CHARACTERISATION, ANTIMALARIAL AND BIOLOGICAL ACTIVITIES OF SECONDARY METABOLITES FROM LEAVES OF *ANONIDIUM MANNII*

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

at

RHODES UNIVERSITY

by

PFUNGWA GERVASE MAKONI

B.Sc. Hons (Unisa)

Supervised by Professor R W Krause

February 2016

DEDICATION

This work is in honour of Maynard Tapfumaneyi Makoni, Elisha Nyamudoka, Regina Nyamudoka nee Chidawanyika, Venencia Mashava nee Makoni and last but not least Enock Chingwe. These all succumbed to the elements of this world (of which the world did not have answers), and the world was not worth them. The nature in which you departed from this world continues to inspire me, you are missed.

ABSTRACT

Anonidium mannii is a plant of the Annonaceae genus which is used traditionally in Africa for the treatment of gonorrhoea, malaria, cancer, skin inflammation and dysentery. In this study we will evaluate antimalarial, antifungal, anti - tuberculosis, antibacterial activities and cytotoxicity of different fractions in order to provide a scientific rationale for the traditional use of *Anonidium mannii* as well as provide possible novel drugs in the treatment of multi drug resistant strains of parasites and bacteria. Extracts from dried leaves were obtained by using solvent extraction and different fractions obtained using column chromatography eluted with solvents of varying polarities to obtain a wide range of metabolites. The antimalarial activity of the various fractions and some pure compounds was evaluated using plasmodium lactate dehydrogenase (pLDH) assay. Cytotoxicity was evaluated using HeLa cells while anti – tuberculosis assay was evaluated using the green fluorescent protein. Antibacterial activity of the extracts was evaluated using micro-dilution assay against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) bacteria and Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria. Antifungal activity was evaluated against *Candida albicans*. The antimalarial assays yielded some fractions with promising IC_{50} values. The selected fractions yielded activities ranging between 0.73 µg/mL and 20.23 µg/mL. The fraction with the best activity was obtained from a hexane/ethyl acetate fraction. **AM1C,** a cholestane, showed the best activity from the pure metabolites that were screened. **AM3C,** stigmasterol, a pure compound gave the best antifungal activity with an MIC of 0.063 µg/mL. **AM9C** another pure compound (sterol) showed the best activity against *S. typhi* with a value of 0.031 µg/mL. **AM2C** a pure compound showed an activity of 0.063 µg/mL against *E. faecalis*. The best cytotoxicity was demonstrated by the fraction **C2AM3P** with a cell viability of 7.1 ± 0.2 % while **AM1C** had a viability of 20.2 ± 1.2 %. Several pure metabolites were isolated and four of these were positively identified as steroids. Of these steroids the structure of three novel metabolites from *A. mannii* was deduced. The study showed promising antibacterial, antifungal, anti – tuberculosis, antimalarial and anticancer activity of *A. mannii*. These results validate the use of *A. manni* against cancer, skin inflammation which is caused by fungus, malaria and bacterial diseases.

ACKNOWLEDGEMENTS

This work would not have amounted to anything if I had not received help from several people and institutions. I want to acknowledge them heartily. First and foremost I want to acknowledge the Creator, the Father and source of all knowledge and wisdom who has enabled me by His grace to be able to complete this work. It is He who gave us the herbs for food and medication. To Him be glory, honour and thanksgiving.

I want to appreciate the guidance, words of wisdom and support that I derived from my supervisor, Prof Rui W Krause. Sir, thank you for accepting me as your students against all odds. You are indeed one of a kind, generous, humble and always willing to listen and give a helping hand. You have been more than a supervisor to me; you took on the role of a father. Your willingness to avail yourself even at odd times still baffles me. I am indeed honoured to have you as a supervisor. I pray God may continue to strengthen you and to sharpen you as you continue to serve the Chemistry community. May God richly bless you and may He never forget your labour of love.

A special thank you goes to Dr Xavier Siwe Noundou for putting up with me and always being there to assist. All those hours you spent teaching me how to operate instruments, to analyse data and to deduce structures did not fall on deaf ears. You are indeed one of a kind, may the Creator favour you and create room for you in the land even as you have been so gracious to me. I also mention particularly my sister Dr Rufaro Madakadze for investing in my development from a distant land. Your patience with me and your ability to take time out of your busy schedule to read through my work is appreciated. I know God is enlarging your footprint and making you sit in uncommon places. Thank you *vamuvhare, ndinotenda shonga, nyati.*

I want to acknowledge the encouragement I received from Dr S. D Khanye, Dr R Klein and all the lecturers from the organic research group. Your wisdom and thought provoking questions during the group meetings is appreciated. I want to acknowledge the entire Chemistry department staff for creating an enabling, peaceful environment for me to embark on this journey. Michelle Isaacs thank you for help with the antimalarial and cytotoxicity bioassays. I also want to acknowledge the financial support I have received from Rhodes for my lab work.

I also acknowledge the family from Rhodes University F22; you are indeed a great bunch, always willing to go the extra mile. You really supported me in your own individual unique ways. I really appreciate you, may God also favour you and help you to produce the results that you have desired.

Lastly but not least I want to acknowledge two very special families, the River of Life members for your spiritual and moral support. Your prayers and confidence in me kept me going. May God make your dreams also come to fruition. To my family, Evie, Maka, Ruru, Pana and mbuya vaMaka thank you guys for loaning me to the lab and allowing me to be

absent from home for many hours. Your confidence in me, your support and unfailing love is appreciated.

Contents

List of Figures.

List of Tables.

List of Graphs

List of abbreviations

^{13}CNMR	Carbon-13 nuclear magnetic resonance spectroscopy
^{1}HNMR	Proton nuclear magnetic resonance spectroscopy
Å	Angstrom
A. mannii	*Anonidium mannii*
Ac	Acetate
ACT	Artemisinin combination therapy
AIDS	Acquired immunodeficiency syndrome
amu	Atomic mass unit
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
CC	Column chromatography
CDC	Centers for Disease Control and Prevention
CE-MS	Capillary electrophoresis- mass spectrometry
COSY	Correlation spectroscopy
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortionless excitation by polarisation transfer
EPA	United States Enviromental Protection Agency
EtOAc	Ethyl acetate
EtOH	Ethanol
GC-MS	Gas chromatography- mass spectrometry
GLC	Gas-Liquid chromatography

HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HPLC	High pressure liquid chromatography
Hsp70	70 kilodaltons heat shock protein
HSQC	Heteronuclear single quantum coherence
IC_{50}	Half maximal inhibitory concentration
IAMAT	International Association for Medical Assistance to Travellers
IR	Infrared spectroscopy
LC-FTIR	Liquid chromatography -fourier transform infrared detector
LC-MS	Liquid chromatography- mass spectrometry
LC-NMR	Liquid chromatography- nuclear magnetic resonance
LC-NMR-MS	Liquid chromatography- nuclear magnetic resonance- mass spectrometry
LC-PDA	Liquid chromatography -photo diode array
LDH	Lactate Dehydrogenase
mAbs	Monoclonal antibodies
MAE	Microwave Assisted Extraction
MIC	Minimal inhibitory concentration
MeOH	Methanol
MS	Mass spectrometry/spectroscopy
^{n}BuOH	Butan-1-ol
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
P.malariae	*Plasmodium malariae*
PfHRP1	*Plasmodium falciparum* Histine-Rich Protein 1
PfHRP2	*Plasmodium falciparum* Histine-Rich Protein 2
PfHRP3	*Plasmodium falciparum* Histine-Rich Protein 3

pLDH	*plasmodium lactate dehydrogenase*
ppm	Parts per million
PSE	Pressurised solvent extraction
PTLC	Preparative thin layer chromatography
RDTs	Rapid diagnostic tests
RP-HPLC	Reverse phase- High pressure liquid chromatography
SEC	Size exclusion chromatography
SFE	Supercritical fluid extraction
SLE	Solid- liquid extraction
SPE	Solid- phase extraction
TLC	Thin layer chromatography
UAE	Ultrasound assisted extraction
UV-Vis	Ultraviolet- Visible
V. rosea	*Vinca rosea*
VLC	Vacuum liquid chromatography
WHO	World Health Organisation
XRD	X-Ray Diffraction

Chapter 1 Introduction

There are several diseases that have posed serious problems to human beings over the years including malaria, cancer, tuberculosis, HIV and AIDS. In the case of tuberculosis, which is caused be *Mycobacterium tuberculosis,* existing drugs can cure it but the chemotherapy requires six to nine months of consistent medication. Often several drugs are taken at the same time. The hurdle to success of the chemotherapy is often in the form of incomplete course of treatment as some patients stop taking medication before the course is done. This then leads to development of resistance against drugs causing multi – drug resistance (Khare., 2013). Global statistics show that in 2014 alone over one million people (four hundred and fifty thousand from Africa) died of tuberculosis, of these 400 thousand were HIV positive (Kanabus., 2016).

Cancer is a collection of related diseases in which body cells begin to divide without stopping resulting in them spreading into surrounding tissues (National Cancer Institute., 2015). According to the World Health Organisation (WHO), in 2012 cancer was the leading cause of morbidity and mortality with approximately fourteen million new cases and eight million cancer related deaths (WHO., 2016). Considering the disease malaria, which is caused by the parasite *Plasmodium,* it was reported by the WHO that over 3.2 billion people are at risk of contracting malaria and 198 million cases of malaria infections were reported in 2013, leading to about 584 000 reported deaths. The same report mentioned that the mortality rate decreased by 47% globally and by about 54% in Africa due to improved prevention and control. In 2013, 90% of the deaths recorded occurred in Africa mostly among children aged less than 5 years and in the poorest countries (WHO., 2013).

1.1 Malaria

Malaria is an intermittent life threatening blood disease caused by protozoan parasite, which invades red blood cells of the host and is transmitted to humans by the female anopheles mosquito in many tropical and subtropical regions. Malaria is caused by the *Plasmodium* parasite. The four main *Plasmodium* species that cause human malaria, which are *Plasmodium falciparum (P. falciparum), Plasmodium vivax (P. vivax), Plasmodium ovale (P. ovale)* and *Plasmodium malariae (P. malariae),* are not equally prevalent around the

world. *P. falciparum* is found in tropical and subtropical areas and is the major contributor to deaths, *P. vivax* is found in Asia and Latin America and it has a dormant stage that can cause relapse, *P. ovale* is common in Africa and the Pacific Islands while *P. malariae* is found worldwide and can cause chronic infection (Balentine., 2015). A fifth species of parasite is *Plasmodium knowlesi (P. knowlesi)* that causes malaria in monkeys and occurs in the forest areas of south-east Asia, but some cases of human malaria cases due to *P. knowlesi* have been reported in recent years (WHO., 2015). *P. falciparum* is the most endemic of these parasites. The African vector species of *Anopheles* mosquito that carries the *P. falciparum* has the longest lifespan and strongest human biting habit, and this account for the high infection and mortality rate in Africa (WHO., 2015). Young children who still have developing immunity and people with compromised immunity especially pregnant women and people living with HIV are at risk of contracting malaria (WHO., 2015). The signs and symptoms of malaria include fever, headache, chills and vomiting. Children with severe malaria develop one or more of these symptoms: severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. In adults multi-organ involvement is also frequent and could lead to organ failure (WHO., 2015).

The regions where malaria is most endemic are shown in Figure 1.1 (IAMAT., 2015).

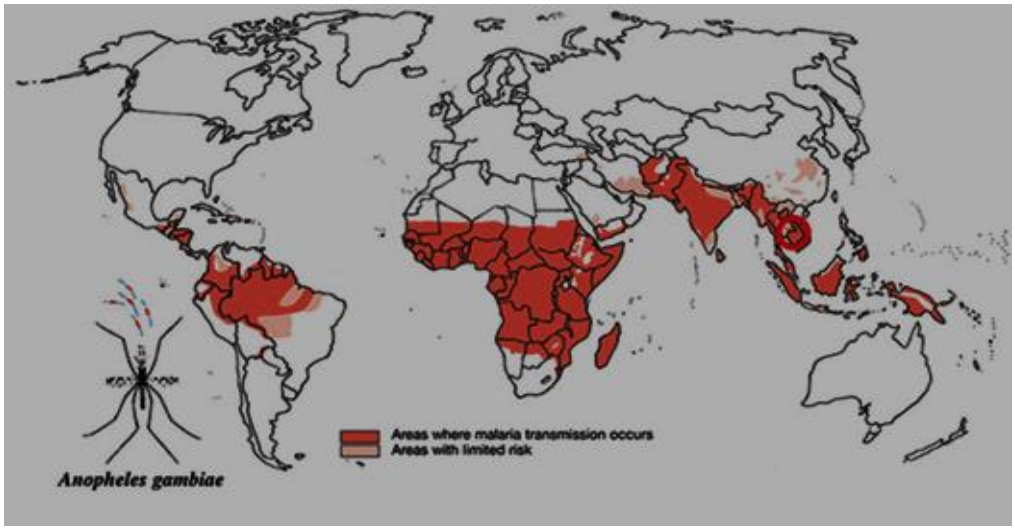


Figure 1.1: World map showing malaria prone zones

In the area around Cambodia-Thailand border (highlighted in Figure 1.1) and in the Democratic Republic of Congo resistance to artemesinin has been reported (WHO., 2015).

The mortality due to malaria for various regions in the world over the last century is shown in Figure 1.2 (Carter., 1999).

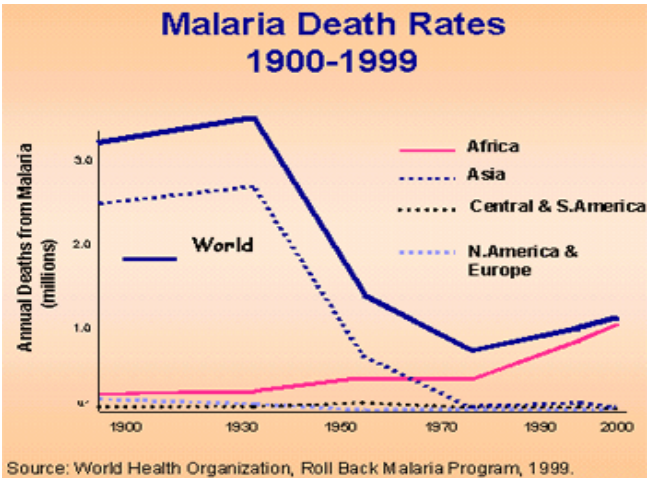


Figure 1.2: Statistics of malaria deaths across the World in the 20th century

The graph shows interesting trends when considering the issue of controlling malaria. It shows that in the early 1900 Asia had higher mortality than Africa, maybe due to the fact that the cases in Africa were not documented. By 1930 the mortality was dropping in Asia and China but continued to rise in Africa. Up to 1930 the major treatment was quinine, and the continued increase in mortality rate shows resistance of parasite to the drug. The drop in Asia after 1930 was due to the use of pesticides, mostly dichlorodiphenyltrichloroethane (DDT); which was developed as the first modern synthetic insecticide to combat malaria, typhus and other insect-borne human diseases, to control the mosquitoes and also the use of more drugs like artemesinin as shown in Figure 1.3 (Katz., 2013; EPA., 2015).

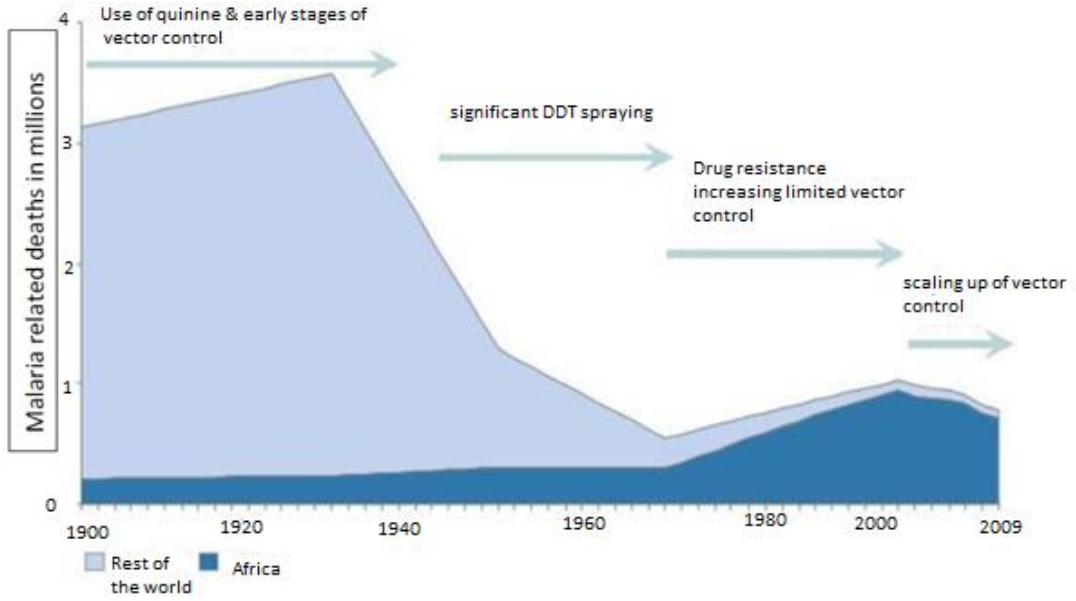


Figure 1.3: The interventions used to remedy malaria and their effect on mortality rate from 1900 to 2009

From 1970 the mortality rate for other continents remained low but continued to rise for Africa despite the use of insecticides like DDT to kill mosquitoes and the use of mosquito nets. This problem may be due to the fact that there was poor education on the use of insecticides, mosquito nets and repellants resulted in parasite developed resistance to artemesinin-monotherapy. In the light of that there is a strong advocacy for the complete removal of artemesinin-monotherapy (WHO., 2015). There also has been a decrease in the use of DDT because of its side effects, in fact some parts of the world have banned the use of DDT although it is still used in South Africa to try and contain the spread of malaria (Malaria Foundation International., 2003; Scientific America., 2009). The use of DDT was therefore sanctioned and is only permissible in the control of malaria and only when used indoors especially in Africa due to the increasing mortality rate as was recommended by the WHO in 2006 (WHO., 2006; EPA., 2015).

