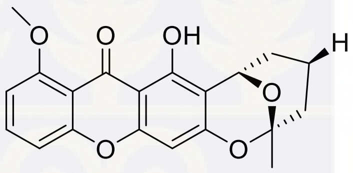


Aspergiolide A

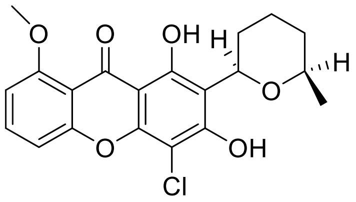
Xanthone-derivatives chaetoxanthones A-C were isolated from the culture extracts of the fungus *Chaetomium sp*. Chaetoxanthones A and B bear a dioxane/tetrahydropyran moiety, which is not so common in natural product chemistry. Chaetoxanthones C in turn is a chlorinated xanthone substituted with a tetrahydropyran ring. Chaetoxanthones B was selectively active against *Plasmodium falciparum* with an IC_{50} value of 0.5 µg/ml and it did not show any cytotoxicity toward cultured eukaryotic cells. On the other hand, chaetoxanthones C was found to be moderately active against *Trypanosoma cruzi* with an IC_{50} value of 1.5 µg/ml.**[111]**



Chaetoxanthones A



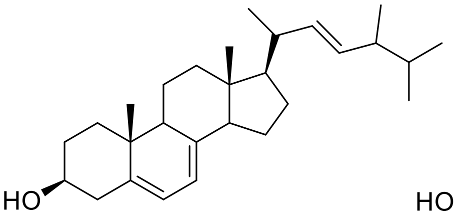
Chaetoxanthones B



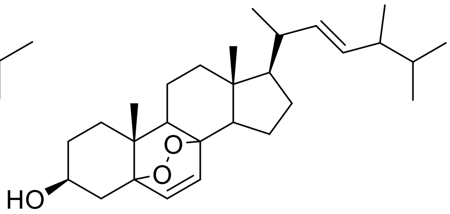
Chaetoxanthones C

2.3.4 Steroids isolated from marine-derived fungi

Steroids constitute an important class of natural products that have produced many drugs currently on the pharmaceutical market. Interestingly, marine derived fungi also produce several new compounds, derivatives or analogues of this class of natural products. For example, ergosterol and its peroxy derivative5α,8α-epidioxy-24(S)-methylcholesta-6,22-diene-3β-ol are common fungal metabolites routinely found and isolated from the liquid broths of *Guignardia* sp. recovered from the marine alga *Undaria pinnatifida*. Ergosterol and 5α,8α-epidioxy-24(S)-methylcholesta-6,22-diene-3β-ol inhibited the growth of the pathogenic yeast cells *Microsporum canis* with MICs of 10 and 20 µg/ml and of *Trichophyton rubrum* with MICs 15 and 20 μg/ml respectively.Ergosterol also showed some activity against *Epidermophyton floccosum* with MIC of 20 μg/ml.[112]

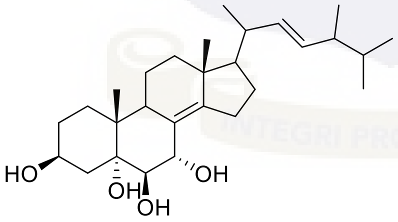


Ergosterol

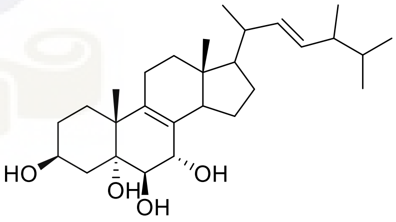


peroxy-Ergosterol

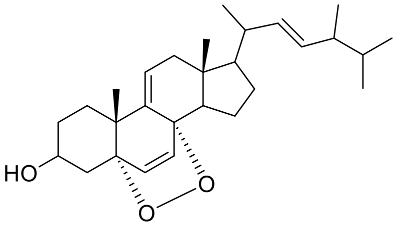
A *Pennicillium* sp. isolated from a strain of moss in the South Pole, produced the steroids ergosta-8(14),22-diene-3,5,6,7-tetraol-(3β,5α,6β,7α,22E), ergosta-8(9),22-diene-3,5,6,7-tetraol-(3β,5α,6β,7α,22E), 5α,8α-epidioxy-24(S)-methylcholesta-6,22-diene-3β-ol, 5α,8α-epidioxy-24(S)-methylcholesta-6,9(11),22-triene-3β-ol and 3β,5α,9α-trihydroxyergosta-7,22-diene-6-one.[113,114]



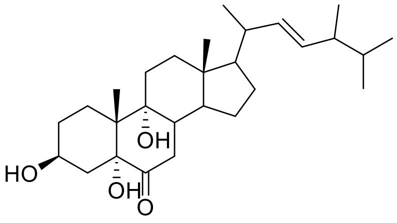
Ergosta-8(14),22-diene-3,5,6,7-tetraol-(3β,5α,6β,7α,22E)



Ergosta-8(9),22-diene-3,5,6,7-tetraol-(3β,5α,6β,7α, 22E)



5α,8α-Epidioxy-24(S)-methylcholesta-6,9(11),22-triene-3β-ol



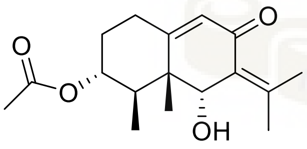
3β,5α,9α-Trihydroxyergosta-7,22-diene-6-one.

These compounds were tested against human liver cancer cell (Hep G2), and all of them exhibited potent activity with ergosta-8(14),22-diene-3,5,6,7-tetraol-(3b,5a,6b,7a,22E) showing the lowest IC_{50} value of 10.4 μg/ml.[113,114]

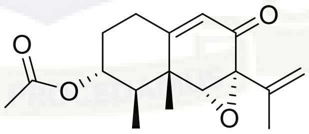
2.3.5 Terpenes isolated from marine-derived fungi

Terpenes are the most numerous and structurally diverse group of natural products. Even though a majority of these compounds have come from plants, marine derived fungi also produce a number of very important terpenes. In some cases, terpenes isolated from plants have been attributed to the endophytic fungi which are directly associated with these plants.

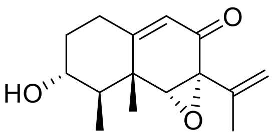
A marine fungus, *Penicillium* sp. BL27-2 produces eremophilane sesquiterpenes, which were characterized as 3-acetyl-9,7(11)-dien-7α-hydroxy-8-oxoeremophilane, 3-acetyl-13-deoxyphomenone, sporogen-AO1, 7-hydroxypetasol, 8α-hydroxy-13-deo-xyphomenone and 6-dehydropetasol.[115]



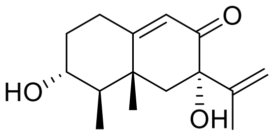
3-Acetyl-9,7(11)-dien-7a-hydroxy
-8-oxoeremophilane



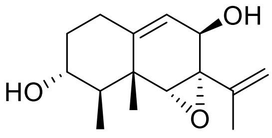
3-Acetyl-13-deoxyphomenone



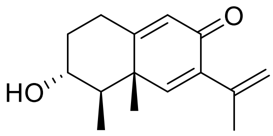
Sporogen-AO1



7-Hydroxypetasol



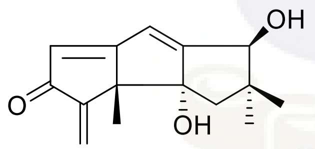
8α-Hydroxy-13-deo-xyphomenone



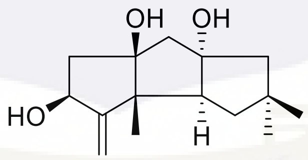
6-Dehydropetasol

Reports indicate that, 3-acetyl-9,7(11)-dien-7α-hydroxy-8-oxoeremophilane, 3-acetyl-13-deoxyphomenone and 8α-hydroxy-13-deo-xyphomenone with epoxide functionalities exhibit cytotoxicity activity for P388, A549, HL60, BEL7402 and K562 cell lines with IC_{50} values ranging between 0.073 to 11.8 µM/L, while the compounds sporogen-AO1, 7-hydroxypetasol and 6-dehydropetasol without epoxide functionalities show weak cytotoxicity activity. These results suggested that, the epoxide rings are absolutely essential for their activity. Also, it appears as if acetylation may enhance activity.[115]

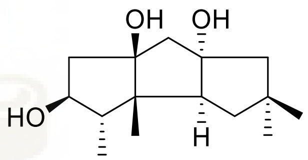
Hirsutanol A, E and F were obtained from the culture extracts of *Chondrostereum* sp. which is a fungus recovered from the soft coral *Sarcophyton tortuosum*. Biological activity tests showed interesting results for only hirsutanol A which showed activity against human colon, human hepatic, human lungs, human breast and human cervical cancer cell lines with IC_{50} values ranging from 0.58 to 8.27 µg/ml.[116]



Hirsutanol A



Hirsutanol E



Hirsutanol F

*The literature reviewed here for natural product compounds produced by marine derived fungi is by no means a comprehensive list of all the molecules obtained from these microbes to-date. However, the literature serves to confirm the following:

- Different species of marine derived flora and fauna indeed harbour within their tissues a wide variety of fungi.

- These different types of fungi produce a very diverse range of secondary metabolites including alkaloids, peptides, polyketides, steroids and terpenes.
- These diverse ranges of metabolites have bioactivity with a large number of them possessing cytotoxicity activity, a few with antimicrobial, anti-parasitic and antifungal activities.
- Chemical investigation of marine-derived fungi is very likely to yield interesting drug leads in many disease areas.

2.4 Terminologies in bioactivity measurements

2.4.1 Half maximal inhibitory concentration (IC_{50})

This is a quantitative measure *in vitro* that indicates how much of a particular substance or drug is needed to inhibit by half a specific biological or biochemical processor a component of that process occurring within a cell.[117-123]

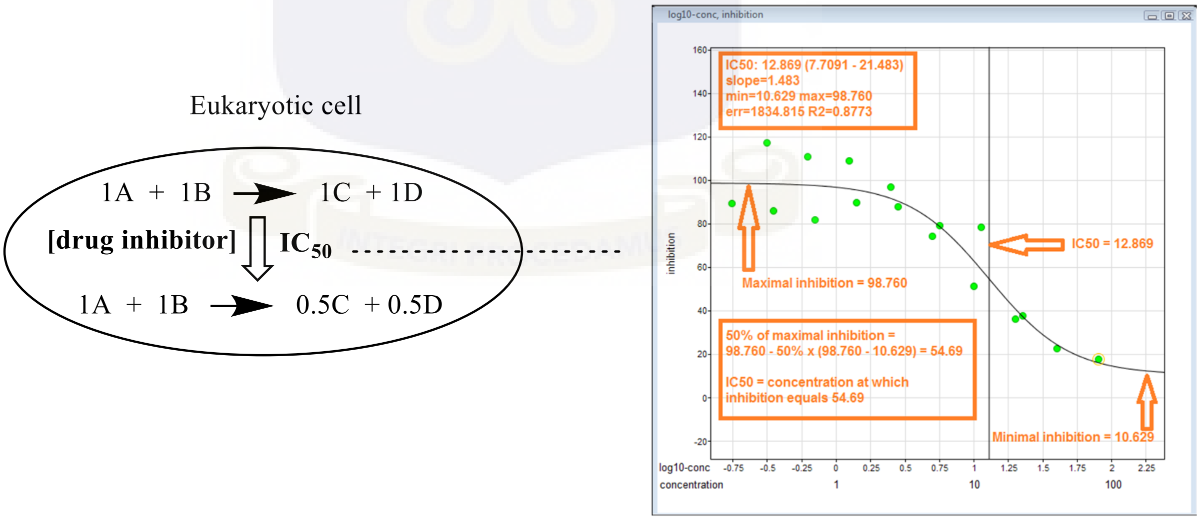


Figure 2.1: Illustration of Half Inhibitory Concentration (IC_{50}).

2.4.2 Half maximal effective concentration (EC_{50})

The term EC_{50} is commonly used to depict or measure the potency of a drug. It represents the concentration of a compound, drug, antibody or toxicant at which 50% of its maximal effect is observed after a specified exposure time.[123-128]

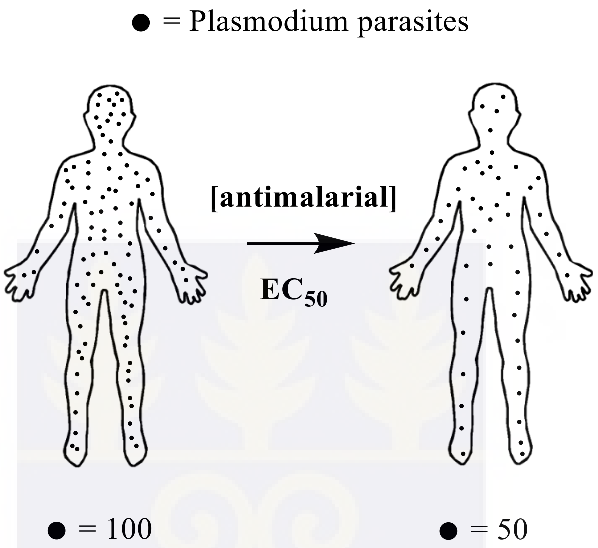


Figure 2.2: Illustration of Half Maximal Effective Concentration (EC_{50}).

2.4.3 Half maximal Effective dose (ED_{50})

This is the dose or amount of drug that produces a specific effect or therapeutic response in some fraction (50%) of the subjects or test sample that have taken it. It is a measure of average effectiveness of the drug or the tolerance level of toxin in a sampled set of individuals.[129-134]

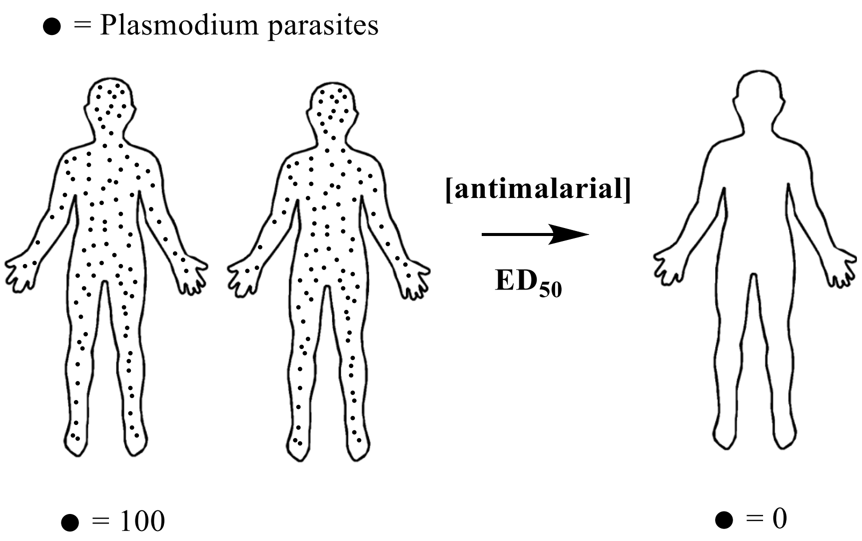


Figure 2.3: Illustration of Effective Dose (ED_{50}).

2.4.4 Lethal dose for 50% (LD_{50})

This is the quantity of a substance (toxin, drug, pesticide etc) which is enough to cause the death of 50% of a group of test animals in a specified period. It is commonly used in bioassay assessment to measure the acute toxicity of a chemically active agent.[135-138]

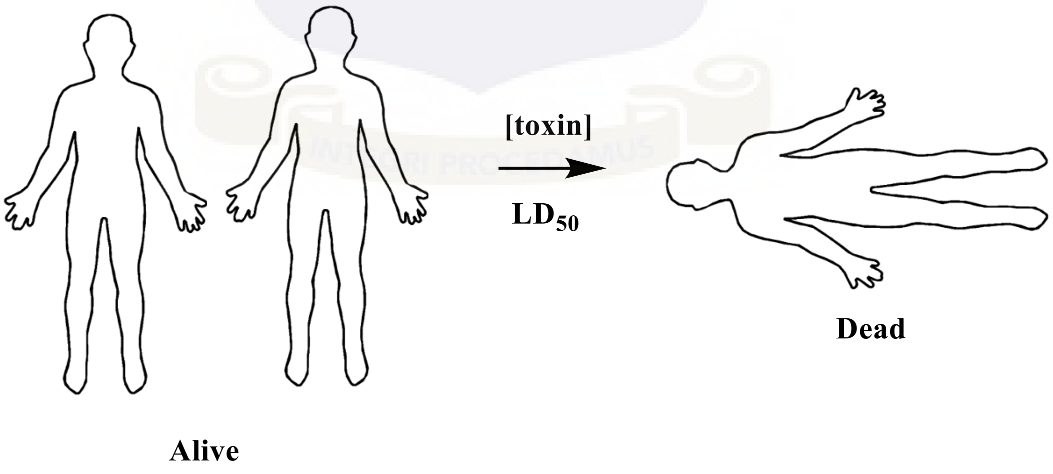


Figure 2.4: Illustration of Lethal Dose (LD_{50}).

2.4.5 Lethal concentration for 50% (LC_{50})

This is the quantity of a substance (toxin, drug, pesticide etc) present in the air or water which is enough to cause the death of 50% of a group of test animals in a specified period.[135,137,139]

2.4.6 Minimum inhibitory concentration (MIC)

This is the minimum concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MICs are important in diagnostic assessments of the potency a new antimicrobial agents or to detect resistance of microbes to already existing antimicrobial agents.[140-143]

2.4.7 Minimum bactericidal concentration (MBC)

This is the minimum concentration of an antimicrobial that will kill and stop the growth of a microorganism after overnight incubation.[144-148]

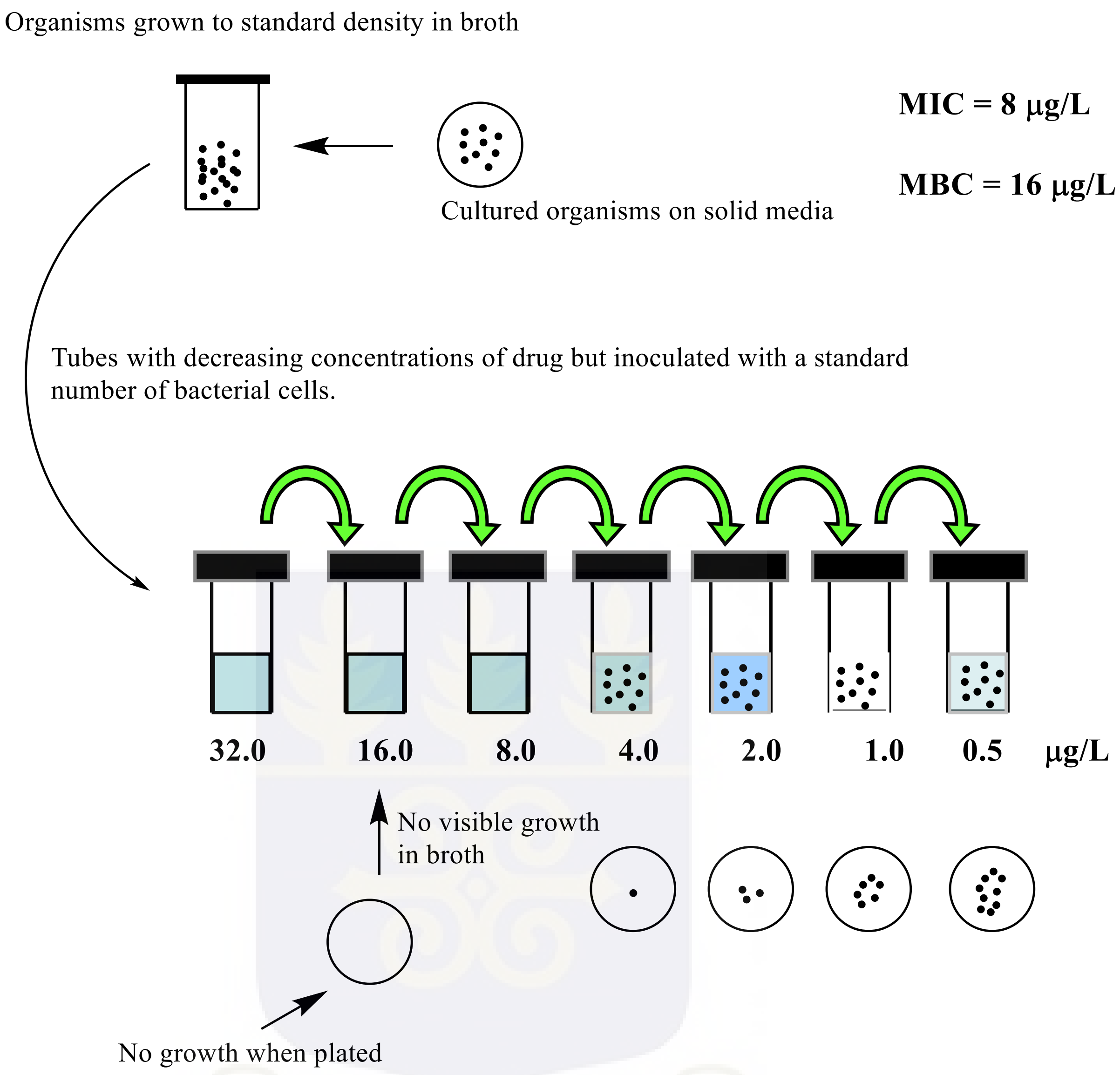


Figure 2.5: Illustration of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

2.4.8 Growth inhibition concentration for 50% (GI_{50})

This is the concentration of a substance or drug that inhibits the growth cells by 50%. For an anticancer agent, it is the concentration of the drug that inhibits the growth of cancer cells by 50% or produces a 50% reduction in cell proliferation.[149,150]

2.5 Malaria

2.5.1 Introduction, statistics and interventions

Parasites are organisms that live within or on other organisms at whose expense they obtain some advantage such as nourishment and shelter.[151]Parasites range from the pathogenic single celled microscopic organisms (protozoans) to larger multi-cellular worms that reach three feet long and are responsible for some of the world's deadliest diseases including leishmaniasis, trypanosomiasis, schistosomiasis, filariasis and enteric protozoans that cause infections such as diarrhoea and dysentery. Among all the parasitic agents causing diseases in the world, Plasmodium parasites continue to be the most destructive by virtue of the widespread occurrence and difficult to avoid nature of its vector, the female Anopheles mosquito.[152]It affects the developing world to a large extent but, almost half of the world's population stand a chance of being infected. Malaria is capable of serious economic damage since it is able to completely weaken its victims and make them completely non-productive.[153] Hence, it is mostly linked to the economic deterioration of most developing countries especially in Sub-Saharan Africa.

The unicellular protozoan parasite Plasmodium belongs to the phylum Apicomplexa which constitute a large group of eukaryotic microorganisms that possess a unique organelle known as the apicoplast and the apical complex that are involved in cell division within the host.[154] The different species of plasmodium that commonly infect humans are *P. falciparum*, P. vivax, *P. ovale*, *P. malariae* and the recently discovered South-East Asian *P. knowlesi*.[155] *P. falciparum* however, is the most virulent and mostly associated with severe cases of malaria and malaria-associated complications or subsequent death.

Currently, malaria is the most prevalent disease in the world, killing 1-2 million people each year.[153]Approximately 3.3 billion people which is about half of the world's population are at risk of being infected with malaria. In 2013, 198 million cases of malaria occurred globally and the disease led to 584,000 deaths. Most of the deaths are amongst children under five who, together with pregnant women, are particularly vulnerable to the disease.[153]In 2013, malaria was one of the causes that led to death of 6.3 million children under the age of five.[156]South and Central America, South and East Asia, the Caribbean, Oceania, Central Asia and the Middle East are all affected by malaria, but the burden is heaviest in Africa where an estimated 90% of malaria deaths occur with most cases in sub-Saharan Africa.[153] The disease does not only significantly affect morbidity and mortality but, the economic burden caused by malaria is estimated to cost Africa US$ 12 billion in lost Gross Domestic Product (GDP). It is estimated to have slowed economic growth in Africa by 1.3% per year as a result of lost life and lower productivity.

The global impact of the disease has caused International agencies and bodies to help eradicate and improve the socio economic challenges associated with it. In 1998, the Group of Eight (G8) nations who met in Birmingham, UK, endorsed an international initiative to control malaria. The leaders agreed to improve mutual cooperation on infectious and parasitic diseases and offered support for the new "Roll Back Malaria" movement to reduce levels of malaria-related mortality by 2010. The G8 leaders sought to achieve the following:

- Collaborate with governments, private sector companies and non-governmental organizations in public-private partnerships to expand malaria interventions and programs.
- Work with African-countries to scale up malaria control interventions, reduce the burden of the disease and eventually defeat malaria on the continent to meet the Abuja target of halving the burden of malaria by 2010.
- Support the development of new, safe and effective drugs or the creation of a vaccine and promote the widest possible availability of prevention and treatment to people in need.
- Support activities of public and private entities to save children from the disease.
- Contribute to the additional US$ 1.5 billion a year needed annually to help ensure access to anti-malarial insecticide treated mosquito nets, adequate and sustainable supplies of combination therapies, preventive treatment for pregnant women and babies and household residual spraying.[157]

2.5.2 Life cycle of the plasmodium parasites

The life cycle of the plasmodium parasite involves both humans and the female Anopheles mosquito hosts. The human part of the life cycle normally begins with the bite of an infected Anopheles mosquito leading to the introduction of the parasites which at this stage are known as sporozoites into the human blood stream. The haploid sporozoites immediately move to the liver and invade a liver cell or hepatocyte which subsequently reproduces by mitotic cell division. The sporozoites within the hepatocytes transform into schizonts. Each schizoint contains thousands of haploid cells called merozoites. The schizonts swell and rupture in about seven (7) days, killing the hepatocytes and hence releasing millions of merozoites into the blood stream. This stage of replication is referred to as asexual exo-erythrocytic schizogony. At the parasite development stages within the liver, hypnozoites which are dormant forms of the parasite that refuse to continue the life cycle but lie dormant for a long time may develop. The development of malaria relapse has been attributed to these latent hepatic forms of the parasite which are mostly characteristic of *P. vivax* and *P. ovale* infections.

The merozoites that are released from the raptured hepatocyte into the bloodstream quicklyinvade erythrocytes initiating the erythyroctic stage of malaria parasitic infections. *P. Falciparum* normally invades the erythrocytes of all ages whereas *P. vivax* and *P. ovale* prefer the erythrocytes of younger people in contrast to *P. malaraiae* which prefers the erythrocytes of older and more matured individuals. Subsequently, the merozoites undergo a period of mitotic amplification during erythrocytic schizogony. The early ring forms of the parasites mature into trophozoites that feed on the haemoglobin found in erythrocytes. Trophozoites then mature into schizonts that contain newly formed merozoites. The schizonts along with the erythrocytes rupture after 2-3 days releasing the merozoites which quickly invade uninfected erythrocytes. The symptoms associated with this continue destruction of red blood cells and the release of toxins into the blood include anaemia, fever and chills.

A portion of the erythrocytic merozoites can then develop into gamete producing cells termed gametocytes. This takes place in the female Anopheles host of the parasite and commence when the Anopheles receives a blood meal containing gametocytes. The gametocytes transform into male and female haploids gametes minutes after they enter the mosquito mid-gut. The male gamete fertilizes the female gamete to produce a diploid zygote which undergoes meiotic division to become an ookinete and travels toward the mosquito gut wall and invade the semi-permeable membrane called the peritrophic matrix. The ookinete upon traversing the gut wall transforms into an oocyst occupying the space between the gut wall and the basal lamina. The oocyst bursts and releases sporozoites into the hemocoel cavity. The sporozoites proceed to invade and establish residence in the salivary glands of the mosquito and are released from the salivary glands of the female Anopheles host to the human host during blood meals and hence completing the cycle.[155]

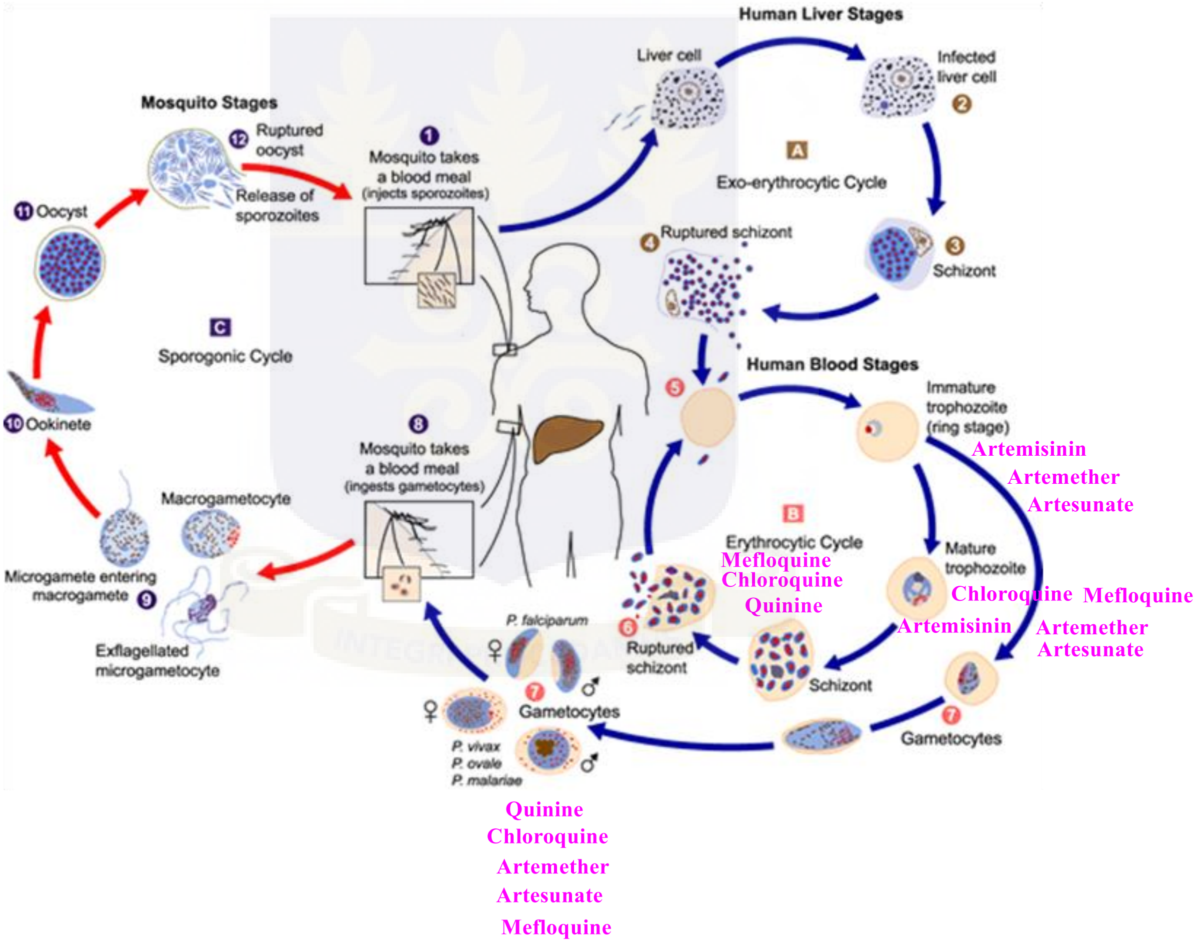


Figure 2.6: Life cycle of Plasmodium parasite showing the stages at which current drugs act.

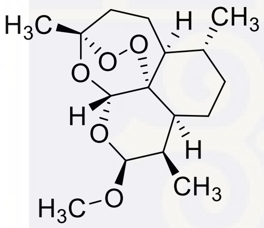
2.5.3 Antimalarials in current use

Chemotherapeutic strategies to combat malaria aim to target different stages of the life cycles in humans or total elimination of the vector which is the female Anopheles mosquito. The matured trophozoites and schizonts at the human blood stage have previously served as good targets for many different antimalarials.[155] Different compound structures have been discovered and used to treat malaria in the past and indeed many different drugs are currently available but, the ability of the parasites to develop resistance and subsequently render useless a particular structure or skeleton at any time demands a continued investigation to discover new drugs.

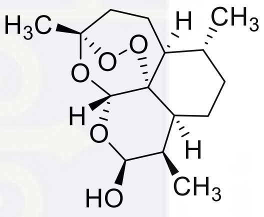
Quinine the first quinoline antimalarial drug acts as a blood schizonticide and weak gametocide against *P. vivax* and *P. malariae*. The drug is also capable of accumulating in the food vacuoles of plasmodium species such as *P.falciparum* and act by inhibiting the heme polymerase enzyme, thus facilitating anaggregation of cytotoxic heme which is not useful to the parasite. Quinine is still used in the treatment of acute cases of severe P. *falciparum* infection and sometimes prescribed as a prophylactic in infants that is taken every Sunday. Until recently, chloroquine was very effective and the drug of choice for over three decades.

Chloroquine is believed to reach high concentration in the vacuoles of the parasites which due to its alkaline nature, raises the internal pH. It inhibits the conversion of toxic heme a by-product of the digestion of haemoglobin to haemozoin by preventing the biocrystallization of haemozoin. The parasites subsequently poison themselves by feeding on human haemoglobin and are thus destroyed. However, the resistance of *P. falciparum* to chloroquine has spread from Asia to Africa making the drug ineffective.[158]

Currently, artemisinin based drugs are the first line of treatment for the parasitic disease and has been proven to be effective against all forms of multi-drug resistant *P. falciparum*. The drug has demonstrated the fastest clearance of all antimalarials currently used and acts primarily on the trophozoite phase of the plasmodium life cycle, thus preventing further progression of the disease. Other derivatives of artemisinin have also proven to be efficacious. For example, artemether is a methyl derivative of dihydroartemesinin and it has a similar mode of action to that of artemesinin but, it has demonstrated a reduced ability as a hypnozoiticidal (hypnozoite-activating) compound, instead acting more significantly to decrease gametocyte carriage.

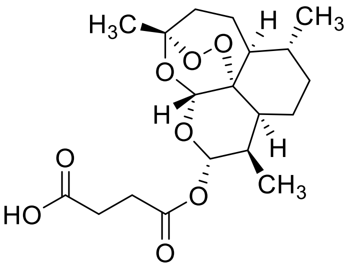


Artemether

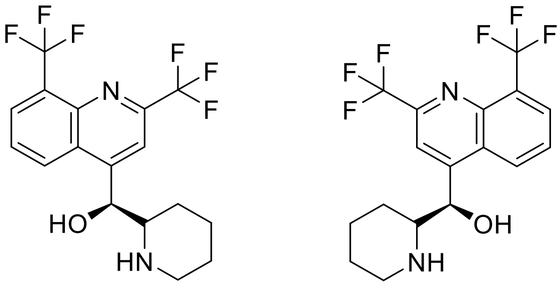


Dehydroartemisinin

Artesunate is a water-soluble semi-synthetic hemi-succinate derivative of artemesinin. It is the most effective because of its instantaneous bioavailability. A combination of mefloquine and artesunate is highly effective in multi-drug resistant malaria.[159]



Artesunate



Mefloquine

2.5.4 Development of drug-resistant plasmodium parasites

The most intimidating problem in the fight against malaria is the widespread occurrence of drug resistance especially in the species of *P. falciparum* which as mentioned earlier, infects all ages in humans. The three other species have no documented resistance apart from the recorded chloroquine resistance in Papua New Guinea and Indonesian regions.[159]

The reasons for this widespread development and spread of resistance are:

- Drug-use pattern such as wrong diagnosis and incorrect dosage.
- Physical and chemical characteristics of the drug itself, for example poor drug quality and poor absorption.
- Human host factors including diet and habits that speed up the quick breakdown of the drug in the blood stream.
- Parasite characteristics, which leads to gene mutation that results in the over-expression of some genes eventually leading to the development of resistance by the parasite.
- Parasite-vector combinations of factors that are not entirely documented enhance the transmission of resistant parasites.
- Environmental factors.[159]

The development of drug resistance is one of the problems that necessitate the continued discovery of antimalarials with novel backbones or structures.

2.5.5 Preliminary screening of antimalarial activity

Most of the preliminary assessments of the antimalarial efficacy of molecules are made *in vitro* using a number of bioassays. Plasmodium parasites require a source of hypoxanthine for nucleic acid synthesis and energy metabolism and they also use ethanolamine, normally derived from serine in the synthesis of glycolipids. Hence, by quantifying parasite uptake of radioactive substrates [^{3}H] hypoxanthine and [^{3}H] ethanolamine as a measure of growth and viability in the presence of the test drug, the effects of many drugs on the parasites were previously studied. These radioactive bioassays were accurate and reliable but, their reliance on very expensive radio-isotopes and multi-step procedures that is increasingly problematic and impractical with large numbers of drugs to be tested, made them gradually unpopular. Also, since the late 1970s, regulations regarding handling of radioactive materials have become considerably more restrictive causing isotopic assays to gradually give way to other *in vitro* tests.

For example, fluorescence based *in vitro* method; the SYBR Green I assay has been designed for the high-throughput screening of the activities of malaria parasites. The advantages of the assay include simplicity, lower costs, robust performance, applicability to automated analysis and speed.[160,161]

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Mangrove plant sample collections

The Western Region of Ghana is noted for its bio-diversely rich indigenous mangrove plants. Out of the six indigenous plants that are characteristic of the mangroves found in Ghana, three of them are particularly common in the Western Region and these are *Conocarpus erectus*, *Laguncularia racemosa* and *Rhizophora racemosa*. Collection of mangrove plants was done along the banks of the River Butre with five (5) sampling sites chosen at 100 m apart from the shore to where the river meets the sea. The three main plant samples collected were *Conocarpus erectus*, *Laguncularia racemosa* and *Rhizophora racemosa* but, different plant parts were sampled at different sections of the river. The plant parts sampled were leaves, buds, submerged roots, aerial roots, aerial stems, fruit shoots and flowers. The exact positions of plants from which different parts were sampled were stored in a GPS and the data uploaded into Google Earth satellite database. The samples were videoed, photographed, bagged, labelled and stored at 4 °C in an ice-chest and transported to the Department of Chemistry, University of Ghana, Legon. Identification of the plants was done at the Department and specimens of all plant parts collected were dried in newspapers for onward submission to the herbarium.

Figure 3.1:Map of Butre River running through communities.

Rhizophora racemosa

Cornacarpus erectus

Langucularia racemosa

Plate 3.1: Pictures of three mangrove plants particularly common in the Western Region of Ghana.

Plate 3.2: Boat riding on the Butre River to the site where samples were collected.

Plate 3.3: Collection and Identification of mangrove plants along the Butre River.

Plate 3.4: Collection and bagging of mangrove plants along Butre River.

Plate 3.5: Butre River running through the mangrove where plants were collected.

3.2 Preparation of culture media

3.2.1 Malt extract solid media

Approximately 15g of malt extract and 15g of bacteriological agar were weighed into a 1L autoclave bottle containing 900 ml of water and stirred to mix with a magnetic stirrer. An autoclave tape was pasted on the bottle and the mixture autoclaved. The autoclaved mixture was cooled to about 55°C and then poured into petri dishes (90 mm) to fill about 2/3 of the volume of each Petri dish under sterile conditions (25 plates for each 900 ml preparation). The plates were left half-open under a clean bench until almost all the water had evaporated from the plates. Dry plates were parafilmed and kept in a fridge at 4°C until needed.

3.2.2 Malt extract liquid media

Approximately 15g of malt extract was weighed into a 1L autoclave bottle containing 900 ml of water and stirred to mix with a magnetic stirrer. An autoclave tape was pasted on the bottle and the mixture autoclaved. The autoclaved media was allowed to cool and then stored in a fridge at 4°C until needed.

3.3.3 Treatment of mangrove plant and culture of endophytic fungi

In the laboratory, pieces of each plant part including leaves, buds, submerged roots, aerial roots, aerial stems, fruit shoots and flowers was surface sterilized under sterile conditions by first rinsing it with sterile artificial sea water (SASW) and then immersing it in 70% ethanol for 1 minute.