Closer to home, Figure 1.4 shows the malaria and malaria-related mortality statistics in South Africa from about 1995 to 2011 (Marahaj., 2013). This shows how the emergency of Chloroquine resistance led to an unprecedented increase in deaths in 1985 (Peters., 1987). The resistance of the mosquitoes to pyrethoid and the parasite to sulphadoxine pyremethamine towards the end of the 20th century also led to increased mortality rate (Phillips., 2001). This led to the re-introduction of DDT as a means of controlling the parasite hosts (mosquitoes) and the introduction of Coartem ™ to control the parasite (Tren et al.,

2004). The major concern is that the parasite and the insect tend to develop resistance and this call for more research into alternative drugs and insecticides so that we will not be caught unaware.

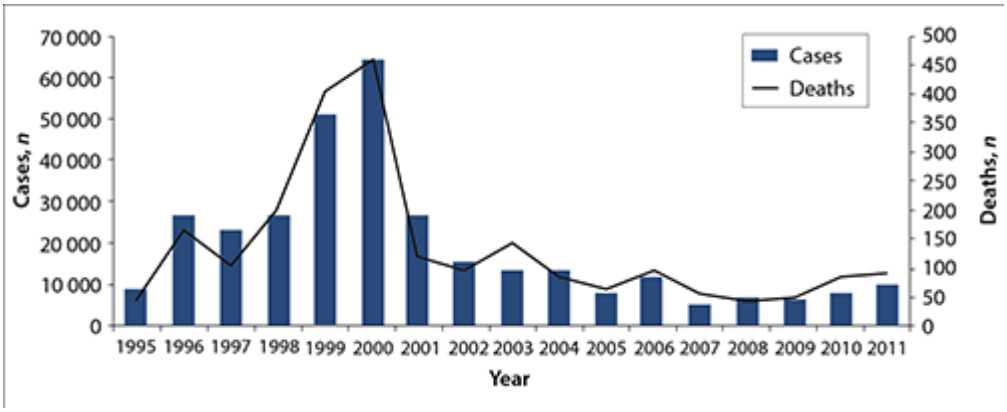


Figure 1.4: Malaria deaths and trends in parasite and insect resistance in South Africa

1.1.1 Life cycle of the malaria parasite

The protozoan that causes malaria has two life cycles, one in the mosquito and the other in the human being. The cycles are shown in the Figure 1.5 (NIH., 2012). The malaria parasite infects two hosts: female *Anopheles* mosquitoes and humans, and this give rise to the two cycles (NIH., 2012). During a blood meal an infected female *Anopheles* mosquito injects sporozoites (parasite) into the human host. These sporozoites infect liver cells and mature into schizonts which rupture and release merozoites, which are daughter parasites. *P. vivax* and *P. ovale* can remain dormant in liver cells for months and may lead to relapse. After replication in the liver the parasite undergoes asexual replication in the erythrocytes. These then invade red blood cells leading to blood stage malaria. Some parasites differentiate into sexual erythrocytic stages (gametocytes).

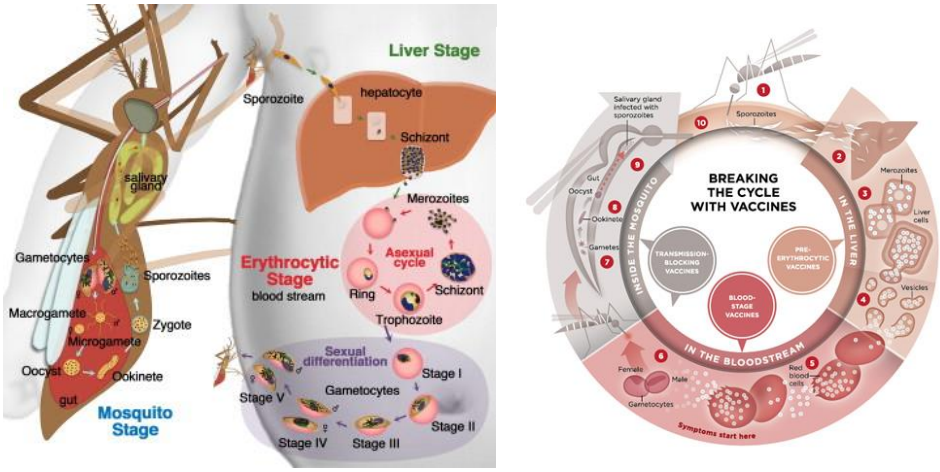


Figure 1.5: The two life cycles of the malaria parasite and vaccine development strategies

Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes are then picked up by *Anopheles* mosquito during a blood meal to start the cycle in the mosquito; this cycle of multiplication while in the mosquito is called the sporogonic cycle. This cycle takes place in the stomach of the mosquito leading to formation of oocysts which grow and rupture releasing sporozoites in the process. These find their way to the mosquito's saliva. This cycle takes 10 to 18 days. From there when the mosquito bites a human the human cycle begins (CDC., 2012).

1.1.2 Detection of malaria parasite

The malaria parasite can be detected in its host by using several methods. These methods are of importance as they are used in the diagnosis of malaria. We will discuss some of the methods that are in use and then concentrate on the ones that we used in our research.

Microscopy has been used for over 100 years and is inexpensive, rapid and relatively sensitive if used appropriately. (Laveran., 1891). In 1999, WHO described microscopy as the gold standard in regards to the diagnosis of malaria. Microscopy requires skilled personnel to interpret the data and is often labour intensive, time consuming and fails to detect sequestered *P. falciparum* cases and low-density parasitaemia (Leke et al., 1999; Kilian et al., 2000; Bell et al., 2005). The key to successful treatment of malaria is early rapid diagnosis and this is being done using rapid diagnostic tests (RDTs). There are three RDTs that focus

on 3 antigens: *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2), plasmodial aldolase and plasmodium lactate dehydrogenase (pLDH).

More than 90% of malaria RDTs use PfHRP2 test target. There are 3 histidine- rich proteins that have been identified (Howard et al., 1986; Parra et al., 1991). PfHRP1 is associated with the cytoskeleton of infected red blood cells in knob positive *P. falciparum.* PfHRP2 was identified in all *P. falciparum* infected red blood cells regardless of phenotype, it is rich in histidine amino acids repeat regions. PfHRP3 like PfHRP2 is rich in histidine amino acids repeat regions and is secreted by parasites and recognised by some of the monoclonal antibodies (mAbs) that detect PfHRP2 in RDTs (Baker et al., 2010a; Maltha et al., 2012). According to a WHO article released in 2003, PfHRP2 is a good marker of *P. falciparum* infection. It is water soluble, heat stable and synthesized by the parasite (Stahl et al., 1985; Howard et al., 1986). There is a positive correlation between the blood concentration of the protein and the parasite biomass (Dondorp et al., 2005; Desakorn et al., 2005). PfHRP2 is expressed in gametocytes and all erythrocytic stages of the parasite. It is released on schizont rupture and therefore found in the supernatants of cultured parasites and in the blood of parasite infected individuals (Howard et al., 1986; Parra et al., 1991; Hayward et al., 2000). PfHRP2 can be detected in sequestered parasites (MacPherson et al., 1985; Goldring et al., 1992; Goldring, 2004) but cannot be used to predict parasite response to therapy because of persistence of antigen in the peripheral blood circulation after parasite clearance (Tjitra et al., 2001; Houzé et al., 2009). PfHRP2 can be used to detect the parasite in pregnant and non-pregnant individuals (Leke et al., 1999; Mueller et al., 2007; Hopkins et al., 2007, 2008) which is very useful as placental parasite causes low child birth weight and leads to high incidences of stillbirths and premature births (Okoko et al., 2002). PfHRP2 actively facilitates the polymerization of toxic haem, resulting from the degradation of host haemoglobin (Sullivan et al., 1996).

Some parasites lack the protein PfHRP2 and for these parasites false negative test results are obtained (Koita et al., 2012; Kumar et al., 2013). Since PfHRP2 does not detect parasite response to therapy we did not use it in our research as we needed to check the biological activity of pure extracts and fractions that we obtained from *Anonidium mannii (A. mannii).*

According to WHO articles produced in 2009, 2010 and 2011 another target for antimalarial RDTs is plasmodial adolase (WHO., 2011). This is a glycolytic enzyme found in numerous tissues in the host and in the malaria parasite. The aldolase has very low sensitivity and so we did not consider using it in our research.

In our research we decided to use the *Plasmodium* lactate dehydrogenase (pLDH) test target as it has a shorter half-life and is only produced by living parasites. Lactate dehydrogenase (LDH) is an essential energy-producing enzyme. It is the last enzyme in the glycolytic pathway. It is soluble in water and produced by both asexual and sexual stages of the parasites. It is also produced by the mature gametocytes of all four human *Plasmodium* species (Brown et al., 2004; Piper et al., 1999). This makes it a better target than PfHRP2 which was only a target for knob positive *P. falciparum*. The parasite and erythrocytic cells in human host lack complete citric acid cycles for mitochondrial ATP, this then make pLDH an important enzyme for energy production in the parasite (Lang-Unnasch & Murphy, 1998; Brown et al., 2004). pLDH uses monoclonal antibodies (mAbs) against a common epitope and so it can detect all human *Plasmodium* species, including mixed infections in circulating blood (Mayxay et al., 2004). The down side to pLDH is that it has poor performance at low parasite density (Ashley et al., 2009; Abba et al., 2011). However, its use as a drug sensitivity test for malaria and viability of parasites makes it a good test target for researchers who are into drug development (Makler et al., 1993). It is therefore good for monitoring treatment and predicting treatment failure (Druile et al., 200l; Fogg et al., 2008).

In the past decades the international strategy against malaria has been disease control measures which were tackled using a two process approach. The first approach has been prevention of mosquito bites while the second has been the use of drug treatment with ACTs (Roll Back Malaria Partnership Programme., 2011). The recent perspective has shifted from disease control to elimination of malaria eventually leading to its eradication (White., 2008; Alonso., 2011). This could be achieved by targeting production of new drugs that inhibit the liver stage reproduction or development of gametocytes. This would lead to directly blocking parasite transmission. Drug screening methods suitable for identification of anti-gametocytes compounds are therefore needed to achieve this goal. One of the methods developed to screen potential drugs against gametocytes measures the parasite

pLDH activity of gametocytes using 96- well plates (D'Alessandro et al., 2013). This further justified the choice of the pLDH assay in this research.

Other RDTs that are still being considered include monitoring the heat shock protein 70 (Hsp70). The parasite is subjected to a range of temperatures in the different cycles during fever, and this leads to the production of high levels of Hsp70 (Morimoto et al., 1994; Kattenberg et al., 2012; Thézénas et al., 2013).

1.1.3 Emergence of multi-drug resistant parasites

The major worrying issue has been the development of resistance to drug by the parasite. The first line of treatment of malaria in the world at the moment is the Artemesinin-based Combination Therapy (ACT). The use of combination therapy has become essential due to the development of drug resistance from parasites. In this case if the parasite develops resistance to one of the drugs in the ACT during treatment chances are that the other drug will be able to control the parasite. If resistance to a partner drug in ACT has been reported then the combination has to be changed as it becomes similar to monotherapy. Other drugs that have been used in the treatment of malaria are chloroquine and sulfadoxine-pyrimethamine. Resistance to artemesinin has been reported in 5 countries namely Cambodia, Laos, Myanmar, Thailand and Viet Nam (WHO., 2013). The resistance of the parasite maybe due to use of artemesinin as monotherapy (WHO., 2011, 2015). The major worry now is that if this resistance spreads to Africa the continent will be at risk as ACT is the first line of treatment of uncomplicated malaria.

It is important to note that both artemesinin and chloroquine were initially obtained from natural products. Artemesinin is produced by *Artemesinin annua,* while quinine is obtained from the bark of *Cinchona* tree. Total synthesis of artemesinin makes the drug very expensive and unaffordable by the poor who are mostly at risk of contracting the disease. Current initiatives include large scale farming of *Artemesinin annua* in Mozambique and East Africa as the source of the drug has been from plantation from outside Africa.

The cost of the drugs that are currently available is high due to the fact that their extraction is costly or their synthesis is complex. In cases where total or semi-synthesis has been

developed the methods and processes are also costly. This makes the drugs out of reach for the poor and vulnerable. The drugs obtained from extraction of natural products are also obtained in very small quantities and that pushes their cost up as well.

The risk posed by malaria and other diseases necessitate for the development and discovery of new, effective drugs. It is reported in the United States that 1 in every 4 deaths is due to cancer. It is also reported that most of the drugs that are currently used in the treatment of cancer were discovered from natural products (Newman et al., 2003; Siegel et al., 2012). It is therefore clear that nature is a good source of drugs that are needed to combat various diseases. This added to the need of investigating other plants that have been used traditionally for medical purposes has necessitated this research.

1.2 Natural Products

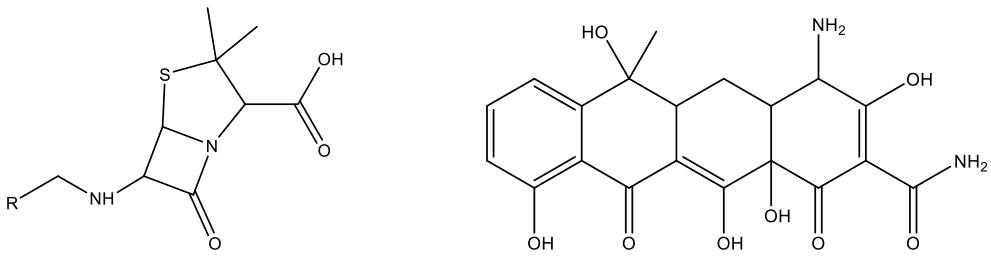
Nature is a great source of new compounds. Understanding the environment and how to use the countless chemical resources produced by living organisms is one key way of advancing civilisation. Organic compounds that are produced by living organisms (plants, microbes, anthropods and marine organisms) can broadly be defined as natural products. There are two major classes of organic compounds that are produced by living organisms: those that are essential for the functioning of the plant and are needed in large quantities and those that are not necessary for the core metabolic functions but serve other mirror roles. The ones that are necessary for core metabolic functioning of the organism are called primary metabolites while those that are not necessary for the core metabolic functioning are called secondary metabolites (Sarker et al., 2006; Bahar et al., 2008).

Natural products have been used for thousands of years in many fields including medicine, agriculture and industry (Ikan., 2008). The term natural products normally refers to secondary metabolites. There are several classes of thousands of compounds referred to as secondary metabolites and some of these are alkaloids, steroids, terpenes and polyketides (Clayden et al., 2001).

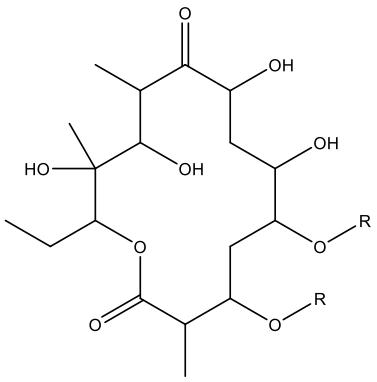
1.2.1 Sources of natural products

Natural products are produced from a variety of sources. One of the most promising sources, which are dates back to the ground breaking work of Louis Pasteur and Victor Babés late in the 19th century, is micro-organisms. These scientists had insights that bacteria somehow secrete defensive chemicals (Sneader., 2005). Since then many natural products have been isolated from bacteria and fungi. A famous example of a commercial compound, which is used as an antibiotic was isolated from *Penicillium sp* and discovered by Fleming is penicillin (Peláez., 2006). Other compounds formed by fungi, actinomycetes and certain bacteria produced or formed the basis of a large portion of antibiotic compounds (Newman et al., 2003). These belong to classes of compounds called β- lactam, macrolides and glycilcyline. Structures of three examples are shown in Figure 1.6, (a) Penicillin (β lactam core) R groups arevariable, (b) Tetracycline (Glycilcyline core), (c) Erythromycin (macrolides core) R groups are pyranose derivatives.