The plant part was then cut transversely at all sides into a smaller piece with a pair of flame sterilized scissors in a bio-safety cabinet. This piece was again sterilized under sterile conditions by first rinsing it with SASW and then immersing it in 70% ethanol for 1 minute. The piece was again cut at all sides into a much smaller piece with a flame sterilized scalpel. This piece was again sterilized by rinsing with SASW, then immersing in 2% sodium hypochlorite for 1 minute and then rinsed again with SASW 3 times. The treated plant piece was afterwards placed on a malt extract agar plate (parent or master plate) and the plate was labelled, parafilmed and incubated at 28 °C for three weeks with daily observations to detect the spring-up of new fungal colonies.

3.3.4 Isolation of pure endophytic fungi

Observation of the parent or master plates obtained for each plant part cultured, starting from day one (1) to day twenty-one (21) saw the appearance on the parent plates of several different colonies of marine endophytic fungi which were subsequently picked one at a time, re-cultured on fresh malt extract plates and kept at an incubation temperature of 28°C. All the colonies initially sub-cultured from the parent or master plates were subsequently sub-cultured until very pure strains were obtained for each species.

3.3.5 Small scale culture of pure endophytic fungi

The malt extract liquid broth prepared earlier was thawed in a water bath and 100ml was transferred into an already autoclaved 250 ml Erlenmeyer flasks with non-absorbent cotton wool stuck in the mouth under sterile conditions. A single colony from one of the pure strains of endophytic fungi obtained was picked and inoculated into the broth under sterile conditions. The inoculated flasks were labelled and incubated in an incubator at 28°C for two weeks. After two weeks, the culture broth was filtered by suction filtration to separate fungal mycelia from the broth itself. The broth was extracted once with ethyl acetate and the mycelia repeatedly and alternatively extracted with methanol and dichloromethane. The ethyl acetate, methanol and dichloromethane extracts were dried under vacuum and subsequently combined to give a total crude extract or TCE. The same steps were repeated for all the pure fungal strains that looked interesting and worthy of chemical investigations.

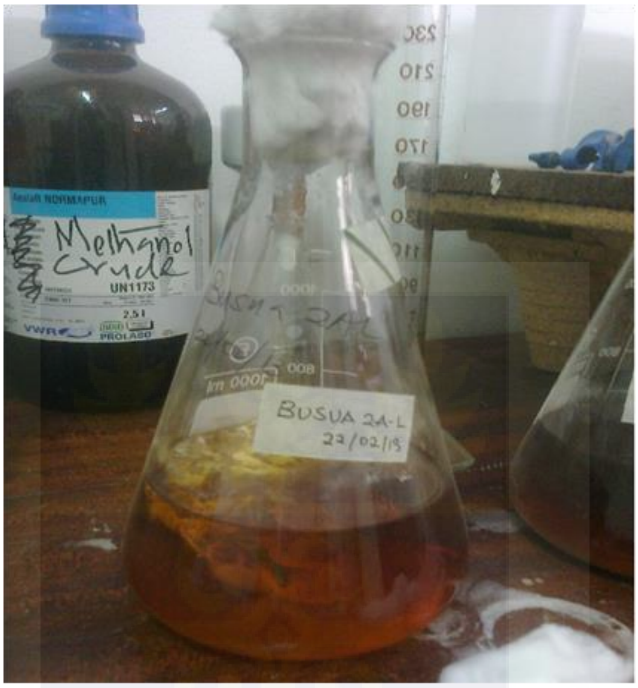


Plate 3.6: Small scale fermentation broth of BUSUA 2A-L.

3.3.6 Chemical profiling and prioritization of pure endophytic fungi

About 1-1.5 mg of TCEs obtained for pure species of mangrove endophytic fungi isolated from different plant parts were dissolved in 1-1.5 ml of HPLC grade methanol and sent to the Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Scotland for HRESI/HPLC-DAD-MS^n analysis.

High resolution mass spectrometric data were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump). The following conditions were used: capillary voltage 45V, capillary temperature 320

°C, auxiliary gas flow rate 10–20 arbitrary units, sheath gas flow rate 40–50 arbitrary units, spray voltage 4.5 kV, mass range 100 2000 amu (maximum resolution 30,000). HPLC separations were carried out using a Phenomenex reversed-phase (C18, 250 6 10 mm, L x i.d.) column connected to an Agilent 1200 series binary pump and monitored using an Agilent photodiode array detector. Detection was carried out at 227 nm.

This data provided the photodiode array profile, mass ion counts, specific UV and fragmentation pattern of all the metabolites present in each TCE. Input of this information into the MarinLit, AntiMarin and Antibase databases provided detailed information on already known compounds and prior information about the structures of all new compounds.

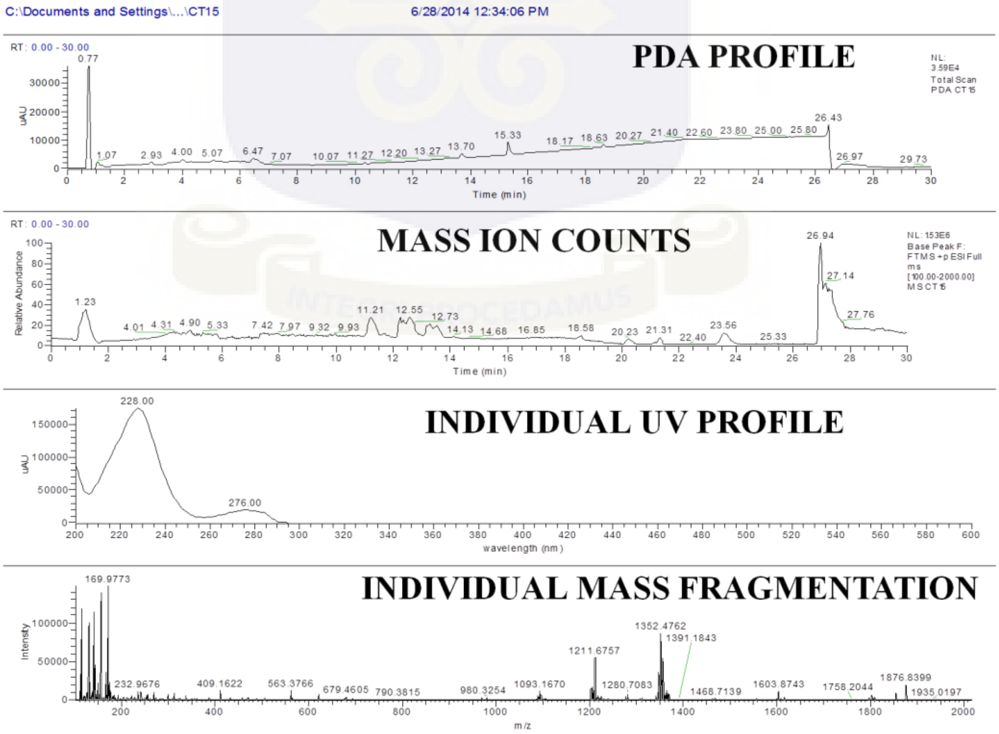


Figure 3.2: Illustration of the HRESI/HPLC-DAD-MS^n spectrum.

3.3.7 Large scale culture of high priority endophytic fungi

After the HRESI/HPLC-DAD-MS^n analysis, it was detected that marine-derived fungi, *Penicillium* sp. BRS2A-AR2 produced interesting compounds. Therefore, large scale fermentation in about 1 L broth was carried out to isolate these compounds. This was done by inoculating two already autoclaved 1 L Erlenmeyer flasks, sealed with non-adsorbent cotton wool, containing 500 ml of malt extract liquid broth with *Penicillium* sp. BRS2A-AR2under sterile conditions. These flasks were labelled and incubated at 28 °C for a period of 12 months. At the end of the incubation period, the cultures were filtered under suction and the mycelia separated from the broth. The broths were extracted once with ethylacetate and the mycelia were combined and repeatedly and alternatively extracted with methanol and dichloromethane. The extracts obtained after drying under vacuum from the ethylacetate, methanol and dichloromethane fractions were combined to give a TCE (1.30 mg).

3.3.8 Solvent partitioning by a modification of Kupchan's method

The next step which followed solvent extraction of the liquid broth and mycelia of *Penicillium* sp. BRS2A-AR2 was solvent partitioning. The procedures adopted were a modification of Kupchan's method (1973),[162] employing a combination of solvent systems with polarities ranging from the relatively non-polar hexane to the extremely polar water solvent. First, the TCE was suspended in water and extracted with an equal volume of dichloromethane three times. The result was two fractions, an aqueous fraction and a dichloromethane fraction. The aqueous fraction was extracted with an equal volume of sec-butanol once. Two separate fractions were obtained, the aqueous fraction from this end was discarded and the sec-butanol layer was rotary evaporated to dryness and labelled BRS2A-AR2-WB (390.04 mg). The dichloromethane fraction obtained initially from the first partitioning process was rotary evaporated to dryness under reduced pressure. This fraction was then suspended in 90% methanol: water and extracted with an equal volume of hexane (1:1) three times. Resulting hexane layer was rotary evaporated to dryness and labelled BRS2A-AR2-FH (248.78 mg). The 90% methanol: water layer was then phase adjusted to 50% methanol: water and extracted with an equal volume of dichloromethane (1:1) three times. Both resultant fractions were rotary evaporated to dryness under reduced pressure and labelled BRS2A-AR2-FD dichloromethane fraction (396.95 mg) and BRS2A-AR2-FM 50% methanol: water fraction (256.48 mg) accordingly. Fractions that separated out in the solvent partitioning process possessed defined polarities.

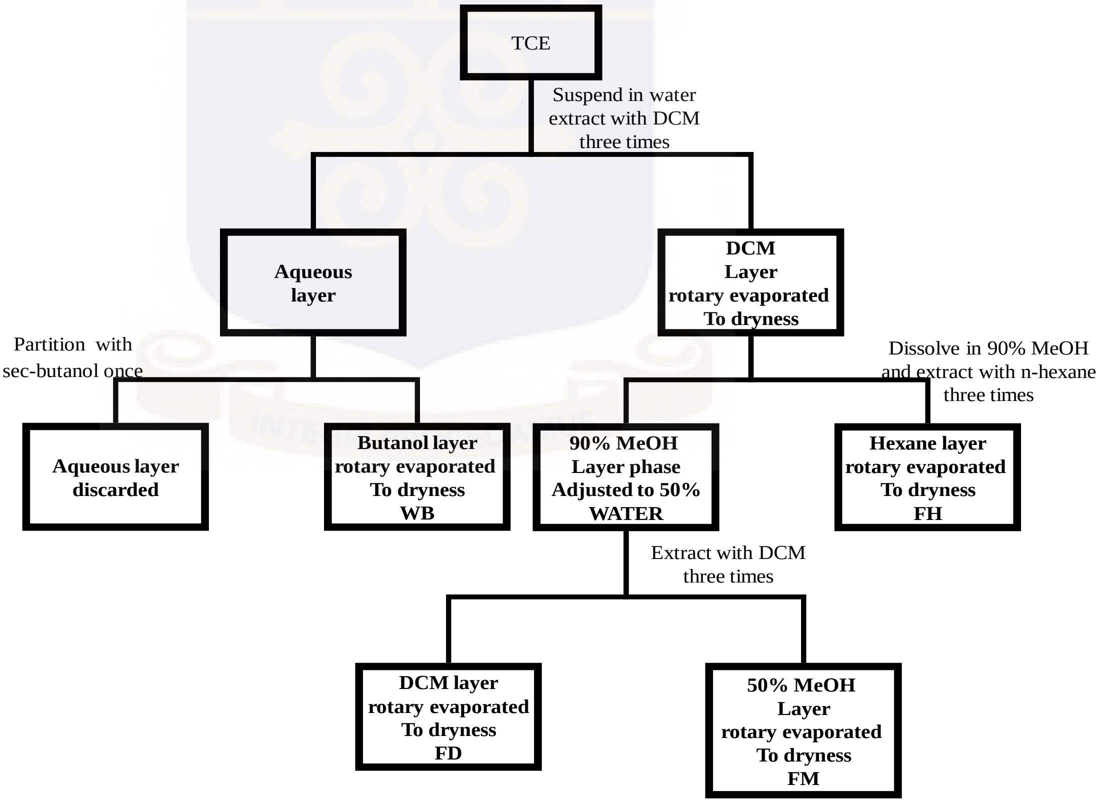


Figure 3.3: Flow chart for the isolation of BRS2A-AR2-FD and FM.

Between 1-1.5 mg of each of the resulting four fractions FH, FD, FM, and WB were then sent to our collaborators in the Aberdeen for HRESI/HPLC-DAD-MS^n analysis to determine which fractions had retained the compounds of interest.

3.3.9 Purification of BRS2A-AR2-FM and isolation of compounds

HRESI/HPLC-DAD-MS^n analysis of the Kupchan solvent partitioning extracts showed that, most of the compounds of interest were in BRS2A-AR2-FM. Hence, this fraction was subjected to a size exclusion column chromatography using Sephadex-LH20 as stationary phase and methanol as mobile phase as shown in the Figure below.

Plate 3.7: Sephadex LH-20 size exclusion chromatography of BRS2A-AR2-FM with fractions starting to separate into different molecular weights.

Five Sephadex LH-20 fractions were collected in all and these fractions were concentrated using a rotary evaporator, transferred into vials and labelled BRS2A-AR2-FM-SF1-5. About 1.0 mg of each fraction was dissolved in 1.5 ml HPLC grade methanol and submitted for HRESI/HPLC-DAD-MS^n analysis. From the analysis, the SF2, SF3, SF4 and SF5 fractions all contained interesting compounds but only the isolation of compounds in SF2 (58.00 mg) is subsequently described in this thesis.

Fraction BRS2A-AR2-FM-SF2 was subjected to alternating HPLC separation and purification using a Phenomenex Luna C18 column (C18 250 × 10 mm, L × i.d.) and column chromatography by gravity using silica as stationary phase and hexane-ethyl acetate mixtures as eluents. Gradients of H_2O:CH_3CN (1:1 in 30 min and hold for 20 min) were used as eluents with column flow rates set at 1.5 mL/min to afford 3.90 mg of Quinolactacin A1 and A2 as an intimate mixture and 3.03 mg of Citrinadin A from 58.00 mg of sample BRS2A-AR2-FM-SF2. About 1.0 mg of each of the fairly pure compounds was subjected to HRESI/HPLC-DAD-MS^n analysis to ascertain the purity, molecular formula and the fragmentation pattern.

3.3.10 1D and 2D NMR analysis of Quinolactacin A1 and A2 and Citrinadin A

NMR data were acquired on a Varian VNMR 600 MHz and Bruker 500 MHz spectrometers with either $CDCl_3$ or CD_3OD as solvents. Data acquired include ^{1}H, ^{13}C, DEPT-135°, ^{1}H-^{1}H-COSY, HSQC, HMBC and gHSQC-TOCSY.

3.3 Biological activity test

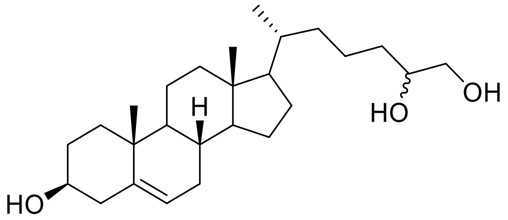
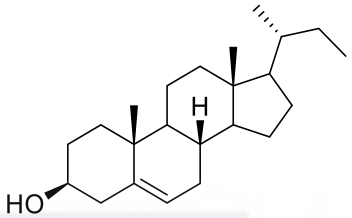
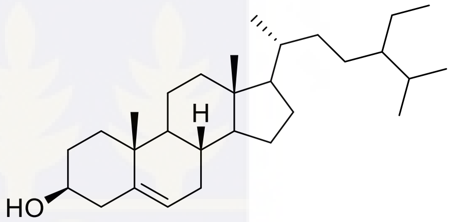
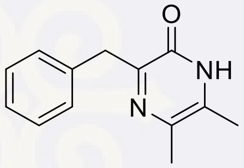
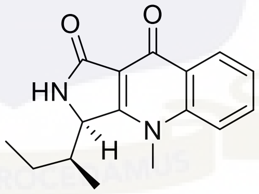
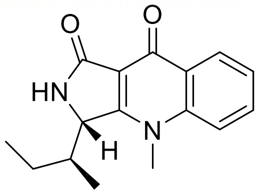
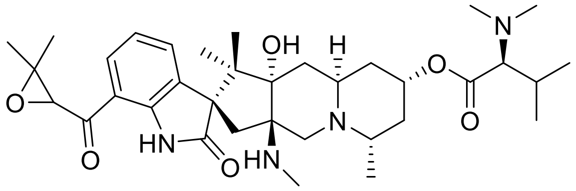
3.3.1 Chemicals and reagents

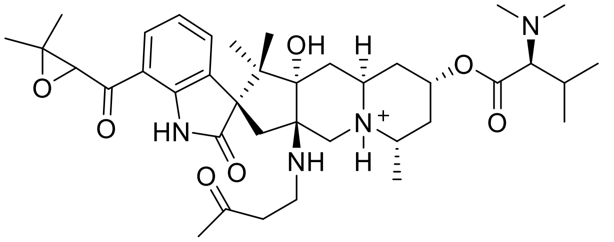
RPMI-1640, HEPES, Dulbecco Modified Eagle’s culture Media (DMEM), Foetal Bovine Serum (FBS),Trypsin, Gentamycin, Penicillin-Streptomycin-L-Glutamine (PSG), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Curcumin, Artesunate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, Dimethyl sulphoxide (DMSO), Sodium citrate, Adenine, Sodium bicarbonate ($NaHCO_3$), AlbuMax II, Sodium chloride (NaCl), Potassium chloride (KCl), Sodium Phosphate Dibasic (Na_2HPO_4), Sodium Phosphate Monobasic (KH_2PO_4), Sodium bicarbonate ($NaHCO_3$), Sodium hydroxide (NaOH), Hydrochloric acid (HCl),5,5’,6,6’-tetrachloro-1, 1’,3,3’-tetramethyl-benzimidalylcarbocyanine iodide and 7-Aminoactinomycin D were purchased from Sigma-Aldrich, USA.Malt extract, and other chemicals and reagents used were of analytical grade or of the highest purity commercially available.

3.3.2 Compounds tested for possible biological activity

Apart from the compounds isolated in the course of this project, some of the compounds previously isolated by other students within the research group were also tested alongside to make the study more interesting. All the compounds tested are therefore summarized in Table3.1 as shown below.

Table 3.1: Name and structure of compounds studied for biological activity

Compound name/Source	Structure
Cholest-5-ene-3,25,26-triol (3□,25□) *Axinella* sp. *new compound **SF2C5HG**	
20-Ethyl-5-pregnen-3-ol *Axinella* sp. **SF2C5HH**	
□-Sitosterol *Axinella* sp. **SF2C5HI**	
Butrepyrazinone *Verrucosispora* sp. K51G *new compound	
Quinolactacin A1 *Penicillium* sp. BRS2A-AR2	
Quinolactacin A2 *Penicillium* sp. BRS2A-AR2	
Citrinadin A *Penicillium* sp. BRS2A-AR2	

Butrecitrinadin *Penicillium* sp. BRS2A-AR2	

3.3.3 Cell lines tested in the cytotoxicity assay

The under listed human cancerous cell lines used in the cytotoxicity assays conducted during the course of this project were kind gifts from Dr. Takuhiro Uto of Nagasaki International University, Japan.

Jurkat	HumanT-lymphoblastic leukemia cells (Suspension cells)
HepG2	Human hepatocellular carcinoma (Adhesive cells)
HL-60	Human promyelocytic leukemia cells (Suspension cells)
LNCap	Human prostate cancer (Adhesive cells)
MCF-7	Human breast cancer (Adhesive cells)
PC-3	Human prostate cancer (Adhesive cells)

3.3.4 Preparation of compounds for bioactivity testing

Stock solutions of all the compounds were prepared at a concentration of 10mM. This was achieved by drying the compounds with nitrogen gas, weighing to ascertain the mass of the compound and dissolving in appropriate amount of DMSO in order to attain desired concentration. The solutions were vortexed and filter sterilized into vials through 0.45µm millipore filters under sterile conditions and stored at -20°C until use.

3.3.5 Cytotoxicity studies

3.3.5.1 Cell culture and cell treatments

Jurkat, HL-60, LNCap and PC-3cells were cultured in RPMI-1640 medium, while the MCF-7 and HepG2 cells were maintained in DMEM medium. All cell cultures were supplemented with 10% FBS, 1% Penicillin-Streptomycin-L-Glutamine and incubated at 37˚C in a 5% CO_2under humidified atmosphericconditions.

All the compounds in addition to Curcumin (used as positive control) were dissolved in DMSO and stored at -20˚C until used. The DMSO concentration in the test wells did not exceed 0.1% (v/v), and the control cells were treated with the same amount of DMSO.

Plate 3.8: Cancer cells in incubation.

3.3.5.2 Cell viability assay

The growth inhibition of the cell lines due to the test compounds or standard was examined using the tetrazolium-based colorimetric assay (MTT Assay) to determine the viability of the cells at any particular time.

PRINCIPLE: The assay is based on the capacity of the cellular mitochondrial reductase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4, 5-dimethylthiazol- 2yl)-2, 5-diphenyl tetrazolium bromide (MTT) into apurple formazan crystals which is soluble in acidified isopropanol. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The colour development from yellow to purple ismonitored at 570nm using a spectrophotometer.

Scheme 3.1: Conversion of yellow MTT dye to dark blue formazan by cellular mitochondrion reductase.

PROCEDURE: The procedures described by Ayisi *et. al.* (1991) were followed with modifications. Dilution of the 10mM stock solution of each compound was made in 1% DMSO to obtain five different concentrations ranging from0to 100µM.[163]

Cultured suspension cells (HL-60 and Jurkat cells) were transferred into centrifuge tubes. The mono-layer adhesive cells (LNCap, PC3, HepG2, and MCF7) in the culture flasks were washed with PBS, detached with trypsin solution and transferred into centrifuge tubes. Tubes were centrifuged at 1000rpm for 5 minutes and the pellets were re-suspended in growth media. Cells were counted using a haemocytometer (MARIENFELD, Germany) and a cell suspension of $1x10^5$ cells/ml was prepared by diluting with a growthmedia.100µL (1×10^5 cells/mL) of cell suspensions were seeded into 96-well plates and incubated overnight before treatment.

10 µL of the compounds at different concentrations were each added to the wells with the cell suspensions and incubated under the condition stated above for 72 hours. Curcumin was used as a positive control in all assays and a colour control plate was also setup for each compound. 20µL of 2.5mg/mL MTT solution was added to the wells and incubated further for4 hours in the CO_2 incubator. 150µL of acidified isopropanol containing Triton-X was then added to stop the reaction. The reaction plates were incubated in the dark at room temperature overnight and absorbance read at 570nm using a micro-plate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria).

Percent Cell viability was determined as:

$$\text{\% Cell viability} = \frac{[A\alpha - A\beta]}{[A\gamma - A\beta]} \times 100$$

A_α= Mean absorbance of treated cells

A_β= Mean absorbance of blank

$A\gamma$ = Mean absorbance of untreated cells

The average percentage cell viability determined at each concentration was plotted as a dose response curve using Graph Pad Prism Version 5.02. The inhibition concentration at fifty percent (IC_{50}) values, that is, concentration of compounds or standard drug inducing 50% inhibition of cells, determined from the dose response curve by nonlinear regression analysis.

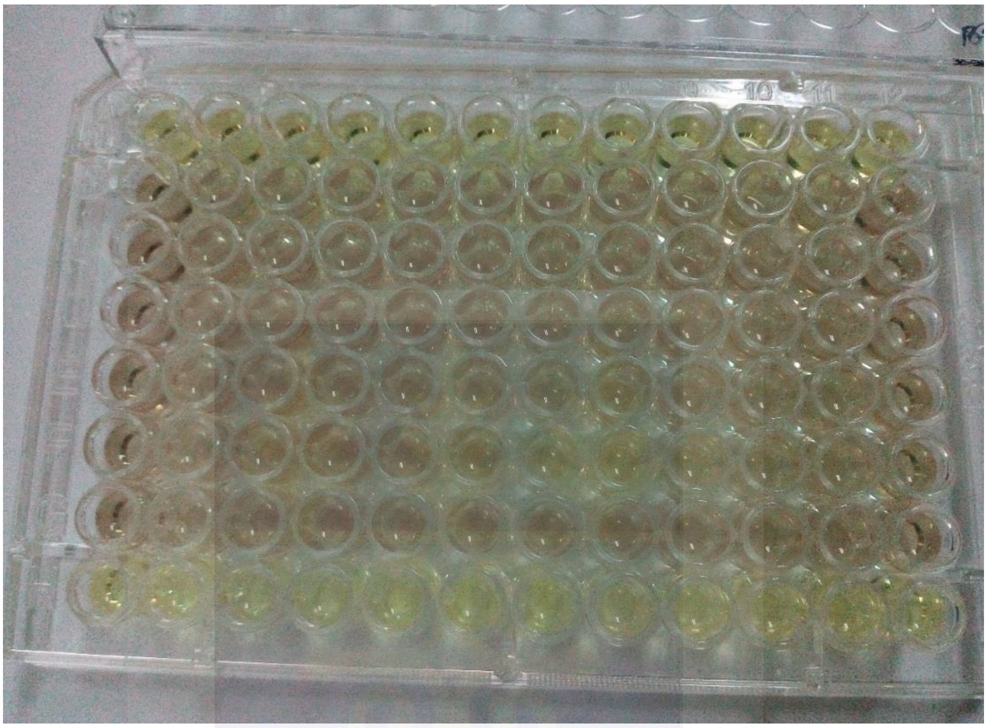


Plate 3.9: 96 well plate showing conversion of yellow MTT to Formazan

3.3.6 Anti-malaria activity study

3.3.6.1 Blood collection and erythrocytes preparation

Erythrocytes were obtained from blood of consented volunteers (Blood group O^+). Venous blood was drawn and collected into containers containing citrate phosphate dextrose (CPD) solution and kept at 4 ^oC overnight. It was centrifuged at 2,000 rpm for 10 minutes to separate the serum and buffy coat. Packed erythrocytes were washed three times with parasite washing medium (RPMI 1640, buffered with,50 µg/mL gentamicin and 2 mM L-glutamine) Each washing step involved addition of wash medium, pipetting up and down thrice, centrifuging at 2,000 rpm for 10 minutes and then discarding suspended medium. After washing, medium was added to the packed erythrocytesand stored at 4 °C until ready for use. Washed RBCs were stored and used for up to 2 weeks maximum after which new blood was collected.

3.3.6.2 Giemsa stained thin blood smear and parasitaemia determination

A drop of infected erythrocytes cultured medium was placed on a microscope slide and spread with the aid of another slide. Slides were air dried, dipped into methanol for some seconds to fix and air dry again. A 10% Giemsa stain was added to cover completely the surface of the fixed slides for at least 10 minutes and then gently rinsed off with running water. Stained slides were air dried and then viewed under a light microscope (OLYMPUS CK30) (with immersion oil at 100x objective) to determine parasitaemia. Percent parasitaemia was determined by counting the number of infected cells in a total of 500 erythrocytes in the Giemsa-stained thin blood smear. Parasitaemia was expressed as a percentage of the number of infected erythrocytes to the total number of erythrocytes counted.

3.3.6.3 In vitro cultivation of Malaria Parasite

Erythrocytic stages of malaria parasite (*Plasmodium falciparum*-chloroquine sensitive strain 3D7) were cultured in 25 cm^2flasks using the method of Trager and Jensen (1976) with modifications. Erythrocytes were maintained at 2% haematocrit (v/v) cell suspension in complete malaria parasite medium (RPMI 1640, buffered with 25 mM HEPES, supplemented with 7.5% $NaHCO_3$, 25 ug/ml gentamycin, 5% heat-inactivated human O^+ serum (from consented subject) and 5 mg/mlAlbuMax II) and incubated at 37°C under gas condition of 2% O_2, 5% CO_2 and 93% N_2.

Parasite growth and development were monitored with Giemsa stained thin blood smear. Parasite culture was purified by using 5% sorbitol to obtain matured erythrocyte parasitic stages (late trophozoites and schizonts) from uninfected cells. The matured erythrocyte parasitic stages (purity > 90%) obtained were used to screen the compounds for anti-malaria activity.

3.3.6.4 Screening for anti-malaria activity using the SYBR Green I assay

A total of six(6) compounds were screened for anti-malaria activity by using the SYBR Green I fluorescence assay as described by Smilktein *et. al.* (2004) with some modifications.[164] Serial dilution of the standard (artesunate) which served as an experimental control and stock solution of compounds to yield final concentrations ranging from 100 ng/ml to 400 ng/ml and 3.13 µg/ml to 25 µg/ml respectively were prepared. The matured erythrocyte parasitic stages were treated with compounds and washed erythrocytes in 96 well plates (Nunc) and incubated with complete malaria parasite medium for 24 hrs.

Slides were then prepared and percent parasitaemia was determined by counting the number of infected cells in a total of 500 erythrocytes in the Giemsa-stained thin blood smear. Briefly an aliquot of 5 µL per each concentration of the standard drug and compounds was dispensed into test wells. 95µL of complete malaria parasite medium with washed erythrocytes at 2% haematocrit and the purified matured erythrocyte parasitic stages (1% parasitaemia) were added, and incubated at 37^{o}C under gaseous conditions as stated above and untreated erythrocytes were used as control.

Wells containing erythrocytes at 2% haematocrit, infected erythrocytes at 2% haematocrit and complete parasite medium alone served as negative controls, positive controls and blank controls respectively. Furthermore, wells containing infected parasites and 0.1% DMSO served as reference controls. Final volume per well was

100 µL. Plates were then incubated for 24 hr as described above in the cultivation of malaria parasites. 100 µL aliquot of 2.5x buffered SYBR Green I (0.25 µL of SYBR Green I/mL of phosphate buffer saline) was added to each well after the incubation period and incubated in the dark for 30 min at 37 °C. Fluorescence was detected by Guava EasyCyte HT FACS machine (Millipore, USA).

Principle: The contrast between host erythrocytes which lack DNA and RNA and the malaria parasites which have DNA and RNA form the basis of the SYBR Green experiment. The plasmodium parasites can easily be stained with the SYBR Green dye and quantified by fluorescence spectroscopy.

3.3.7 Apoptosis study

3.3.7.1 Mitochondrial Potential

Determination of apoptosis-inducing capabilities of compounds as indication of high anti- malaria activities was done by the use of the Guava MitoPotential$^{(R)}$ kit (Guava Technologies, USA) as described by the manufacturer's instructions. A cationic dye, 5,5',6,6'-tetrachloro-1, 1',3,3'-tetrathylbenzimidalylcarbocyanine iodide (JC-1), was used to evaluate mitochondrial membrane potential changes and 7-Aminoactinomycin D (7-AAD), a cell-impermeant DNA intercalator, was also used to monitor cell membrane permeability changes. Synchronized culture parasites and preparation of 96-well micro-titer plates were taken through the same procedure described above for the screening of the compounds to. 2x staining solution (4 µL of 50x staining solution/100 µL of complete parasite medium) of the Guava MitoPotential$^{(R)}$ kit was then added to each well in a ratio of 1:1 (v/v) and incubated for 30 min at 37 °C in the dark. Stained cells were analyzed on a Guava EasyCyte HT system.

3.3.8 Anti-buruli ulcer activity assay

3.3.8.1 Resazurin Microtiter Assays (REMA)

Preparation of Inoculum: The Resazurin based assay as described by Yemoa *et. al.* (2011) was followed.[165]The assay used in detecting growth inhibition of microorganisms including mycobacteria was used to access the ability of the compounds to exhibit inhibition against *Mycobacterium ulcerans*.The inoculum was prepared by making a direct broth suspension of a full loop of isolated colonies selected from a 6 weeks Middlebrook 7H9 solid medium fresh *Mycobacterium ulcerans* MN209 characterized isolate culture adjusted to achieve a turbidity of 1.0 McFarland turbidity standard which is equivalent to 3 x 10^8 colony forming units (CFU)/ml. The adjusted inoculum suspension was further diluted 1:1000 in 7H9broth up to approximately 1 x 10^5 CFU/ml.

Inoculation: Within 15 minutes after the inoculum has been standardized as described above, 100 µL of the adjusted inoculum was added to each tube containing 4 µL of the compounds in the dilution series with a positive control well containing only broth and the plates were incubated.

Procedure: After 15 days of incubation, resazurin was added and a colour change observed after 48 hours incubation.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Dereplication of marine-derived endophytic fungi crude extracts

Dereplication is a process in natural product drug discovery that is designed to maximise all laboratory efforts and prioritise the isolation of new or novel chemistry from crude extracts of nature's flora or fauna.[166,167] When structural novelty is not a priority but, biological activity constitute the main driving force for a specific natural product drug discovery project, biological activity guided screening of crude extracts is preferred. In some cases, both techniques have been used hand-in-hand to yield compounds that have both structural novelty and biological activity.

In all, twenty-four (24) different species of marine-derived endophytic fungi were isolated from various plant parts of three major mangrove plants growing in and along the banks of the Butre River in the Western Region of Ghana. The three (3) main plants sampled were *Conocarpus erectus*, *Laguncularia racemosa* and *Rhizophora racemosa*. Six (6) different species out of the twenty-four (24) isolated fungi were selected for chemical profiling, prioritization, identification and subsequent isolation of compounds from high priority extracts.

After small scale culture of these six (6) endophytic fungal species with measurement and analysis of HRESI/HPLC-DAD-MS^n data, two fungi i.e. Strain BRS2A-AR andBRS2A-AR2 were prioritized for identification and further isolation and characterization of secondary metabolites.

However, due to time constraints, only *Penicillium* sp. BRS2A-AR2 was further fractionated to isolate some of the component secondary metabolites.

It is important to note that, the remaining four (4) species were also interesting but, set aside for two main reasons:

- These species did not make a wide range of secondary metabolites. They seem to possess low biosynthetic prowess and ingenuity.
- The secondary metabolites detected in their small scale culture extracts were interesting but already known compounds.

4.1.1 Dereplication of crude extracts of BRS3A-T2

The marine-derived fungi BRS3A-T2 (Plate4.1) was isolated as a fast-growing fluffy white microbe with granules of dark stones interspersed within its structural matrix. Careful analysis of the crude extracts obtained from small scale culture of this species showed a rather low biosynthetic prowess. This observation was true for this species, at least for the malt extract medium in which it was cultured.

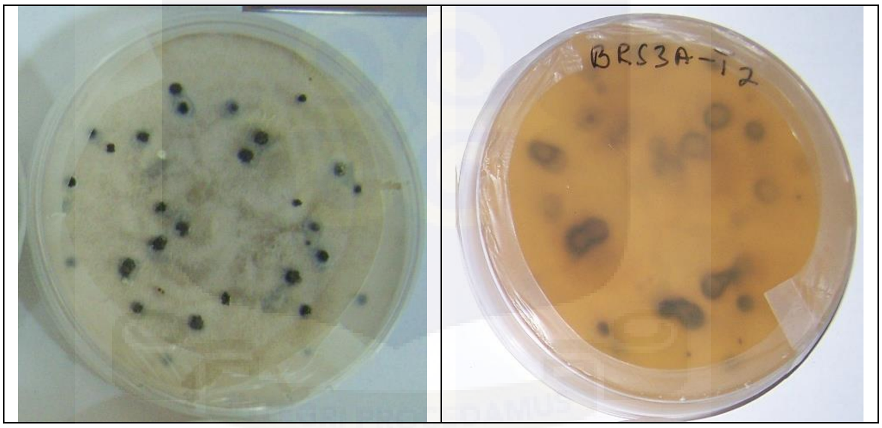
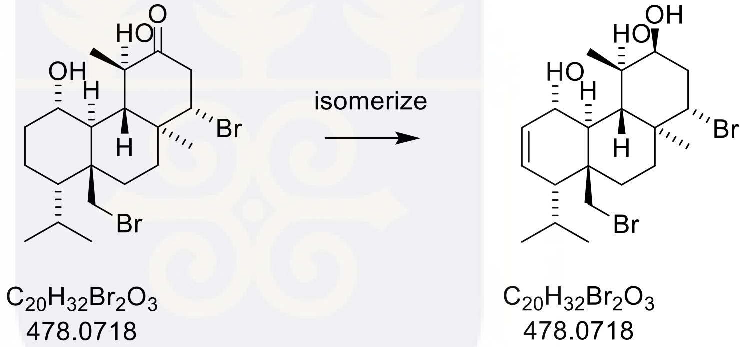


Plate 4.1: Pure endophytic fungi BRS3A-T2 isolated from the trunk of an indigenous mangrove plant sampled at Butre River.

After small scale culture of BRS3A-T2, the culture was suction filtered and the mycelium was separated from the broth as outlined in detail in Chapter 3. The broth was extracted once in a separating funnel with ethyl acetate while the mycelium was extracted with methanol. The two extracts were then dried under vacuum and 1.0 mg/ml of each was submitted for the HRESI/HPLC-DAD-MS^n analysis. The

MarinLit database search for the two measurements shown in Figures 4.1 and 4.2 revealed that, BRS3A-T2 predominantly makes the already isolated and characterized brominated diterpene 14S-bromo-1S-hydroxy-1,2,13,14-tetrahydrosphaerococenol A which was previously isolated from the red alga *Sphaerococcus coronopifolius* (Scheme 4.1).**[168]** In this article, Smyrniotopoulos *et. al.* (2008) obtained five other derivatives of this compound all of which were based essentially on the brominated diterpene backbone. These compounds were tested and found to possess antibacterial activity against a panel of multidrug-resistant and methicillin-resistant *Staphylococcus aureus* with MICs in the range of 0.5-128 µg/ml.**[168]**



14S-Bromo-1S-hydroxy-1,2,13,14-tetrahydrosphaerococenol A

Scheme 4.1: Proposed structures for the mass spectrometry fragments of 14S-bromo-1S-hydroxy-1,2,13,14-tetrahydrosphaerococenol A.

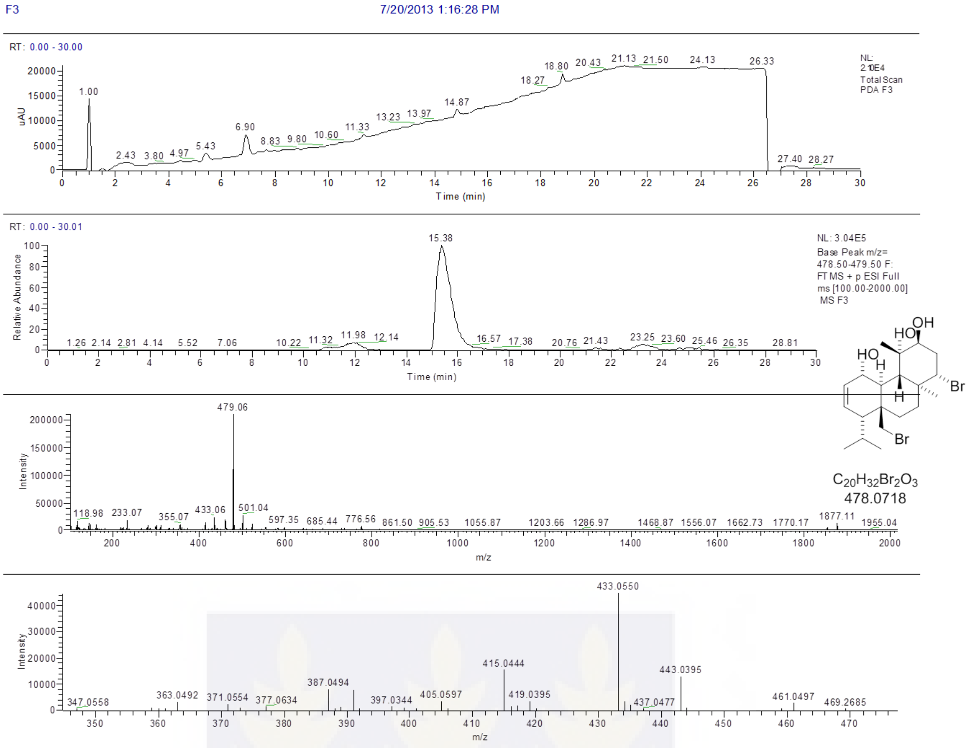


Figure 4.1: HRESI/HPLC-DAD-MSⁿ profile of the ethyl acetate extract of BRS3A-T2 liquid culture in malt extract.

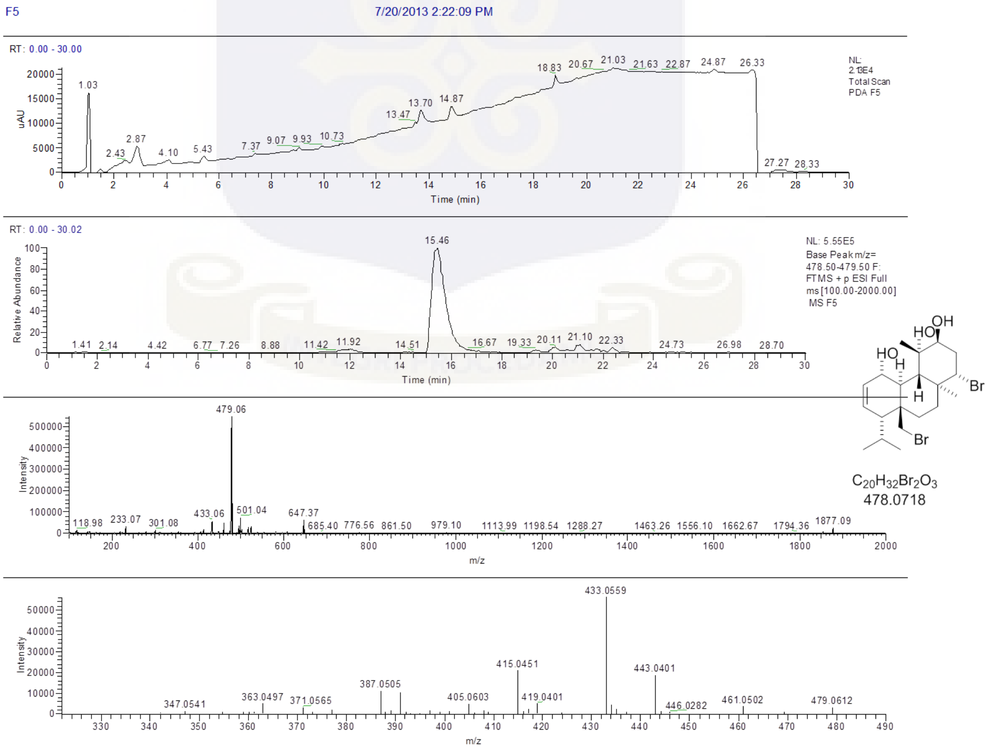
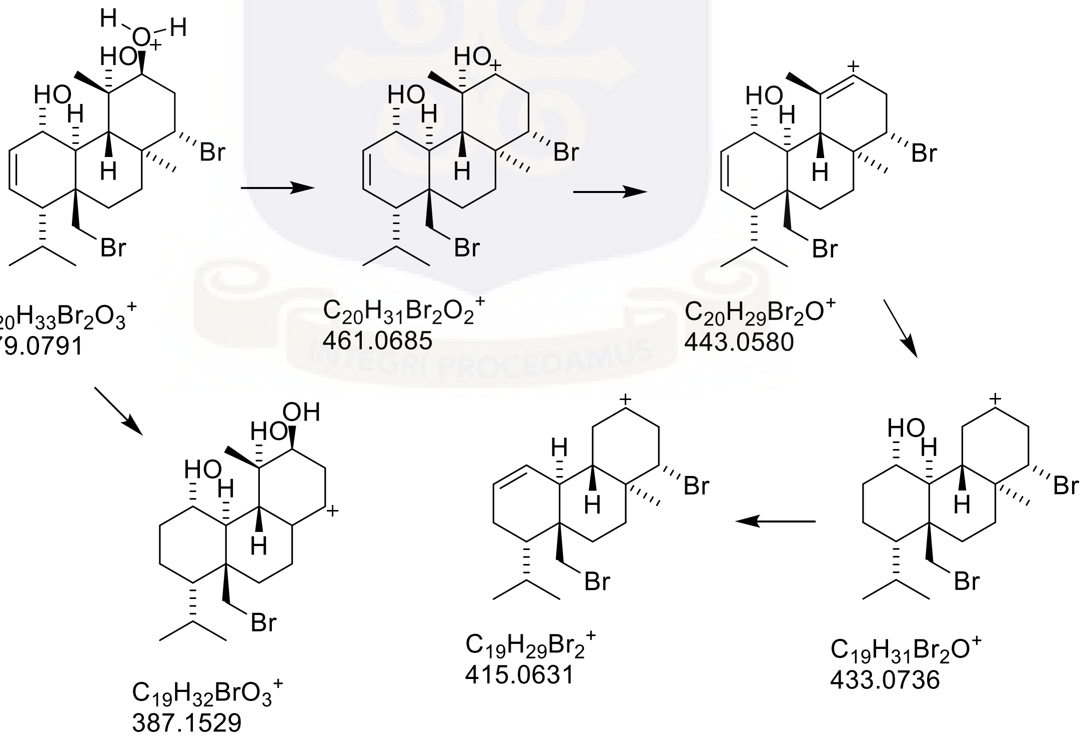


Figure 4.2: HRESI/HPLC-DAD-MSⁿ profile of the methanol extract of mycelia of BRS3A-T2 cultured in malt extract.

In order to confirm the identity of this known compound, the fragmentation pattern produced in theHRESI/HPLC-DAD-MS^n was investigated and sub-structures were proposed as shown in Scheme 4.2.

This species BRS3A-T2 was therefore abandoned for fear of re-isolating a bunch of already characterized marine-derived fungal metabolites. However, the very important discovery here is that, it appears as if the red alga *Sphaerococcus coronopifolius* is not really responsible for the biosynthesis of 14S-bromo-1S-hydroxy-1,2,13,14-tetrahydrosphaerococenol A or any of its derivatives. Rather, marine-derived endophytic fungi colonizing the inner tissues of the red alga might be responsible for making these compounds as an attempt to protect their host which in this case is *Sphaerococcus coronopifolius*.



Scheme 4.2: Biosynthesis of 14S-bromo-1S-hydroxy-1,2,13,14-tetrahydrosphaerococenol.

4.1.2 Dereplication of crude extracts of BRS2A-AR

The species BRS2A-AR was purified and found to grow as a crispy dark green carpet which formed mostly uniform lawn on the agar plates (Plate4.4). Small scale culture of the marine-derived endophytic fungal species BRS2A-AR followed by a work-up process as described above, gave two extracts; an ethyl acetate extract of the broth and methanol extract of the mycelia. 1.5 mg/ml of these two extracts were submitted for HRESI/HPLC-DAD-MS^n analysis. The data obtained showed that, BRS2A-ARpredominantly makes two main compounds at m/z 456.2260 and 421.1258 (Figure 4.3 and 4.4). The accurate masses and fragmentation patterns of these two molecules did not give any hits in the MarinLit, Antimarin and Antibase databases. This species was therefore important and characterized as high priority for further large scale culture and subsequent isolation and characterization of compounds.

Plate 4.2: Pure endophytic fungi BRS2A-AR isolated from the aerial root of an indigenous mangrove plant sampled at Butre River.

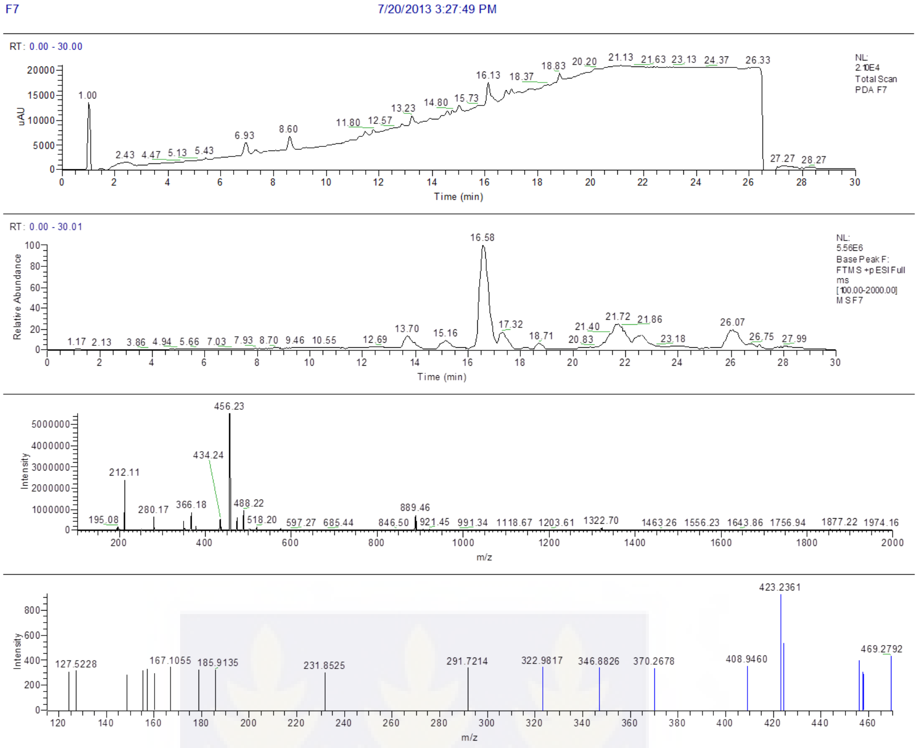


Figure 4.3:HRESI/HPLC-DAD-MS^n profile of the ethyl acetate extract of BRS2A-AR liquid culture in malt extract.

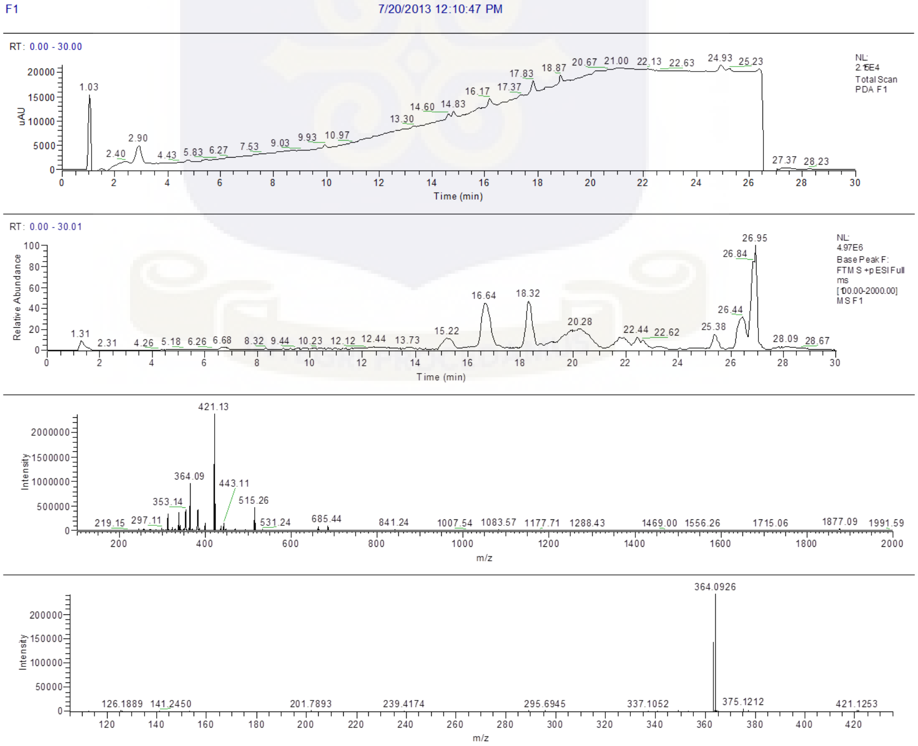
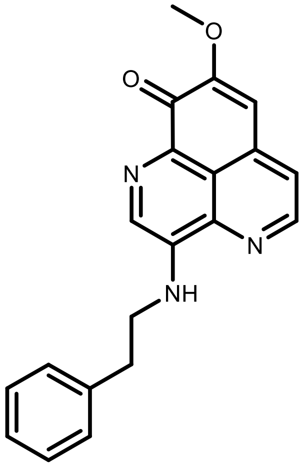


Figure 4.4: HRESI/HPLC-DAD-MS^n profile of the methanol extract of mycelia of BRS2A-AR cultured in malt extract.

4.1.3 Dereplication of crude extracts of BRS1A-B

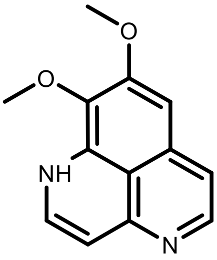
The marine-derived fungi BRS1A-B was very similar to BRS3A-T2 in morphology but, the two produced entirely different secondary metabolites (Plate4.7). Ethyl acetate and methanol extracts of BRS1A-B were dominated by the known metabolite3-(phenethylamino)demethyl(oxy)aaptamine and its derivatives (Figures 4.5-4.7). This compound along with the first member of the group to be characterized which is aaptaminewas previously isolated by Shaari *et. al.* (2009) from the sea sponge *Aaptos aaptos* belonging to the Phylum Porifera.**[169]** It is most surprising therefore to find out that, these metabolites constitute the main secondary metabolites produced by the marine-derived endophytic fungus BRS1A-B. It is therefore possible that, the marine sponge *Aaptos aaptos* is not responsible for the biosynthesis of aaptamine and its derivatives but, harbours endophytic fungi which biosynthesise these secondary metabolites to protect their host which in this case is the sponge.Aaptamine, 3-(phenethylamino)demethyl(oxy)aaptamine and their derivatives were found to be cytotoxic to CEM-SS cells which are T-lymphoblastic leukemia cells with IC_{50} ranging from 5-15 µg/ml.**[169]**

Further confirmation of the known structure of 3-(phenethylamino)demethyl(oxy)aaptamine was achieved through the interpretation of the fragmentation pattern of this compound in Scheme4.3.



Exact Mass: 331.1321

3-(Phenethylamino)demethyl(oxy)aaptamine



Exact Mass: 228.0899

Aaptamine

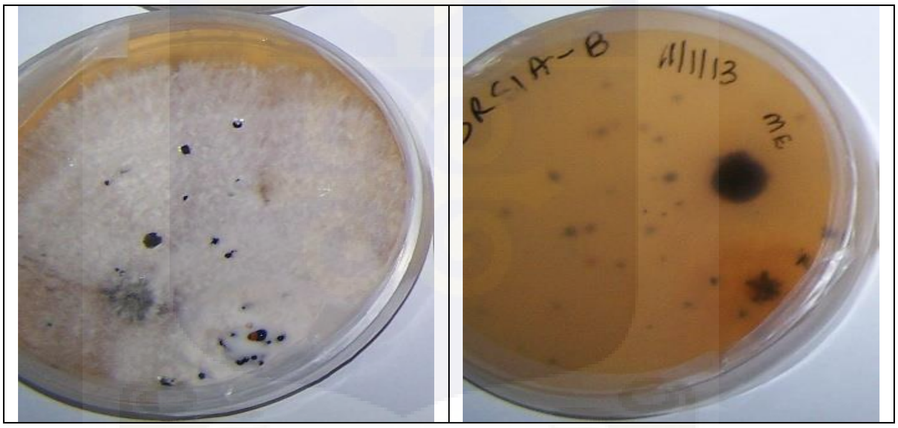


Plate 4.3:Pure endophytic fungi BRS1A-B isolated from the bud of an indigenous mangrove plant sampled at Butre River.

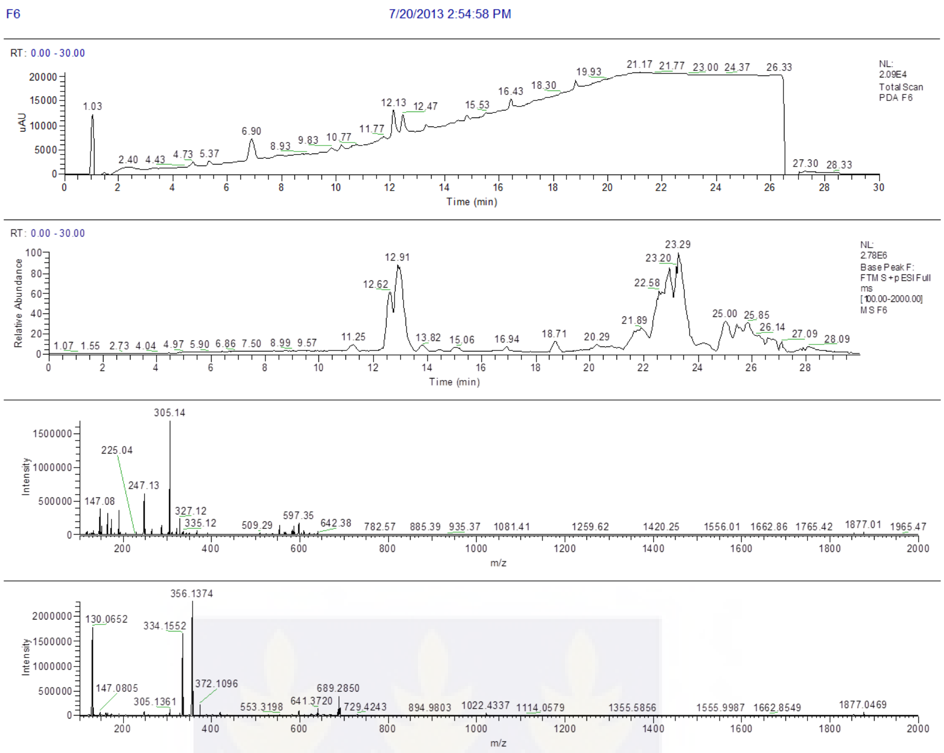


Figure 4.5: HRESI/HPLC-DAD-MS^n profile of the ethyl acetate extract of BRS1A-B liquid culture in malt extract.

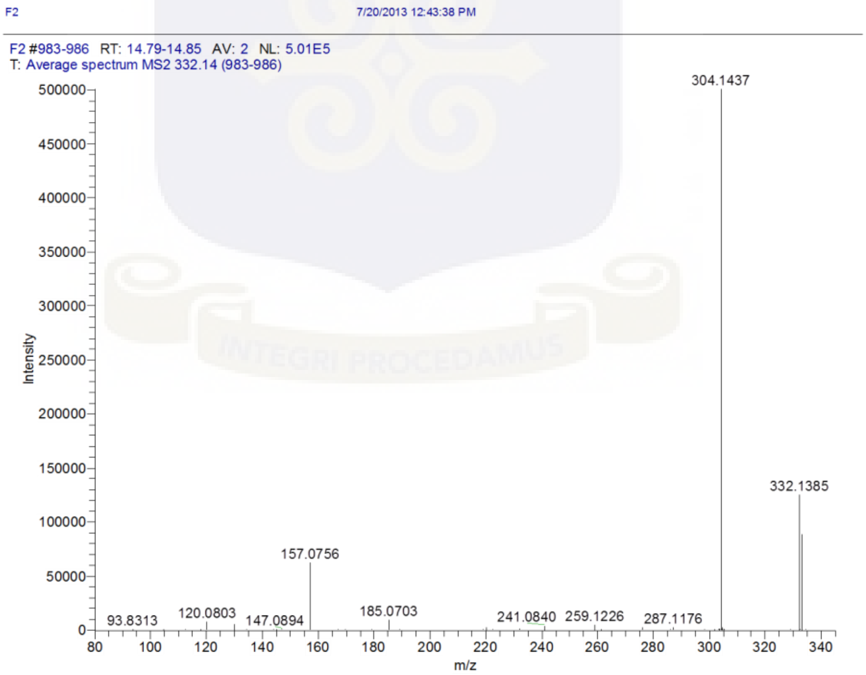


Figure 4.6: MS^n fragmentation pattern of m/z 331.1321 seen in both the ethyl acetate and methanol extracts of BRS1A-B.

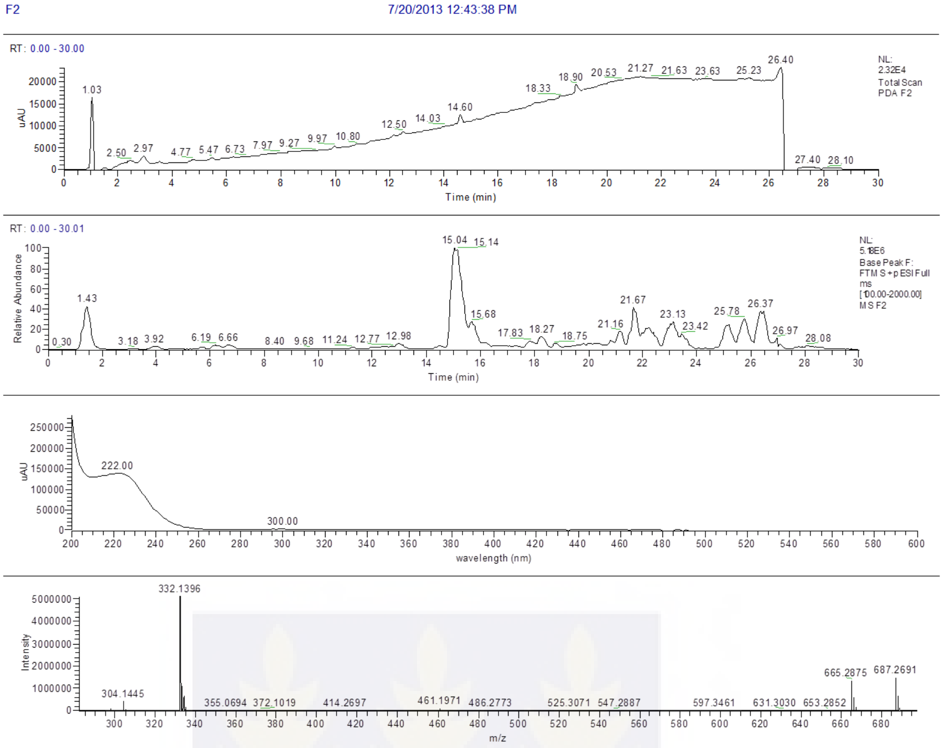
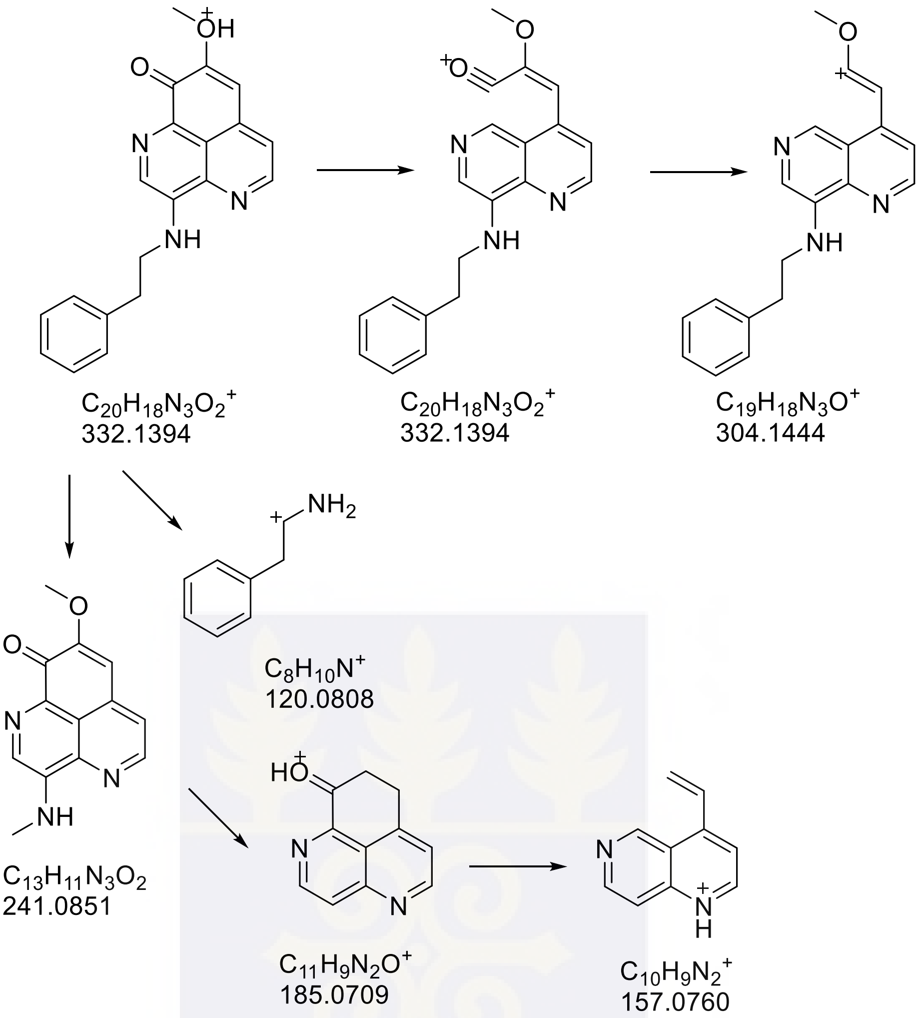


Figure 4.7: HRESI/HPLC-DAD-MS^n profile of the methanol extract of mycelia of BRS1A-B cultured in malt extract.



Scheme 4.3: Biosynthesis of 3-(phenethylamino)demethyl(oxy)aaptamine.

4.1.4 Dereplication of crude extracts of BUSUA2B-T

Marine-derived endophytic fungus BUSUA2B-T was isolated as pure species which grew as a thick grey carpet on solid media (Plate4.4). Crude ethyl acetate and methanol extracts of this fungus did not show the presence of any interesting secondary metabolites (Figures 4.8 and 4.9). Further investigation of BUSUA2B-T was therefore abandoned.

Plate 4.4:Pure endophytic fungi BUSUA 2B-T isolated from the trunk of an indigenous mangrove plant sampled at Busia beach.

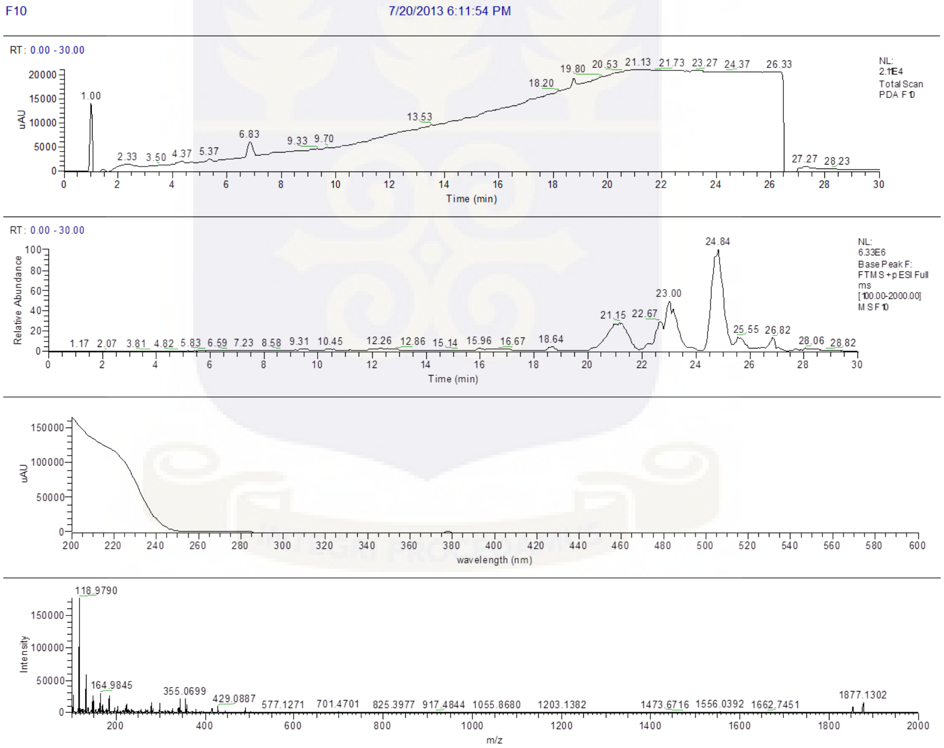


Figure 4.8: HRESI/HPLC-DAD-MS^n profile of the ethyl acetate extract of BUSUA2B-T liquid culture in malt extract.

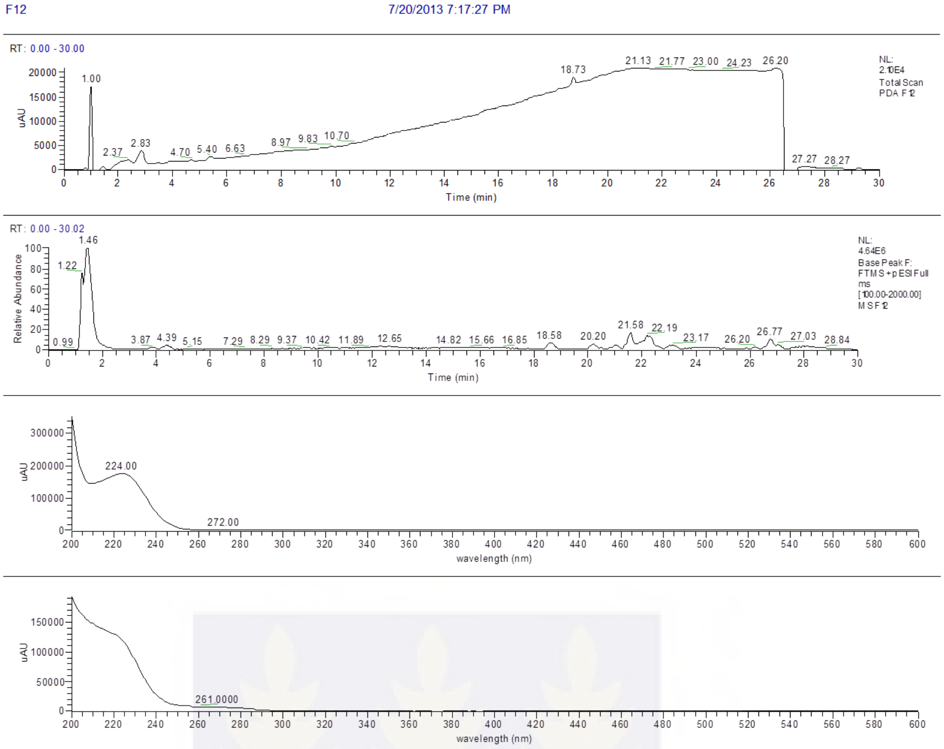


Figure 4.9: HRESI/HPLC-DAD-MS^n profile of the methanol extract of mycelia of BUSUA2B-T cultured in malt extract.

4.1.5 Dereplication of crude extracts of BUSUA2B-F

The species BUSUA2B-F after purification was seen as a dark brown carpet on the malt extract agar plates (Plate4.5). Mass spectrometry analysis of the ethyl acetate and methanol extracts showed that, BUSUA2B-F produced predominantly the mycosporine amino acid N-methylpalythine-serine (Figures 4.10 and 4.16). This compound was previously isolated and characterized by Teai *et. al.*(1997) from a stony coral *Pocillopora eydouxi*. The article did not describe any form of bioactivity test for this compound or its derivatives but, mycosporine amino acids are widespread in marine chemistry and known to act as sun screens amongst other biological activities. Therefore, further chemical characterization of BUSUA2B-F was immediately abandoned.[170]

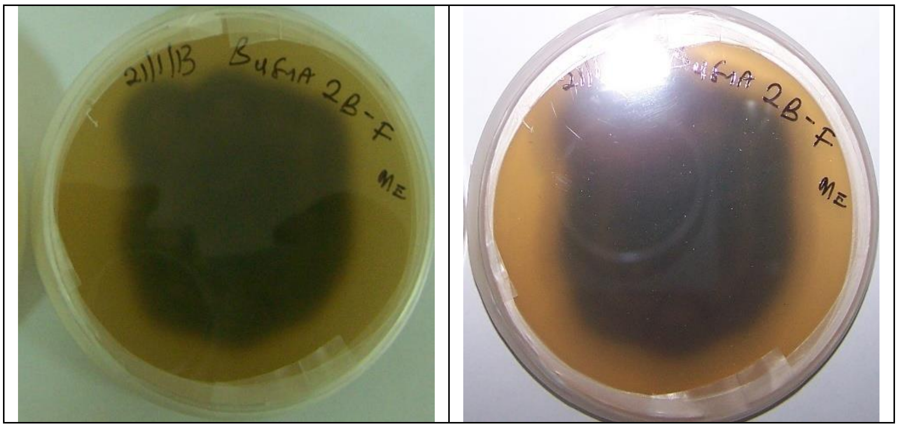


Plate 4.5: Pure endophytic fungi BUSUA 2B-F isolated from the fruit of an indigenous mangrove plant sampled at Busia beach.

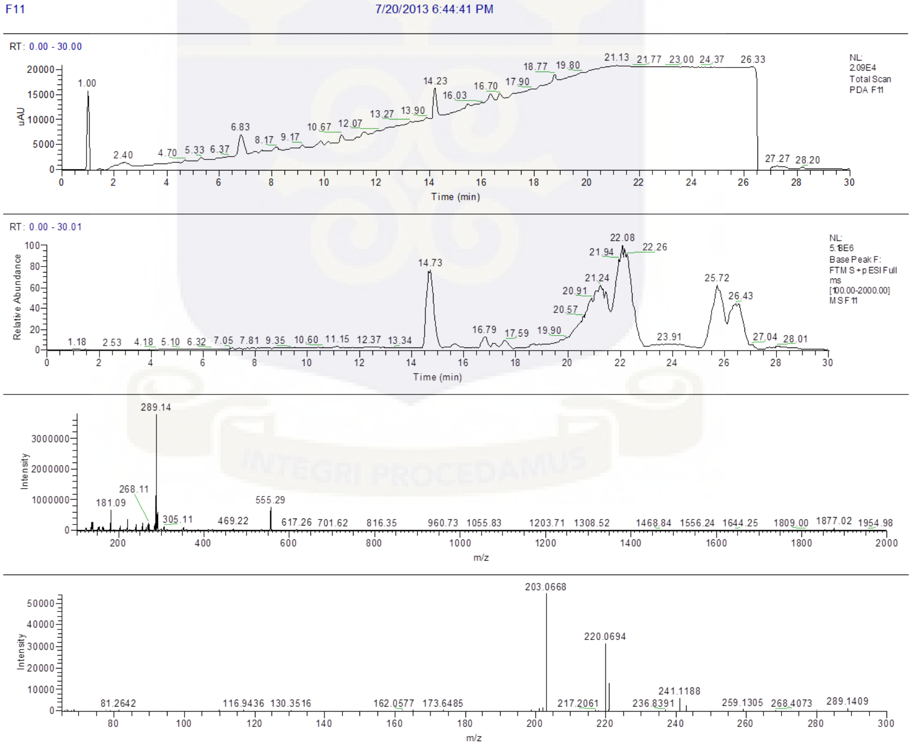
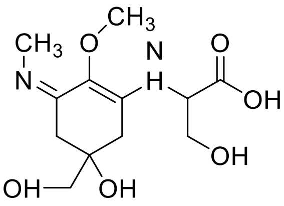


Figure 4.10: HRESI/HPLC-DAD-MS^n profile of the ethyl acetate extract of BUSUA2B-F liquid culture in malt extract.



Exact Mass: 288.1321

N-methylpalythine-serine

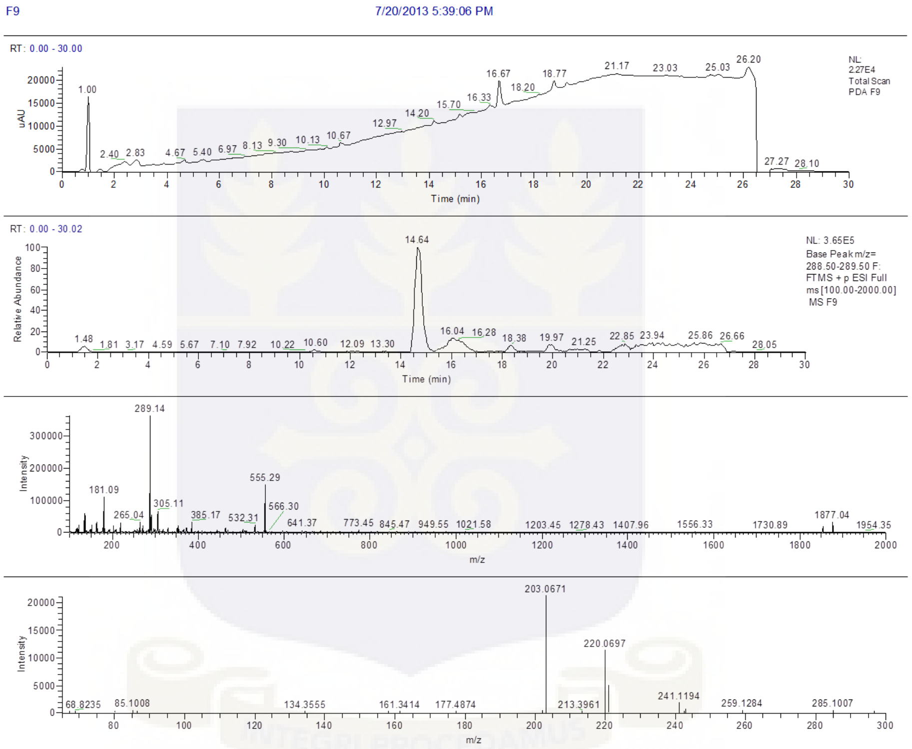


Figure 4.11: HRESI/HPLC-DAD-MS^n profile of the methanol extract of mycelia of BUSUA2B-F cultured in malt extract.

4.1.6 Dereplication of crude extracts of BRS2A-AR2

The marine derived specie BRS2A-AR2 was arguably the most interesting of the six (6) species studied in this project. On solid media, dark stretches of mycelia could be found growing within the agar and on top of the agar was a thick yellowish puss (Plate4.6). Coincidentally, these morphological features are very characteristic of the genus Penicillium and BRS2A-AR2 was noted as one of such species of endophytic fungi although its full characterization is yet to be determined.

Plate 4.6:Pure endophytic fungi BRS2A-AR2 isolated from the aerial root of an indigenous mangrove plant sampled at Butre River.

Analysis of the mass spectrometry data for ethyl acetate and methanol extracts of BRS2A-AR2 revealed this species as very talented for two main reasons:

- It makes quite a number of secondary metabolites as seen in the chromatograms shown in Figure4.12 and 4.13.
- The structural skeletons of the metabolites seen in the chromatogram of Figures4.12 and 4.13 are not the same. This was detected by comparing the fragmentation patterns of all the compounds present in the mass spectrometry data.

Interesting masses were detected at m/z values of 478.2562, 641.2723, 625.3962 (681.4227), 293.1262, 563.2631. All these masses did not come up in the database searches with MarinLit, Antimarin and Antibase with the exception of 625.3962 which came up as the novel pentacyclic alkaloid initially isolated and characterized by Tsuda *et. al.* from the marine-derived fungus *Penicillium citrinum.*[171]

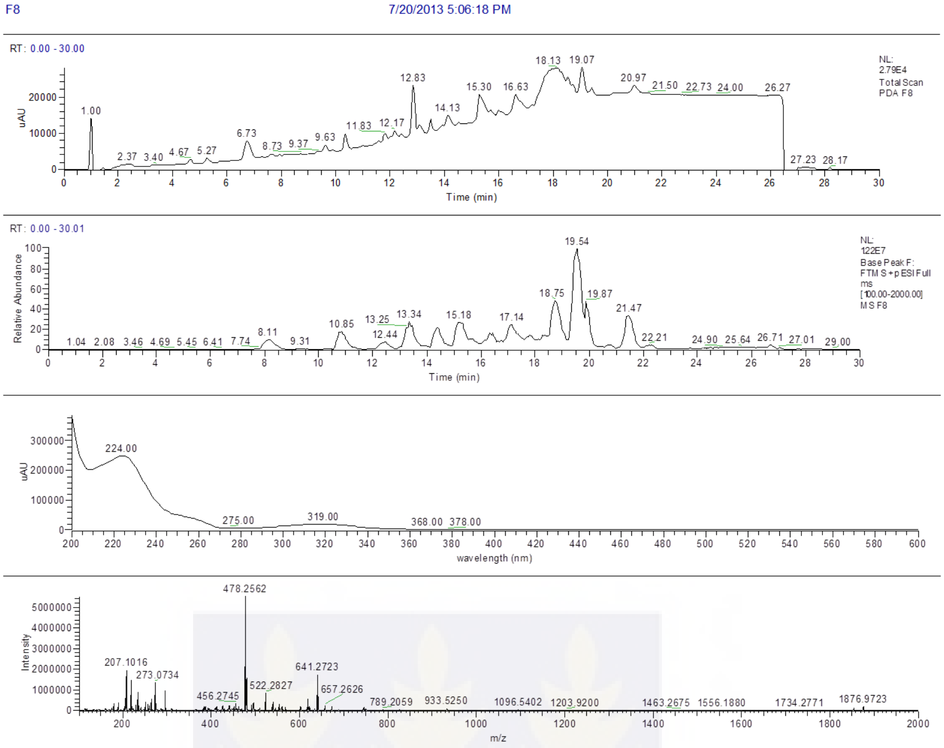


Figure 4.12: HRESI/HPLC-DAD-MS^n profile of the ethyl acetate extract of BRS2A-AR2 liquid culture in malt extract.