.



a b



c

Figure 1.6: Some of antiobiotics that were extracted from micro-organism.

Natural products can also be isolated from mammalian tissues. Common examples of commercial compounds isolated from mammalian tissues are cod liver oil, hormone therapy like epinephrine, adrenaline, dopamine, insulin, progesterone, corticosterone, vitamins and related co-factors (Sneader., 2005). Anthropods and insects produce cytotoxins, nemotoxins, antibiotics, antiviral peptides and pheromones as commercial compounds (Inscoe et al., 1990; Dossey., 2011). Natural products are also obtained from marine based organisms and plants. The marine environment has varied environmental stimuli and contains micro-organisms algae, sponges, phytoplankton and molluscs (Blunt et al., 2011). Potential antitumor compounds have been isolated from marine organisms (Wender et al., 1998; Evans et al., 1999; Trost et al., 2008).

Plants are perhaps the largest source of important compounds for both commercial and medicinal value; there are collections of ethno-botanical data on various plants in different parts of the world (Thirumalai., 2009). This coupled with ease of extraction, ease of cultivation and accessibility makes this one of the most attractive source of natural products (Dixon., 2001). As a result thousands of compounds have been extracted from plants. The common classes of compounds that have been isolated from plants are: phenols, phenylpropanoids, coumarins, stillbenes and pyrones (Croteau., 2000). Plants produce such a wide range of secondary metabolites and use these compounds for defence against predators, infections, for reproduction and for communication. Some important medicinal compounds that have been developed from plants include aspirin, which is produced from salicyclic acid which is a natural product, an analgesic and anti-inflammatory drug (also known as acetylsalicyclic acid). Salicyclic acid was isolated from the bark of willow tree and myrtle, quinine an antimalarial drug extracted from the bark of cinchona tree, artemesinin which is also an antimalarial drug extracted from *Artemesinin annua*. The structures of these compounds are shown in figure 1.7, (a) is aspirin, (b) is chloroquine, (c) is artemesinin, (d) is quinine and (e) is salicyclic acid.

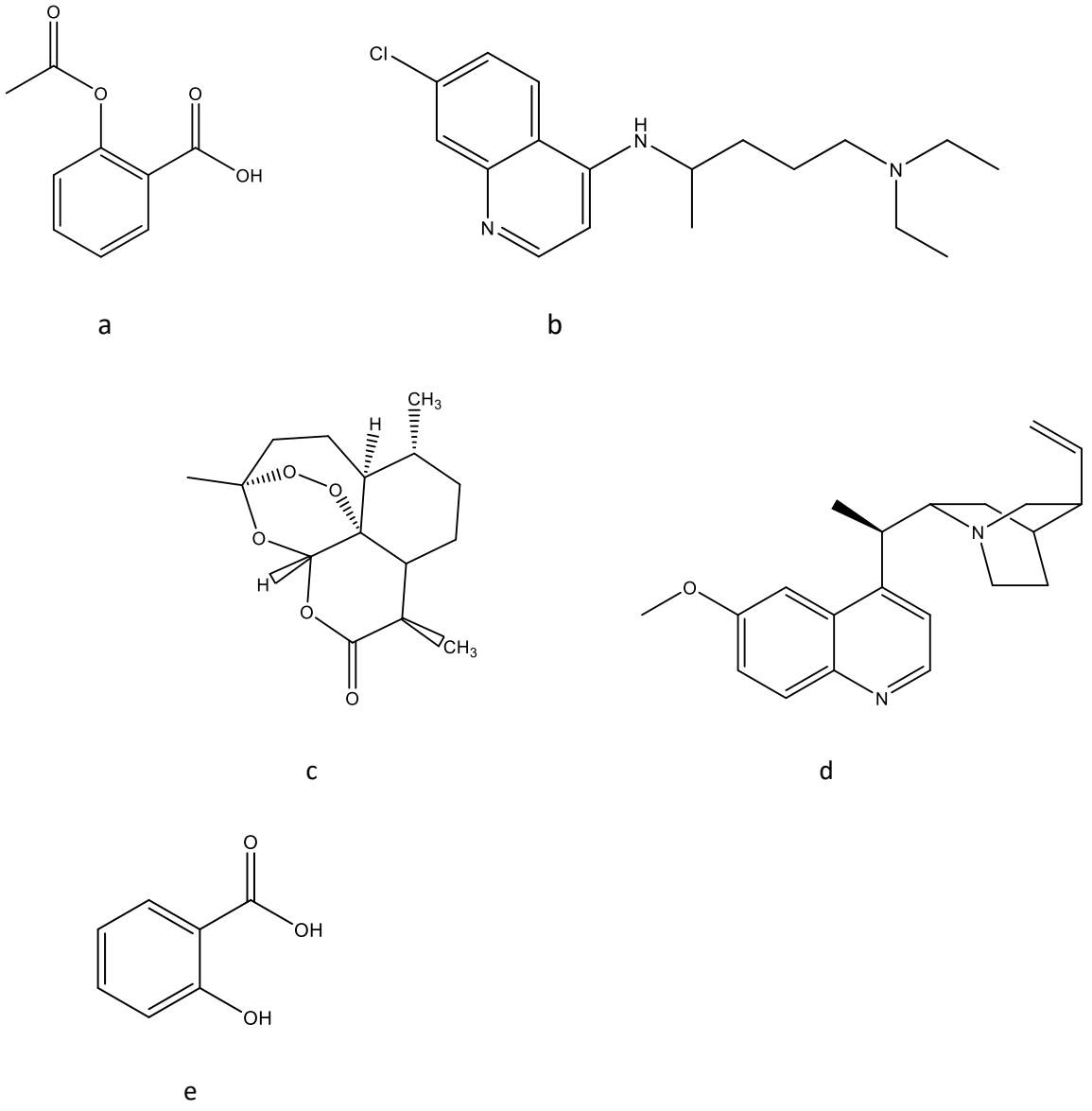


Figure 1.7: Some drugs which have been isolated from plants or derived from plant extracts

We will concentrate on the natural products produced by plants in this particular research because of some of the reasons mentioned above. We will focus on application, isolation, identification and screening of the metabolites for antimalarial, microbial, antifungal and cancer activity.

1.2.2 Uses of secondary metabolites

Natural products have been used for three major roles, namely:

- Food supplements also called nutraceuticals. In this class are plants like garlic, ginger and *Anonidium mannii* which is commonly called *Junglesop* in Cameroon (Dzoyem et al., 2014). A common nutraceutical is Pycnogenol® which is believed to be the gold standard of nutraceuticals. It is natural plant extract obtained from the bark of maritime pines trees growing on the coast of Southwest France (Vitamin Retailer., 2012). It has beneficial effects in cardiovascular health, osteoarthritis, skincare, cognitive function, diabetes, inflammation, sports nutrition, asthma, menstrual disorders and allergy. Another example is Polycan™ a patented soluble compound obtained from novel strain of black yeast clinically proven to support bone health, mobility and healthy immune systems (Kim et al., 2011).
- As conventional, complementary and alternative medicines. We are going to concentrate on this use of natural products especially in the search for antimalarial drugs.
- Other uses include as colourants, fragrances and preservatives.

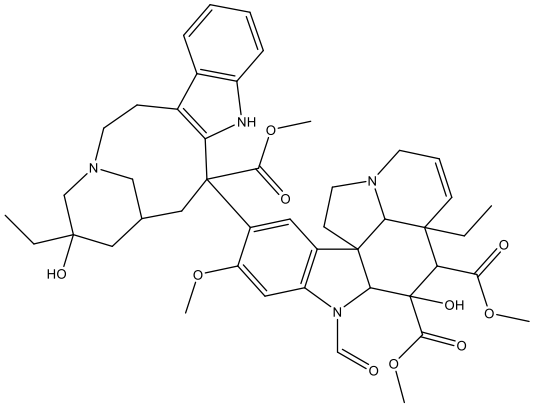


Figure 1.8: An anticancer and antimalarial drug, Vincristine natural products

There are three distinctive ways in which natural products can contribute to search for new drugs and we will be considering all the three ways. These ways are:

- Acting as new drugs. In this case the compounds are used as they are without any modification after extraction and purification. Examples of compounds that have

been extracted from natural products and that are used as they are include vincristine obtained from *Catharanthus roseus (Vinta rosea)* and artemesinin extracted from *Artemesinin annua.* Vincristine (figure 1.8) is an anticancer drug while artemesinin malaria is used as an antimalarial drug.

- Acting as scaffolds for the development of new drugs. Examples in this case are diosgenin from *Dioscorea floribunda* and artemether from *Artemesinin annua.* Diosgenin is used in oral contraceptives while artemether is used in artemesinin combination therapy against malaria (Dewick., 2002).
- Indicating new modes of pharmacological action that allow complete synthesis of novel analogs. Examples in this group are the analogs of penicillin from *Penicillium notatum* (Sarker et al., 2006).

Some of the advantages of using natural products are:

- Natural products have structural diversity
- Most natural products are relatively small in size, less than 2000amu and
- Natural products can be absorbed and metabolised. These are important drug like properties (Sarker et al., 2006). We will carry out research on plants that have been used traditionally in the treatment of malaria and other diseases.

1.2.3 Discovery of secondary metabolites

The method used to discover secondary metabolites depends on the source organism of the compound. The techniques used for the extraction of the secondary metabolites are also dependent on the source of the metabolites. Techniques used to extract metabolites from plants are different from those used to extract metabolites from animal tissue or sponges. However, in all cases a generalised approach to discovery of secondary metabolites involves collection, selection and identification of the parts of organism to be used during the extraction. Since in our research we used plants as source of the secondary metabolites, care was taken during the selection, collection and identification in a bid to ensure that the phytochemical research is reproducible. Plants form the foundation of traditional pharmacopeia and as such several pharmaceutical drugs were and are still being obtained

from plants (Sarker et al., 2006). Secondary metabolites accumulate in specific plant parts, for example some metabolites may be more concentrated in the bark, the root or the leaves of the plant. The selection process is often informed by traditional use as food, medicine or poison. This is often available in literature reviews, interviews or surveys. Sometimes it is based on phylogenetic relationships to species known to produce the secondary metabolite of interest. In other cases selection is informed by reports of biological activities reported in literature. During the collection process phytochemical, ethical and legal issues associated with intellectual properties are also careful considered and followed. Pre-isolation analysis can now also be done thanks to the availability of a range of hyphenated techniques like GC-MS, LC-PDA, LC-MS, LC-FTIR, LC-NMR, LC-NMR-MS and CE-MS. These methods have also made online detection and chemical fingerprinting possible (Sarker et al., 2006).

Plants can be selected by a number of strategies for study, including:

- Random screens of certain areas are carried out.
- Ethno-botanical and Ethno-pharmacological knowledge.
- Observation of biological activities of the species or related species. (Sarker et al., 2006).

In the case of *A. mannii* the selection was based on ethno-pharmacological knowledge and some pre-screening which was done following surveys on its traditional uses (Kuete et al., 2010).

The extraction protocol, purification or fractionation protocols are also vital in the discovery of secondary metabolites. These will affect the quality of results and identification of secondary metabolites. The following discussions will highlight some of the isolation, purification and characterisation protocols used in our research.

1.2.4 Isolation of secondary metabolites

After collection of the material, it must be processed; this usually involves drying, grinding, homogenizing fresh plant material or maceration of total plant parts in a solvent in preparation for extraction (Sarker et al., 2006). Extraction is the process of removing one or

more substances from another substance by transferring them into another liquid phase for which the substances have a higher affinity (Noble et al., 2004). This type of extraction depends on differences in solubility and density of the two phases. Other extraction techniques depend on differences in boiling points or volatility of the substances.

The most economical way of recovering secondary metabolites is by cold pressing but this often produces very low yields. Volatile oils can be recovered by conventional vacuum distillation or hydro-distillation. The most common techniques are steam distillation and solvent extraction. Since we used solvent extraction in our research we will discuss a few points on the process.

There is a broad spectrum of solid-liquid extraction (SLE) methods used in the extraction of natural products from plants and living organisms. SLE can be classified into traditional and recent/modern techniques. Traditional techniques include: Soxhlex extraction, maceration, sonication, percolation and turbo-extraction while recent techniques include: supercritical fluid extraction (SFE), microwave assisted extraction (MAE); ultrasound assisted extraction (UAE) and pressurized solvent extraction (PSE). The traditional methods are time-consuming and use large quantities of solvents whereas the modern techniques are fast and efficient in extracting natural products from solid matrixes.

The choice of the solvent to use often follows the principle of like dissolves like. Some factors are also considered when choosing solvents for use in extraction of metabolites and these include the purpose of the extract. The polarity of the metabolites that are being extracted if known also influences the solvents used. In most cases where the required metabolites are not known a mixture of solvents with a wide range of polarity is used to ensure that a wide range of metabolites are extracted. The polarity of the solvents used and the number of repetitions of extractions also play a pivotal role in increasing yield of the metabolites extracted. Concentration is done using rotary evaporators.

The crude extract is further processed by fractionation. At this stage the crude extract is passed through chromatographic media to separate the secondary metabolites into different groups that hopefully also represent distinct classes. The classes depend on the polarities of the metabolites, so the fractions could be discrete divisions such as the two

phases of liquid-liquid extractions or the continuous eluants from a column chromatography e.g vacuum liquid chromatography (VLC), column chromatography (CC), size-exclusion chromatography (SEC) and solid-phase extraction (SPE). The initial fractions obtained from such processes are collected as large volumes of eluent to minimise the spreading of metabolites between different fractions (Sarker., 2006). When isolating a known compound from the same species or family literature can then be used to design the isolation protocol. However, the design for an unknown compound can be very taxing. In this case it might be advisable to carry out qualitative tests for specific classes of compounds such as phenolics, steroids, alkaloids and terpenes. The fractions obtained can further be purified using appropriate chromatographic techniques.

The isolation protocol is often monitored using standards if the metabolite in question is known, or using activity-guided approach if the pathogens for which the extracts are active are known. The isolation is structured in such a way that the fraction with the desired properties is carried through until the metabolite in question is separated. These two examples of isolation protocols have been used before and will illustrate the points outlined above.

Figure 1.9 shows a summary of the work that was done by Huang and co-workers in extracting metabolites from *Siraitia grossvenorrii* which was screened based on ethno-botanical knowledge, similar to this project (Zheng., 2011). The extraction process involved heating the leaves under reflux in ethanol followed by separation of different metabolites from this crude extract using solvents of increasing polarities. Silica gel column chromatography was used for purification of the metabolites, which is a common stationary phase. A total of nine metabolites were obtained and screened against common oral microbes, compounds 7 and 9 in their case gave the highest antibacterial activity.