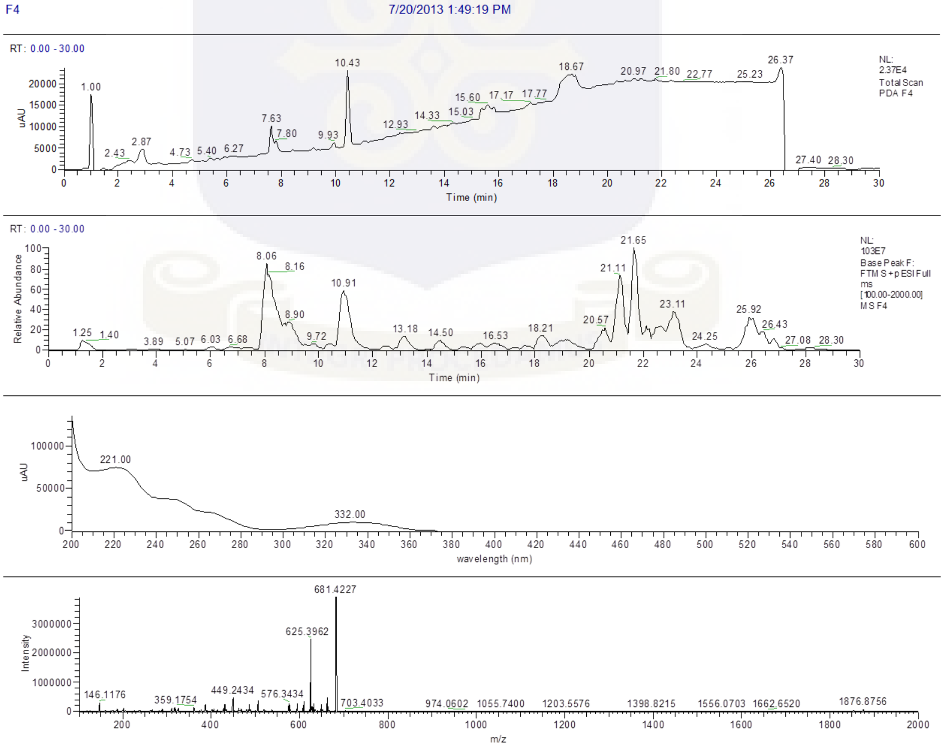


Figure 4.13: HRESI/HPLC-DAD-MS^n profile of the methanol extract of mycelia of BRS2A-AR2 cultured in malt extract.

Further fractionation and subsequent isolation of some of these metabolites are described in sections of the methodology outlined in Chapter 3 and the results are analysed here in this section.

4.2 Structure determination of Citrinadin A and Butrecitrinadin

Citrinadin A was isolated as a very deep yellow crystalline substance that was soluble in both dichloromethane and methanol. This compound showed prominent wavelength absorption maxima at λ_{max} 221, 250, 270 and 332 nm in a 0.1% formic acid methanol solution. The HRESIMS of this compound gave $m/z = 625.3962$ for $[M + H]^+$ corresponding to a molecular formula of $C_{35}H_{52}N_4O_6$ ($\Delta = 0.2$ ppm) with twelve (12) degrees of unsaturation.

Due to the limited amount of the compound isolated from the crude extracts, it was difficult to get a good ^{13}C NMR data for citrinadin A. Hence, the ^{13}C chemical shifts were extracted from the gHSQC and HMBC data. Analysis of the ^{1}H, ^{13}C and multiplicity edited gHSQC spectra, suggested the presence of 11 quaternary carbons, 9 methine, 5 methylene and 10 methyl carbons.

The huge molecular weight of 625.3962 with about 31% of the carbon atoms being quaternary alongside the observation of only a few correlations in the ^{1}H-^{1}H-gCOSY spectrum was direct indication of the presence of several very short spin systems which is typical of the citrinadin A backbone. The only substructures obtained by analysis of ^{1}H-^{1}H-gCOSY data are as shown in Figure 4.14.

The aromatic proton at δ_H 7.20 (1H, ov., H5) showed correlations to protons δ_H 7.76 (1H, d, $J = 7.2$, H6) and 7.65 (1H, d, $J = 7.2$, H4) (substructure A). Correlations from the proton at δ_H 5.28 (1H, m, H14) to protons at 1.94 (1H, m, H13)/1.91 (1H, m, H13') and 1.94 (1H, m, H15)/1.91 (1H, m, H15') confirmed substructure B with 3.75

(1H, ov., H12) also correlating to 1.94 (1H, m, H13)/1.91 (1H, m, H13') and 1.94 (1H, m, H15)/1.91 (1H, m, H15').

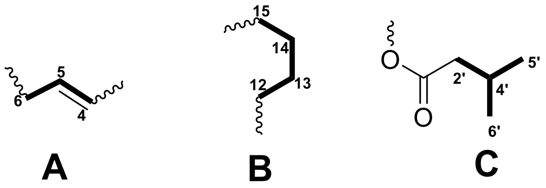


Figure 4.14: Substructures obtained solely from the analysis of ^{1}H-^{1}H-gCOSY data of citrinadin A.

Evidence for substructure C involved correlations that were seen from δ_H 2.04 (1H, ov., H-4') to 2.75 (1H, d, J = 10.8, H2'), 1.00 (3H, d, J = 6.4, H5') and 0.90 (3H, d, J = 6.8, H6'). Also correlations such as 3.75 (1H, ov., H10) to 3.26 (1H, d, J = 11.6, H10') were rather confirmatory of the presence of diastereotopic protons at carbon positions C10, C8, C13, C15 and C17.

Unlike the ^{1}H-^{1}H-gCOSY data, the HMBC was very detailed and provided a lot of correlations to complete a large part of the structure. The aromatic benzene ring portion of the indolinone ring (substructure A) was completed with the aid of HMBC correlations from C6-H4, C7-H5, C3a-H5, C7a-H6, C7a-H4 and C4-H6. There were no ^{1}H-^{1}H-gCOSY or HMBC data observed for the pentacyclic portion of the indolinone ring but, the observations of the carbons C2 (δ_C 185.3) and C3 (δ_C 64.4) provided concrete evidence of the existence of an indolinone ring. Substructure C which was meant to be part of the cyclopenta [b] quinolizidine moiety was also extended with the aid of crucial HMBC correlations C18-H29, C19-H28, C9-H29, C9-H28, C28-H29, C18-H28, C9-H8 and C18-H8. The full HMBC and ^{1}H-^{1}H-gCOSY data are as summarized in Figures 4.15 and 4.16.

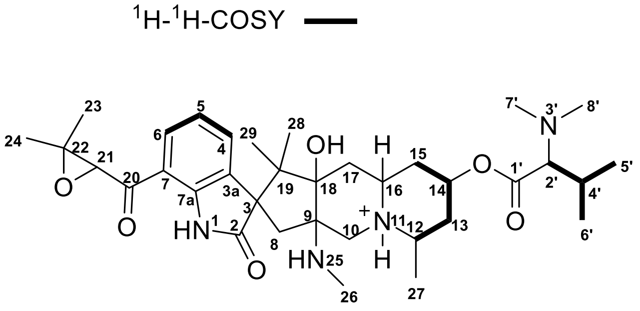


Figure 4.15:Full ^{1}H-^{1}H-gCOSY data of citrinadin A.

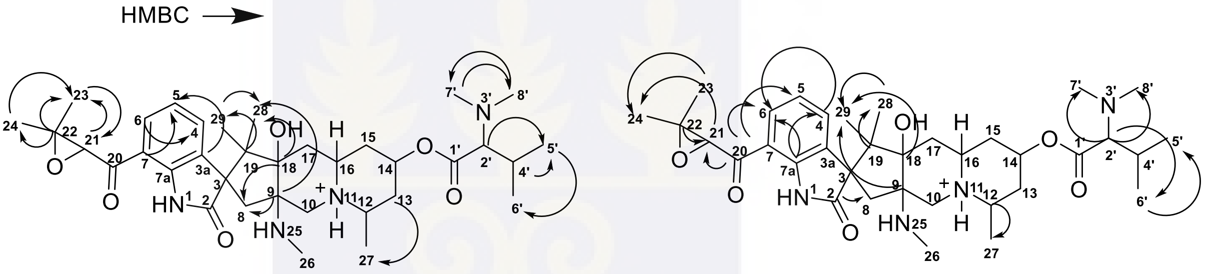


Figure 4.16: Full HMBC correlation data of citrinadin A provides extension for the substructures obtained from ^{1}H-^{1}H-gCOSY data analysis.

HMBC correlations C24-H23, C23-H24, C22-H23, C22-H24, C21-H23 and C23-H21 provided proof of the presence of the epoxy propane ring and its substituents while HMBC correlations C2'-H7', C2'-H8', C2'-H6', C2'-H5', C5'-H6', C7'-H8', C8'-H7' and C4'-H5' made it obvious that, the N, N-dimethylvaline residue was present in this structure.

In order to confirm the structural assignments made by ^{1}H-^{1}H-gCOSY and HMBC data, a detailed interpretation of the 2D-TOCSY of citrinadin A was made. Here further confirmation of all the spin systems made it obvious that, the structure in question was citrinadin A. A detailed illustration of the data obtained from the 2D-TOCSY is shown in Figure 4.17. All 1D and 2D NMR data are summarized in Table 4.1 for citrinadin A and raw data can be found in the Appendix.

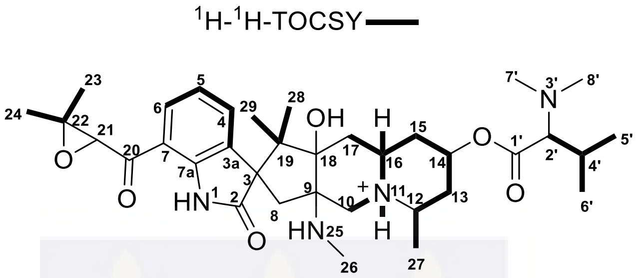


Figure 4.17: Full 2D TOCSY correlations of citrinadin A.

Table 4.1:^{1}H and ^{13}C NMR data of citrinadins in CD3OD.

ATOM	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	1H-1H COSY	HMBC	2D-TOCSY
1'	166.8	C					
20	194.8	C				H6, H5, H21	
2	185.3	C				H8	
7a	142.7	C				H6, H4	
3a	134.6	C				H5	
4	133.2	CH	7.65	d, 7.2	H5	H6	H6
6	127.5	CH	7.76	d, 7.2	H5	H4	H4
5	122.6	CH	7.20	ov.	H6, H4		
7	117.6	C				H5	
18	82.3	C				H8, H29, H28	
2'	74.4	CH	2.75	d, 10.8	H4'	H7', H8', H5', H6'	H6', H5', H4'
14	65.7	CH	5.28	m	H13, H15		H16, H12, H27, H17, H15, H13
3	64.4	C					
21	64.1	CH	4.06	s		H23, H24	
9	63.2	C				H8', H29, H28	
22	61.6	C				H21, H23, H24	
12	56.5	CH	3.75	ov.	H13, H15	H27	H14, H15, H13
19	51.2	C				H29, H28	
10	50.8	CH2	3.75 3.26	ov. d, 11.6	H10, H10'		11-NH
16	47.5	CH	4.02	ov.			11-NH, H14, H17, H15, H13
8	41.7	CH2	2.21 2.13	ov. ov.			
7'	41.4	CH3	2.33	s		H8'	
8'	41.4	CH3	2.33	s		H7'	
15	34.3	CH2	1.94 1.91	m m	H14, H12		H14, H16, H12, H27, H17
13	33.0	CH2	1.94 1.91	m m	H14, H12	H27	H14, H16, H12, H27, H17
17	31.5	CH2	1.71 1.67	m m			H14, H16, H15, H13
26	30.0	CH3	2.45	s			
29	28.1	CH3	1.39	s		H28	H28
4'	27.5	CH	2.04	ov.	H2', H5', H6'	H5'	H2', H6', H5'
23	24.3	CH3	1.61	s		H24, H21	H24
28	22.1	CH3	1.04	s		H29	H29
6'	20.0	CH3	0.90	d, 6.8	H4'	H5'	H2', H4', H5'
5'	19.2	CH3	1.00	d, 6.4	H4'	H6'	H2', H4', H6'
24	18.5	CH3	1.25	s		H23	H23
27	15.1	CH3	1.55	m			H14, H15', H13'
1-NH			9.65	s, br.			
25-NH							
11-NH			11.23	s, br.			H10, H10', H16

Interestingly, some aspects of the spectrometric and spectroscopic data of BRS2A-AR2 extracts cannot be overlooked. The mass spectrometry data of the crude extract of this strain (Figure 4.13) showed the presence of *m/z* 625.3962 and 681.4226 in a ratio of 1:1.5. The UV of the two compounds were virtually identical and their mass fragmentation patterns were also very similar. Also, the HPLC chromatogram of a mixture of the two compounds on preparative HPLC showed very poor resolution between the two compounds. Hence both compounds co-eluted with the same retention time and were collected together. The mass spectrometry analysis of the purified compounds gave the profile shown in Figure 4.24 below.

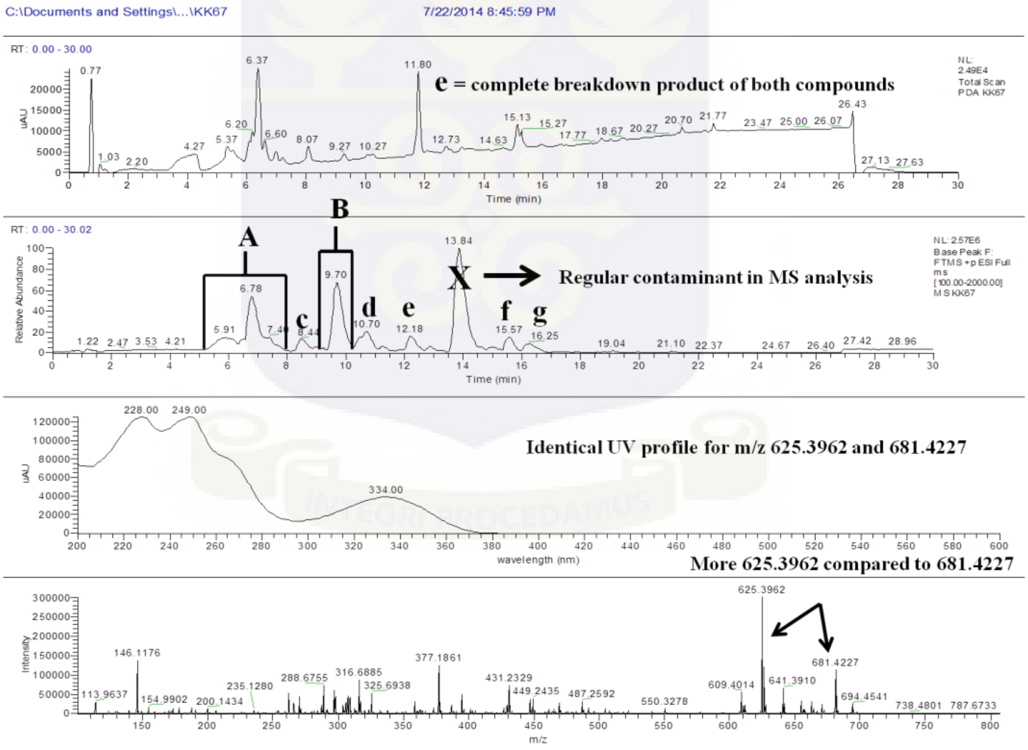


Figure 4.18:HRESI/HPLC-DAD-MS^n profile of citrinadin A and butrecitrinadin.

Careful look at the data in Figure 4.18 showed peaks **c**, **d**, **e** and **f** as break down products of the two main compounds present and a reversal of the amounts of *m/z*625.3962 and 681.4226 present to give a ratio of 1:0.5. This reversal of the amounts of these two compounds in the mass spectrometry data was indicative of the fact that:

- Both compounds did degrade to some extent during the various purification procedures applied to the compound.
- *m/z* 681.4226 suffered more from the effects of degradation than 625.3962 hence a decrease in the percentage amount of the compound present.

Fortunately, the main final degraded product of the two compounds was visible in Figure 4.18 and labelled as peak B. The mass fragmentation pattern of citrinadin A which has already been shown to be *m/z* 625.3962 was analysed into detail and chemical structures were proposed for the various fragments as shown in Figure 4.19 and Scheme 4.4.

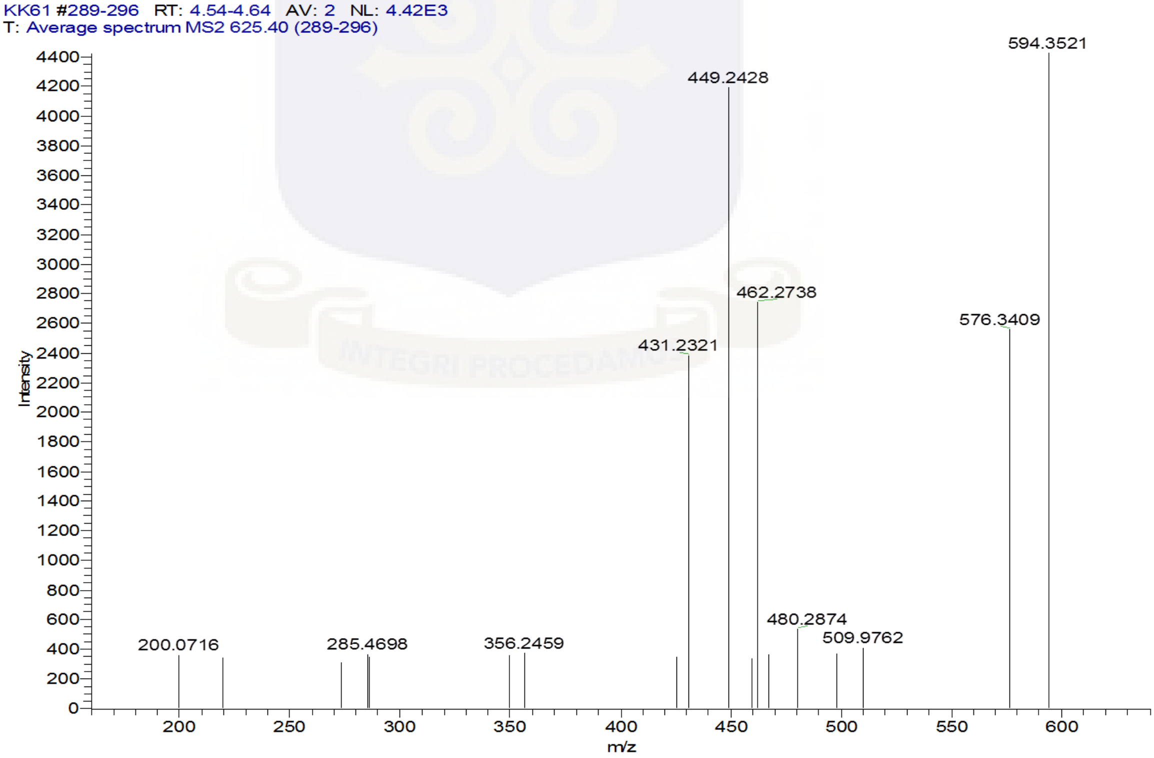
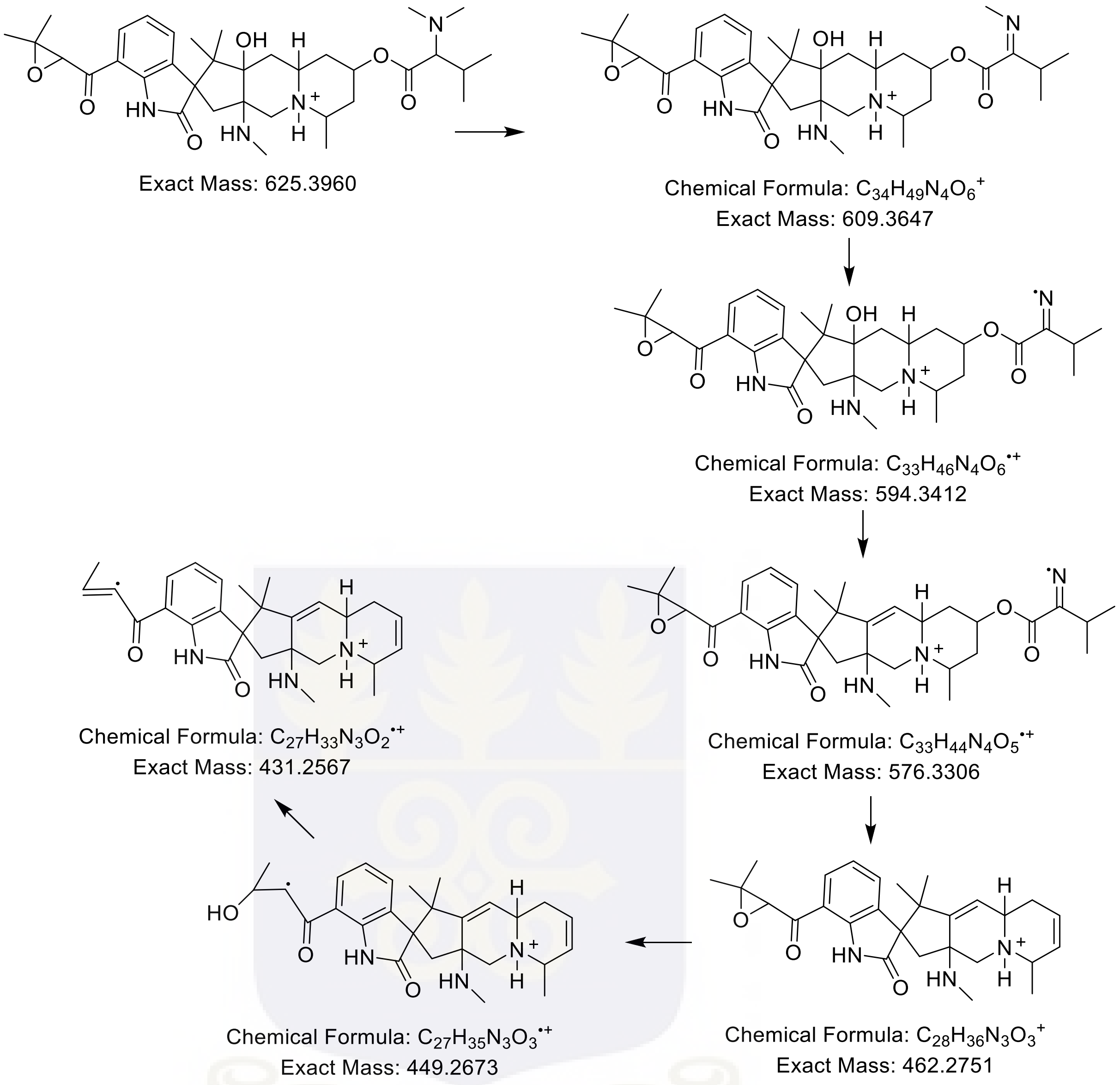
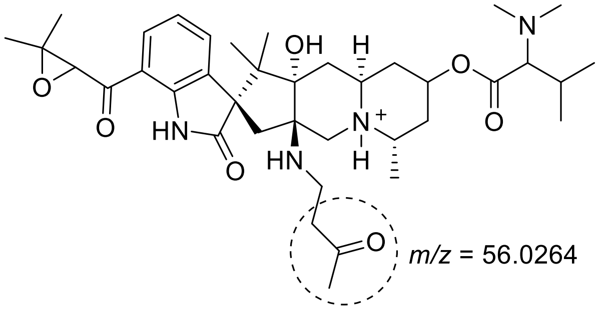


Figure 4.19: Mass fragmentation pattern of citrinadin A m/z = 625.3962.



Scheme 4.4: Proposed structures for the mass fragments of citrinadin A = 625.3962

Mathematically, the difference between *m/z* 625.3962 and 681.4226 was found to be 56.0264. Surprisingly, this consistent mass difference was seen throughout the fragmentation of *m/z* 681.4226 as compared to 625.3962 fragments and this is indicated in Figures 4.20 and Scheme 4.5. Clearly, the compound whose structure was responsible for the *m/z* 681.4226 has a structure as shown below and it was named butrecitrinadin after the river which produced the source organism.



Exact Mass: 681.4222

butrecitrinadin

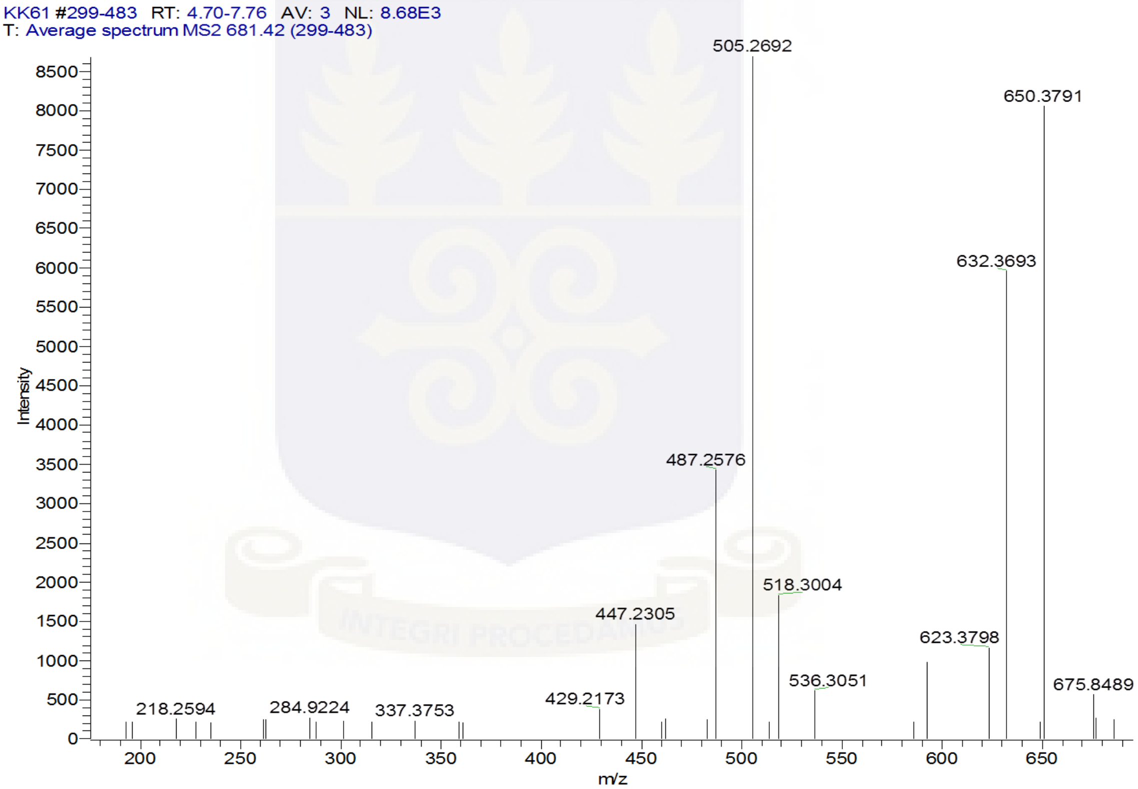
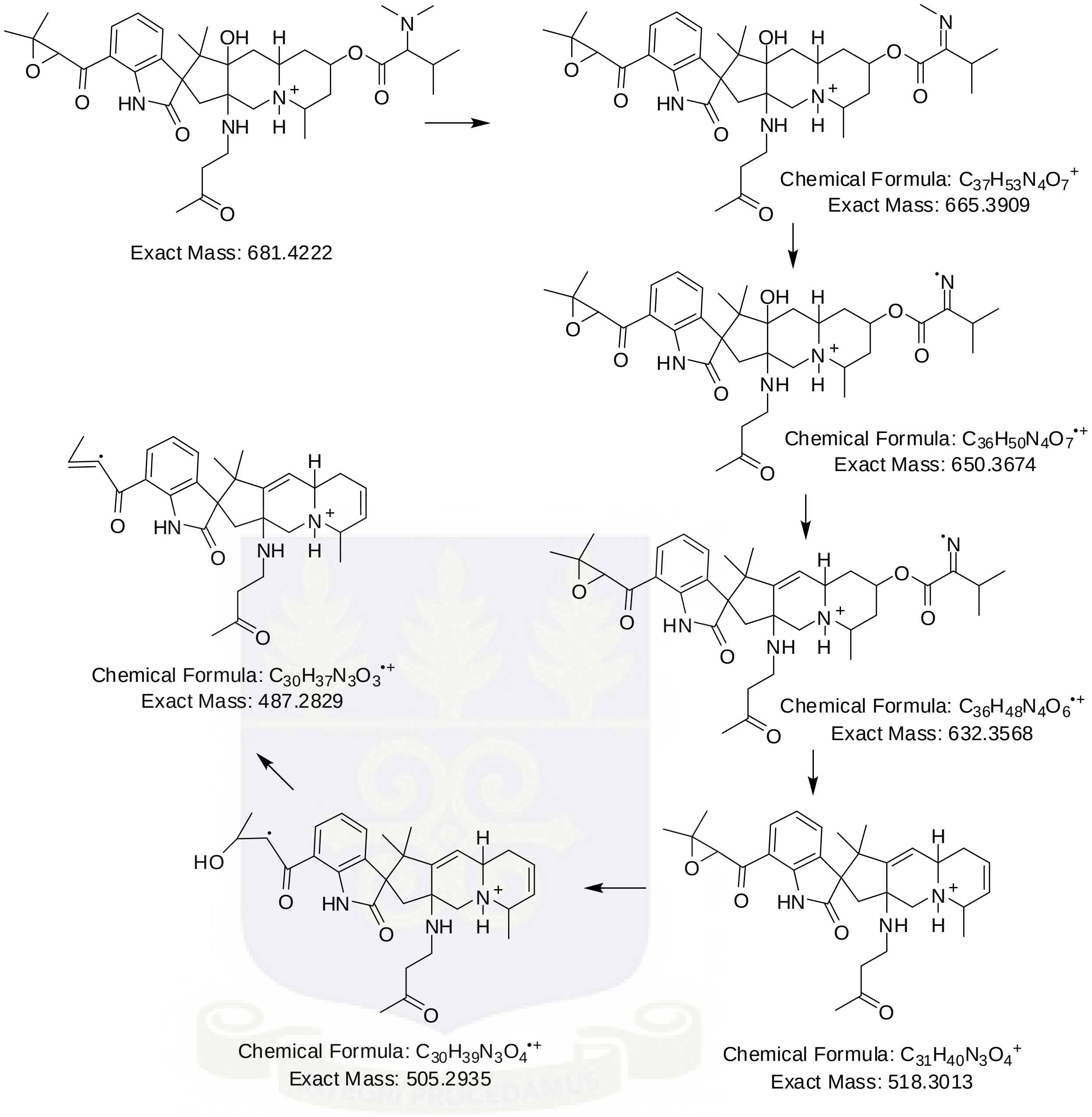
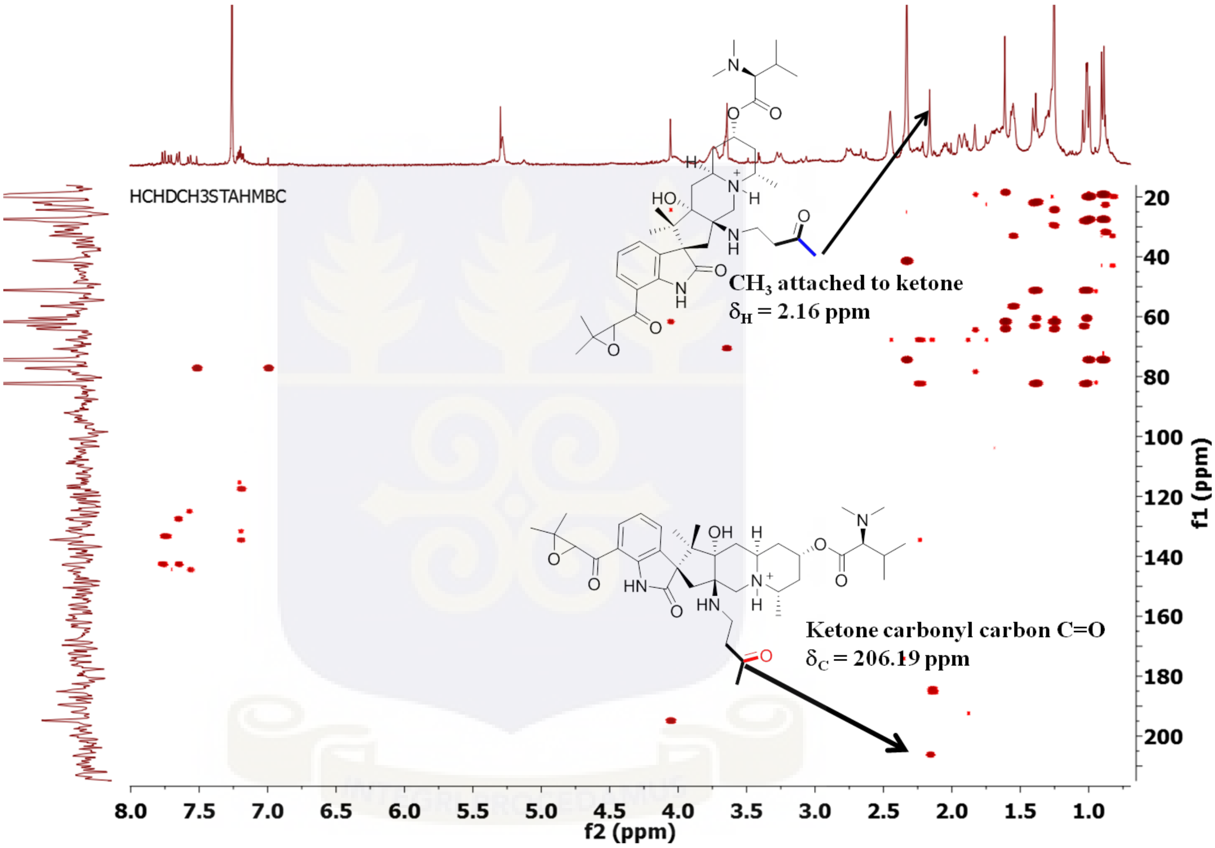


Figure 4.20: Mass fragmentation pattern of butrecitrinadin m/z = 681.4226.



Scheme 4.5: Proposed structures for the mass fragments of butrecitrinadin = 681.4246

Furthermore, evidence from NMR spectroscopy was used to confirm the structure of butrecitrinadin as shown in Figure 4.21 and 4.22. The carbonyl carbon of the proposed 3-butanone side chain attached to the citrinadin backbone was observed at δ_C206.2 ppm which is what is comparable to the values that were calculated theoretically for such a ketone. The methyl protons of the terminal carbon attached to the ketone was also observed at δ_H 2.00 (3H, s) and the corresponding carbon atom at δ_C 33.0 ppm which is consistent with theoretical values. Hence, it appears that, the nature of the 3-butanone side chain makes it difficult to get correlations that link it to the rest of the citrinadin backbone but, butrecitrinadin undoubtedly is present with citrinadin A in the purified sample.



CH$_3$ attached to ketone $\delta_H = 2.16$ ppm

Ketone carbonyl carbon C=O $\delta_C = 206.19$ ppm

Figure 4.21: HMBC spectrum of butrecitrinadin

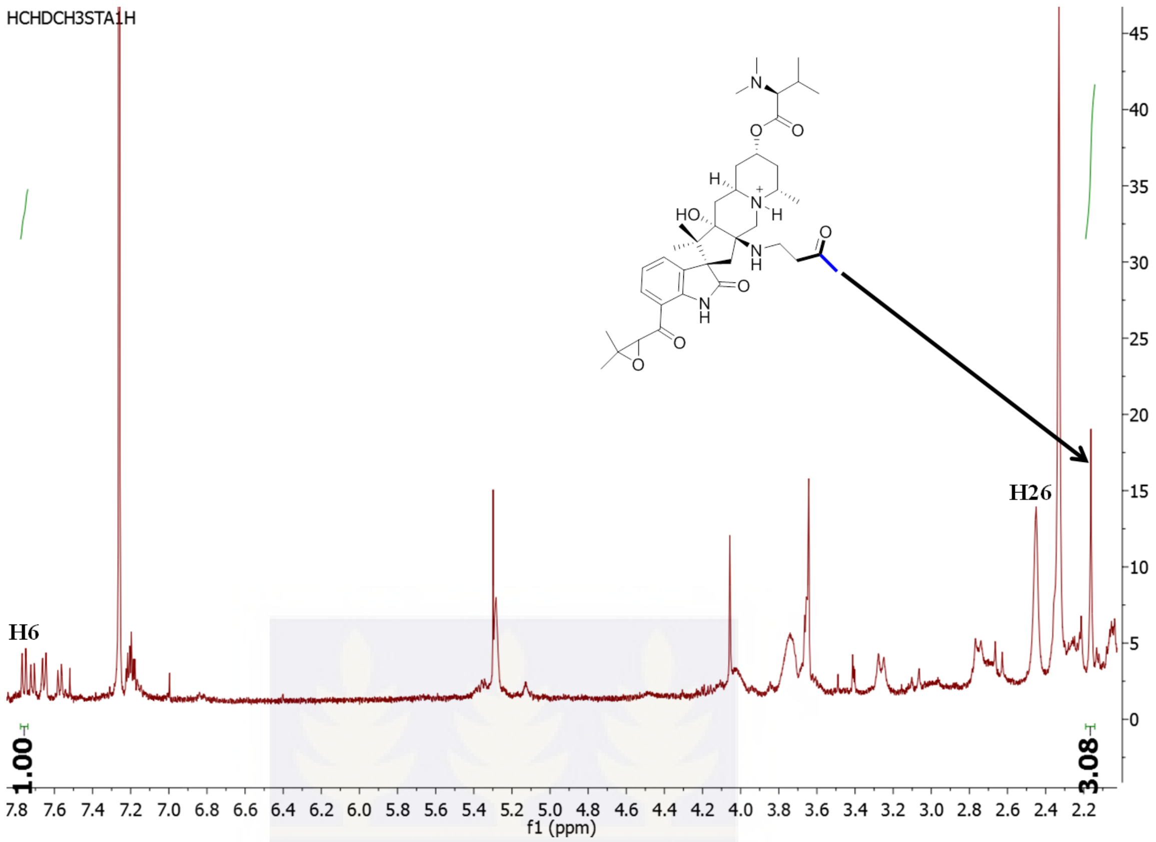


Figure 4.22:^{1}H NMR spectrum of butrecitrinadin

4.3 Structure determination of Quinolactacins A1 and A2

The quinolactacins A1 and A2 were isolated as a very light yellow substance which on drying turned into very white and flaky bits that easily became airborne. Apparently the light yellow portion was only remnants of impurities from the crude extracts. The compounds were soluble both in dichloromethane and methanol. Their UV profile was very interesting with wavelength absorption maxima at λ_{max}221, 248, 255, 315 and 328 nm0.1% formic acid methanol solution. The mass spectrometry data of quinolactacins A1 and A2 was very interesting and actually played a part to conceal the identity of these compounds until late in the structure elucidation process. Two peaks 293.1262 and 563.2631 were very consistent in the mass spectrometry data of the crude extracts as well as pure fractions containing quinolactacins A1 and

A2. Initially, during the dereplication stage, these masses were entered into the databases but they returned with no hits and rightfully so. It turned out after the analysis of 1D and 2D NMR data that, 293.1262 was $[M + Na]^+$ and 563.2631 was $[2M + Na]^+$. The molecular weight of the isolated quinolactacins was therefore 270.1362 representing a molecular formula of $C_{16}H_{18}N_2O_2$ ((Δ = 0.1 ppm) and nine (9) degrees of unsaturation.