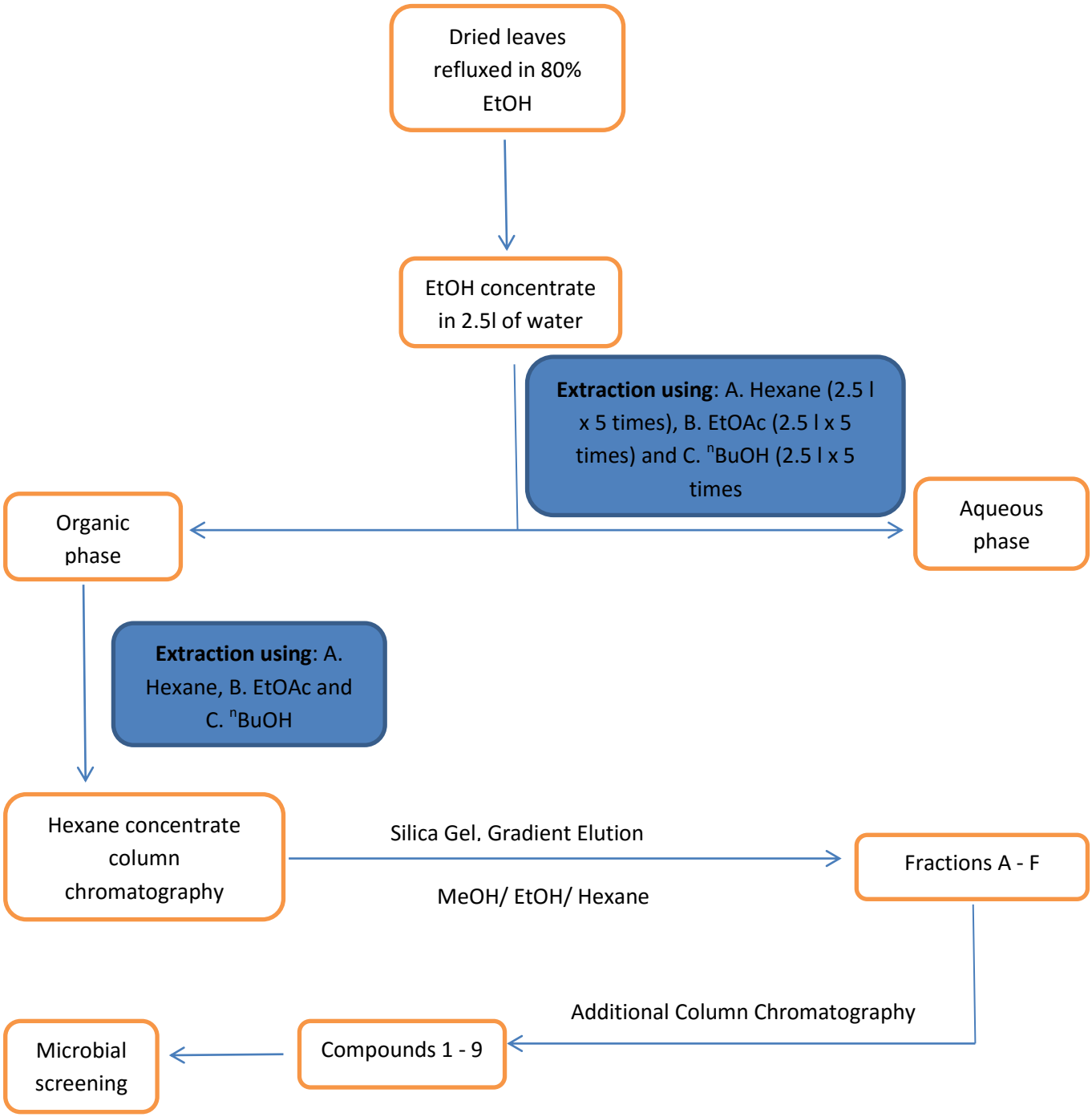
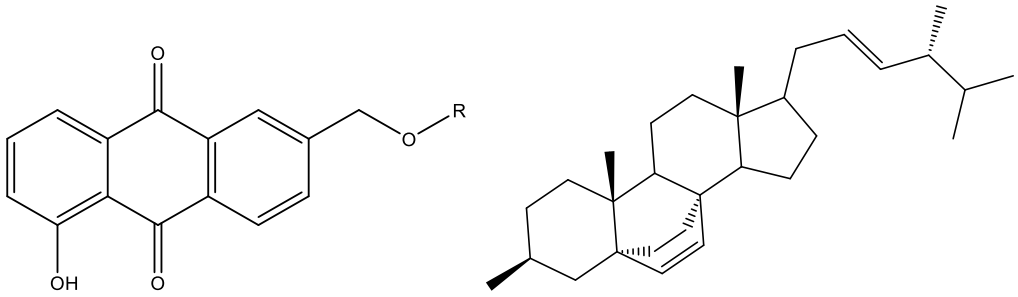


Figure 1.9: Isolation of antibacterial compounds from *Siraitia grossvenorrii*

Compounds 7, 8 and 9 obtained by Huang and co-workers are shown in Figure 1.10.



For 7, R = H; 8, R = Ac 9

Figure 1.10: Some of the antibacterial metabolites obtained by Huang and co-workers.

The name of compound 7 is 1 hydroxy – 6 – (hydroxymethyl) anthracene – 9, 10 – dione; and compound 8 is 6 – (ethoxymethyl) – 1 – hdroxyantracene – 9, 10 – dione.

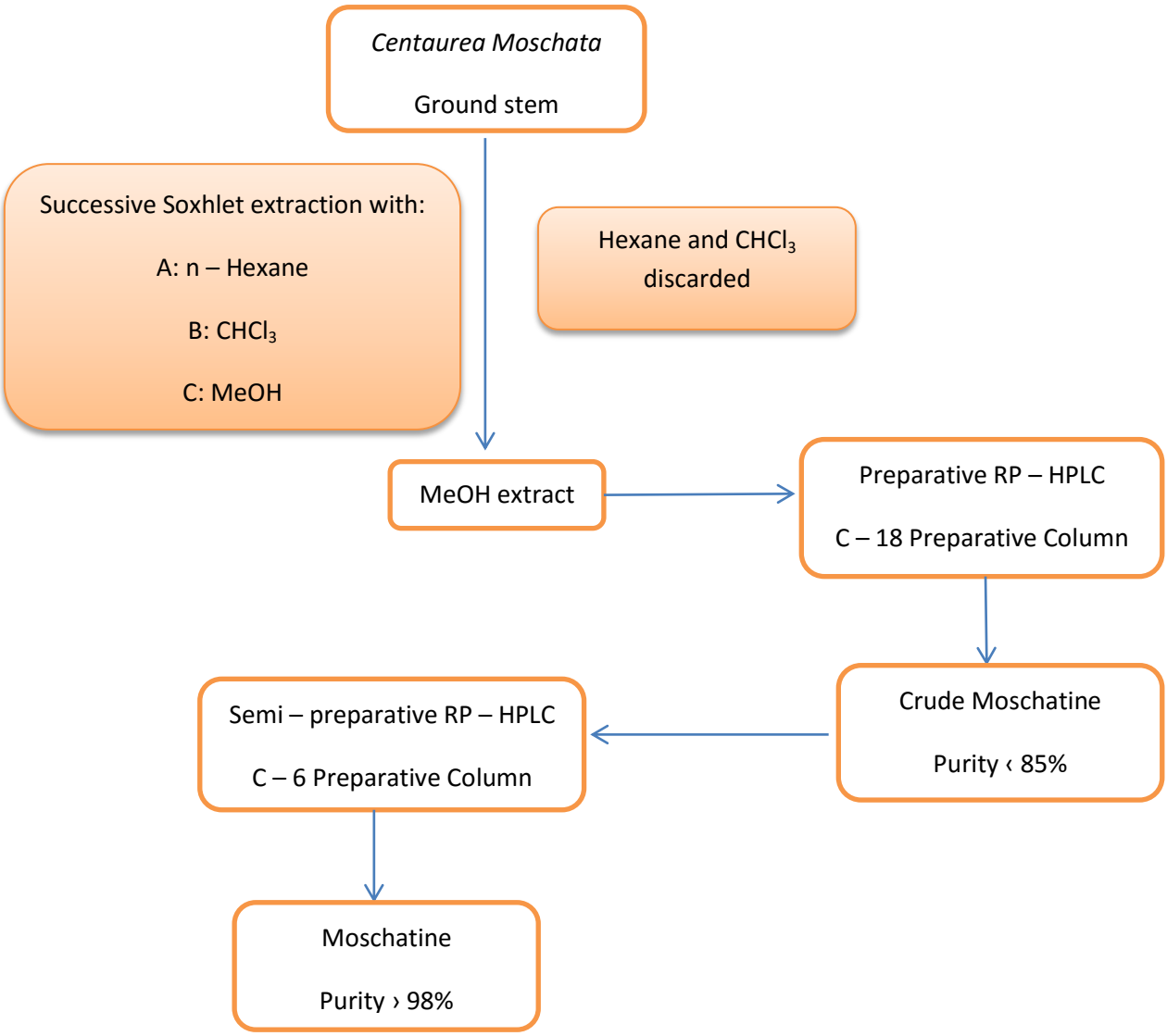


Figure 1.11: The isolation protocol of moschatine a steroidal glycoside.

Moschatine a steroidal glycoside was isolated from *Centaurea moschata* using the protocol shown in Figure 1.11 (Sarker., 1998, 2006).

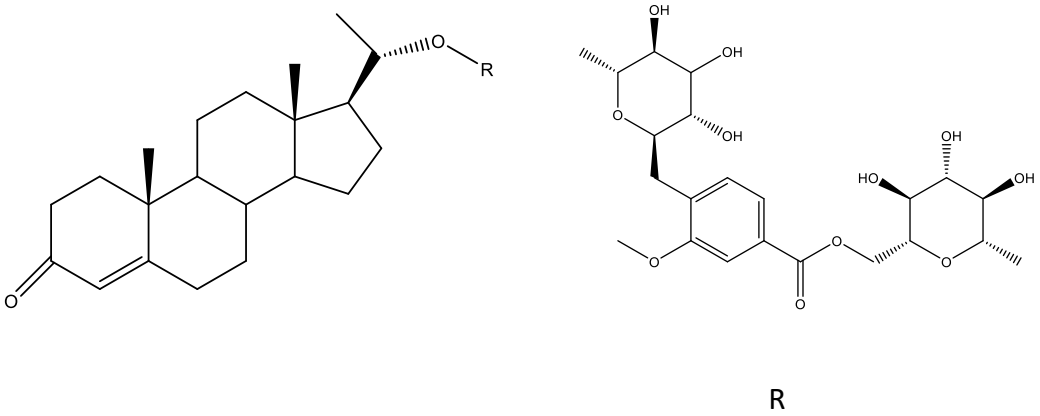


Figure 1.12: The structure of moschatine and the R group.

As secondary metabolites are produced in small quantities, it is important to quantify the yield after isolation and purification. Estimation of recovery can be achieved by making use of a standard, if it's available, with the aid of various analytical techniques (Sarker., 2006). One such technique which has been used to estimate given metabolite yields is ^{1}H-NMR. This is achieved by using calibrated integration of specific peaks of a standard of known concentration and comparing them with the integral of the same peaks from the crude extract or fraction under consideration.

This is shown in Figure 1. 13 where part of a ^{1}H-NMR was used to determine the amount of scutellarin from *Scutellaria incana* extract (Bruker publications., 2011). In this particular case the number of moles of scutellarin was found to be 32.8 mM through a match against a spectral base of potential botanical extracts.

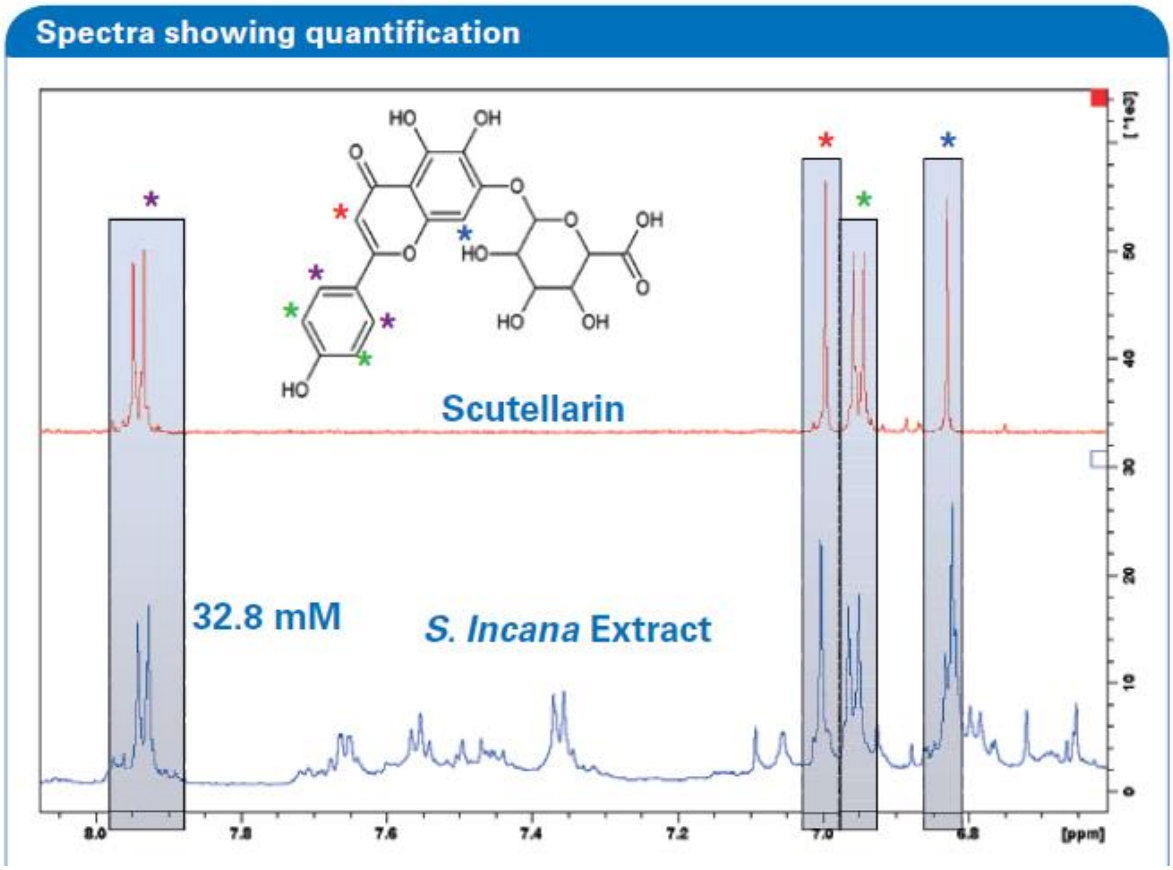


Figure 1.13: Quantification of scutellarin from Skull Cap extract of *Scutellaria incana* using quantitative ^{1}HNMR and a standard

The recovery of the metabolites can be assessed using various other techniques. One of the techniques that provide a clear idea of the recovery of the active compounds is "quantitative bioactivity". In this particular case the bioactivity of the crude/fraction is determined before fractionation and then determined for each fraction after fractionation. If several fractions give some bioactivity then the activity was due to several compounds or the active compound is spread over several fractions. If the activity is lost this could be due to several reasons which include: compounds being retained in the column, active compounds being unstable and breaking down under the conditions used for the isolation process, the extract being prepared in a solvent which is incompatible with mobile phase so that the extract precipitates when loaded on column or the activity could be based on synergy among a number of compounds which would not be active individually (Sarker., 2006).

Often large amounts of plants are used to obtain only a small quantity of the active ingredient. For example thirty (30) grams of vincristine were obtained from fifteen tonnes of dried leaves of *V. rosea,* while 1.9 kilograms of Taxol® were obtained from 6000 trees of *Taxus brevifolia* (Sarker., 2006). Therefore, once some metabolites have shown good activity it is essential to try and find methods of semi-synthesis or total synthesis. The other option would be to start large scale farming of the plants.

1.2.5 Characterisation of secondary metabolites

After natural products have been isolated, purified and their bioactivity determined the next crucial stage is the identification of the compounds or the elucidation of the conclusive structure of the compounds. If the target compounds are known it is easy to compare preliminary spectroscopic data with data from literature. If the data matches then the structure can be deduced.

If the compounds are unknown then a comprehensive and systematic approach which uses physical, chemical and spectroscopic techniques is used. The process can be made easier by using information from the genus if that is available as this gives additional hints regarding the possible chemical classes of the metabolites. Some techniques that are used include:

- Elemental analysis. This helps to deduce empirical formula and can be used together with information from other techniques to deduce the exact chemical formula of the metabolites.
- Ultraviolet-visible (UV-vis) spectroscopy. This provides information on chromophores that are available in the compound. This can be used to predict some families of compounds like coumarins, flavonoids and isoquinoline alkaloids which have characteristic absorption bands.
- Infra-red (IR) spectroscopy, this provides valuable information on the functional groups that are present in the compounds especially aldehydes, alkenes, hydroxyl and aromatic systems.
- Mass spectrometry (MS), this gives information on the accurate molar mass and fragmentation patterns which could be useful when assembling the structure.

- X-ray diffraction (XRD), this is a very useful and conclusive technique in cases where the metabolite forms crystals. XRD can be used to give the absolute structure and confirm stereochemical arrangement of groups in the compound.

Table 1: NMR experiments used in structural elucidation

Experiment	Coupling	Correlation	Application
^{1}H			Determines proton's environment including number of neighbouring protons
^{13}C			Determines number of carbons and the chemical environment of the carbons
^{13}C DEPT135			Determination of methyl, methylene, methine and quaternary carbons
COSY	^{1}H-^{1}H	Geminal and vicinal proton coupling through bond	Backbone structure; atom linkage
HSQC	^{1}H-^{13}C	Coupling of attached proton and carbon	Backbone structure, join related spin systems
HMBC	^{1}H-^{13}C	Coupling of protons to carbons separated by 2-3 bonds	Backbone structure, spin related systems and stereochemistry
NOESY	^{1}H-^{1}H	Interaction of protons that are less than 5Å in distance through space	Relative stereochemistry, join related spin systems

- Nuclear magnetic resonance spectroscopy (NMR). NMR can be used to deduce the structure and put the different pieces together if the whole array of one dimensional and two dimensional experiments are carried out. It also gives the relative stereochemical configuration at stereogenic centres. The NMR experiments which were used in this research are tabulated in table 1 and their significance elaborated.

The use of the chemical shifts obtained from the ^{1}H and the ^{13}C NMR provides information on functional groups present, degree of saturation and insights into the degree of oxidation. This information is often enough to classify the compound (Nadja., 2005).