The ^1H, ^{13}C and multiplicity edited gHSQC spectra, suggested the presence of 6 quaternary carbons, 6 methine, 1methylene and 3 methyl carbons. Analysis of the ^1H-^1H COSY data gave the correlations 7.68 (1H, ddd, *J* = 8.0, 7.2, 4.0, H6) to 7.50 (1H, dd, *J* = 8.8, 4.4, H5), 7.68 (1H, ddd, *J* = 8.0, 7.2, 4.0, H6) to 7.40 (1H, ddd, *J* = 8.0, 7.2, 6.8 H7), 8.39 (1H, ddd, *J* = 8.8, 6.4, 4.8 H8) to 7.40 (1H, ddd, *J* = 8.0, 7.2, 6.8 H7), 7.40 (1H, ddd, *J* = 8.0, 7.2, 6.8 H7) to 8.39 (1H, ddd, *J* = 8.8, 6.4, 4.8 H8), 7.40 (1H, ddd, *J* = 8.0, 7.2, 6.8 H7) to 7.68 (1H, ddd, *J* = 8.0, 7.2, 4.0, H6) and 7.50 (1H, dd, *J* = 8.8, 4.4, H5) to 7.68 (1H, ddd, *J* = 8.0, 7.2, 4.0, H6) confirmed the first spin system as indicated by substructure A.Also, ^1H-^1H COSY correlations 2.12 (1H, ov., H1') to 5.98 (1H, d, *J* = 45.2, NH), 4.76 (1H, s, H3), 0.53 (3H, *J* = 6.8, 1'-CH_3) and additional correlations from 1.63 (1H, m, H2') to 4.76 (1H, s, H3), 4.76 (1H, s, H3) to 1.63 (1H, m, H2')/1.49 (1H, m, H2''), 5.98 (1H, d, *J* = 45.2, NH) to 2.12 (1H, ov., H1') and 0.53 (3H, *J* = 6.8, 1'-CH_3) to 2.12 (1H, ov., H1') confirmed the substructure B as indicated in Figure 4.23 below.

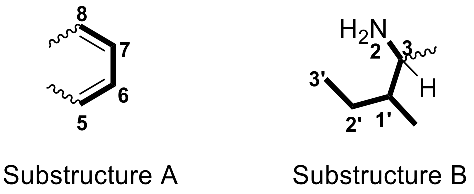


Figure 4.23: Substructures obtained solely from the analysis of ^{1}H-^{1}H-gCOSY data of quinolactacin A1 and A2.

Subsequent extensions of substructure A and B were aided by several HMBC correlations including C9-H8, C3a-4-CH_3, C4a-H8, H6 and 4-CH_3, C6-H8, C8a-H5 and H7, C8-H6, C7-H5, C5-H7 that helped to construct fully the unique N-methyl quinolone moiety that is fused to a lactam ring. HMBC correlations from C3-H2' and 1'-CH_3, C1'-H2', H2'', H3' and 1'-CH_3 provided evidence for the sec-butyl group attached at C3 which is characteristic to quinolactacins A1 and A2. Illustrations of all COSY and HMBC data are shown in Figures 4.24 and 4.25.

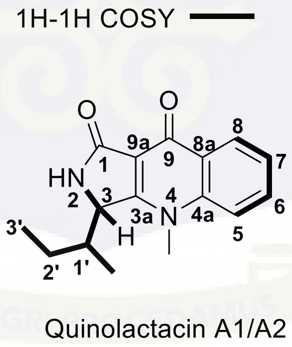
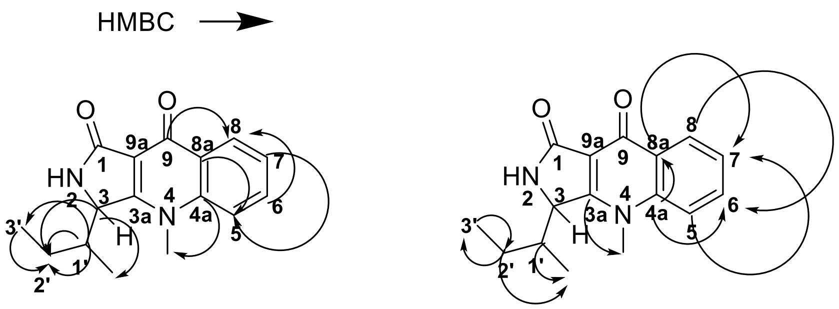


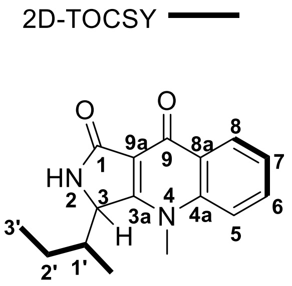
Figure 4.24: Full ^{1}H-^{1}H-gCOSY data of quinolactacin A1/A2.



Quinolactacin A1/A2 Quinolactacin A1/A2

Figure 4.25: Full HMBC correlation data of quinolactacin A1/A2 provides extension for the substructures obtained from ^{1}H-^{1}H-gCOSY data analysis.

In order to confirm the structure obtained from all ^{1}H-^{1}H COSY and HMBC data, a 2D-TOCSY experiment was conducted. Correlations from the 2D-TOCSY experiment were rather few probably due to the short acquisition time of five (5) hours. However, the few correlations obtained only served to further confirm those that were obtained previously from the HMBC and ^{1}H-^{1}H COSY. All 2D-TOCSY data are summarized in Figure 4.26 and detailed 1D and 2D NMR data can be found in Table 4.2 with raw data in the Appendix. All the NMR data obtained in the current project was consistent with those documented in the literature.[172]



Quinolactacin A1/A2

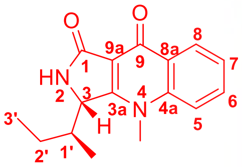
Figure 4.26:Full 2D TOCSY correlations of quinolactacin A1/A2.

Table 4.2:^{1}H and ^{13}C NMR data of quinolactacins in CD3OD.

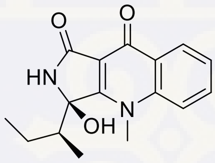
ATOM	δ ^{13}C (ppm)	^{13}C mult	δ ^{1}H (ppm)	Mult (Hz)	^{1}H-^{1}H COSY	HMBC	2D-TOCSY
9	173.5	C				H8	
1	170.6	C					
3a	164.1	C				4-CH_3	
4a	141.3	C				H8, H6, 4-CH_3	
6	133.0	CH	7.68	ddd, 8.0, 7.2, 4.0	H5, H7	H8	H8
8a	128.2	C				H5, H7	
8	127.1	CH	8.39	ddd, 8.8, 6.4, 4.8	H7	H6	H6
7	125.0	CH	7.40	ddd, 8.0, 7.2, 6.8	H8, H6	H5	
5	115.7	CH	7.50	dd, 8.8, 4.4	H6	H7	
9a	110.9	C					
3	58.5	CH	4.76	s, broad		H2', 1'-CH_3	
1'	36.9	CH	2.12	ov.	NH, H3, 1'-CH_3	H2', H2", H3', 1'-CH_3	
4-CH_3	36.1	CH_3	3.79	s			
2'	28.1	CH_2	1.63 1.49	m m	H3', H3'	H3', 1'-CH_3	
3'	12.2	CH_3	1.05	t, 7.2	H2', H2"	H2', H2"	H2', H2"
1'-CH_3	11.8	CH_3	0.53	d, 6.8	H1'		H1', H2', H2", H3'
NH			5.98	d, 45.2	H1'		

4.3.1 Acid-base rapid epimerization of quinolactacins

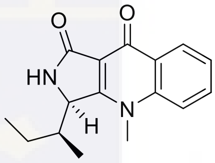
Only thirteen (13) quinolactacins have been isolated and characterized to-date but, their structural novelty and biological activity has inspired a lot of research. Interestingly, out of the thirteen molecules described so far, only quinolactacins A2 and B2 are genuine or native natural product molecules with the remaining eleven (11) described structures the result of very quick acid-base catalysed epimerization reactions.[172] These reactions lead to the formation of quinolonimide and then eventually to quinolonic acid. Clark *et. al.* (2006) proposed mechanisms for these acid-base catalysed epimerization reactions, an example of which is shown in Figure4.27 for the transformation of quinolactacin A2 to A1 and vice versa.



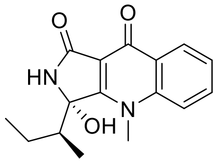
Quinolactacin A2



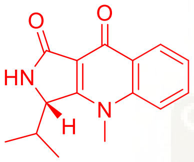
Quinolactacin C2



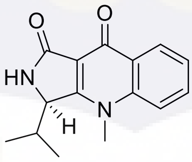
Quinolactacin A1



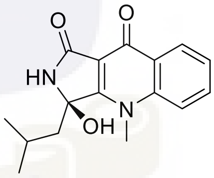
Quinolactacin C1



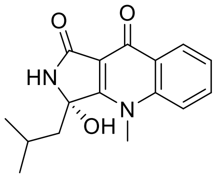
Quinolactacin B2



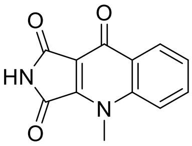
Quinolactacin B1



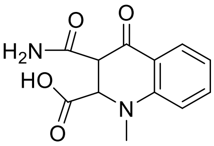
Quinolactacin D2



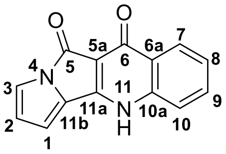
Quinolactacin D1



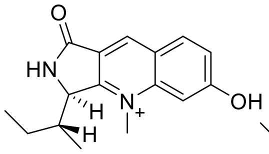
Quinolonimide



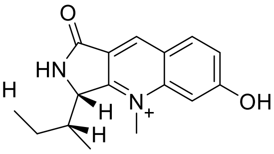
Quinolonic acid



Quinolactacide



Quinocitrinine A



Quinocitrinine B

These reactions are the reason why it is very difficult to have pure solutions of one quinolactacin. While we are confident that the current research led to the isolation of quinolactacin A, we are not sure which of the epimers predominates and therefore we assume an equilibrium mixture of both as can be seen from the very broad 1H NMR signals that were observed in this project.

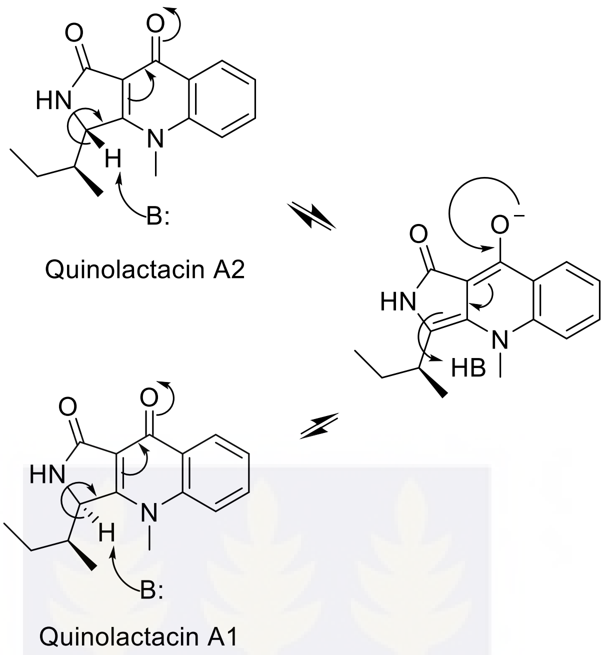


Figure 4.27: Acid-base epimerization interconversions of quinolactacin A2 to A1.

4.4 Anti-proliferative activity of compounds

The anti-proliferative activities of six compounds cholest-5-ene-3,25,26-triol (3β,25ξ) (SF2C5HG), 20-ethyl-5-pregnen-3-ol (SF2C5HH), β-sitosterol (SF2C5HI), butrepyrazinone, quinolactacin A1/A2, citrinadin A/butrecitrinadin were tested on human cancer cell lines as shown in Figure 4.28A-F. Quinolactacin A1 and A2 were tested as an intimate mixture of both compounds in the ratio of 1:1 while citrinadin A and butrecitrinadin were tested as an intimate mixture in the ratio of 1.5:1. The compounds showed some level of anti-proliferative activity against the cell lines compared to curcumin as a positive control (Figure 4.28A-F). The cell growth-inhibitory potencies of compounds, expressed as IC$_{50}$values, are shown in Table 4.3. Two of the steroids labelled SF2C5HH and SF2C5HI showed high inhibition (IC$_{50}$ of 5.58 and 5.28μM**,** respectively) against the human prostate cancer cells (LNCap) than the positive control, curcumin (IC_{50} of 6.15 µM) used. However, SF2C5HG, quinolactacins, citranadins and butrepyrazinone showed moderate anti-proliferative activity against LNCap cells. In addition, only the SF2C5HH showed a comparable IC_{50} of 24.54µM to that of curcumin (20.68µM) on the Human breast cancer cell line MCF-7.

Curcumin is known to be a potent inhibitor of cancer cell lines but, in this case, the inhibitory effect ofSF2C5HH and SF2C5HI was stronger than curcumin. SF2C5HH and SF2C5HI exerted a high anti-proliferative activity on the Human Prostate cancer cell line LNCap than the effect of SF2C5HG. SF2C5HG has hydroxyl groups on the C26 and C27 which is absent in the other compounds. Garry *et al.*, (2002), published a report which indicated that, hydroxyl groups are required to decrease viability in LNCap cells.[173] This could account for why SF2C5HG had a lowered inhibitory activity as compared to SF2C5HH and SF2C5HI.

Naturally occurring steroids in the human body are sometimes used to treat prostate cancer that has spread and not responding to hormone therapy. Body steroids are used to manage cancers by shrinking and inhibiting growth. The ability of the steroids SF2C5HH and SF2C5HI to inhibit the human prostate cancer cell line supports the fact that steroids have anti-prostate cancer activity. The fact that these compounds were selective against LNCap as compared to PC-3 cell lines (Table 4.3) even though they are both Human Prostate Cancer cells showed some level of selectivity and hence their potential as leads that could be developed asanti-prostate cancer drugs. The compounds may be acting on the androgen receptors since the LNCap cell line is an androgen dependent prostate cancer cell and has an androgen receptor whiles PC-3 cell line is an androgen independent prostate cancer cell. One of the causes of prostate cancer is an androgenic disorder which arises mainly by the ill effects of testosterone metabolism and its conversion to dihydrotestosterone in the presence of 5-alpha reductase.[174]

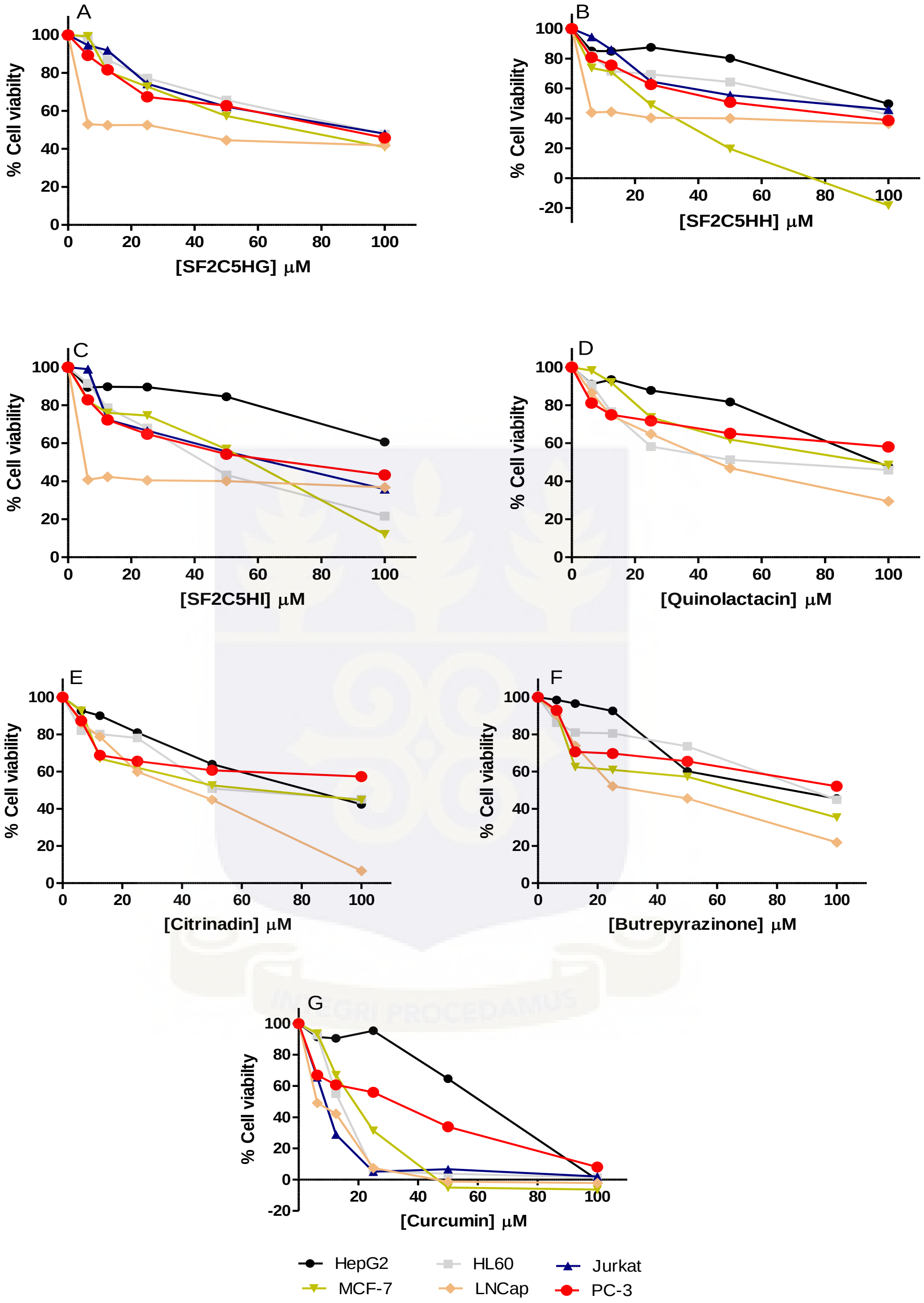


Figure 4.28A-F: Anti-proliferative activities of pure compounds and standard on human cancer cell lines.

Table 4.3: Cell growth-inhibitory potencies of pure compounds expressed as IC$_{50}$values.

Cell line	**Origin**	**Compound IC$_{50}$ (µM)**						
		SF2C5HG	SF2C5HH	SF2C5HI	Quinolactacin	Citrinadin,	Butrepyrazinone	**Curcumin**
HEPG2	Human Hepatocellular Carcinoma	ND	99.56	>100	96.54	82.51	84.74	61.38
HL-60	Human Promyelocytic Leukemia	93.85	82.95	43.20	54.47	57.23	91.06	13.78
Jurkat	Human T-Lymphoblastic Leukemia	92.79	78.74	64.08	ND	ND	ND	8.91
MCF-7	Human Breast Cancer	72.46	24.54	57.79	94.49	66.07	66.62	20.68
LNCap	Human Prostate Cancer	32.88	5.58	5.28	45.71	41.42	33.36	6.15
PC-3	Human Prostate Cancer	87.59	53.21	69.40	>100	>100	>100	20.72

Data presented are mean of two experiments done in triplicates. ND: Not Determined
IC$_{50}$ (µM) = Concentration that inhibits cell growth by 50%

4.5 Anti-Plasmodial Activity of Compounds

4.5.1 Anti-Plasmodial activity ofcompounds to chloroquine sensitive*Plasmodium falciparum* 3D7strain

The six compounds cholest-5-ene-3,25,26-triol (3β,25ξ) (SF2C5HG), 20-ethyl-5-pregnen-3-ol (SF2C5HH), β-sitosterol (SF2C5HI), butrepyrazinone, quinolactacin A1/A2, citrinadin A/butrecitrinadin were also tested against the chloroquine sensitive *Plasmodium falciparum* 3D7 strain for the anti-plasmodial activity as shown in Figure4.29. Only the quinolactacin showed anti-plasmodial activity within the concentration range tested.

Table 4.4 shows the Inhibition Concentration (IC_{50}) of the compounds on the plasmodial strain. Quinolactacins gave an IC_{50} of 24.80 µM, all the other compounds gave values greater than 25 µM.

Table 4.4: Effective concentration of the six compounds on the 3D7 plasmodial strain.

Strain	**Compounds EC_{50} (µM)**						
	SF2C5HG	SF2C5HH	SF2C5HI	Quinolactacins	Citrinadins	Butrepyrazinone	Artesunate
3D7	>25	>25	>25	24.80	>25	>25	0.074

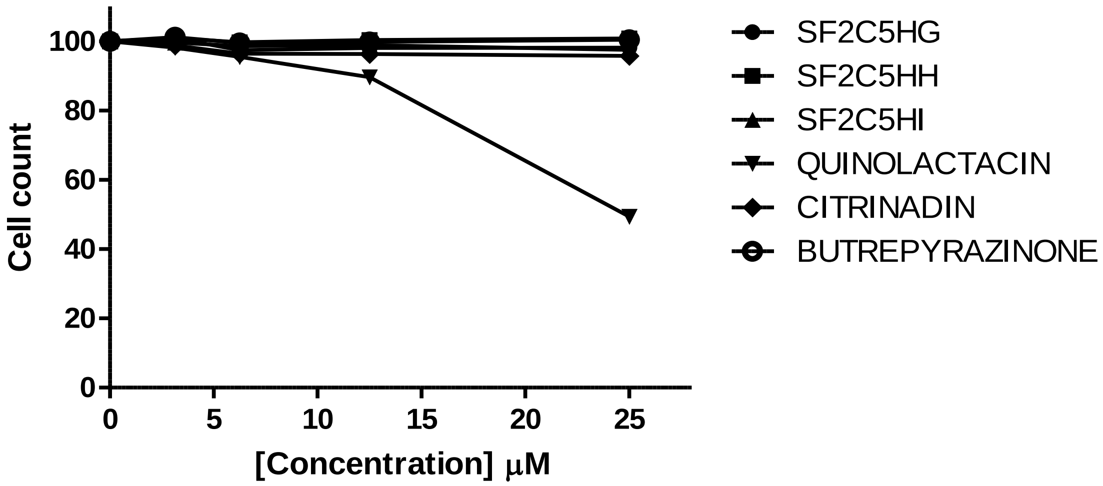


Figure 4.29: Anti-plasmodial activity of compounds on 3D7 plasmodial strain.

Quinolactacins A1/A2 exhibited anti-plasmodial activity *in vitro* with IC$_{50}$ of 24.80 µM. This represents the first study of the anti-plasmodial activity of quinolactacins in general and the results proved very encouraging. Interestingly, citrinadins also alkaloids isolated from the same endophytic fungi did not show anti-plasmodial activity. Structurally, quinolactacins have a backbone comprising of a quinolone moiety fused to a lactam ring. Compounds containing the quinolone skeleton have been found to exhibit a variety of biological activity including cytotoxicity,[175]anti-HIV,[176]and ability to remedy certain conditions of Alzheimers.[177] However, their inhibitory role against malaria parasites has not been fully explored except for a few known antibiotics such as ciprofloxacin, nalidixic acid and ofloxacin.[178-180]In one study, some4-quinolone compounds were shown to have moderate antiplasmodial activities against a chloroquine-sensitive *Plasmodium falciparum*strain with IC$_{50}$ values in the micro molar range.[181]Studies by other research groups have shown that changing the substituents on the nitrogen atom present in the quinolone affects the anti-plasmodium activity of these compounds.[182]

The antimalarial activities of lactams have not been studied into detail. The few synthesized β-lactams tested for their antimalarial potential against chloroquine sensitive *Plasmodium falciparum* D10 strain did not show any considerable antimalarial activity.**[183]** However, in some studies, introduction of suitable substituents on the nitrogen atom present in the lactams have helped to fine tune the antimalarial activity of lactams.**[183]**

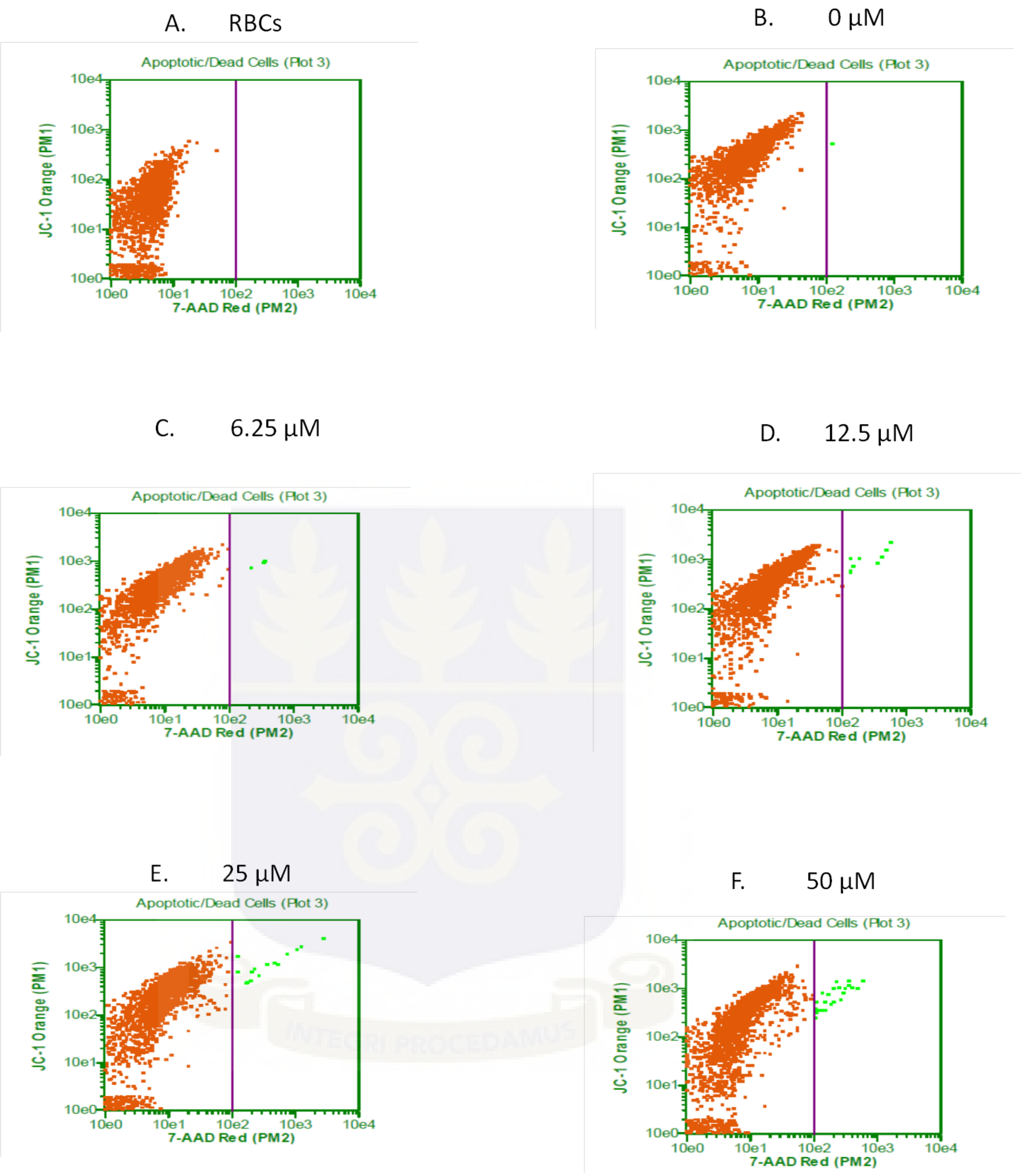
Results obtained for antimalarial studies of the quinolactacins suggest that these compounds could act as good scaffolds for the development to antimalarials if they are able to effectively induce apoptosis.

4.6 Plasmodial Apoptotic Activity of Quinolactacins A1/A2

The apoptotic activity of the quinolactacins was evaluated on the strain by studying the effect of the compound on the mitochondrion membrane potential of the parasite. The effect of the compound on the loss of mitochondrion membrane potential of the strain treated with 0 µM, 6.25 µM, 12.5 µM, 25 µM and 50 µM concentrations of the intimate mixture of quinolactacins A1 and A2 for 24 h were analysed by flow cytometry after staining with JC-1 as shown in Figure 4.30A-Fand 4.31 shows the percentage of apoptotic cells on the parasites strain after treatment with the compound. The Figures show a concentration dependent activity on the parasite.

Apoptosis is a highly regulated programmed cell death in cells which occur widely in multicellular organisms as well as unicellular parasites and is essential for normal development and immune defences. The process of apoptosis is initiated by the activation of death receptors, or by intracellular stress conditions. This leads to a series of genetically controlled and ordered biochemical changes, resulting in morphological changes to the cell. The process of apoptosis is characterised by condensing of chromatin, DNA breakdown, mitochondrial alterations, membrane changes, shrinkage of the cell and finally and the formation of apoptotic bodies. Picot *et. al.*, in (1997) first highlighted the relationship that exists between Plasmodium drug resistance and apoptosis.**[184]**Quinolactacins used in this study exhibited histogram displacement (Figure 4.30A-F) indicating dissipation of the mitochondrial membrane potential($\Delta\Psi m$) and abolition of probe accumulation in a concentration dependent manner (Figure 4.31).Mitochondria play an essential role in apoptosis induction by a variety of death stimuli therefore, the ability of quinolactacins to compromise mitochondrial changes leading to the loss of the $\Delta\Psi m$ which result in cytochrome C release from the mitochondria to the cytosol.

Literature provides evidence that one of the mechanisms of malaria drug resistance is mediated through a decrease in apoptosis susceptibility. However, quinolactacins may provide evidence of apoptotic death in the stages of *Plasmodium falciparum* development.



A. RBCs

B. 0 µM

C. 6.25 µM

D. 12.5 µM

E. 25 µM

F. 50 µM

Figure 4.30A-F: Effect of Quinolactacin A1/A2 on the loss of mitochondrion membrane potential.

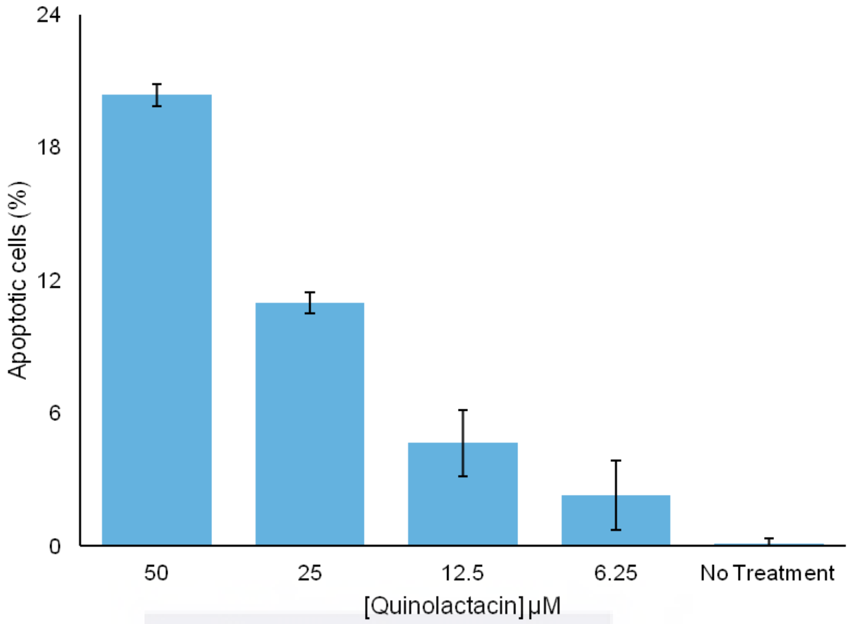


Figure 4.31: Apoptotic effect of quinolactacins on the plasmodial strain

4.7 Anti-Buruli ulcer Activity of Compounds

The six compounds cholest-5-ene-3,25,26-triol (3β,25ξ) (SF2C5HG), 20-ethyl-5-pregnen-3-ol (SF2C5HH), β-sitosterol (SF2C5HI), butrepyrazinone, quinolactacin A1/A2, citrinadin A/butrecitrinadin were tested for Anti-buruli ulcer activity on *Mycobacterium ulcerans* MN209 characterised isolates and the MICs are shown in Table 4.5. All the compounds showed no Minimum Inhibition Concentration (MIC) within the concentration range tested

.

Table 4.5 Minimum Inhibition Concentration of the compounds on Mycobacterium ulcerans MN209.

Strain	**MIC (µM)**						
	SF2C5HG	SF2C5HH	SF2C5HI	Quinolactacins	Citrinadins	Butrepyrazinone	Artesunate
Mu MN209	>10	>10	>10	>10	>10	>10	>10

The compounds were tested for their anti-buruli activity but none of the compounds were able to inhibit the *Mycobacterium ulcerans* MN209 characterised isolates. This is the first time each of the compounds was tested for their activity against *Mycobacterium ulcerans* MN209.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The Ghanaian mangrove ecosystem is underexplored for the isolation of important natural products with key pharmacophore. This thesis proves the potential of isolating new and novel compounds from endophytic fungi from Ghanaian mangrove plants.

The mangrove plants sampled along the banks of River Butre were investigated for new or novel secondary metabolites. The HRESI/HPLC-DAD-MS^n was an appropriate and efficient dereplication technique and also very useful in identifying and isolating new secondary metabolites from the mangrove plants.

With the aid of the MarinLit, AntiMarin and Antibase database, detailed information on already known compounds and prior information about the structures of all new compounds was known.

The HRESI/HPLC-DAD-MS^n analysis of the total crude extracts showed that the marine-derived fungi, *Penicillium* sp. BRS2A-AR2 produced interesting compounds and therefore, large scale fermentation was carried out to isolate these compounds. Quinolactacin A1/A2 and Citrinadin A and the new Butrecitrinadin were isolated and characterized using appropriate techniques.

The compounds isolated as well as other compounds from the research group were studied for their biological activity against the proliferation of cancerous cells lines, their anti-plasmodial activity as well as their anti-buruli ulcer potential.

In the search of new compounds with inhibitory abilities, 20-Ethyl-5-pregnen-3-ol and β-sitosterol proved strong inhibitory activity against human cancer prostate LNCap cells and quinolactacins also showed inhibitory activity against chloroquine sensitive *Plasmodium falciparum* 3D7 strain as its apoptotic activity via the loss of mitochondrion membrane potential of the parasite. These compounds are therefore lead for anti-cancer and anti-malarial candidates respectively.

5.2 Recommendations

Since this is the first attempt to isolate metabolites from endophytic fungi from Ghanaian marine habitat, this work should serve as a baseline for the investigation of natural products from mangrove habitats in Ghana.

The remaining four species isolated in the course of the study should be investigated for the isolation of new or novel secondary metabolites by culturing them in a different media. In time past, scientists have mainly focused on the identification of endophytic fungi and isolation of metabolites however, knowledge in genetic engineering, microbial fermentation amongst other techniques should be employed to increase yield in compounds generated from endophytic fungi with efficient activity.

The mechanism of action of 20-Ethyl-5-pregnen-3-ol and β-sitosterol against human cancer prostate LNCap cells should be studied to enhance the possibility of being potential anti prostate cancer agents. The *in vivo* activity of quinolactacins should be studied to fully understand their mode of action. The compounds that showed some level of anti-proliferative activity against the cancerous cell line should be modified

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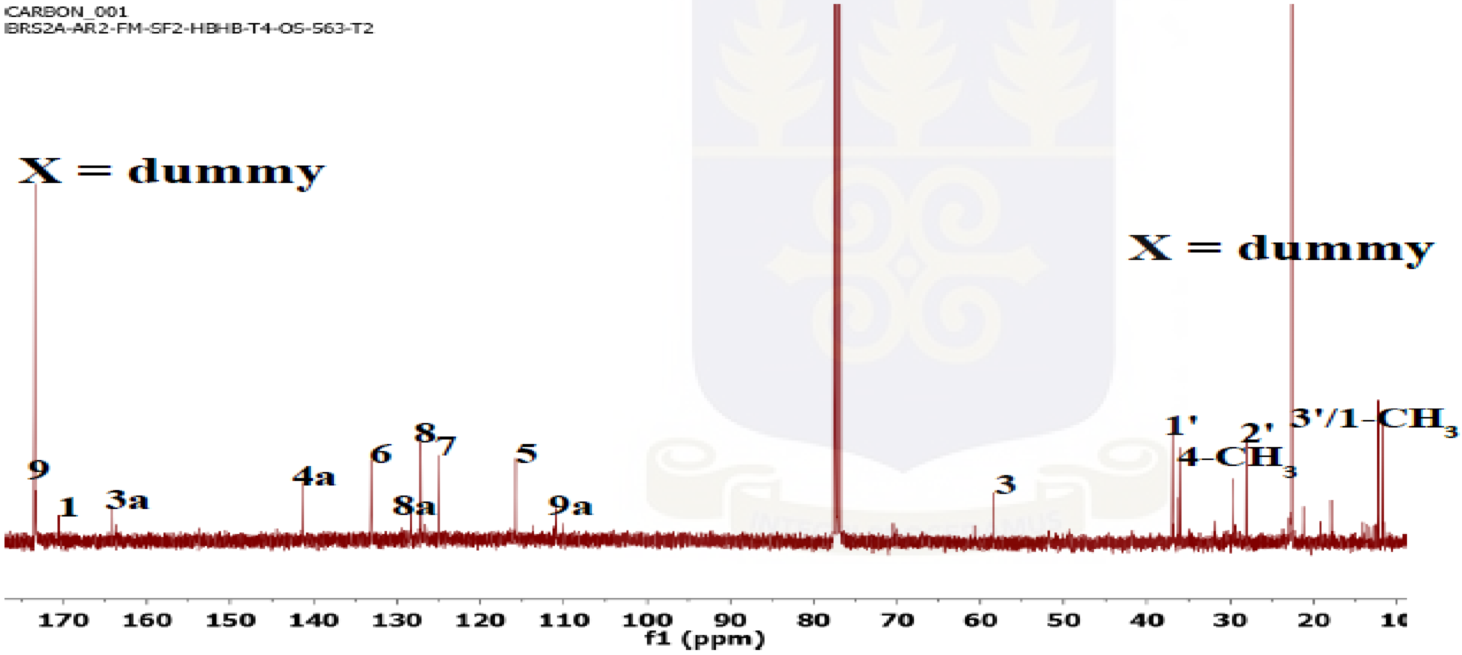
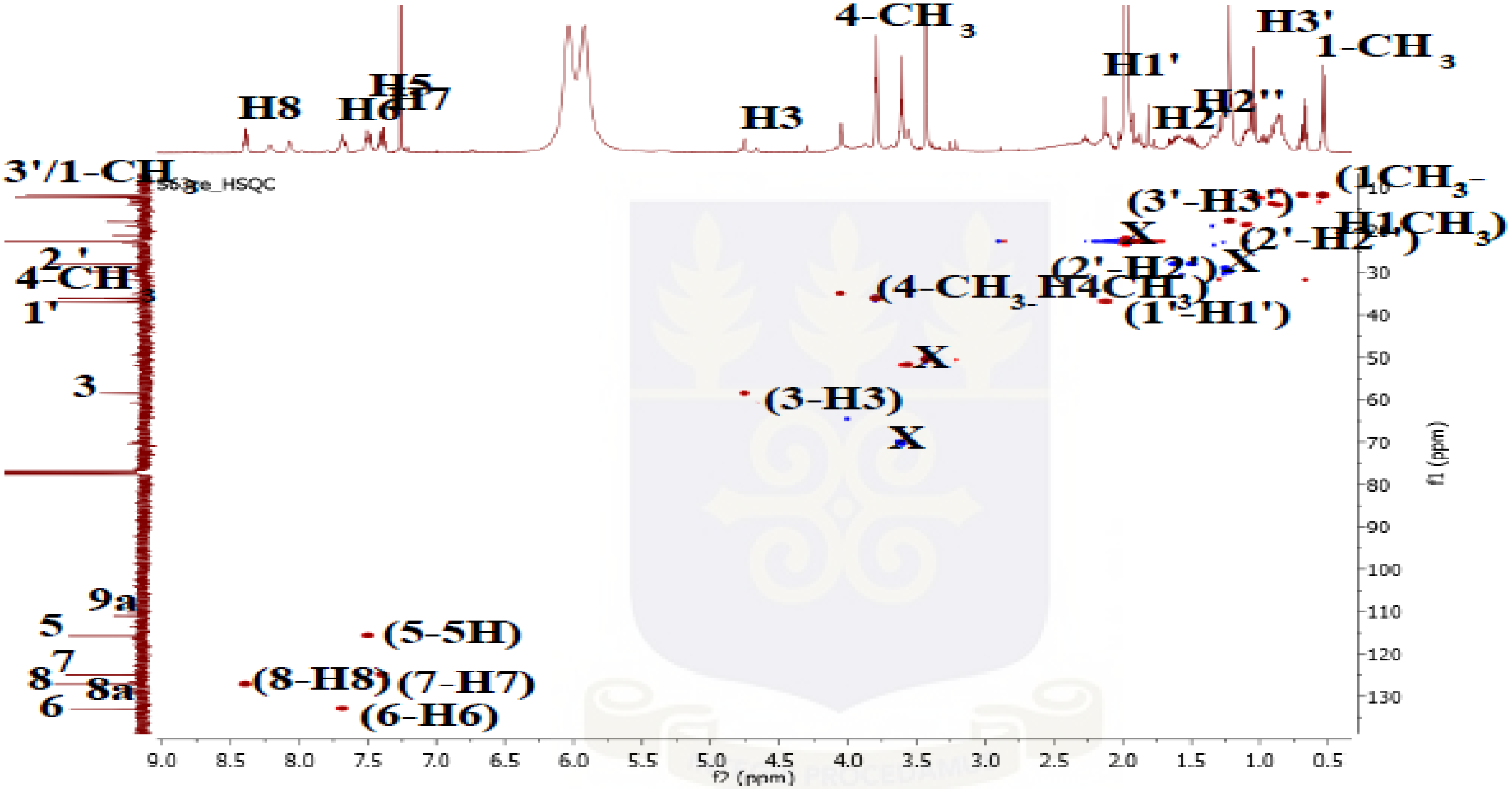
APPENDICES

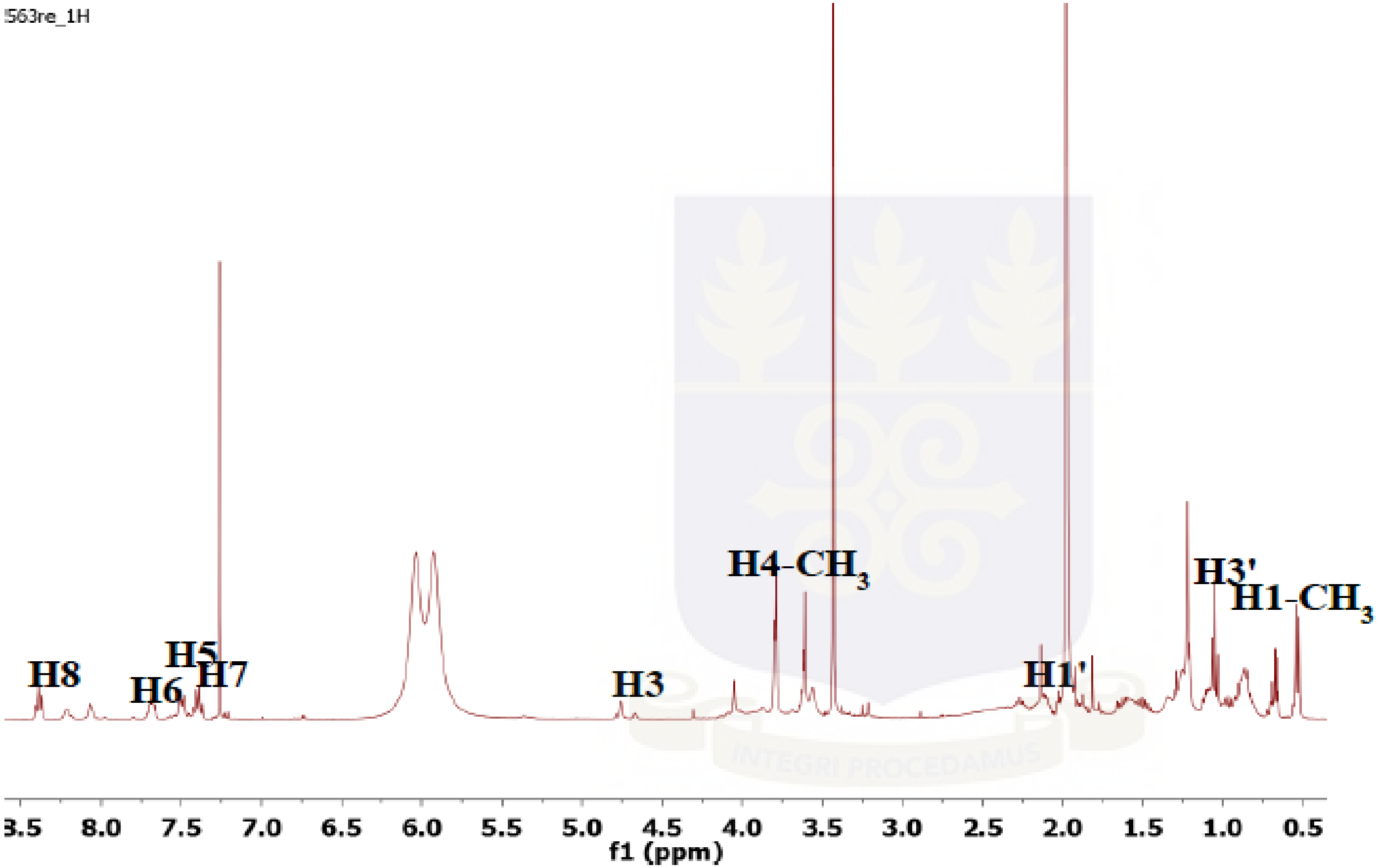
Appendix 1a: ^{13}CNMR spectrum of quinolactacins



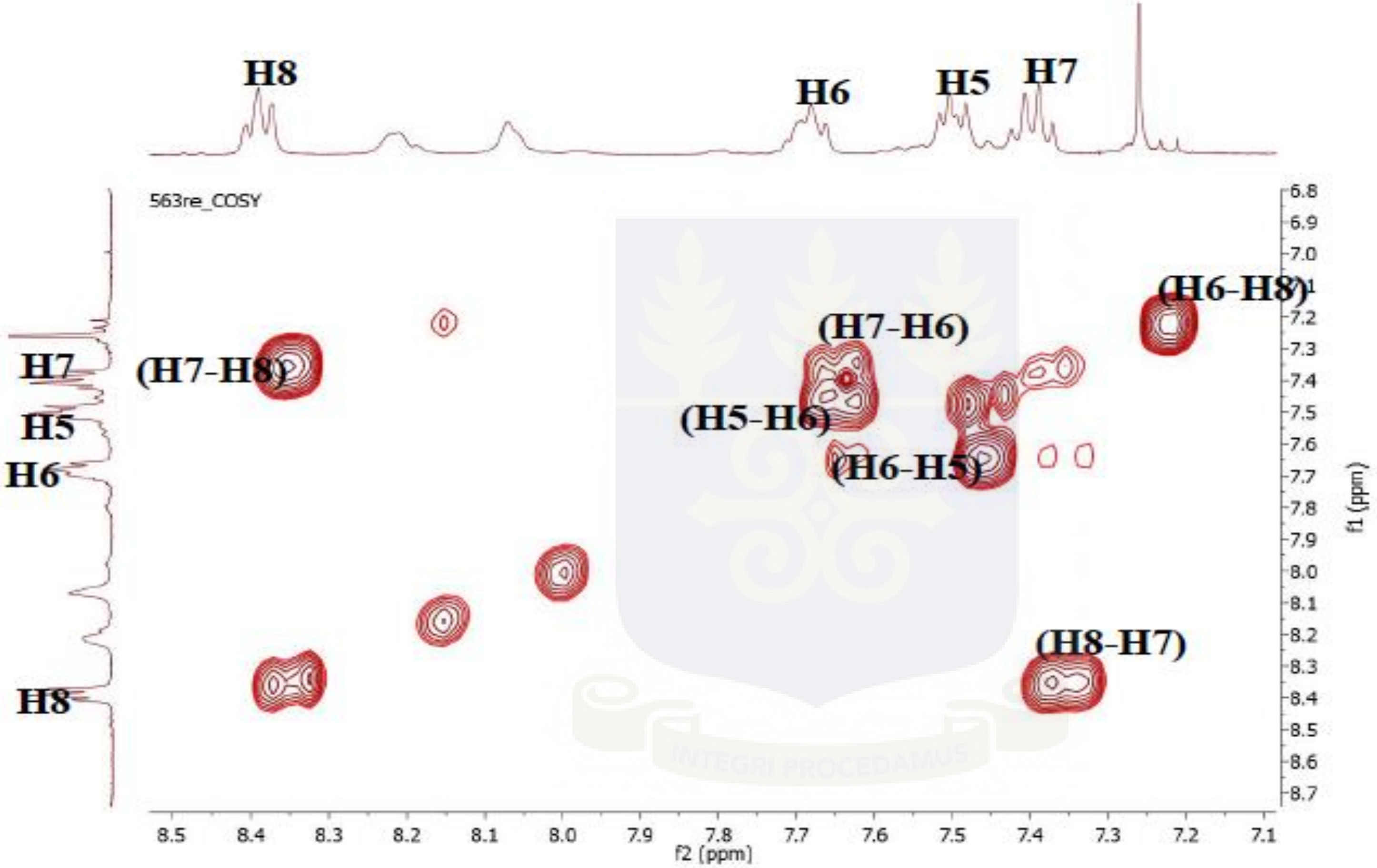
Appendix 1b: HSQC spectrumof quinolactacins



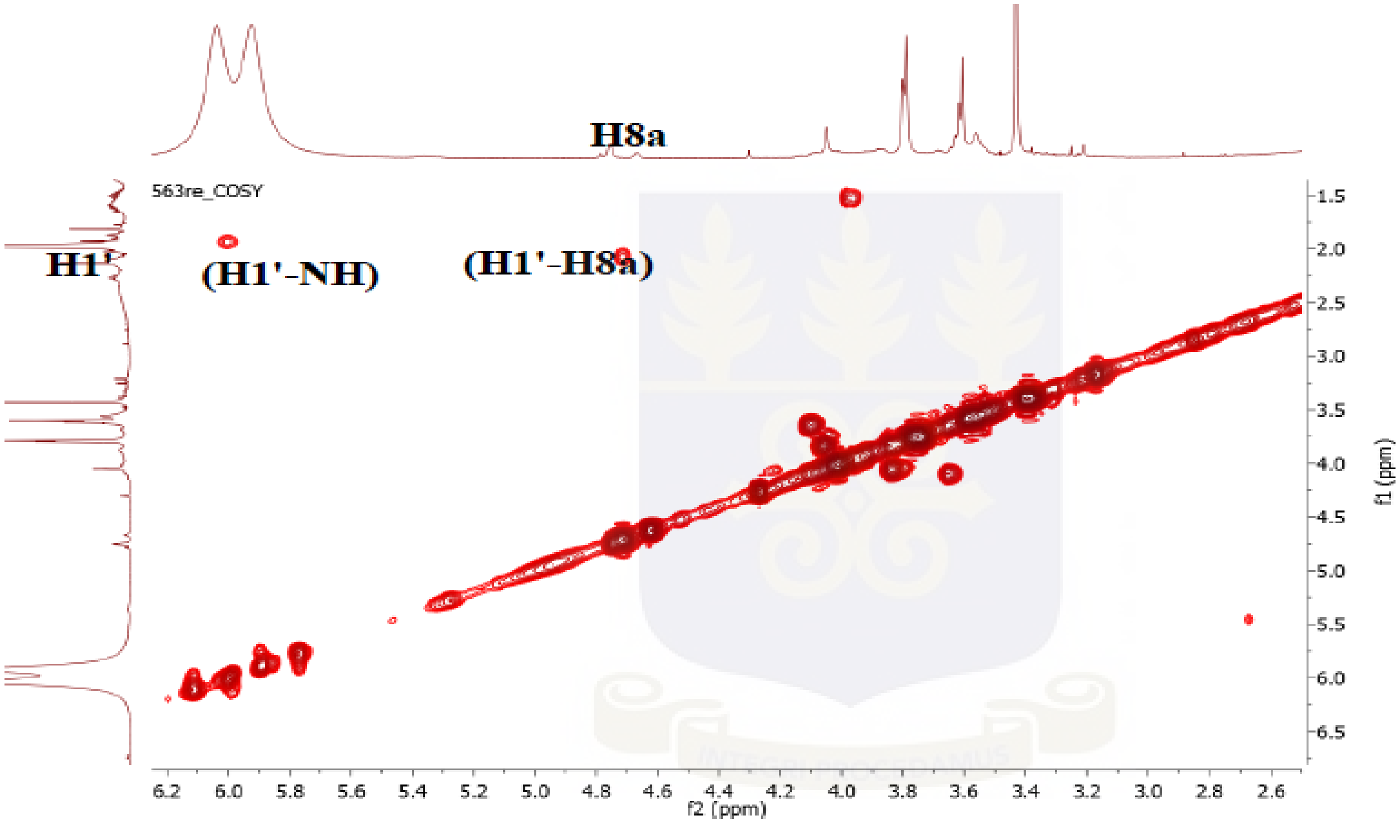
Appendix 1c: ^{1}H NMR spectrum of quinolactacins



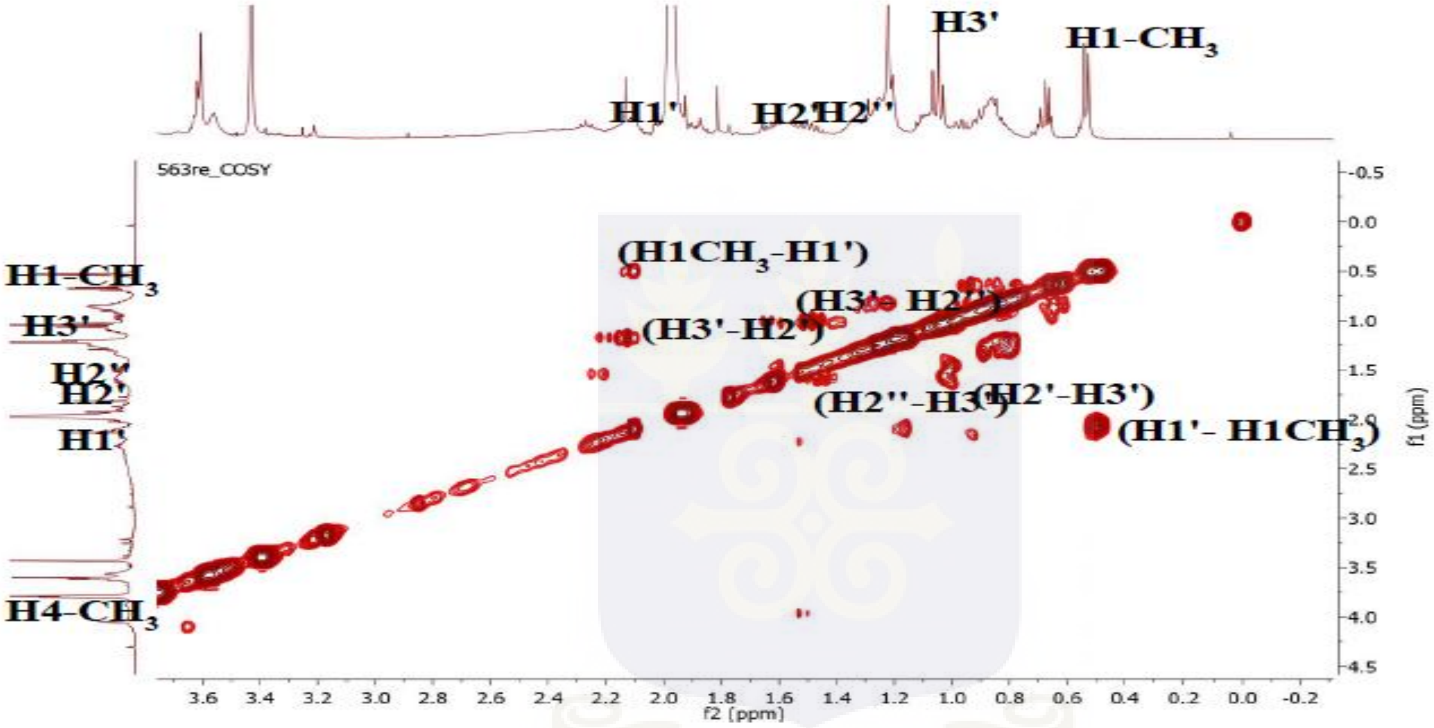
Appendix 1d: ^1H-^1H COSY spectrum of quinolactacins



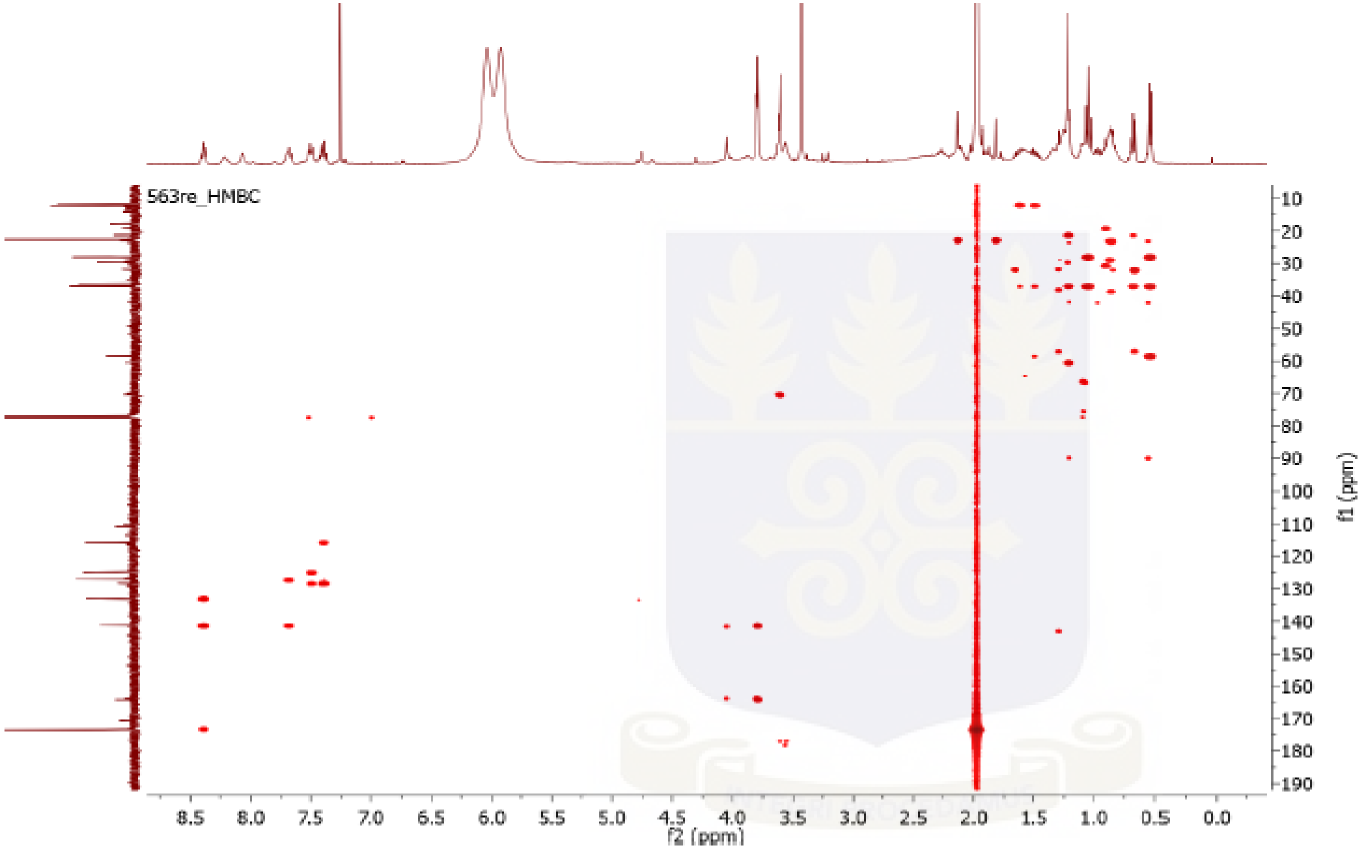
Appendix 1e: ^{1}H-^{1}H COSY spectrum of quinolactacins



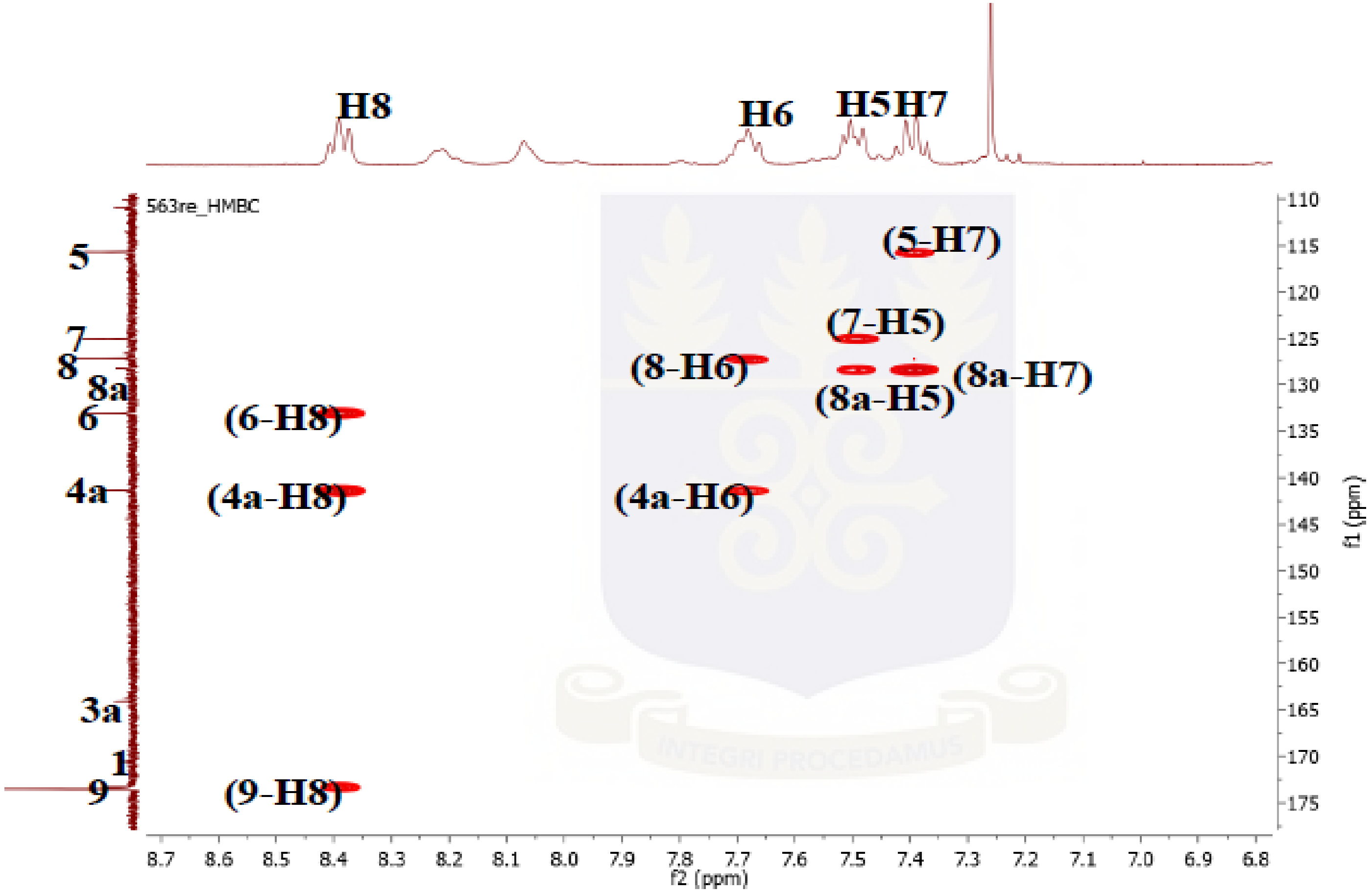
Appendix 1f: ^{1}H-^{1}H COSY spectrum of quinolactacins



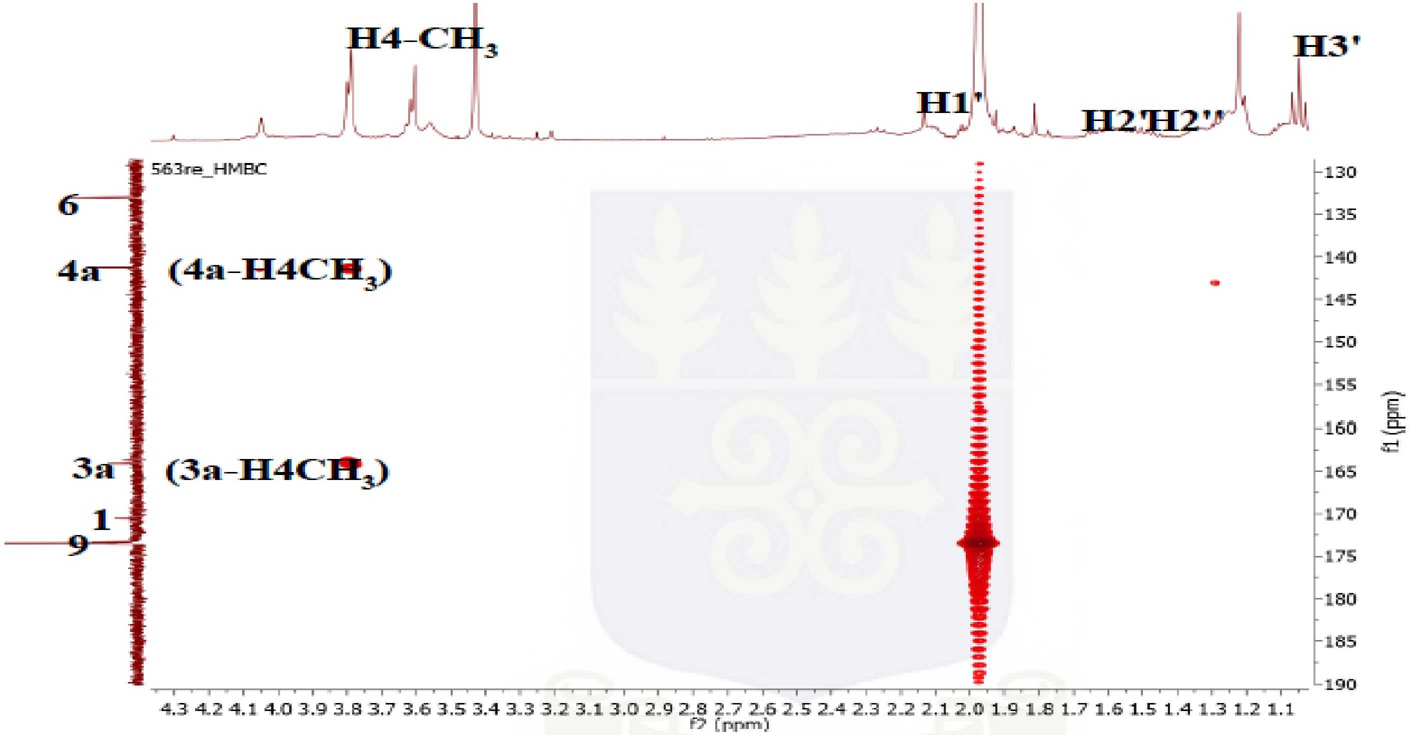
Appendix 1g: HMBC spectrum of quinolactacins



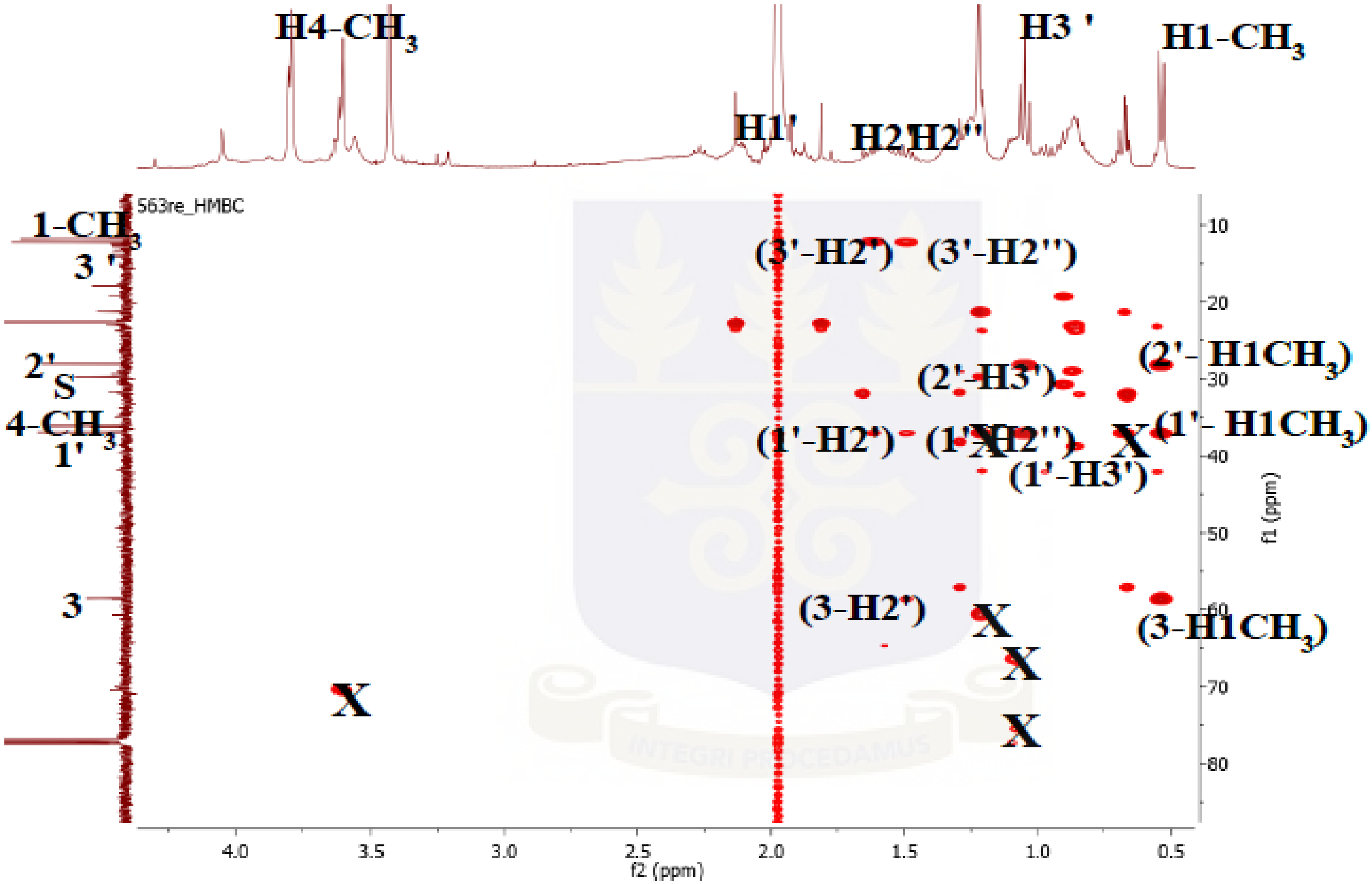
Appendix 1h: HMBC spectrum of quinolactacins



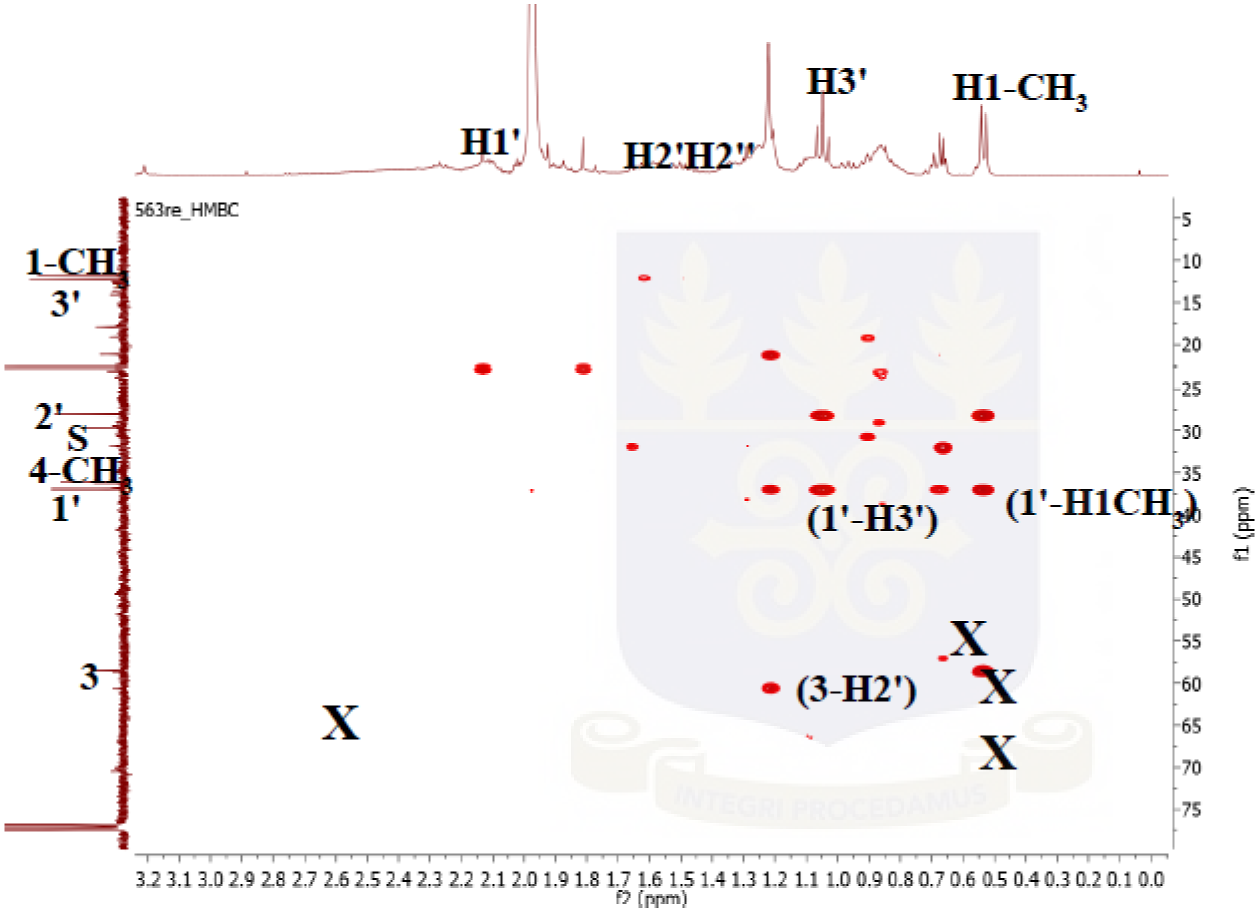
Appendix 1i: HMBC spectrum of quinolactacins



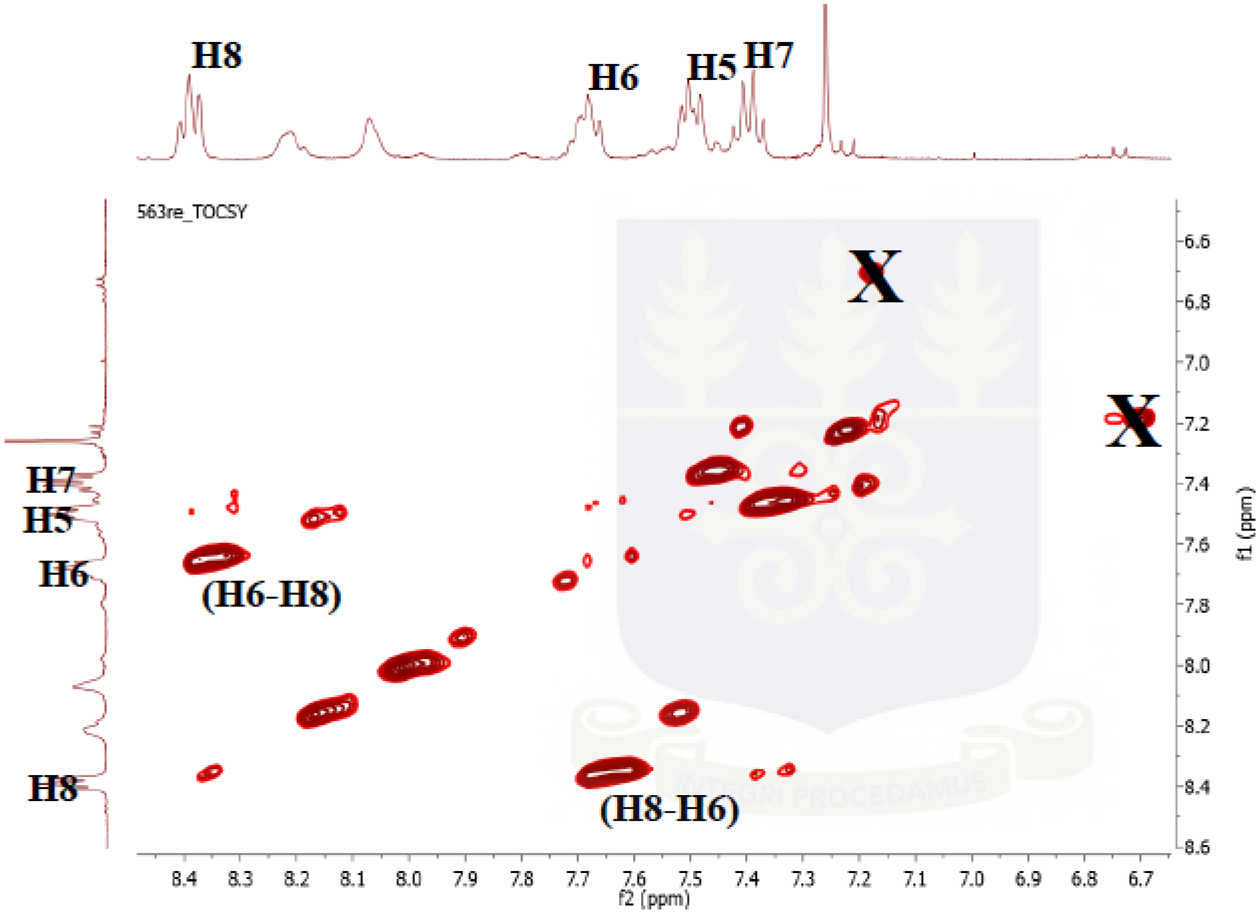
Appendix 1j: HMBC spectrum of quinolactacins