Comparison of this information with those in databases can be used to quickly obtain the structure of the compounds (Elyashberg., 2002). The correlations from COSY and HSQC are useful in grouping atoms in similar spin systems leading to the determination of the backbone of the compound. The links between related spin systems can be established using NOESY, HSQC and HMBC while relative stereochemistry can be assigned using NOESY and HMBC.

1.2.6 Biological assays

In order for the activity of a target compound to be pinpointed chemical, physical or biological assays must be included in the isolation protocol. Chemical assays are used in the case where the compounds of interest are known or to determine the class of compound, whereas physical assays can be used if standards for the target compound are available. Some physical assays include melting point, TLC, HPLC, LC-NMR or other spectroscopic techniques. Chemical assays include the use of chemical tests to identify specific classes of compounds, such as triterpenes and steroids. Biological assays (bioassays) involve the use of a biological system to detect properties of the extract. The properties that are investigated include antibacterial, antifungal, anticancer and cytotoxicity to mention a few. In order for an assay to be reputable the following conditions must be taken into consideration:

1. Samples must be completely soluble especially if the solvent used for the assay is different from the extraction solvent, otherwise all undissolved particles must be filtered off.
2. Acidified or basic samples must be readjusted to their original pH to prevent interference with the assay.
3. Positive and negative assays must be included in the test.
4. The minimum requirement for the assay is that it should be semi-quantitative otherwise serial dilution can be used.

Bioassays can involve *in vivo* trials were whole animals are used or *in vitro* assays were cultured cells are used. Other assays which could be used include *ex vivo* where only the relevant organ or tissue from an animal is isolated and used. *In vivo* assays are relevant at pre - clinical trial level as they tend to be costly. Such assays can also provide toxicity information. *In vitro* assays are faster and small amounts of test compounds are used but these may not be relevant for clinical trials.

In this particular research antimalarial, cytotoxicity, antibacterial and anti-tuberculosis assays were run using serial dilutions. The pLDH test targets were used to evaluate bioactivity of *P. falciparum* parasite, while HeLa cells were used to evaluate cytotoxicity. Bioassays were also run for both gram positive and gram negative bacteria and some fungi. There are many bioassays that are available and most of them are cell viability and cytotoxicity assays. These assays are useful for drug screening and cytotoxicity tests of chemicals. Figure 1.14 shows some of the reagents used in cell viability tests (Dojindo., 2013).

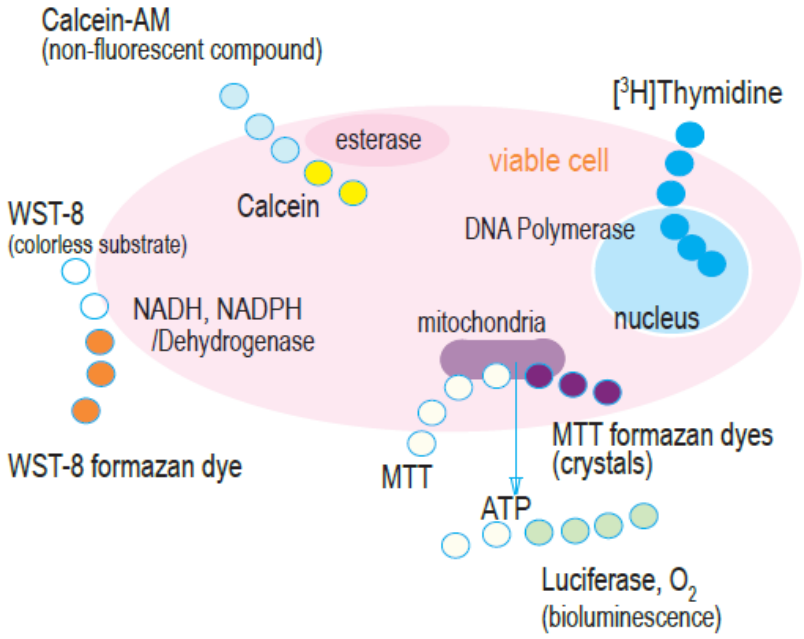


Figure 1.14: Some reagents that are used to test for cell viability

These various reagents target different cell functions such as enzyme activity, cell membrane permeability, cell adherence, Adinosine triphosphate (ATP) production, co-

enzyme production and nucleotide uptake activity. The reagents use established methods to count the number of cells that are alive like the colony formation method, tritium-labelled thymine uptake, MTT and water-soluble tetrazolium (WST) method. In this case we will discuss the MTT and WST methods briefly.

Cellular enzymes such as lactate dehydrogenase (LDH), adenylate kinase and glucose – 6 – phosphate dehydrogenase are also used as cell death markers. Of these three the LDH is the most stable and sensitive as it does not lose its activity during cell death assays. MTT and WST methods are enzyme – based assays which rely on a reductive colouring reagent and dehydrogenase in a viable cell to determine cell viability in a colorimetric method. The MTT method is most suitable for determination of mitochondrial dehydrogenase activities in living cells (Dojindo., 2013). MTT is reduced by NADH to purple formazan which forms needle like crystals which need to be solubilised so an organic solvent is added as these crystals would make it tough to remove cell culture media from the plate walls. This results in significant well to well errors in the assay. On the other hand the WST method developed by Dojindo is suitable for cell proliferation and cytotoxicity assays. In this method viable cells release electrons to WST resulting in the formation of yellow, orange or purple formazan dyes. These dyes produced are then used to count viable cells; this can be achieved by measuring the absorbance of light at a given wavelength directly from the 96 – well plate.

1.3 *Anonidium mannii*

Anonidium mannii (Oliv). Eng. & Diels is a medium sized fast growing tropical plant that grows naturally in Central Africa. It is commonly known as *Junglesop*, *obệ*, *mubệ*, *taku* and mobei by the people in the region were it grows (PlantUser contributors., 2015). The plant belongs to the broad family called Annona and to a smaller class in that family called Annonaceae. The plant grows to between 8 to 30 metres and produces fruit after about 10 years. The fruit it produces can be as long as a man's forearm and as thick as a man's legs. It weighs between 4 to 6 kilograms but masses of up to 15 kilograms have been reported. It is edible and is often sold at high prices due to high demand. Leaves from the plant range from 20 to 40 cm and are often used as decoctions in the treatment of ailments like malaria.

Figure 1.15 shows some images of the leaves and its fruit (Sillans., 1953; Worldplantsmarket., n.d) (courtesy of P. Latham for first two pictures).

a b c

Figure 1:15: *Anonidium mannii* plant showing (a) leaves and the fruit in (b) and (c)

Anonidium mannii is used traditionally for many different purposes, including the treatment of spider and snake bites, bronchitis, dysentery, gastroenteritis, syphilis, gonorrhea, diarrhoea, malaria, skin inflammation, female infertility and cancer (Tsabang., 2012; Djeussi., 2013). The decoctions used traditionally are from the stem, bark and the leaves. In the case of the bark it is reported that about 500 grams of the bark obtained by scraping with a *machete* is soaked in three litres of water and the extract concentrated to two-thirds initial volume before being administered to the patient orally (Tsabang., 2012).

Previous phytochemical studies on *A. mannii* reported that the plant produces alkaloids, phenols, polyphenols, saponins, tannins and steroids. Yields from methanol extracts have been quoted in the range of about 3% (Djeussi., 2013). Some metabolites that have been reported from the plant include a number of isopentenyl indoles shown in Figure 1.16. The prenylated indoles and bisindole alkaloids isolated from the stem bark have been reported to have the following biological activities: antibacterial, immunosuppressive and radical scavenging (Southon et al., 1989). The isopentenyl indoles called anonidines were obtained from petroleum ether/ethyl acetate fractions as needles with m.p. 106-108^{o}C and molar mass 368.52 g/mol (Figure 1.15) (Southon et al., 1989).

Previous results from crude methanolic extracts of *A. mannii* by Djeussi et al in 2013 showed no significant antibacterial activity against *Escherichia Coli, Klebsiella pneumonia, Pseudomonas aeruginosa*. Anticancer results from *A. mannii* leaves methanol extracts

showed some activity with an MIC value of 9.14 µg/mL against U87MG.ΔEGFR cells while 40 g/mL extract when used to screen against CCRF-CEM leukemia a growth of the cells of about 31 % was recorded (Kuete., 2013).

Anonidine A with R group being 7-indolyl, for Anonidine B the R group is 3- indolyl.

Anonidine C the R group is 7-indolyl, When R group is 6-indolyl then we have Anonidine D. Anonidine E is similar to Anonidine C with

Figure 1.16: Five Isopentenyl indoles isolated from *A. mannii.*

1.4 Thesis scope

This brief introduction has highlighted the need for further research into drugs to deal with existing conditions like malaria and to prepare against multi-drug resistant strains of parasites and bacteria. The threat posed by emerging resistance to artemesinin therapy, for example, in the treatment of malaria highlights this urgent need for new drugs. The role of natural products in drug discovery has also been highlighted. The fact that cancer, the third leading cause of deaths in the world trailing behind cardiovascular diseases, HIV and AIDS, and the rise of tuberculosis also means that more research needs to be carried out in the discovery of new drugs which either have the same targets or have different targets and modes of action (Kuete., 2013).

Previous research into extracts from *A. mannii* have shown that there is value in further study into the secondary metabolites from the plant as they showed some notable biological activities during pre-screening. Ethno-botanical and ethno-medicinal information

has established that *A. mannii* has antimalarial, anticancer, antibacterial and antifungal activities (Djeuss., 2013). This research was therefore carried out with four specific aims and these are:

1. To extract, isolate, purify and identify the secondary metabolites in *A. mannii.* This will also lead to profiling of the metabolites in *A. mannii* leaves.

2. To evaluate the biological activities of the pure metabolites as well as the various fractions obtained during extraction especially against malaria, cancer, bacteria, fungi and tuberculosis. This also includes an evaluation of the cytotoxicity of the metabolites and fractions.

3. To validate the ethno-pharmacological uses of *A. mannii.*

1.5 References

1. World Health Organisation (WHO) **2013** reviewed in **2015**. Malaria. Fact sheet. http://www.who.int/mediacentre/factsheets/fs094/en/. Accessed on 30/04/2015.
2. Siegel, R.; Naishadham, D.; Jemal, A. **2012.** *A Cancer Journal for Clinicians.* 62, 10-29.
3. Newman, D. J.; Cragg, G. M.; Snader, K. M. **2003.** *Journal of Natural Products*. 66, pp1022-1037.
4. Sarker, S. D.; Latif, Z.; Gray, A. I. **2006.** Natural Products isolation: an overview. In *Natural Products Isolation, 2nd Ed;* Sarker, S. D.; Latif, Z.; Gray, A. I., Eds.; Methods in Biotechnology, 20, Humana Press: Totowa.
5. Bahar,M.; Deng, Y.; Fletcher, J. N.; Kinghorn, A. D. **2008.** Plant-Derived Natural Products in Drug Discovery and Development: An Overview. In *Selected Topics in the Chemistry of Natural Products,* Ikan, R.; World Scientific: Singapore, 11-48.
6. Ikan, R. **2008.** The Origin and the Nature of Natural Products. In *Selected Topics in the Chemistry of Natural Products;* Ikan, R.; World Scientific: Singapore, 1-9.

7. Clayden, J., Greeves, N., Warren, S,. Wothers, P. **2001.** Organic Chemistry. Oxford University Press, 1412-1414.
8. Sneader, W. **2005.** *Drug Discovery: A History.* John Wiley & Sons. West Sussex, 287-318.
9. Paláez, F. **2006.** *Biochemical Pharmacology*. 71(7). 981-990.
10. Newman, D. J.; Cragg, G. M.; Snader, K. M. **2003.** *Journal of Natural Products*. 66(7), 1022-1037.
11. Sneader, W. **2005.** *Drug Discovery: A History;* John Wiley & Sons: West Sussex, 155-184.
12. Dossey, A. T. **2010.** Insects and their new Chemical weaponry. New potential for drug discovery. *Natural Products Report*. 27, 1737-1757.
13. Inscoe, M. N.; Leonhardt, B. A.; Ridgway, R. L. **1990**. Commercial availability of insect pheromones and other attractants. *Behavior-Modifying Chemicals for Insect Management.* Ridgway, R. L; Silverstein, R. M; Inscoe, M. N., *Eds*., Amsterdam: Elsevier, 631-715.
14. Alonso, P. L., Brown, G., Arevalo-Herrera, M et al. **2011**. A research agenda to underpin malaria eradication. *PLoS Med.* 8, e1000406.
15. Kanabus, A. **2016.** Information about Tuberculosis. Global Health Education. Accessed online from www.tbfacts.org on 13/04/2016.
16. Khare, G., Kumar, P., Tyagi, A K. **2013.** Whole Cell screening – Based Identification of Inhibitors against Intraphagosomal Survival of *Mycobacterium tuberculosis. Antimicrobial Agents and Chemotherapy* 57(12), 6372 – 6377.
17. White, N. J. **2008.** The role of antimalarial drugs in eliminating malaria. *Malaria Journal.* 7, supp 1: S8.
18. Roll Back Malaria Partnership. **2011.** Refined/updated Global Malaria Action Plan objectives, targets, milestones and priorities beyond 2011. http://www.rbm.who.int/gmap/gmap2011update.pdf. (last accessed 7 May 2014).
19. Blunt, J. W.; Copp, B. R.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. **2011.** *Natural Products Report.* 28, 196-268.
20. Wender, P. A.; de Brabander, J.; Harran, P. G.; Jimenez, J. M.; Koehler, M. F. T.; Lippa, B.; Park, C. M.; Shiozaki, M. **1998.** *Journal American Chemical Society.* 120, 4534-4535.

21. Evans, D. A.; Carter, P. H.; Carreira, E. M.; Charette, A. B.; Prunet, J. A.; Lautens, M. **1999.** *Journal of American Chemical Society.* 121, 7540-7552.
22. Trost, B. M.; Dong, G. **2008.** *Nature*. 456,485.
23. Dixon, R. A. **2001.** *Nature*. 411, 843.
24. Croteau, R.; Kutchan, T. M.; Lewis, N. G. **2000.** Natural Products (Secondary Metabolites). *Biochemistry & Molecular Biology of Plants;* Buchanan, B.; Gruissem, W.; Jones, R., *Eds*.; John Wiley & Sons: Somerset, 1250- 1318.
25. Abba, K., Deeks, J. J., Olliaro, P. L., Naing, C. M., Jackson, S. M., Takwoingi, Y., Donegan, S. and Garner, P. **2011**. Rapid diagnostic tests for diagnosing uncomplicated *P. falciparum* malaria in endemic countries.
26. Baker, J., Ho, M., Pelecanos, A., Gatton, M., Chen, N., Abdulla, S., Albertini, A., Ariey, F., Barnwell, J. **2010.** Global sequence variation in the histidine-rich proteins 2 and 3 of *P. falciparum*: implications for the performance of malaria rapid diagnostic tests. *Malaria Journal.* 9, 129-135.
27. Bell, D. R., Wilson, D. W. and Martin, L. B. **2005**. False-positive results of a *P. falciparum* histidine-rich protein 2-detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. *The American Journal of Tropical Medicine and Hygiene.* 73, 199-203.
28. Brown, W. M., Yoell, C. A., Hoard, A., Vander Jagt, T. A., Hunsaker, L. A., Deck,L. M., Royer, R. E., Piper, R. C., Dame, J. B. and other authors **2004**. Comparative structural analysis and kinetic properties of lactate dehydrogenases from the four species of human malarial parasites. *Biochemistry.* 43, 6219-6229.
29. Centers for Disease Control and Prevention. **2012**. Review. Malaria: Biology. www.cdc.gov/malaria/about/biology/. Accessed 30/04/2015.
30. Desakorn, V., Dondorp, A. M., Silamut, K., Pongtavornpinyo, W., Sahassananda, D., Chotivanich, K., Pitisuttithum, P., Smithyman, A. M., Day, N. P. and White, N. J. **2005**. Stage-dependent production and release of histidine-rich protein 2 by *P. falciparum. Transactions of the Royal Society of Tropical Medicine and Hygiene.* 99, 517-524.
31. Ashley, E. A., Touabi, M., Ahrer, M., Hutagalung, R., Htun, K., Luchavez, J., Dureza, C., Proux, S., Leimanis, M. and other authors. **2009**. Evaluation of three parasite lactate dehydrogenase-based rapid diagnostic tests for the diagnosis of *falciparum* and *vivax* malaria. *Malaria Journal.* 8, 241-248.