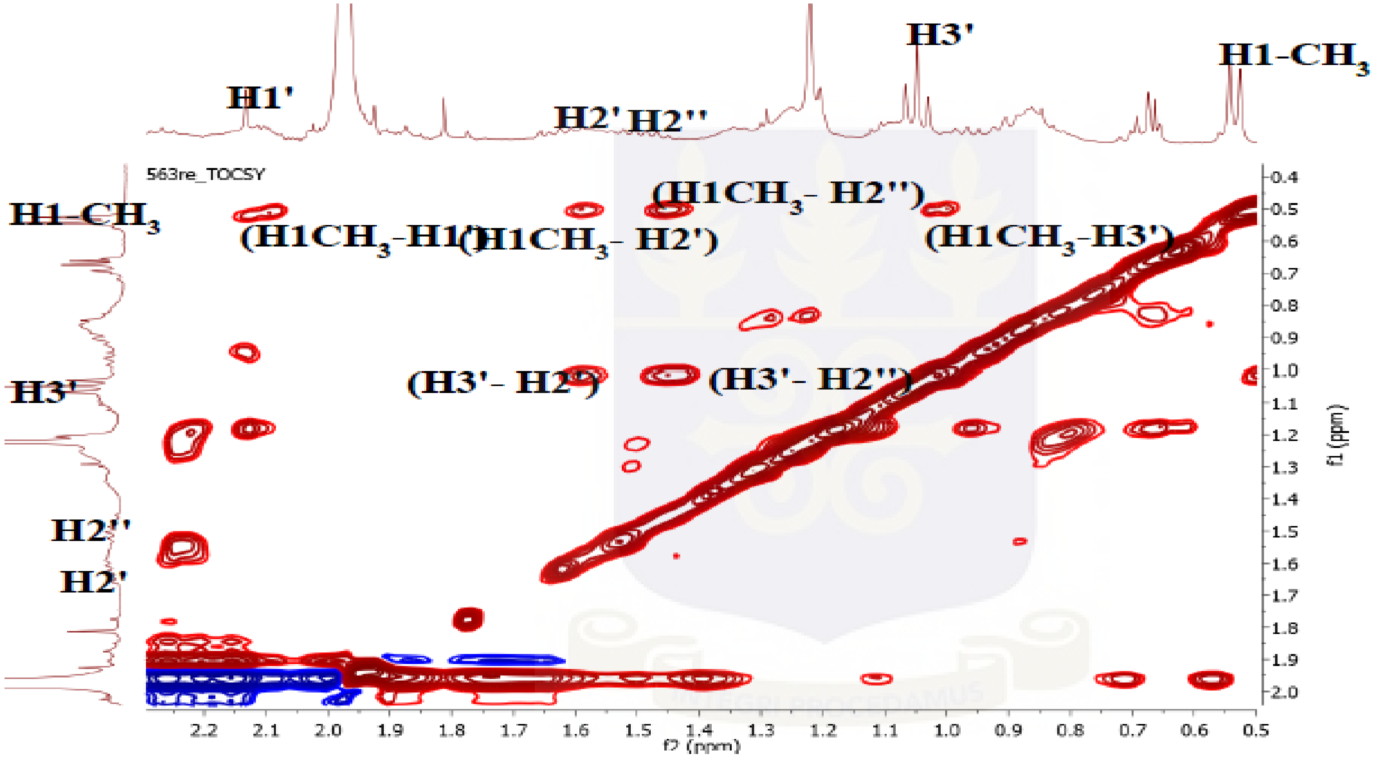
Appendix 1k: HMBC spectrum of quinolactacins



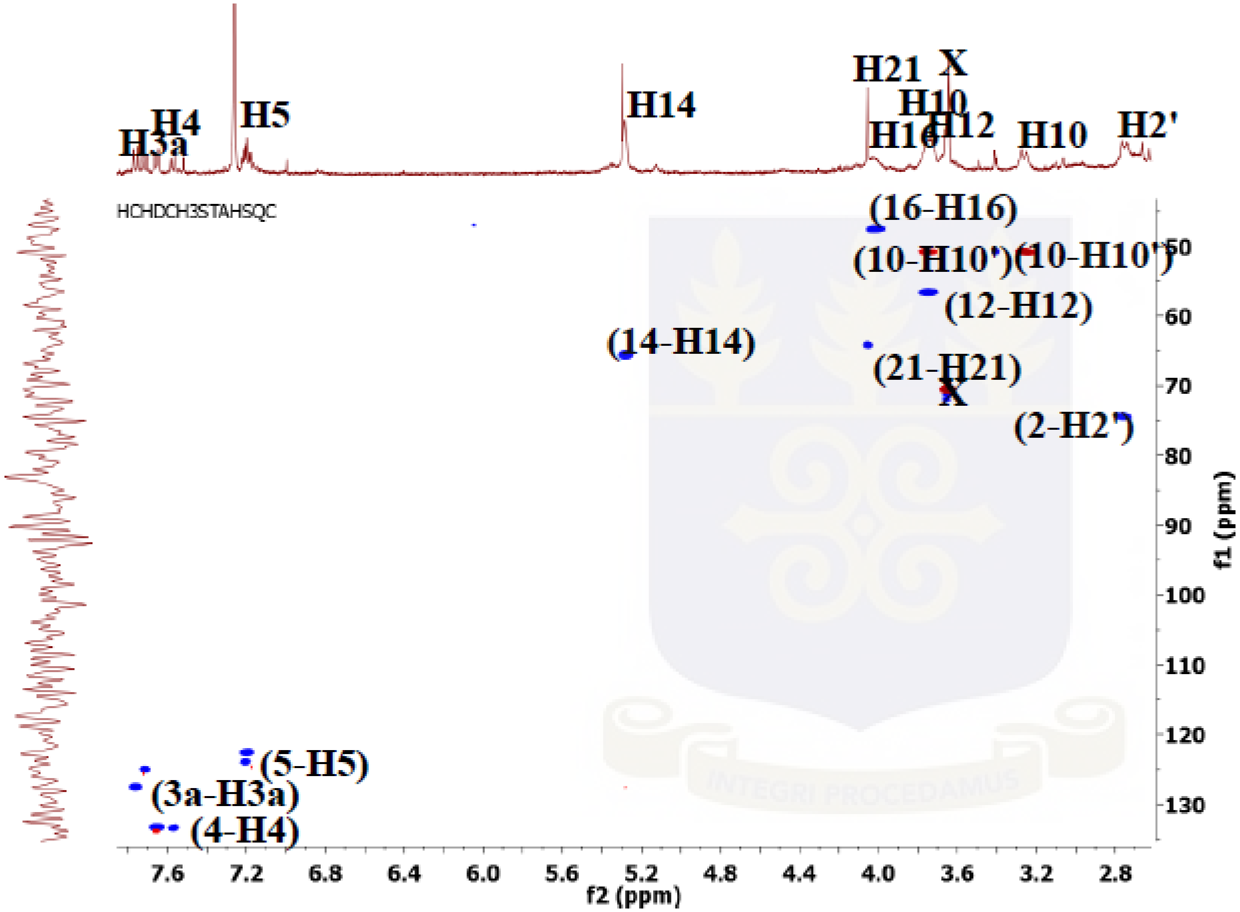
Appendix 1l: ^{1}H-^{1}H TOCSY spectrum quinolactacin



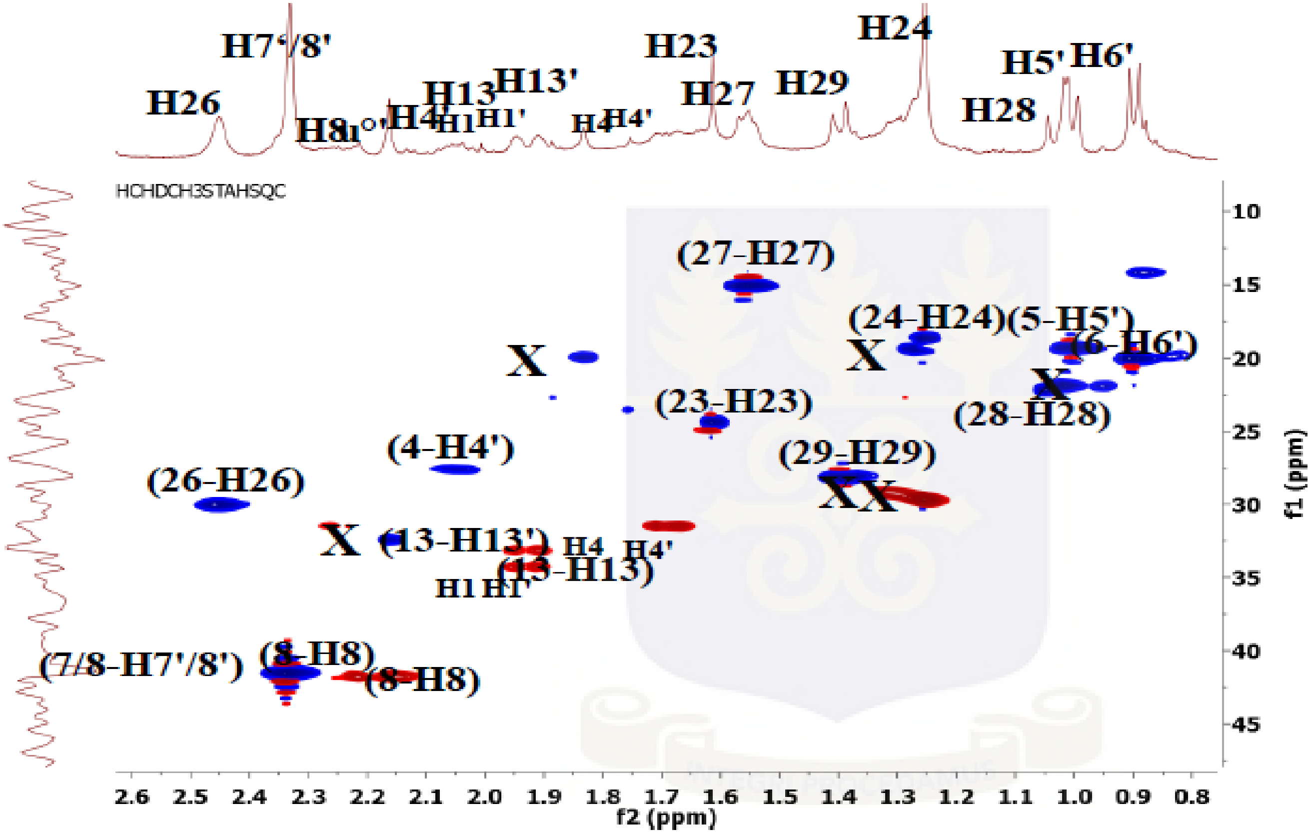
Appendix 1m: ^{1}H-^{1}H TOCSY spectrum quinolactacin



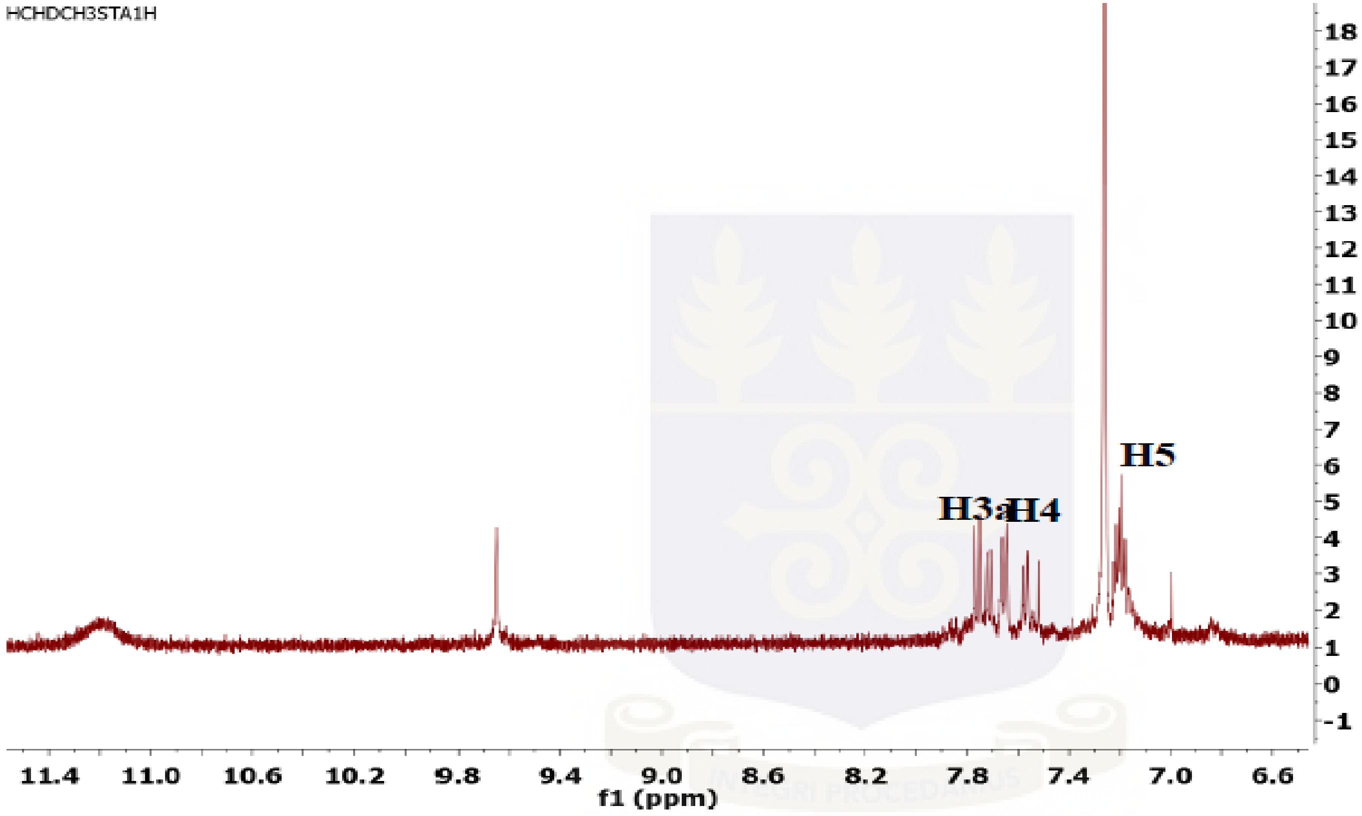
Appendix 2a: HSQCspectrum of citrinadins



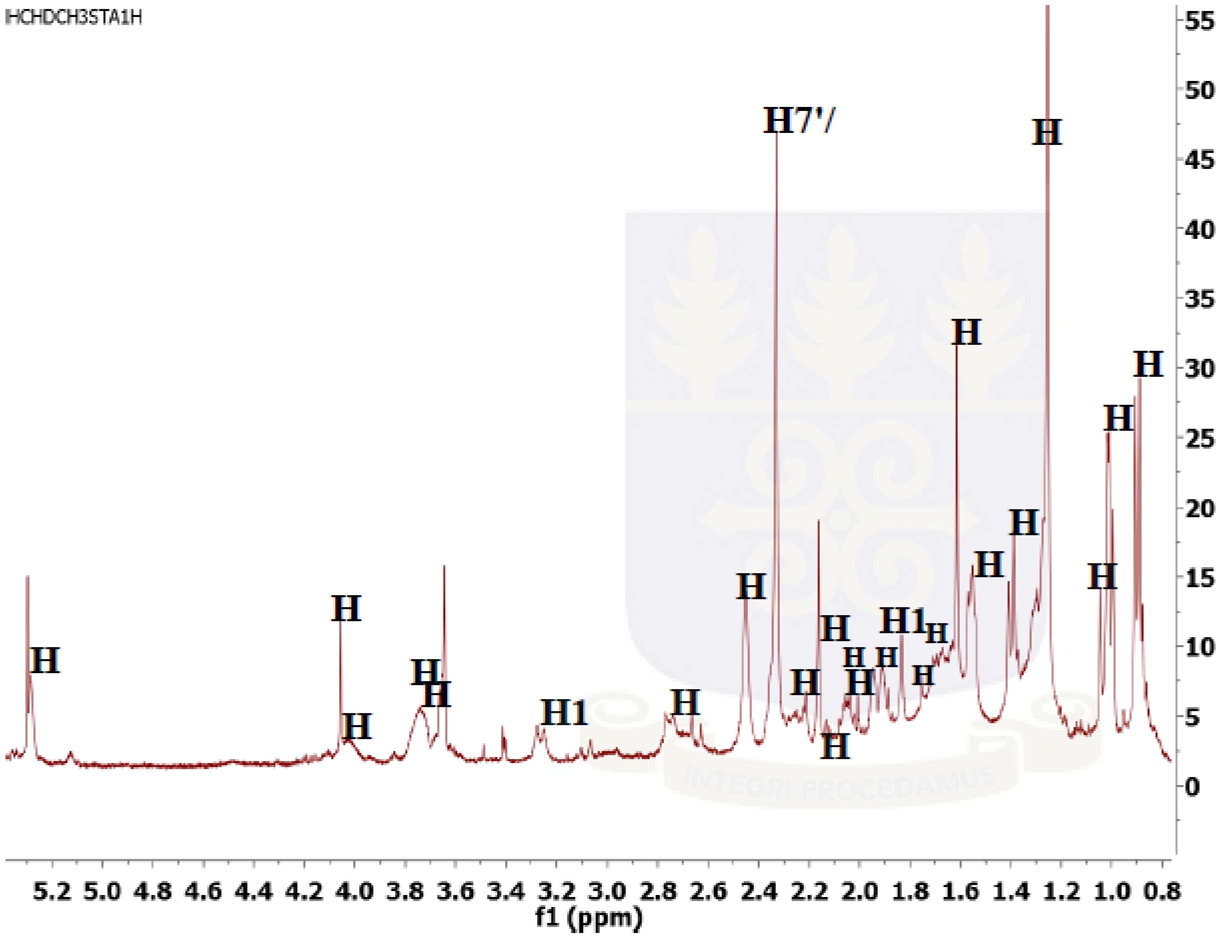
Appendix 2b: HSQCspectrum of citrinadins



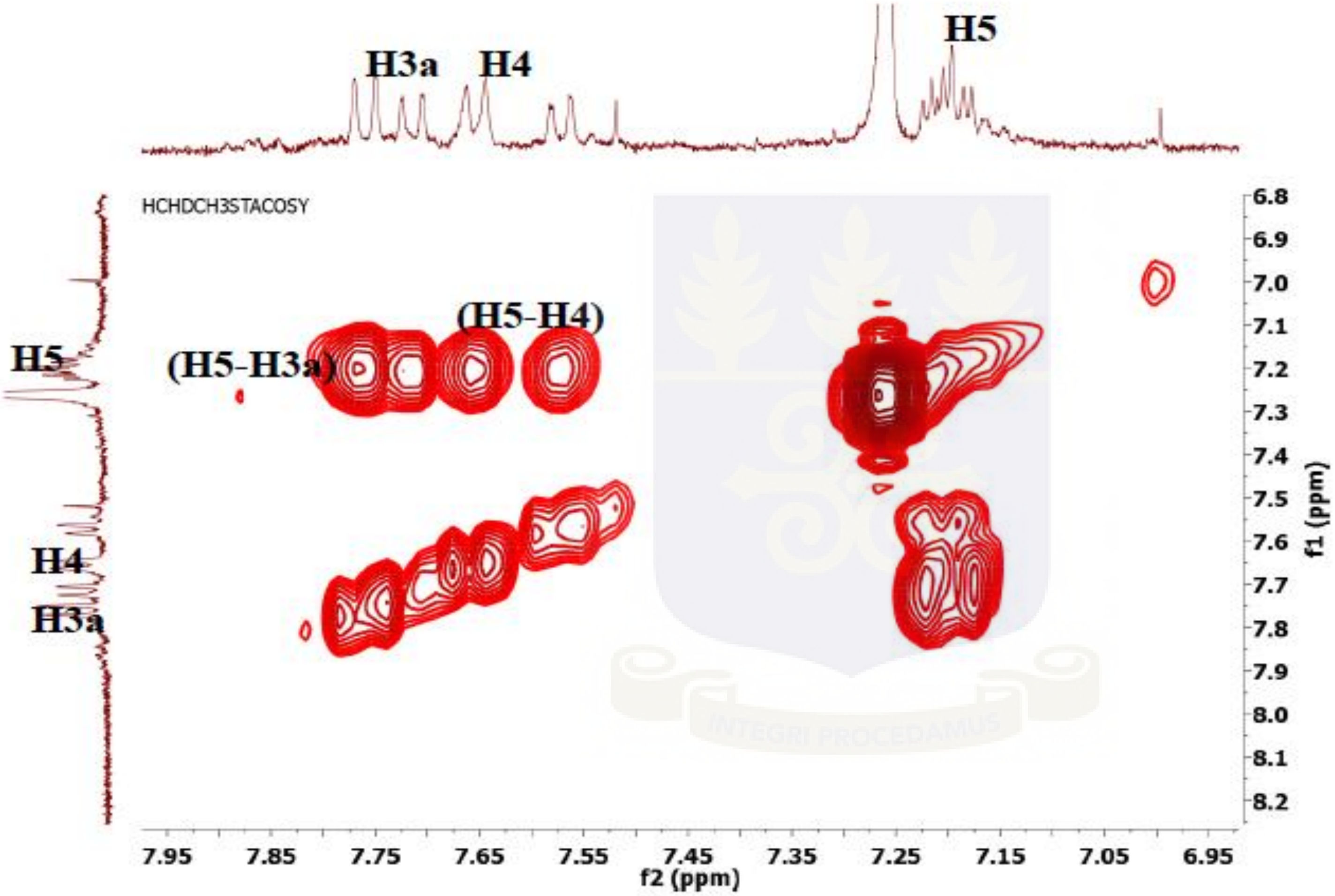
Appendix 2c: ^{1}H NMR spectrum of citrinadins



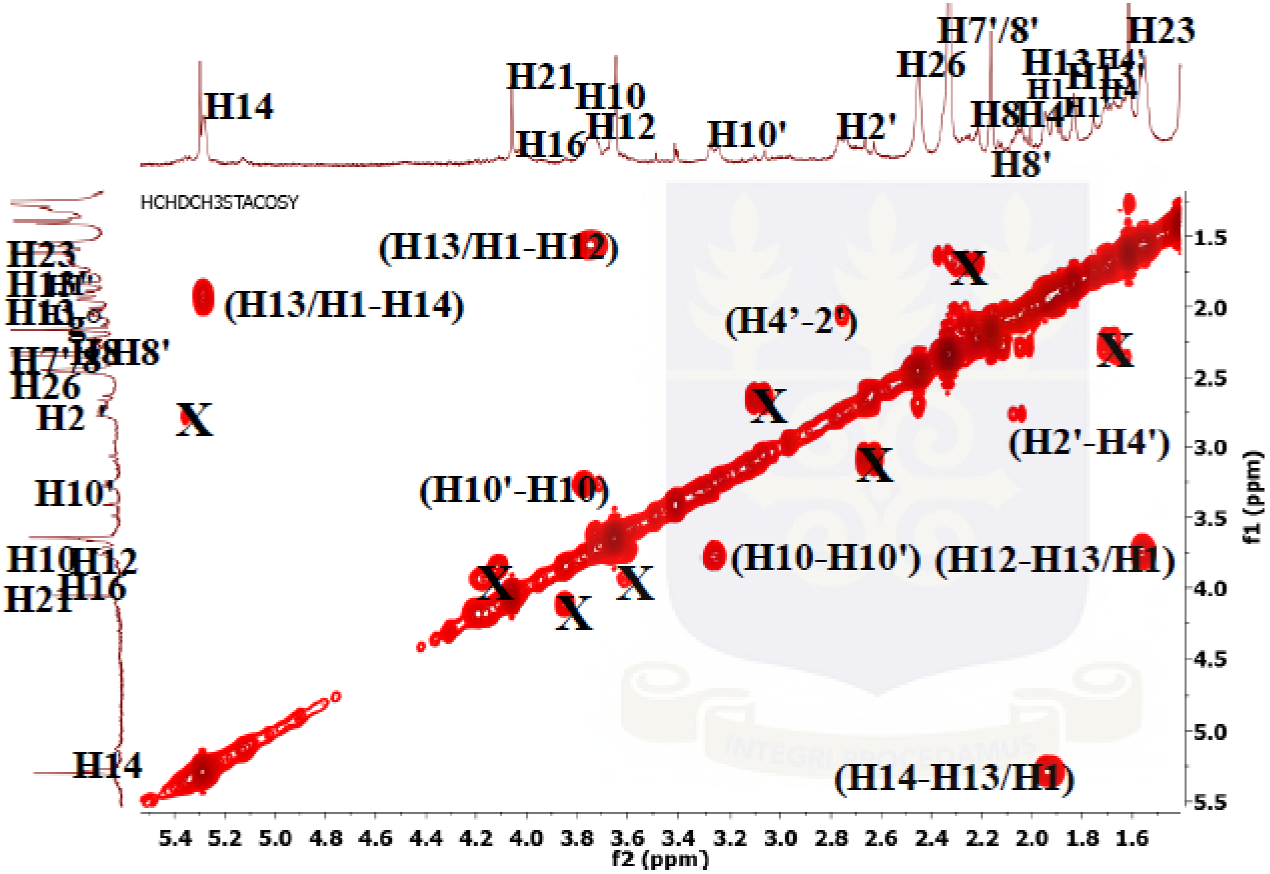
Appendix 2d: ^{1}H NMR spectrum of citrinadins



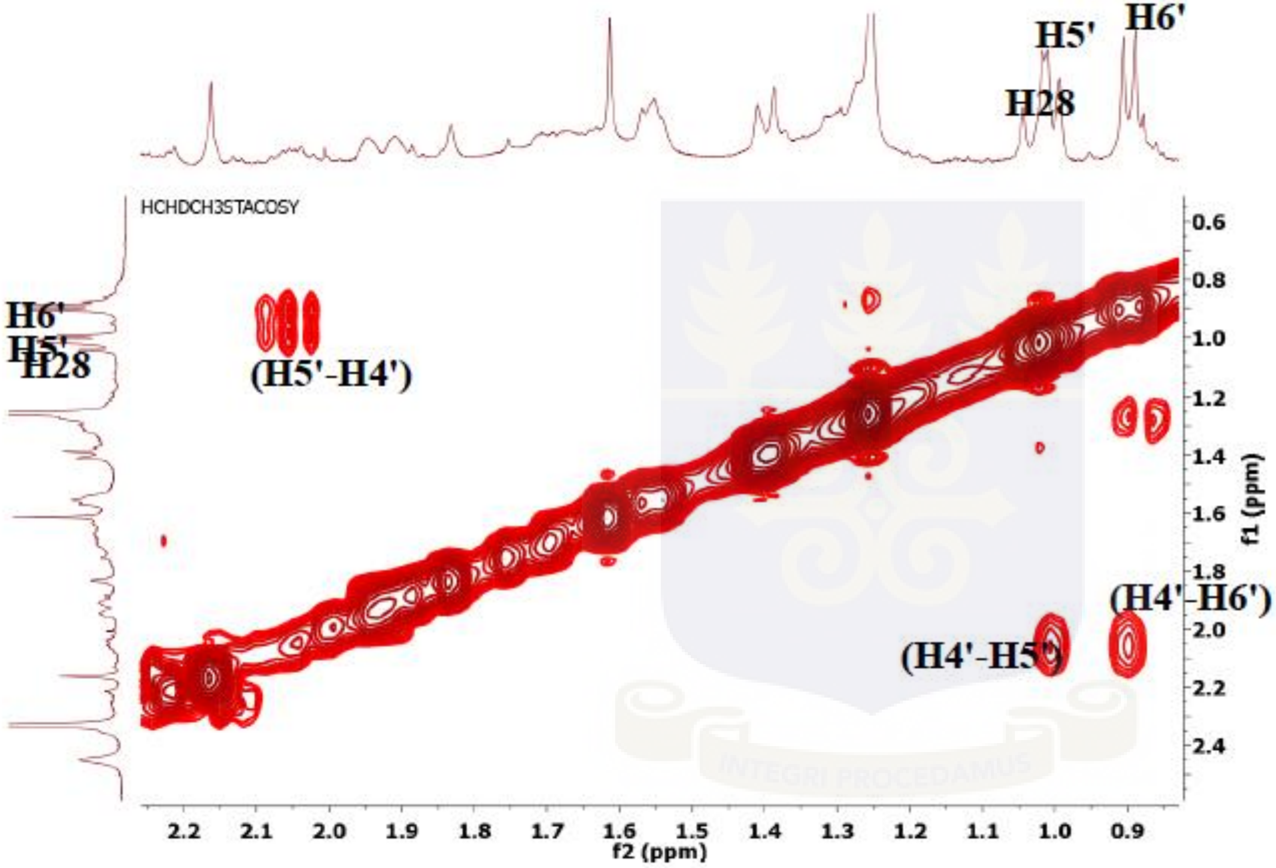
Appendix 2e: ^{1}H-^{1}H COSY spectrum of citrinadins



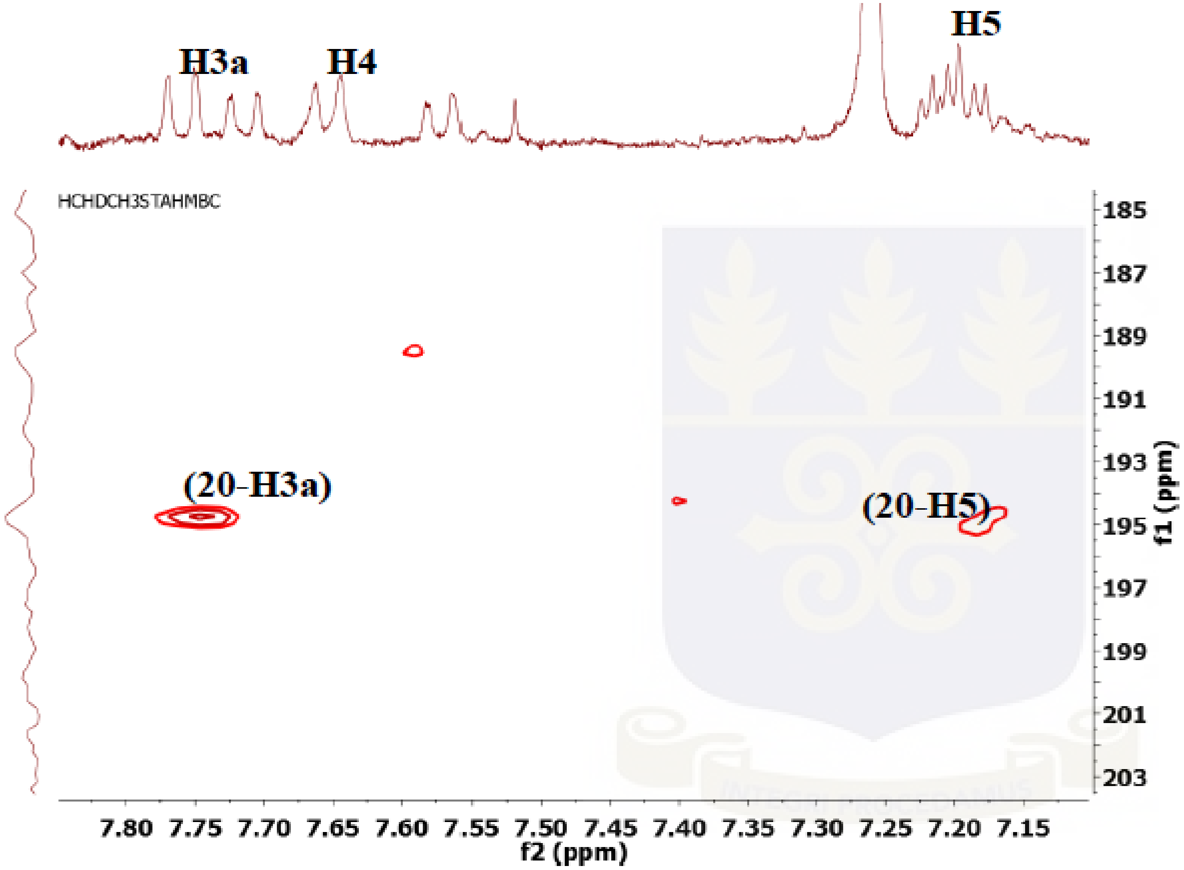
Appendix 2f: ^{1}H-^{1}H COSY spectrum of citrinadins



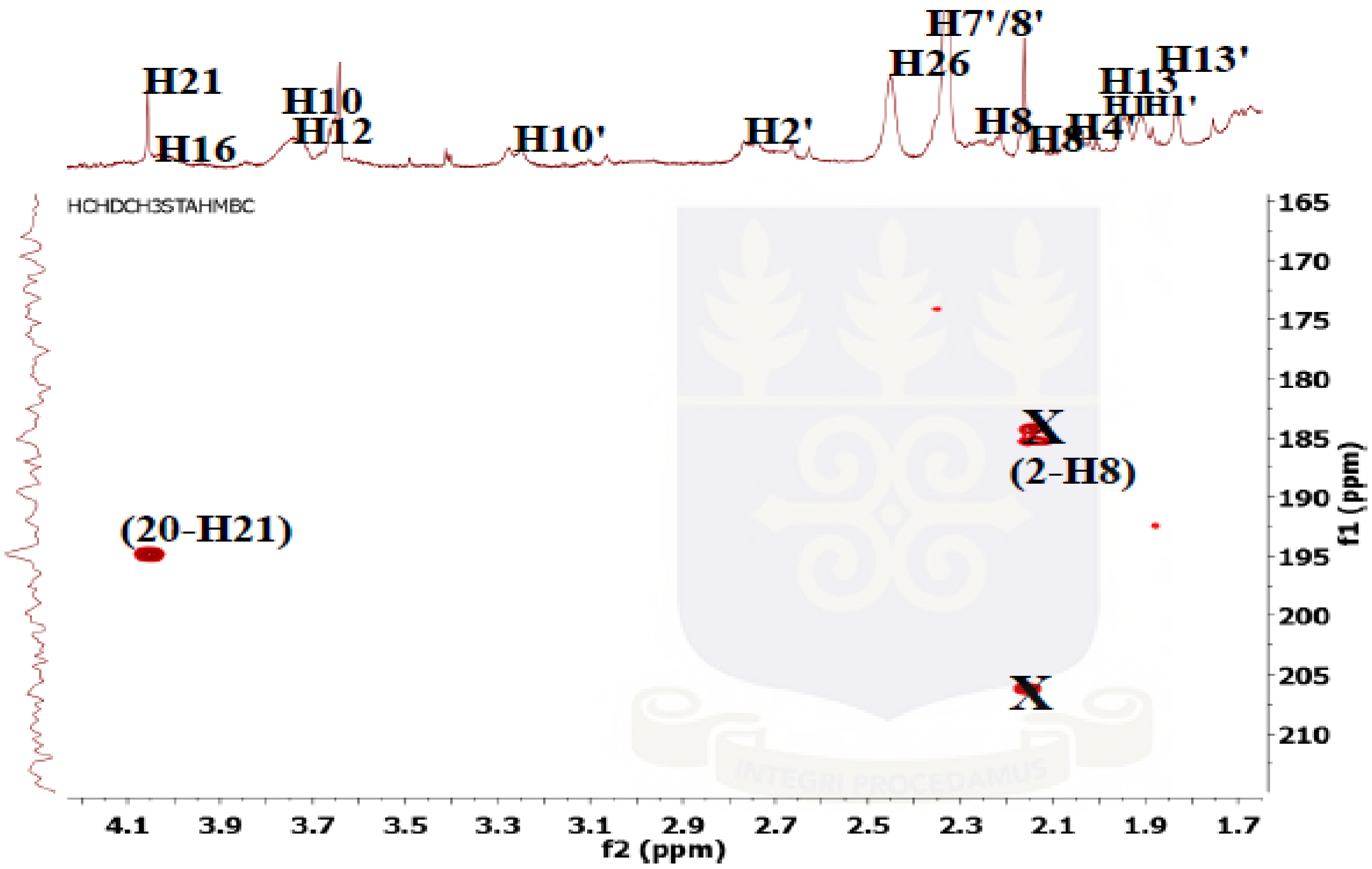
Appendix 2g: ^{1}H-^{1}H COSY spectrum of citrinadin



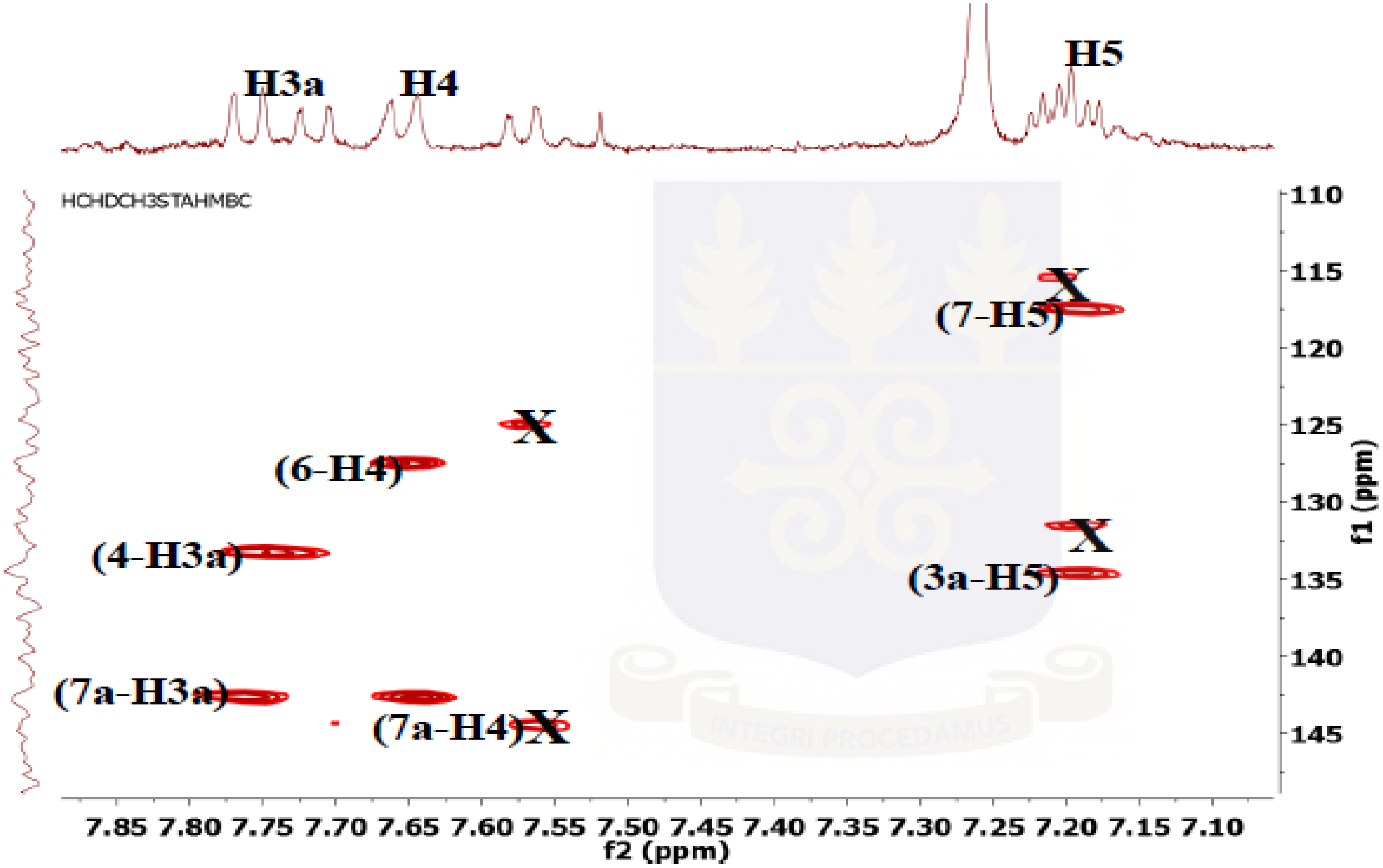
Appendix 2h: HMBC spectrum of quinolactacin