32. D'Alessandro, S., Silvestrin, F., Dechering, K., Corbett, Y., Parapini, S., Timmerman, M., Galastri, L., Basilico, N., Sauerwein, R., Alano, P. and Taramelli, D. **2013**. A *P. falciparum* screening assay for anti-gametocytes drugs based on pLDH detection. *Journal of Antimicrobial Chemotherapy.* 68. 2048-2058.
33. Dondorp, A. M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., Newton, P. N., Pitisuttithum, P., Smithyman, A. M. and other authors. **2005**. Estimation of the total parasite biomass in acute falciparum malaria PfHRP2. *PLoS Medicine* 2, e204.
34. Druilhe, P., Moreno, A., Blanc, C., Brasseur, P. H. and Jacquier, P. **2001**. A colorimetric *in vitro* drug sensitivity assay for *P. falciparum* based on a highly sensitive double-site lactate dehydrogenase antigen capture enzyme-linked immunosorbent assay. *American Journal of Tropical Medicine and Hygiene.* 64, 233-241.
35. United States Environmental Protection Agency (EPA). **2015.** DDT- A brief history and status. http://www2.epa.gov/ingredients-used-pesticide-products/ddt-brief-history-and-status. Accessed on 30/06/2015.
36. Fogg, C., Twesigye, R., Batwala, V., Piola, P., Nabasumba, C., Kiguli, J., Mutebi, F., Hook, C., Guillerm, M. and other authors. **2008**. Assessment of three new parasite lactate dehydrogenase (pan-pLDH) tests for diagnosis of uncomplicated malaria. *Transactions of the Royal Society of Tropical Medicines and Hygiene.* 102, 25-31.
37. Goldring, J. D., Molyneux, M. E., Taylor, T., Wirima, J. and Hommel, M. **1992**. *P. falciparum:* diversity of isolates from Malawi in their cytoadherence to melanoma cells and monocytes *in vitro*. *British Journal of Haematology.* 81, 413-418.
38. Goldring, J. P. D. **2004**. Evaluation of immunotherapy to reverse sequestration in the treatment of severe *P. falciparum* malaria. *Immunology and Cell Biology.* 82, 447-452.
39. Hayward, R, E., Sullivan, D. J. and Day, K. P. **2000**. *P. falciparum:* histidine-rich protein II is expressed during gametocytes development. *Experimental Parasitology.* 96, 139-146.
40. Hopkins, H., Bebell, L., Kambale, W., Dokomajilar, C., Rosenthal, P. J. and Dorsey, G. **2008**. Rapid diagnostic tests for malaria at sites of varying transmission intensity in Uganda. *Journal of Infectious Diseases.* 197, 510-518.

41. Hopkins, H., Kambale, W., Kamya, M. R., Staedke, S. G., Dorsey, G and Rosenthal, P. J. **2007**. Comparison of HRP2- and pLDH-based rapid diagnostic tests for malaria with longitudinal follow-up in Kampala, Uganda. *American Journal of Tropical Medicines and Hygiene.* 76, 1092-1097.
42. Houzé, S., Boly, M. D., Le Bras, J., Deloron, P. and Faucher, J. F. **2009**. PfHRP2 and PfLDH antigen detection for monitoring the efficacy of artemesinin-based combination therapy (ACT) in the treatment of uncomplicated falciparum malaria. *Malaria Journal.* 8, 211-218.
43. Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., Wellems, T. E., Rener, J. and Taylor, D. W. **1986**. Secretion of malarial histidine-rich protein (PfHRP II) from *P. falciparum*-infected erythrocytes. *Journal of Cell Biology.* 103, 1269-1277.
44. IAMAT. **2015**. World Malaria Risk Chart. Geographical distribution of malaria risk areas, *P. falciparum* drug-resistant areas, principle mosquito vectors and guidelines for suppressive medication by country. https://www.iamat.org/elibrary/view/id/1376. Pdf downloads. Accessed on 13/06/2015.
45. Kattenberg, J. H., Tahita, C. M., Versteeg, I. A. J., Tinto, H., Traoré-Coulibaly, M., Schallig, H. D. F. H. and Mens, P. F. **2012**. Antigen persistence of rapid diagnostic tests in pregnant women in Nanoro, Burkina Faso, and the implications for the diagnosis of malaria in pregnancy. *Tropical Medicine & International Health.* 17, 550-557.
46. Killian, A. H., Metzeger, W. G., Mutschelknauss, E. J., Kabagambe, G., Langi, P., Korte, R. and von Sonnenburg, F. **2000**. Reliability of malaria microscopy in epidemiological studies: results of quality control. *Tropical Medicine & International Health.* 5, 3-8.
47. Koita, O. A., Doumbo, O. K., Outtara, A., Tall, L. K., Konaré, A., Diakaté, M., Diallo, M., Sagara, I., Masinde, G. L. and other authors. **2012**. False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of HRP2 gene. *American Journal of Tropical Medicine and Hygiene.* 86, 194-198.
48. Kumar, N., Pande, V., Bhatt, R. M., Shah, N. K., Mishra, N., Srivastava, B., Valecha, N. and Anvikar, A. R. **2013**. Genetic deletion of HRP2 and HRP3 in Indian *P. falciparum* population and false negative malaria rapid diagnostic test. *Acta Tropica.* 125, 119-121.

49. Kumar, N., Singh, J. P., Pande, V., Mishra, N., Srivastava, B., Kapoor, R., Valecha, N. and Anvikar, A. R. **2012**. Genetic variation in histidine-rich proteins among Indian *P. falciparum* population: possible causes of variable sensistivity of malaria rapid diagnostic tests. *Malaria Journal.* 11, 298-304.
50. Lang-Unnasch, N. and Murphy, A. D. **1998**. Metabolic changes of the malaria parasite during the transition from the human to the mosquito host. *Annual Review of Microbiology*. 52, 561-590.
51. Laveran, A. **1891**. *Traité des fiévres palustres*. Paris: Masson.
52. Leke, R. F. G., Djokam, R. R., Mbu, R., Leke, R. J., Fogako, J., Megnekou, R., Metenou, S., Sama, G., Zhou, Y. and other authors. **1999**. Detection of *P. falciparum* antigen histidine-rich protein 2 in blood of pregnant women: implications for diagnosing placental malaria. *Journal of Clinical Microbiology.* 37, 2992-2996.
53. MacPherson, G. G., Warell, M. J., White, N. J., Looareesuwan, S and Warell, D. A. **1985**. Human celebral malaria. Aquantitative ultrastructural analysis of parasitized erythrocyte sequestration. *American Journal of Pathology.* 119, 385-401.
54. Maharaj, R., Raman, J., Morris, N., Moonasar, D., Durrheim, D N., Seocharan, I., Kruger, P., Shandukani, B., Kleinschmidt, I. **2013**. Epidemiology of Malaria in South Africa: From Control to Elimination. *The South African Medical Journal*. 103 (10), 779 – 783.
55. Makler, M. T. and Hinrichs, D. J. **1993**. Measurement of lactate dehydrogenase activity of *P. falciparum* as an assessment of parasitemia. *American Journal of Tropical Medicine and Hygiene.* 48, 205-210.
56. Malaria Foundation International **2003**. FAQs: Is DDT Still Effective and Needed in Malaria Control? www.malaria.org/DDTcosts.htmL. Accessed on 5/08/2015.
57. Maltha, J., Gamboa, D., Bebdezu, J., Sanchez, L., Cnops, L., Gillet, P. and Jacobs, J. **2012**. Rapid diagnostic test for malaria diagnosis in the Peruvian Amazon: Impact of *pfhrp2* gene deletions and cross-reactions. *PLoS ONE.* 7, e43094.
58. Mayxay, M., Pukrittayakamee, S., Newton, P. N. and White, N. J. **2004**. Mixed species malaria infections in humans. *Trends in Parasitology.* 20, 233-240.
59. Morimoto, R. I., Tissiéres, A and Georgopoulos, C. (editors). **1994**. *The Biology of Heat Shock Proteins and Molecular Chaperones.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

60. Mueller, I., Betuela, I., Ginny, M., Reeder, J. C. and Genton, B. **2007**. The sensistivity of OptiMSL rapid diagnostic test to the presence of *P. falciparum* gametocytes compromises its ability to monitor treatment outcomes in an area of Papua New Guinea in which malaria is endemic. *Journal of Clinical Microbiology.* 45, 627-630.
61. Okoko, B. J., Ota, M. O., Yamuah, L. K., Idiong, D., Mkpanam, S. N., Avieka, A., Banya, W. A. and Osinusi, K. **2002**. Influence of placental malaria infection on foetal outcome in Gambia: twenty years after Ian McGregor. *Journal of Health, Population and Nutrition.* 20, 4-11.
62. Parra, M. E., Evans, C. B. and Taylor, D. W. **1991**. Identification of *P. falciparum* histidine-rich protein 2 in the plasma of humans with malaria. *Journal of Clinical Microbiology*. 29, 1629-1634.
63. Piper, R. C., Wentworth, L. L., Cooke, A. H., Houze, S., Chiodini, P. and Makler, M. **1999**. A capture diagnostic for assay for malaria using *Plasmodium* lactate dehydrogenase (pLDH). *American Journal of Tropical Medicine and Hygiene.* 60, 109-118.
64. Scientific American. **2009**. Review article by Marla Cone. Should DDT be Used to Combat Malaria? www.scientificamerican.com/article/ddt-use-to-combat-malaria/. Accessed 5/08/2015.
65. Stahl, H. D., Kemp, D. J., Crewther, P. E., Scanlon, D. B., Woodrow, G., Brown, G. V., Bianco, A. E., Anders, R. F. and Coppel, R. L. **1985**. Sequence of a cDNA encoding a small polymorphic histsidine- and alaninie-rich protein from *P. falciparum. Nucleic Acids Research.* 13, 7837-7846.
66. Sullivan, D. J., Jr, Gkuzman, I. Y. and Goldberg, D. E. **1996**. *Plasmodium* hemozoin formation mediated by histidine-rich proteins. *Science.* 271, 219-222.
67. Thézénas, M. L., Huang, H., Njie, M., Ramaprasad, A., Nwakanma, D. C., Fischer, R., Digleria, K., Walther, M., Conway, D. J. and other authors **2013**. PfHPRT: a new biomarker candidate of acute *P. falciparum* infection. *Journal of Proteome Research.* 12, 1211-1222.
68. Thirumalai, T., Kelumalai, E., Senthilkumar, B., David, E. **2009**. Ethnobotanical study of medicinal plants used by local people in Vellore District, Tamilnadu, India. Ethnobotanical leaflets 13, 1302 - 1311

69. Tjitra, E., Suprianto, S., McBroom, J., Currie, B. J. and Anstey, N. M. **2001**. Persistent ICT malaria *P.falciparum/P.vivax* panmalarial and HRP2 antigen reactivity after treatment of *P. falciparum* malaria is associated with gametocytemia and results in false-positive diagnosis of *P. vivax* in convalescence. *Journal of Clinical Microbiology.* 39, 1025-1031.
70. WHO. **1999**. New Perspectives: Malaria Diagnosis. Report of joint WHO/USAID informal consultation 25-27 October 1999. Report No. WHO/CDS/RBM/2000.14, WHO/MAL/2000.1091. Geneva: World Health Organization. http://wholibdoc.who.int/hq/2000/WHO_CDS_RBM_2000.14.pdf. Accessed 25/06/2015.
71. WHO. **2003**. Malaria Rapid Diagnosis: Making it Work. Meeting report 20-23 January 2003. Manila: World Health Organization. http://www.who.int/malaria/publications/atoz/rdt2/en/index.htmL. Accessed 25/06/2015.
72. WHO **2006.** WHO gives indoor use of DDT a clean bill of health for controlling malaria. News. 15 September 2006. http://www.who.int/mediacentre/news/releases/2006/pr50/en/. Accessed 03/08/2015.
73. WHO. **2009**. Malaria Rapid Diagnostic Test Performance: Results of WHO Product Testing of Malaria RDTs: Round 1 (2008). Geneva: World Health Organization. http://www.who.int/tdr/news/documents/executive-summary-malaria.RDTs.pdf. Accessed 25/06/2015.
74. WHO. **2010**. Malaria Rapid Diagnostic Test Performance: Results of WHO Product Testing of Malaria RDTs: Round 2 (2009). Geneva: World Health Organization. http://www.who.int/malaria/publications/atoz/9789241599467/en/index.htmL. Accessed 25/06/2015.
75. WHO. **2011**. Global Plan For Artemesinin Resistance Containment. http://apps.who.int/iris/bitstream/10665/44482/1/9789241500838_eng.pdf?ua=1 . Accessed 25/06/2015.
76. WHO. **2011b**. Malaria Rapid Diagnostic Test Performance: Summary results of WHO Malaria RDT Product Testing: Rounds 1-3 (2008-2011). Geneva: World Health

Organization. http://www2.wpro.who.int/NR/rdonlyres/005E574B-FFCD-484C-BEDE-C580324AC2CF/0/RDTMalariaRd3_FINAL11Oct.pdf. Accessed 25/06/2015.

77. WHO. **2015**. Malaria: Question and answers. http://who.int/malaria/media/artemisinin_resistance_qa/en/ Accessed 25/06/2015.
78. WHO. **2015.** Cancer. Facts Sheet Number 297 http://www.who.int/mediacentre/factsheets/fs297. Accessed 13/4/2016.
79. NIH. **2012**. Understanding Malaria: Life cycle of Malaria parasite: www.niaid.nih.gov/topics/malaria/Pages/lifecycle.aspx. Accessed 15/05/2015.
80. Balentine, J **2015.** What causes malaria? http://www.medicinenet.com/malaria_facts/page2.htm#what_causes_malaria. Accessed on 15/08/2015.
81. Katz, C. **2013.** Malaria Mosquitoes Gain Ground as Search for New Defenses Intensifies. Environmental Health News. Scientific American. http://www.scientificamerican.com/article/malaria-mosquitoes-gain-ground-as-search-for-new-defenses-intensifies/. Accessed 15/08/2015.
82. Bruker publications. **2011.** Analysis of Natural Products. https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/MagneticResonance/NMR/brochures/nat-prod_broch_0310.pdf. Accessed 17/08/2015.
83. Sarker, S. D., Sik, V., Dinan, L. and Rees, H. H. **1998.** Moschatine: an unusual steroidal glycoside from *Centaurea moschata. Phytochemistry. 48,* 1039-1043.
84. Bross-Walch, N., Kühn, T., Moskau, D. and Zerbe, O. **2005.** *Chemistry & Biodiversity.* 2, 147-177.
85. Elyashberg, M. E., Blinov, K. E., Williams, A. J., Martirosian, E. R., Molodtsov, S. G. **2002.** *Journal of Natural Products.* 65, 693-703.
86. Frausin. G., Lima. R. B. S., Hidalgo. A. D. F., Maas. P., Pohlit. A. M. **2014.** Plants of the Annonaceaue traditionally used as antimalarials. A Review. Accessed online www.scielo.br/pdf/rbf/v36nspe1a38.pdf. On 01/01/2016.
87. Sillans. R. **1953.** Replublique Centrafricaine. PHARMELZ data bank. Accessed online www.africanmuseum.be/collections/external/prelude. On 01/01/2016.
88. Plant Use Contibutors. **2015.** *A. mannii* (PROTA). http://uses.plantnet-project.org/e/index.php? Title = Anonidium_mannii_(PROTA). Accessed 01/01/2016.

89. Neergheen-Bhujun. V. S. **2013.** Underestimating the toxicology challenges associated with the use of Herbal Medicinal Products in Developing Countries. *Biomedical Research International.* 2013. 1-10.

90. Termote. C. **2012.** Wild Edible Plant use in Tsopo District, DRC. PhD thesis 12,108-118. Accessed www.tropicallab.ugent.be/thesisceline.pdf on 01/01/2016.

91. Laatsch. H. **1990.** Book Review. Dictionary of Alkaloids. 2nd Edition. Eds. Southon. I. W., Buckingham. J.

92. Achenbach. H., Renner. C. **1985.**Prenylindole alkaloids from *A. mannii. Heterocycles***.** 23. 2075-2079.

93. Peters. W. **1987**. Chemotherapy and Drug Resistance in Malaria. Volume 2. London: Academic Press. Resistance in Human Malaria IV: 4 aminoquinolines and multiple Resistances. 659-786.

94. Phillips. R. S. **2001.** Current Status of Malaria and Potential for Control. *Clinical Microbiology Reviews.* 14(1). 208-226.

95. Tren. R., Bate. R. **2004.** South Africa's War Against Malaria: Lessons for the Developing World. Policy Analysis. 513. 1-20.

96. South African Department of Health. **2004.** National Malaria Update.

97. Dzoyem. J. P., Kuete. V., McGaw. L. T., Eloff. J. M., **2014.** The 15- lipoxygenase inhibitory, antioxidant, antimycobacterial activity and cytotoxicity of fourteen ethnomedically used African Species and Culinary Herbs. Repository.up.ac.za/bitstream/handle/2263/45856/Dzoyem.

98. Vitamin Retailer. **2012.** 2012. "History Lessons". Company Profiles. Digitaledition.qwinc.com/article/2012+ "History Lessons". Accessed 01/01/2016.

99. Dewick. P. M. **2002.** Medicinal Natural Products: A Biosynthetic Approach. 2nd Edition. Wiley & sons. 198-337.

100. Kuete. V., Efferth. T. **2010.** Cameroonian Medicinal Plants: Pharmacology and Derived Natural Products. *Frontiers In Pharmacology.* 123-128.

101. Kim. D. S., Chae. H. J. **2011.** Effects of Polycan™ on Bone Metabolism Improvement – Results from Randomised, Double – Blind Human Clinical Trial. *Korean Journal of Clinical Pharmacology.* 21(4). 297-304.

102. Djeuss. D. E., Noumedem. J. A. K., Seukep. J. A., Fankman. A. G., Voukeng. I. K., Tankeo. S. B., Nkuete. A. H. L and Kuete. V. **2013.** Antibacterial activities of

selected edible plant extracts against multi-drug resistant Gram-negative bacteria. *BMC Complementary Alternative Medicine.* 13. 164-169.

103. www.dojindo.com/Protocol/. **2013**. Accessed 11/01/2016.

CHAPTER 2: RESULTS AND DISCUSSION

2.1 Introduction

A. mannii leaves were collected in Cameroon and identified by a botanist from the Herbier National du Cameroun in Yaoundé where a voucher specimen, 45582 HNC, was deposited. The leaves were air dried for preservation. Mass of dried *A. mannii* used during extraction was 274g. This was subjected to three consecutive extractions in DCM/methanol 1:1 (v/v) of 72 hours each to give a combined mass of crude extract of 13.5g (4.92% w/w of dried material). This percentage yield is an increase from the 3.39% which was reported for one extraction after soaking in methanol for 48 hours (Djeuss et al., 2013). The increase in yield is attributed to the solvent system used DCM/MeOH (1:1; v/v) and also to the three extractions carried out for 72 hours for each. The inclusion of DCM would extract other less polar metabolites. The schematic diagram (figure 2.1) shows the stages at which the pure secondary metabolites were obtained. The pure secondary metabolites are labelled **AM1C** up to **AM9C** and **AM9P**. Compound **AM7C** is notably in the schematic diagram as it was discovered accidentally after fractions had been combined and before washing up vials white crystals were observed in some of the vials, these crystals gave **AM7C**.

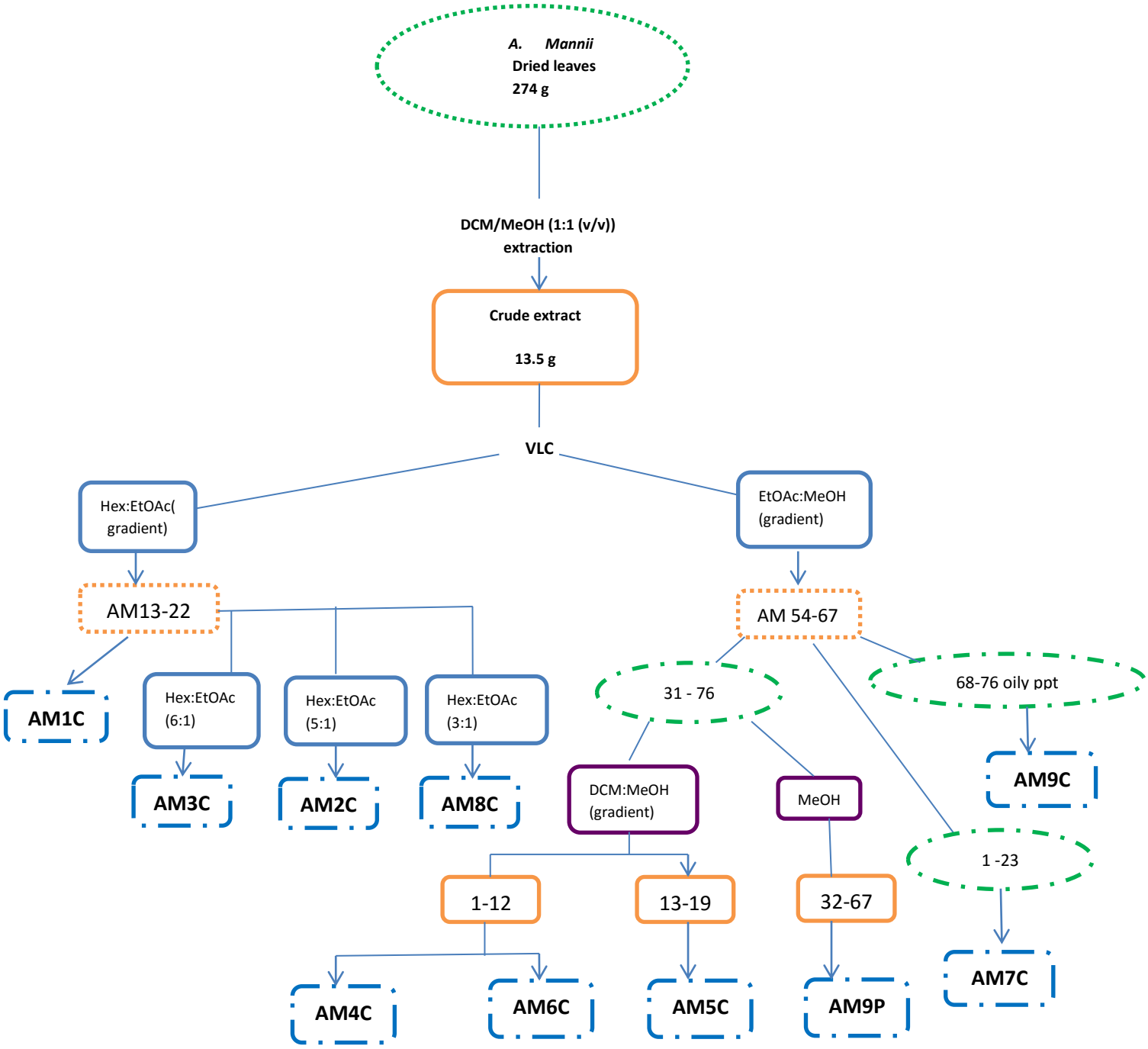


Figure 2.1: Flow diagram showing the pure metabolites as they were obtained

2.2 Pure metabolites obtained; their yields and general discussion

The table 2.1 shows the pure metabolites obtained, their masses and percentage yields as compared to both the crude extract as well as the plant material used.

Table 2.1: Pure metabolites obtained from *A. mannii* leaves and their yields

Metabolite	mass/mg	% yield of crude	% yield from plant material used
AM1C	261.9	1.94	0.095
AM2C	142.7	1.06	3.86×10^{-4}
AM3C	45.0	0.33	1.20×10^{-4}
AM4C	110.4	0.82	2.97×10^{-4}
AM5C	72.12	0.53	1.93×10^{-4}
AM6C	145.7	1.07	3.90×10^{-4}
AM7C	22.12	0.16	5.83×10^{-5}
AM8C	43.34	0.32	1.17×10^{-4}
AM9C	99.5	0.74	2.69×10^{-4}
AM9P	88.45	0.65	2.36×10^{-4}
$^{5}C_{5}$	47.65	0.35	1.27×10^{-4}

The Liebermann Burchard test (Kandati et al., 2012; Raju et al., 2012)confirmed that **AM1C**, **AM2C, AM3C** and **AM9C** are steroids. There are several classes of steroids, some of which are anabolic steroids and corticosteroids (Anabolicsteroids.net., 2013). Anabolic steroids are known to have harmful effects whereas corticosteroids have medicinal properties and can be used to treat a variety of disease including vasculitis (inflammation of blood vessels), myositis (inflammation of muscles, rheumatoid arthritis, lupus and gout to mention a few (Lupus news., 2010). Steroids contain four rings of carbon atoms and act as hormones in the body. They have a general skeleton similar to the one shown in figure 2.2 (a). Most steroids are thought to be derived from cholesterol also shown in figure 2.2 (b).

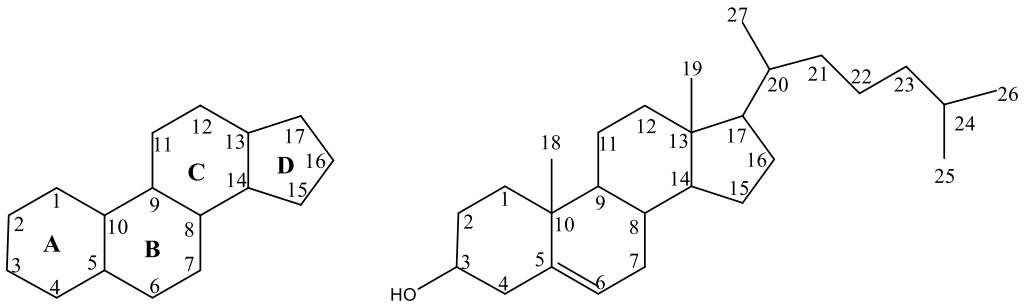
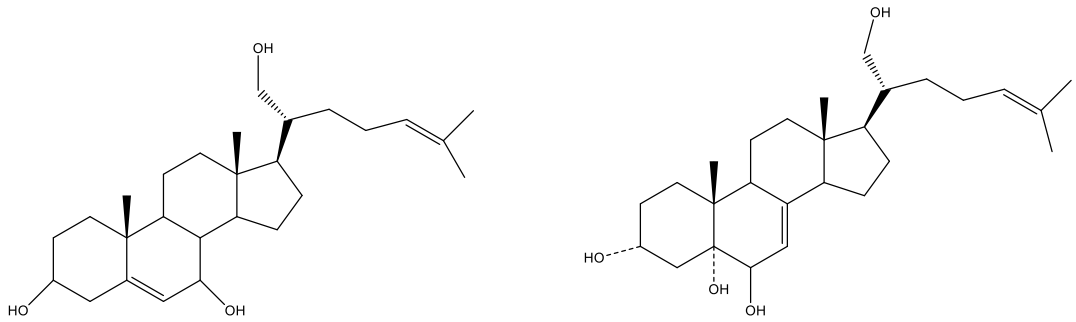


Figure 2.2: The backbone structure of steroids (a) and the structure of cholesterol (b)

The NMR, MS and FT-IR data was used to deduce the structures of the three compounds These compounds belong to a class called cholestanes and lanostanes.

AM1C and **AM2C** are hydroxyl cholestanes. Poly-hydroxyl and tri-hydroxyl cholestanes have been isolated in marine organisms before and in other species. Some examples that have been extracted from marine pulmonate, *Trismusculus peruvianus (T. peruvianus)* and are shown in figure 2.3.

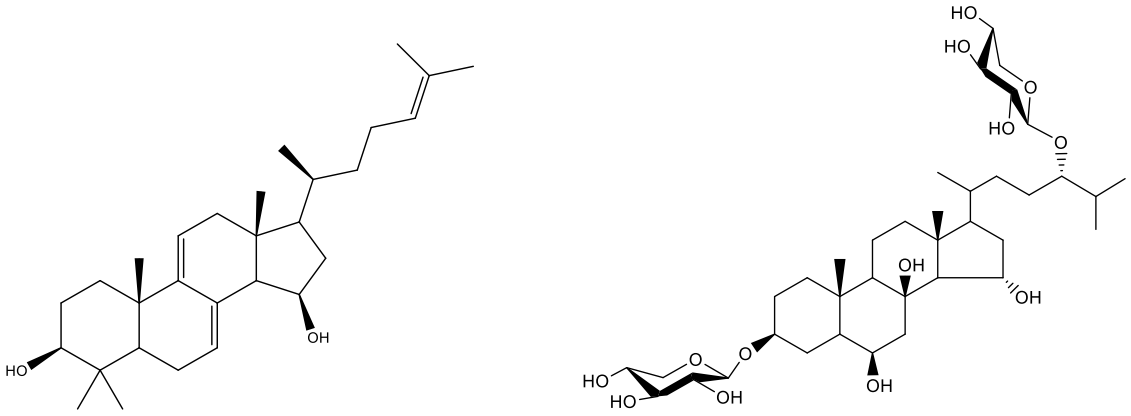


(20R)-cholest-5,24-diene -3β, 7β, 21-triol (20R)-cholest-7,24-diene- 3α, 5α, 6β-tetrol.

Figure 2.3: Examples of polyhydroxyl cholestanes that have been extracted from *T. peruvianus*

These two poly-hydroxyl cholestanes have some structural similarities with both **AM1C** and **AM2C**. The two metabolites from marine organisms (figure 2.3) have been reported to have moderate in-vitro cytotoxic activity against human colon carcinoma cell line (Diaz-Marrero et al., 2003). Two other dihydroxyl- and trihdroxyl-cholestane that have been isolated and are similar in structure to **AM1C** and **AM2C** (LIPID Maps., 2009). This further emphasises the

fact that the cholestane moiety can be hydroxylated on position 15 and can have several carbon-carbon double bonds, in this particular case three as in the metabolites isolated from *A. mannii* albeit in different positions. However, the positions shown in the structures have been known to have pi-bonds as shown in the structures from the marine organisms and the ones in figure 2.4.



Evasterioside E

Figure 2.4: A di and tri-hydroxylated cholestane that are hydroxylated on C-15 as reported in literature

Another steroid that has been reported in literature is lanosta – 7, 9(11), 24 – triene -3, 15 – diol, this has a C – 13 NMR spectra which is so closely related and whose NMR data almost matches that of **AM1C (**Chemspider., no date)**.** Its structure is shown in figure 2.5.

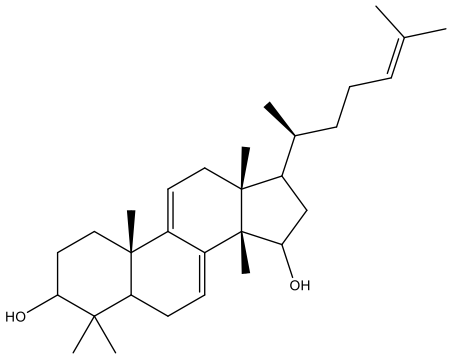
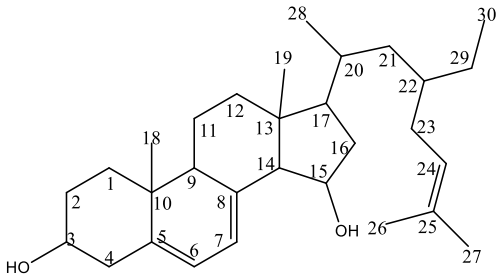


Figure 2.5: The structure of Lanosta– 7, 9(11), 24 – triene -3, 15 – diol

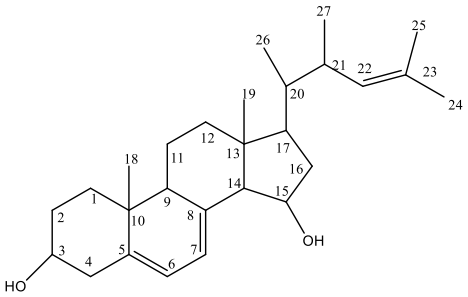
The structures given above were very useful in deducing the structures of three of the steroid that were isolated.

2.3 Structural elucidation of some isolated metabolites

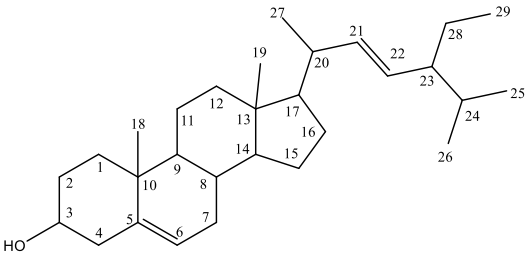
The structures of three of the steroids were deduced and are shown in figure 2.6.



AM1C



AM2C



AM3C

Figure 2.6: The suggested structures of the steroids isolated from *A. mannii.*

2.3.1 Structure elucidation of AM1C

AM1C, was isolated as a white powder with a melting point between 172-174 ^{o}C. **AM1C** was isolated using hexane/ethyl acetate, 1:3 (v/v) as mobile phase from the VLC of the crude extract. Elementary analysis data gave an empirical formula of $C_{15}H_{24}O$. The mass spectral data of the compound gave a molecular formula of $C_{30}H_{48}O_2$, with seven degrees of unsaturation. The ESI-MS data of the compound gave a molecular formula of $C_{30}H_{48}O_2$, with seven degrees of unsaturation. The molecular formula was supported by ESI-MS, FT-IR and ^{13}C spectral data. The ESI-MS (positive) showed the following characteristic peaks at *m*/*z* values of: 457.3 amu, 415 amu, 340 amu, 306 amu and 294 amu (Appendix, Figure 4.6).The FT-IR spectrum (Figure 4.7) showed the characteristic absorbance for OH group at 3428 cm^{-1} and C-H stretch between 2850 and 2970 cm^{-1}. The FT-IR spectra did not show any absorbance in the regions typical for the C = O and CO_2H groups. Data from the ^{1}H NMR

spectrum (figure 2.7, Table 2.2) showed the presence of six signals for methyl group, four of them singlets at δ_H = 0.59 ppm, 0.86 ppm, 0.92 ppm and 1.24 ppm; one methyl doublet that appeared at δ_H = 1.07 ppm (J = 6.6 Hz) corresponding to protons on C-28.