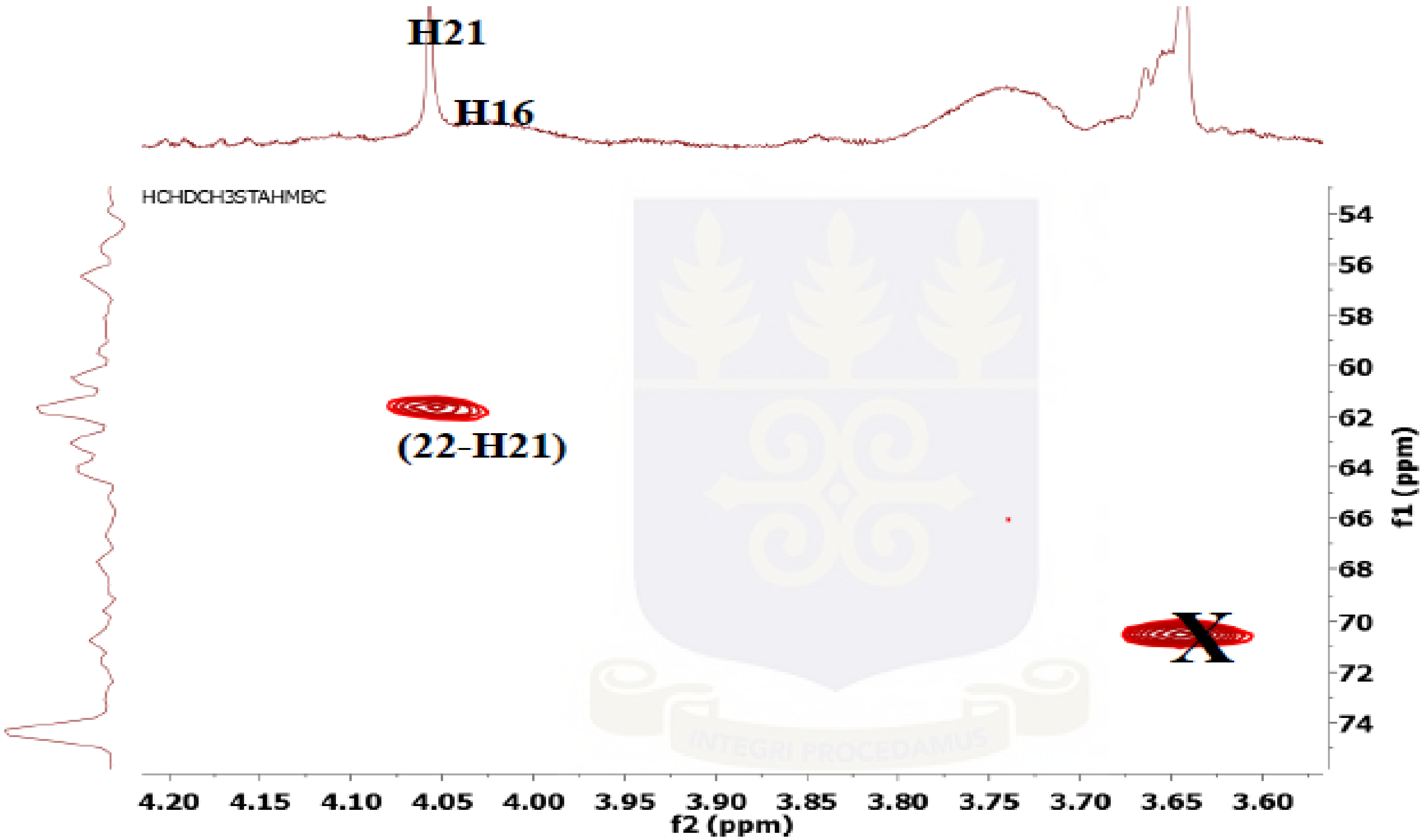
Appendix 2i: HMBC spectrum of citrinadins



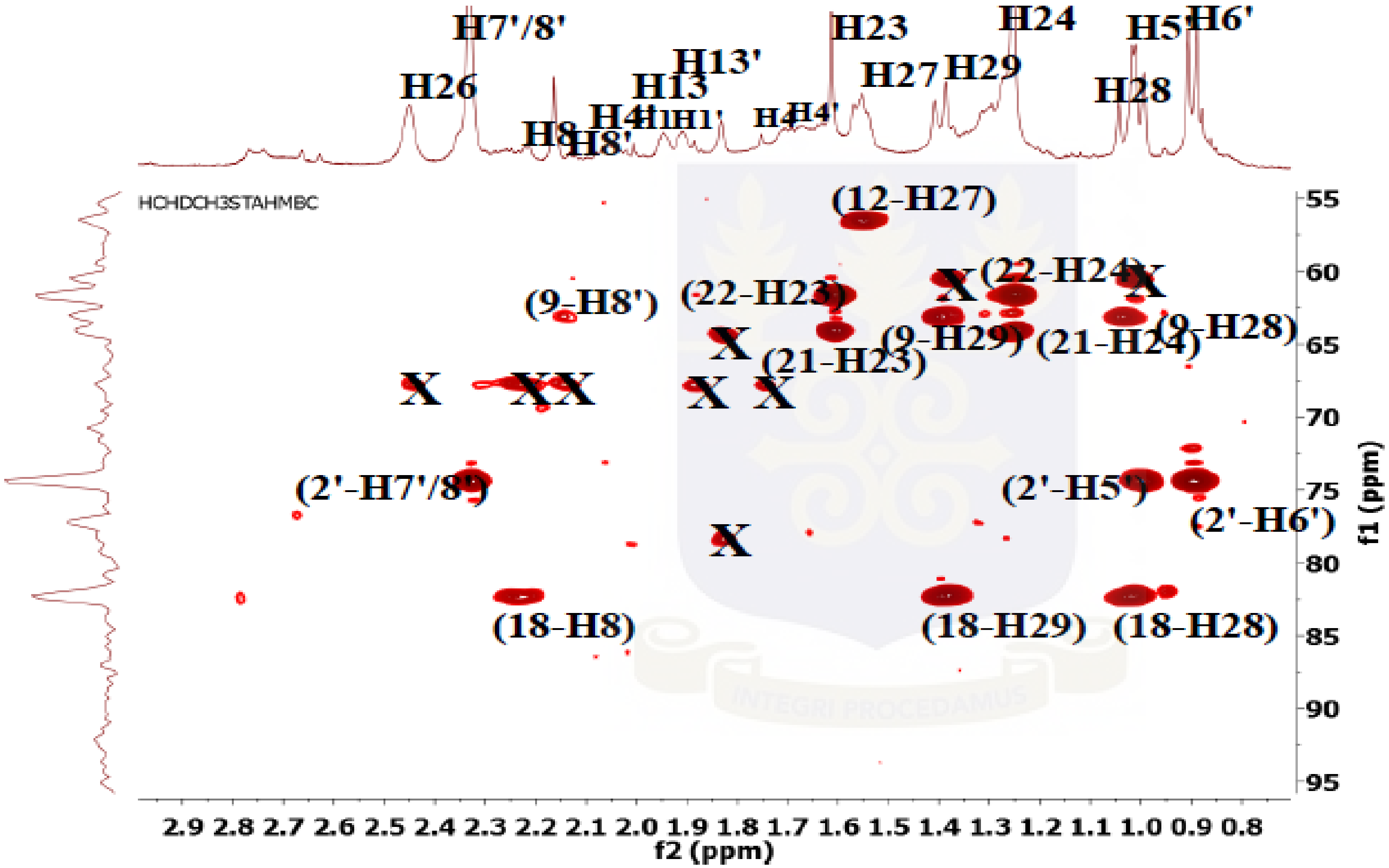
Appendix 2j: HMBC spectrum of citrinadins



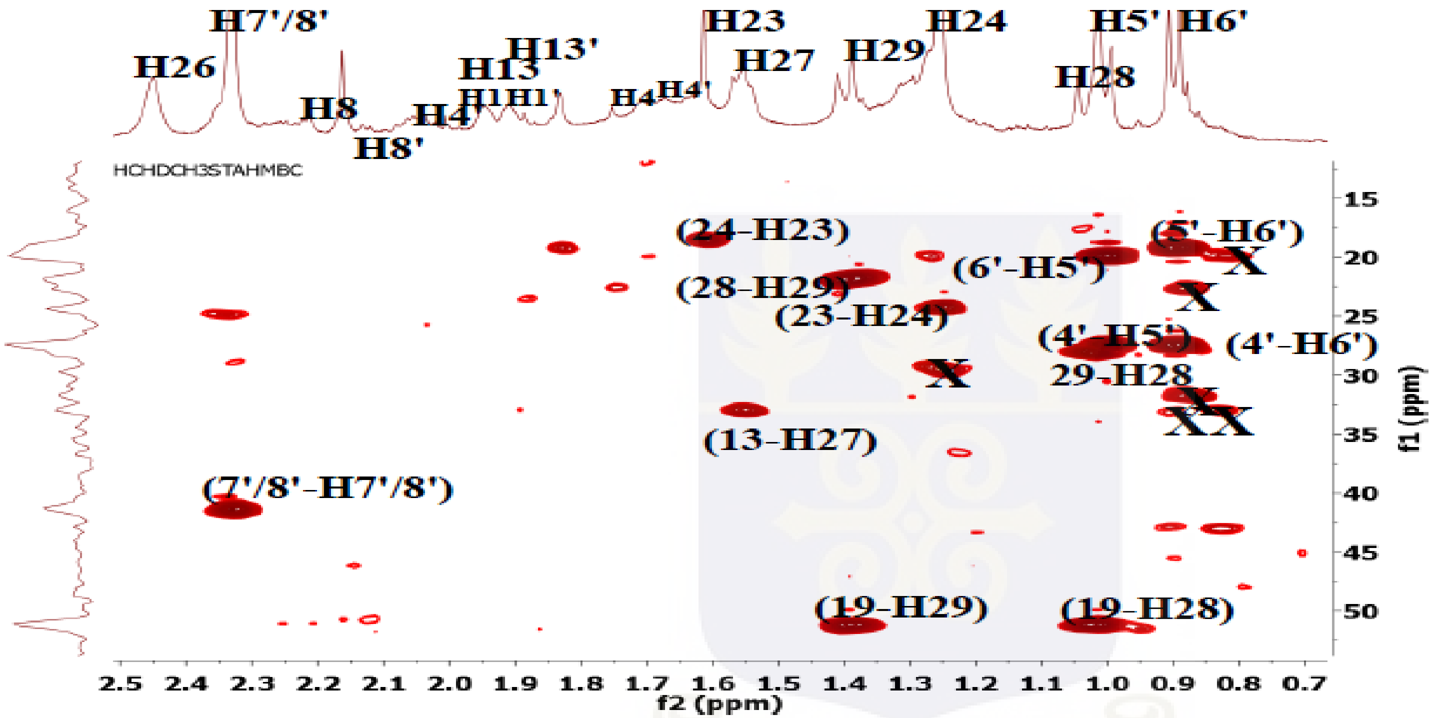
Appendix 2k: HMBC spectrum of citrinadins



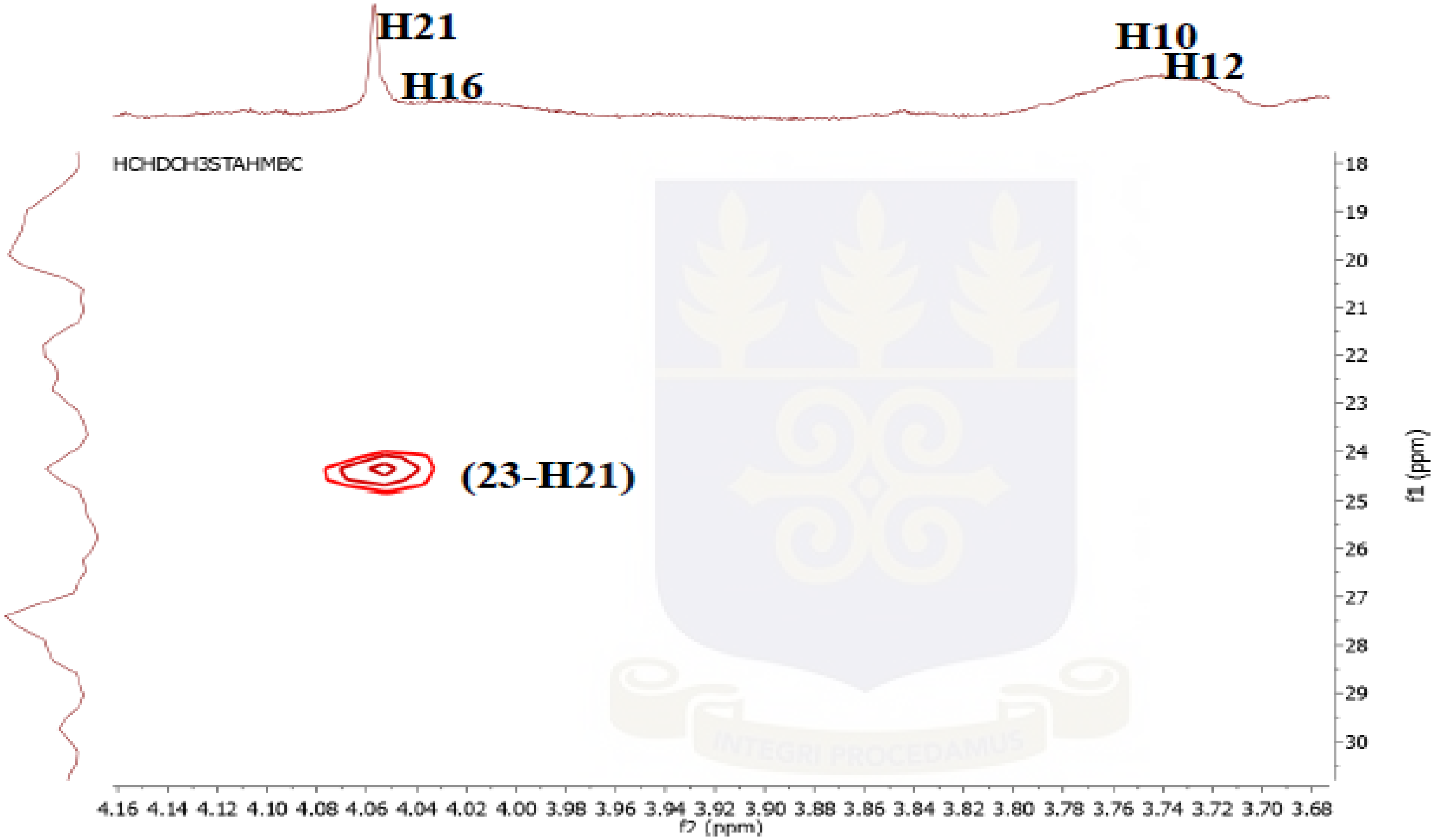
Appendix 2l: HMBC spectrum of citrinadins



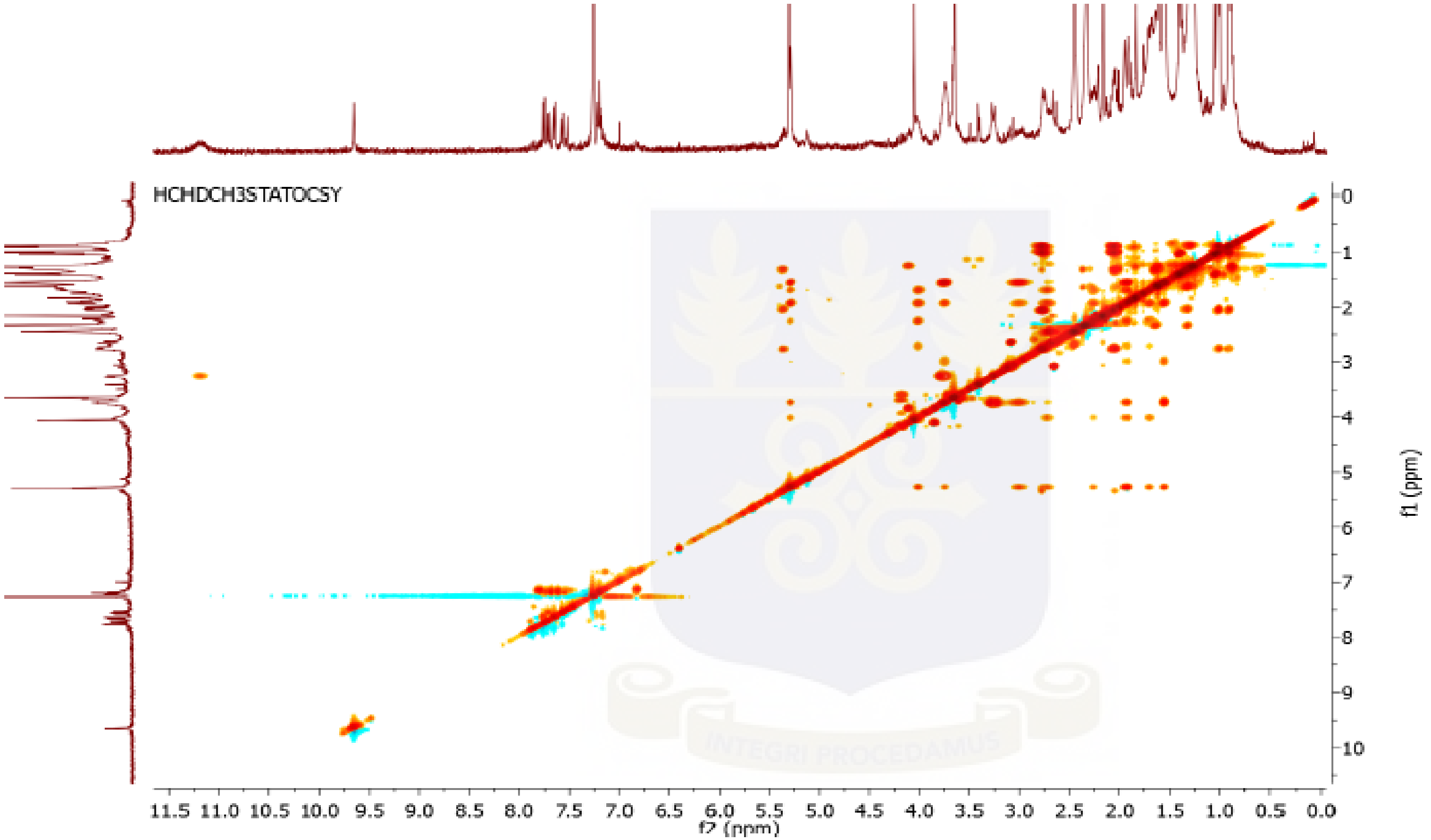
Appendix 2m: HMBC spectrum of citrinadins



Appendix 2n: HMBC spectrum of citrinadins

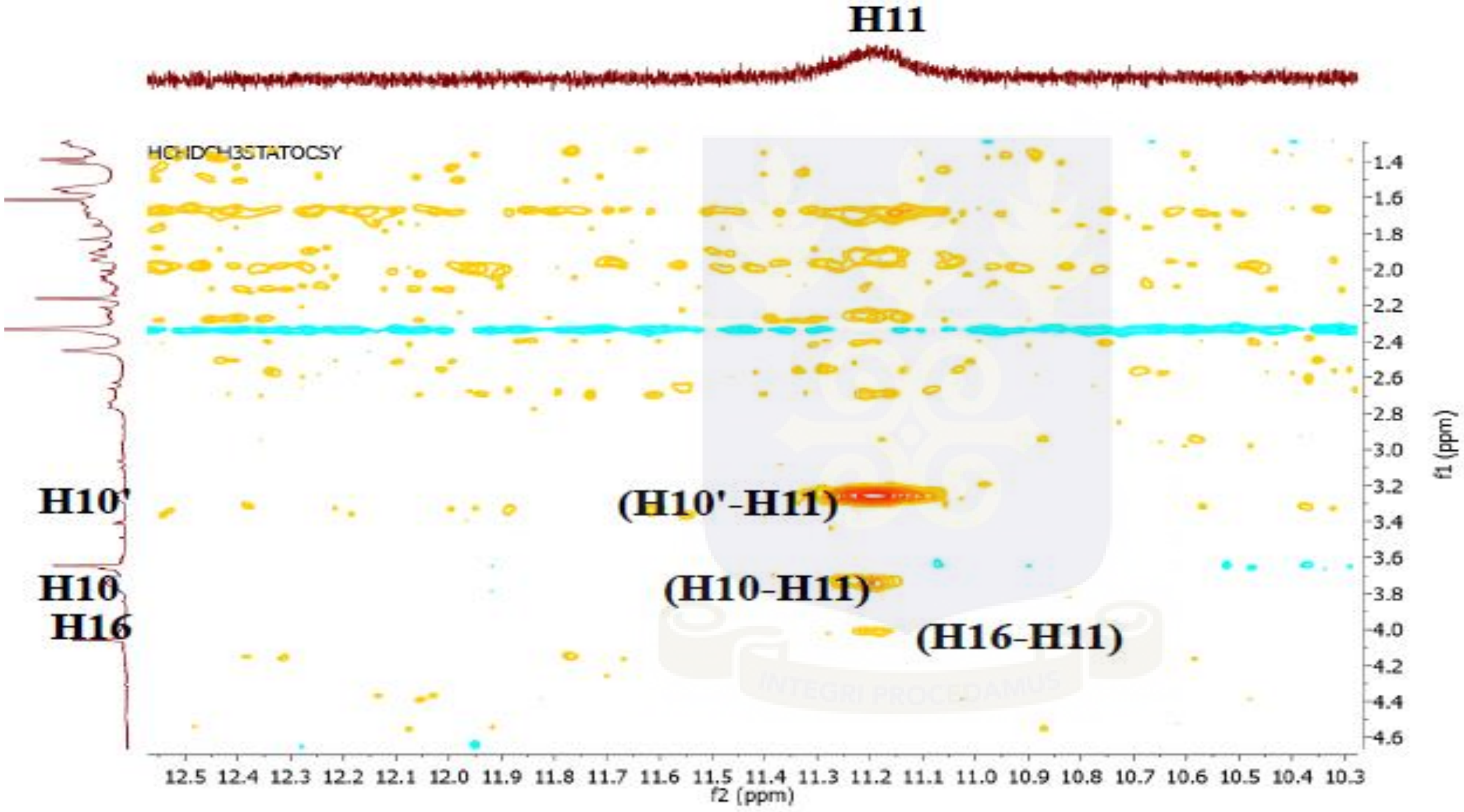


Appendix 2o: TOCSY spectrum of citrinadin

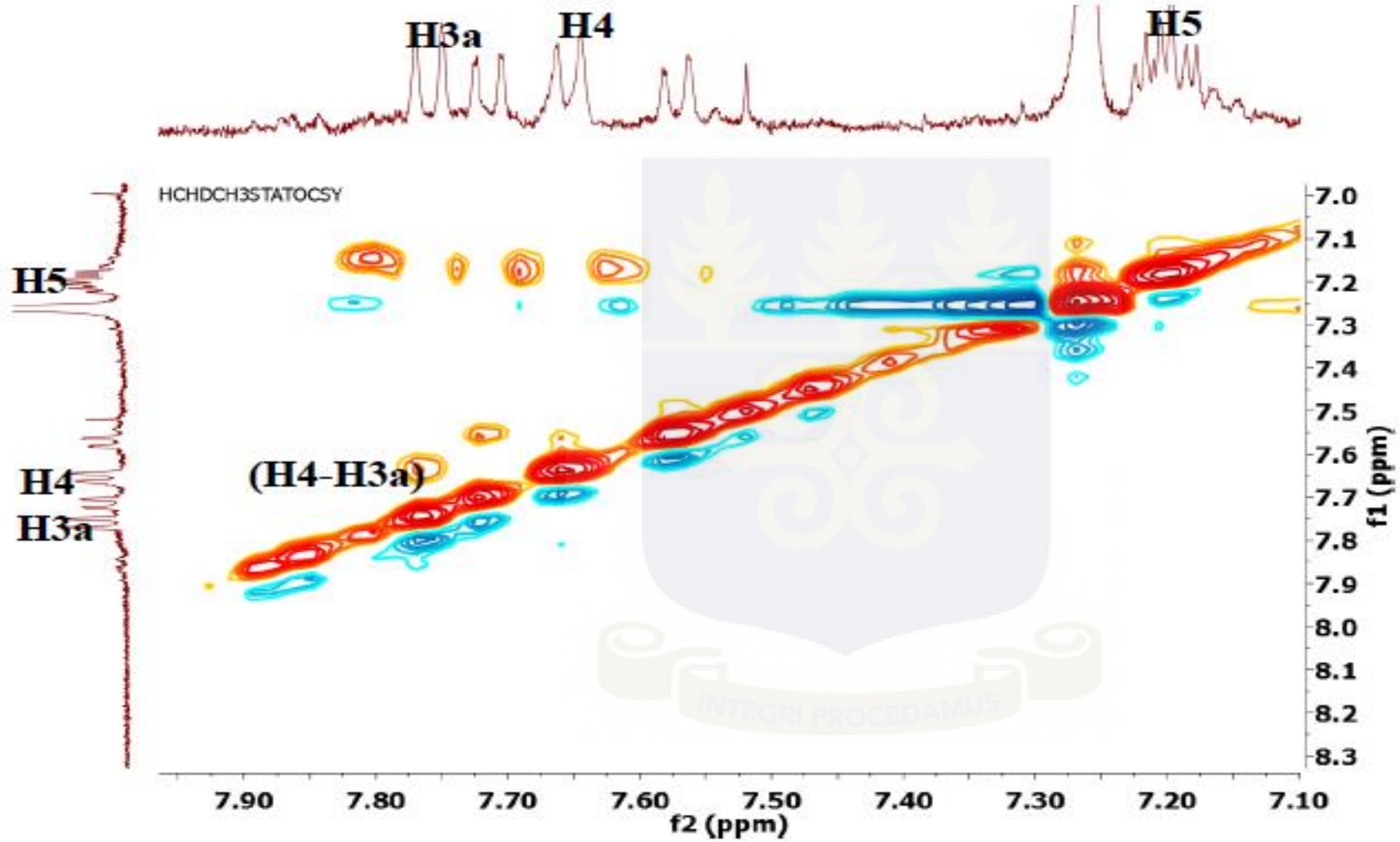


AAGIIF F FFF

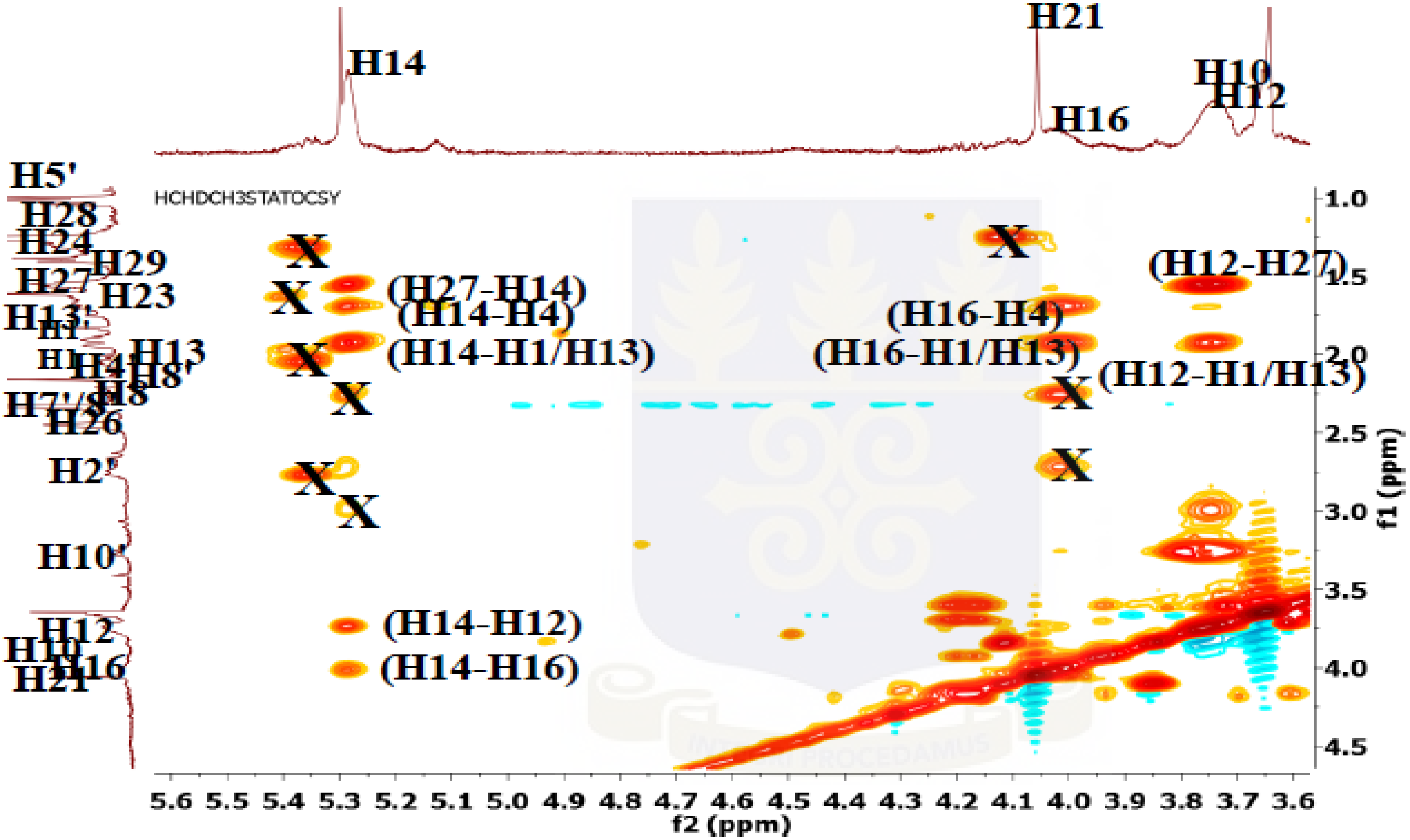
Appendix 2p: HMBC spectrum of citrinadins



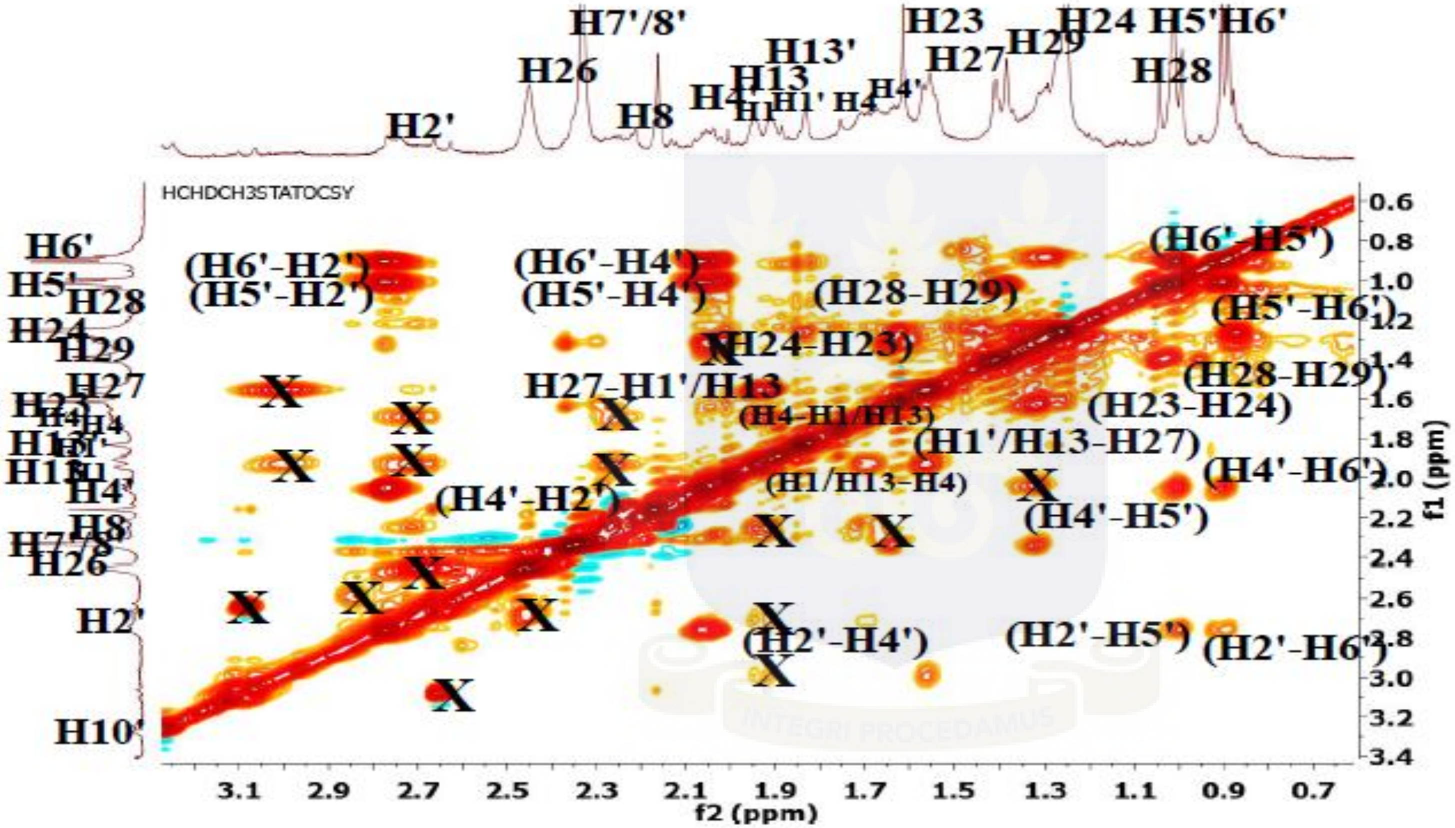
Appendix 2q: TOCSY spectrum of citrinadins



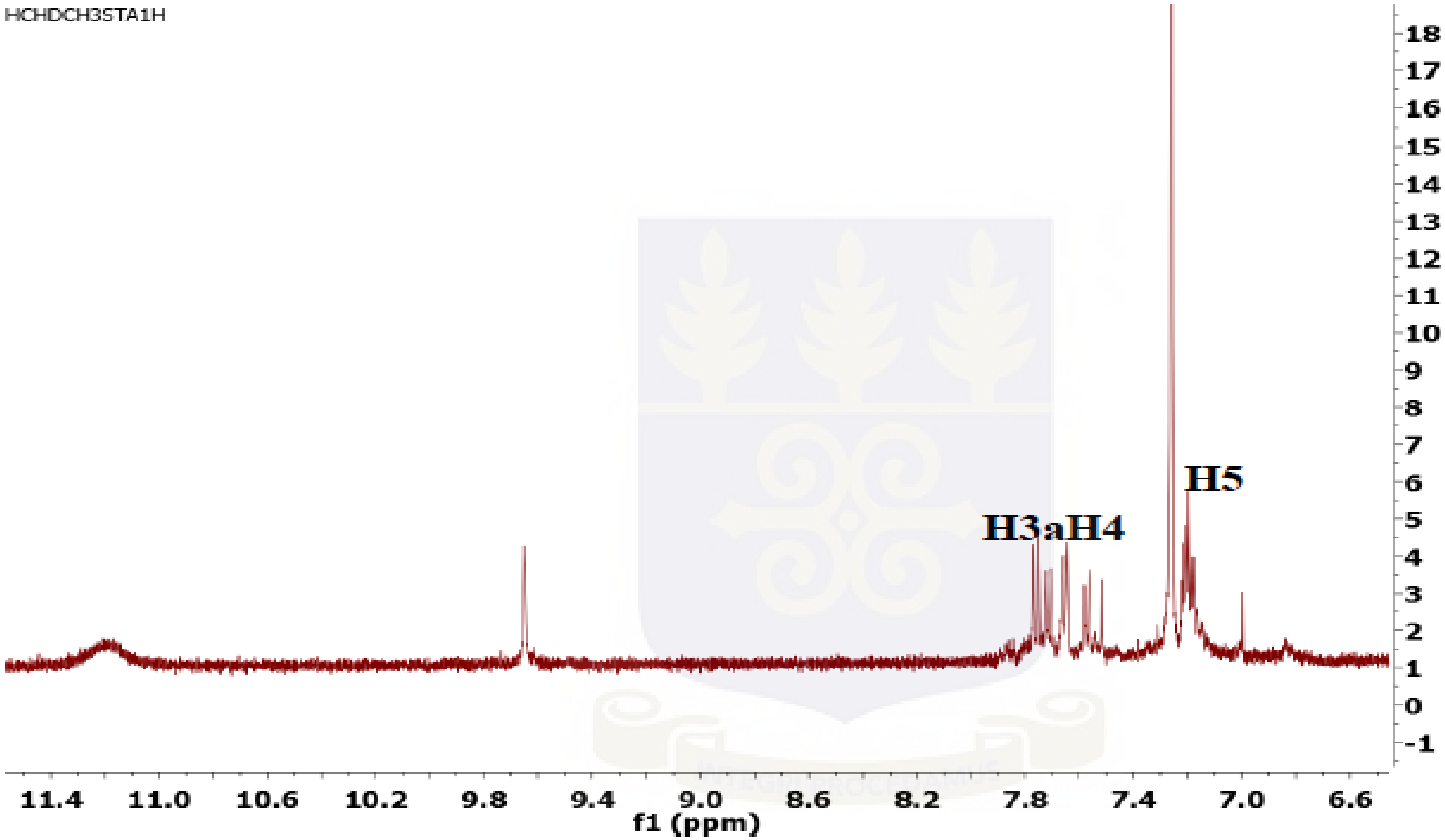
Appendix 2r: TOCSY spectrum of citrinadins



Appendix 2s: ^1H-^1H TOCSY spectrum of citrinadin



Appendix 2t: ^{1}H NMR spectrum of citrinadin



3. Preparation of reagents used for MTT assay

3a. Preparation of DMEM

4.75 g of DMEM powder was weighed and 300mL of sterile distilled water added and stirred for 30 mins. Sterile distilled water was added to top up to 500 mL and transferred into an autoclave flask for autoclaving. After autoclaving, the content was cooled to 37ºC and 5 mL of $NaHCO_3$, 5 mL of PSG, and 5 mL of L-glutamate are added under sterile conditions. The medium was stored at 4ºC for later use.

3b. Preparation of Acidified Isopropanol

1.7 mL HCl was added to 500mL of isopropanol in a reagent bottle and mixed Five millilitre of the solution was pipetted off and replaced with 5 mL of triton X and stored at room temperature.

3c. Preparation of Phosphate buffered saline (PBS)

4 g of NaCl, 0.1 g of KCl, 0.72 g of $Na_2HPO_4$and 0.12 g of KH_2PO_4 were weighed and dissolved with 450 mL of distilled water in a 500 mL beaker. The pH was adjusted to 7.4 using NaOH and HCl. The solution was transferred into a 500 mL measuring cylinder and topped up to the 500 mL mark. The solution was then transferred into a 1L autoclave bottle and sterilised. After cooling, it was labelled and stored at 4ºC.

3d. Preparation of MTT solution

0.125 g of MTT was weighed into a tube covered with aluminium foil. A volume of 50 µL of PBS was added and mixed to dissolve completely the MTT salt. After that the solution was filtered using a 50 mL syringe barrel and 0.45µM Millipore filter. It was labelled and wrapped in a 50 mL centrifuge tube container with aluminium foil and stored at 4°C.

4. CELL CULTURE

4a. Cell recovery

Cryopreserved cells in -80 °C refrigeratorwas thawed in a water bath at 37°C for 5 min and suspended in 10 mL of completemedia in a falcon tube. The falcon tube was spun at 1000 rpm for 5 min.Discard supernatant was resuspended pellets in 1 mL of completemedia and transfer cells into 25cc culture flask containing 5 mL of complete DMEM and incubatein a humidified chamber at 37°C in the presence of 5% CO_2.

5a. Breast Cancer cell Line (MCF-7)

Passage No. 6

Cell count: 35/35/25/36

Cell density: $\frac{131}{4}$X10^4

= 13.1 X 10^5cells/mL

For MTT Assay, 1X10^5 cells/mL required;

For 10 mL cell suspension; $\frac{10}{13.1}$ = 0.76 mL

= 0.76 mL cells + 9.24 mL DMEM

5b. Prostate Cancer cell Line (PC-3)

Passage No. 7

Cell count: 22/13/17/12

Cell density: $\frac{64}{4}$X10^4

= 6.4 X 10^5cells/mL

For MTT Assay, 1X10^5 cells/mL required;

For 10 mL cell suspension; $\frac{10}{6.4}$ = 0.76 mL

= 1.56 mL cells + 8.44 mL DMEM

5c. Human Prostate Cancer cell Line (LNCap)

Passage No. 10

Cell count: 83/73/60/57

Cell density: $\frac{273}{4}$X10^4

= 27.3 X 10^5cells/mL

For MTT Assay, 1X10^5 cells/mL required;

For 10 mL cell suspension; $\frac{10}{27.3}$ = 0.37 mL

= 0.37 mL cells + 9.63 mL RPMI

5d. Human promyelocytic leukemia cells

Passage No. 6

Cell count: 104/107/84/95

Cell density: $\frac{390}{4}$X10^4

= 39.0 X 10^5cells/mL

For MTT Assay, 1X10^5 cells/mL required;

For 10 mL cell suspension; $\frac{10}{39.0}$ = 0.26 mL

= l0.26 mL cells + 9.74 mL RPMI

5e. Human T-lymphoblastic leukemia cells

Passage No. 4

Cell count: 93/85/94/107

Cell density: $\frac{379}{4}$X10^{4}

= 37.9 X 10^{5}cells/mL

For MTT Assay, 1X10^{5} cells/mL required;

For 10 mL cell suspension; $\frac{10}{37.9}$ = 0.26 mL

= 0.26 mL cells + 9.74 mL RPMI