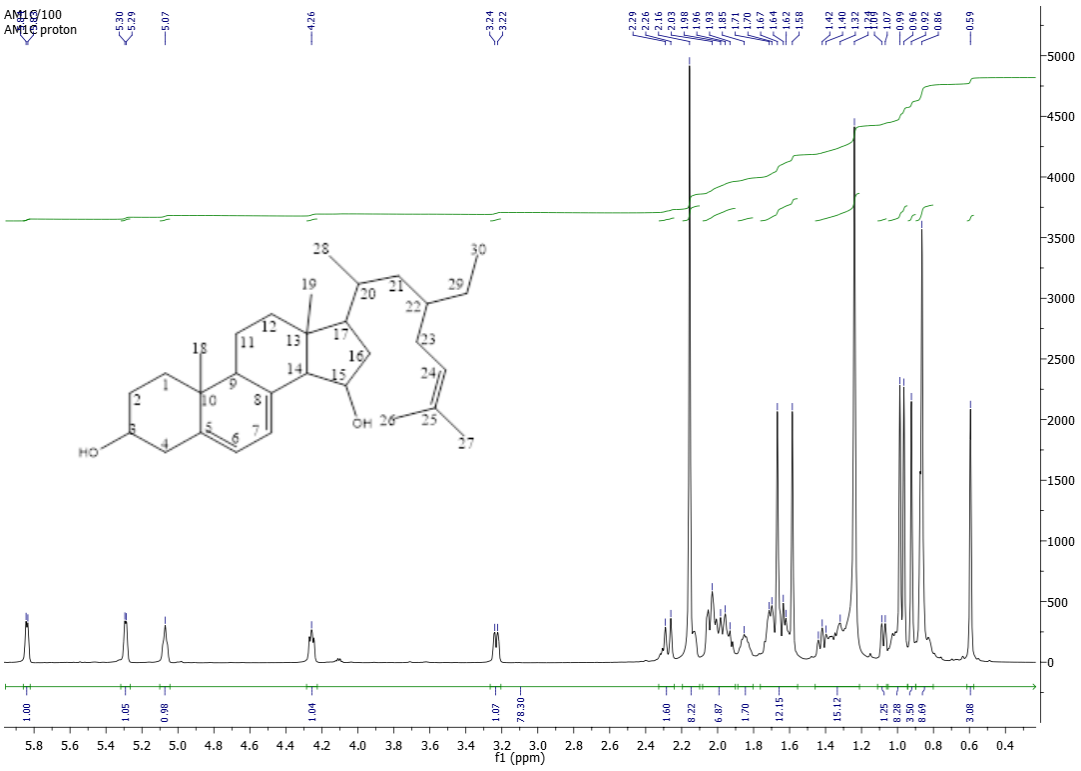


Figure 2.7: ^{1}H NMR spectra of AM1C in $CDCl_3$

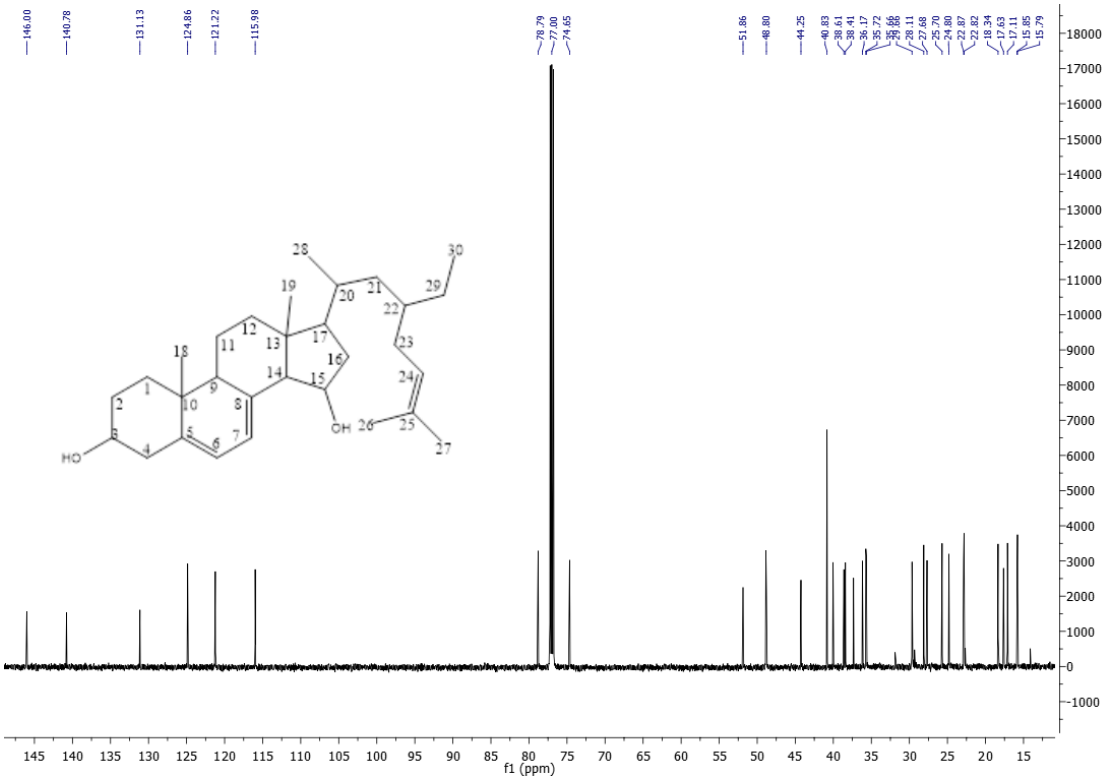


Figure 2.8: ^{13}C NMR of AM1C in $CDCl_3$

The spectrum exhibited another signal for methyl triplet corresponding to C-30 appearing at δ_H= 0.99ppm with a typical coupling constant of 7.1Hz. The ^{1}H NMR spectrum for **AM1C** also displayed signals integrating for three protons corresponding to that of di-substituted and tri-substituted olefinic bonds at δ_H= 5.07ppm (triplet), 5.29ppm (doublet, J = 6.46 Hz) and 5.84ppm (doublet, J = 6.80 Hz). These corresponded to the protons on C-24, C-7 and C-6, respectively. The appearance of the two doublet of doublets at δ_H = 4.25 ppm and 3.24 ppm for H-3 and H-15 confirmed the attachment positions of the hydroxyl (OH) groups in the sterol moiety on C – 3 and C - 15. The broad multiplet appearing at δ_H = 4.10 ppm corresponds to the proton on the hydroxyl groups. The ^{13}C NMR spectrum (Figure 2.5) and DEPT 135 spectrum (Appendix, Figure 4.1) showed the six well resolved methyl groups described above, nine methylene carbons, ten methines and five quartenary carbons. The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned using H-H COSY, HSQC and HMBC correlations as they are shown in Table 2.3 below. All this data supported the presence of the sterol skeleton having hydroxyl groups at C-3 and C-15 and three double bonds at C-5/C-6, C-7/C-8 and C-24/C-25. The structure was also supported by the COSY and HMBC correlations, which were also used to locate the remaining functionalities.

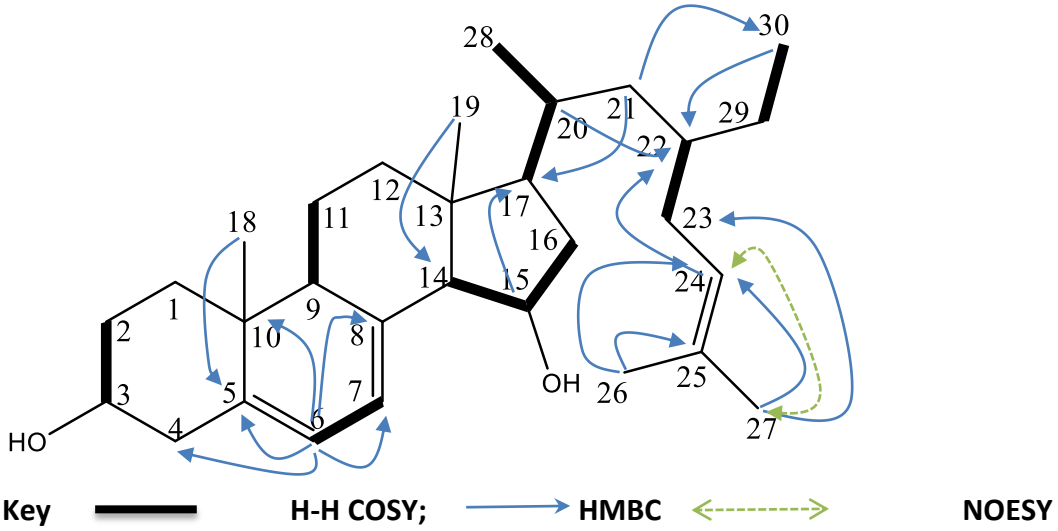


Figure 2.9: HMBC NOESY and H-H COSY correlations for the side chain of AM1C

The HMBC and COSY correlations are shown in Figure 2.9. The NOESY cross-peaks between H-24 and H-27; H-15 and H-16 confirmed the relative stereochemistry in the compound.

AM1C was thus confirmed as, 17- (4-ethyl-7-methyloct-6-ene-2-yl)-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17- dodecahydro-1 *H*-cyclopenta [α] phenanthren-3,15-ol. To the best of our knowledge this is the first time that **AM1C** has been reported in literature and as a metabolite extracted from *A. mannii*.

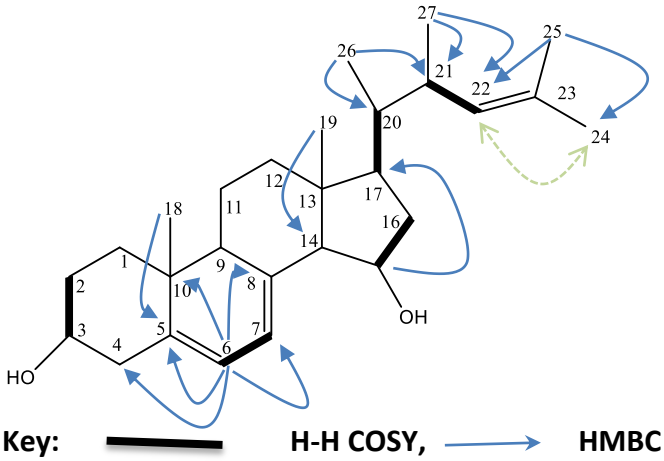
Table 2.2: ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), COSY, HMBC and NOESY data of AM1C in $CDCl_3$

Position	δC ppm	δH ppm; [m,J(Hz)]	COSY	HMBC	NOESY
1	37.3	1.06m, 1.84m		141	
2	36,2	1.58m, 1.84m	4.25	74.7, 40	
3	74.7	4.25dd (9.15, 5.81)	1.58		
4	40	2.28d	4.25	74.7,141, 121	
5	141				
6	121	5.84d (6.80)		146,35.7	
7	116	5.29d (6.46)		141, 35.7	
8	146				
9	48.9	1.67m			
10	35.7				
11	22.8	1.41m, 1.15m		48.4, 51.9	
12	38.4	1.23m, 1.94m			
13	44.3				
14	51.9	1.99m			
15	78.9	3.24dd (11.57, 4.29)	1.70		
16	35.66	1.70m		78.8	
17	48.8	1.40m			
18	17.6	0.86			
19	15.8	0.59		44.3	
20	44.3	1.31	1.07	38.6	
21	40	1.06d		15.9	
22	38.6	1.33m	2.00		
23	27.88	2.00m	1.33		
24	125	5.07	1.67	38.6	0.92
25	131				
26	17.1	1.24		125, 131	
27	28.11	0.92		125, 27.9	5.07
28	18.3	1.07d	1.31		
29	29.66	1.23	0.99		
30	15.9	0.99t	1.23	38.6	

2.3.2 Structure elucidation of AM2C

AM2C, was isolated as a white powder with a melting point between 168-172 ^{o}C from a column chromatography using hexane/ethyl acetate (1:3; v/v) as eluent. The ESI-MS data of

the compound gave a molecular formula of $C_{27}H_{42}O_2$, with 7 degrees of unsaturation. The molecular formula was supported by ESI-MS (negative) and ^{13}C spectral data. The ESI-MS obtained using the Advion mass spectrometer with the TLC interface showed the pseudo molecular ion peak at *m/z* value of 399.2 (M+1 peak (Appendix, Figure 4.16). Data from the FT-IR spectrum (Appendix: Figure 4.15) showed the presence of the free hydroxyl groups as confirmed by the absorption bands around 3352 cm^{-1}. Data from the ^1H NMR spectrum (Appendix, Figure 4.6, Table 2.3) showed the presence of six methyl groups, four of them singlets at δ_H = 0.61 ppm, 0.94 ppm, 1.60 ppm and 1.68 ppm; two methyl doublets that appeared at δ_H = 0.88 ppm and 0.98 (J = 6.6 Hz) corresponding to protons on C-28. The ^1H NMR spectrum for **AM2C** also showed three protons corresponding to that of di-substituted and tri-substituted olefinic bonds at δ_H = 5.07 ppm, 5.29 ppm and 5.84 ppm. These corresponded to the protons on C-22, C-7 and C-6 respectively. The appearance of the two doublet of doublets at δ_H = 4.25 and 3.24 ppm for H-3 and H-15 confirmed the positions of the hydroxyl (OH) groups in the sterol moiety.



Key: **H-H COSY,** **HMBC correlations** **NOESY**

Figure 2.10: Pertinent H-H COSY, NOESY and HMBC correlations in AM2C

The broad multiplet appearing on δ_H = 4.10 ppm was assigned to the proton on the hydroxyl groups as this peak disappeared when a few drops of D_2O were added and another ^{1}HNMR spectrum acquired. The ^{13}C NMR spectrum (Appendix, Figure 4.7) and DEPT 135 spectrum (Appendix, Figure 4.8) showed the six well resolved methyl groups described above, six methylene carbons, ten methines and five quaternary carbons. The ^1H and ^{13}C NMR values for all the protons and carbons were assigned using H-H COSY, HSQC and HMBC correlations

Table 2.3: ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), COSY, HMBC and NOESY data of AM2C in $CDCl_3$

Position	δC ppm	δH ppm [m, J(Hz)]	COSY	HMBC	NOESY
1	37.3	1.06m, 1.84m		141	
2	36,2	1.58m, 1.84m	4.25	74.7, 40	
3	74.7	4.25dd (9.15, 5.81)	1.70; 1.58		
4	40	2.28d	4.25	74.7,141, 121	
5	141				
6	121	5.84d (6.80)	5.29	146,35.7, 116	
7	116	5.29d (6.46)	5.84	141, 35.7	
8	146				
9	48.9	1.67m			
10	35.7				
11	22.8	1.41m, 1.15m		48.4, 51.9	
12	38.4	1.23m, 1.94m			
13	44.3				
14	51.9	1.99m			
15	78.9	3.24dd (11.57, 4.29)	1.70; 1.40	48.8	
16	35.66	1.70m	3.24	78.8	
17	48.8	1.40m	1.30		
18	17.6	0.86			
19	15.8	0.94		44.3	
20	44.3	1.30	1.40		
21	38.6	1.33m	5.07		
22	125	5.07	1.67, 1.33		1.67
23	131				
24	17.1	1.67			5.07
25	28.11	1.58		125, 17.1	
26	18.3	0.96d		44.3, 38.6	
27	15.9	1.03d		38.6, 125	

as they are shown in Table 2.4. All this data supported the presence of the sterol skeleton having hydroxyl groups at C-3 and C-15 and three double bonds at C-5/C-6, C-7/C-8 and C-22/C-23.

The structure was also supported by the COSY and HMBC correlations, which were also used to locate the remaining functionalities. These correlations are shown in Figure 2.10. **AM2C** was thus confirmed as 17-(3,5-dimethylhex-4-en-2-yl)-10,13–dimethyl-2,3,4,9,10,11,12,13,14,15,16,17- dodecahydro-1H-cyclopenta[α]phenanthrene-3,15-diol. To the best of our knowledge this is the first time that **AM2C** has been reported in literature and it is the first time it is reported as a metabolite extracted from *A. mannii*.

2.3.3 Structural elucidation of AM3C

AM3C was isolated as a white powder from a hexane/ethyl acetate (6:1 v/v) mobile phase. The ESI MS spectrum data (Appendix, Figure 4.25) gave a molecular formula of $C_{29}H_{48}O$ showing six degrees of unsaturation, which was supported by the ^{13}C NMR spectral data. The ESI-MS (positive) exhibited the pseudo molecular ion peak at *m/z* values of: 413.3 (M+1). The melting point was determined to be 140-142 ^{o}C.

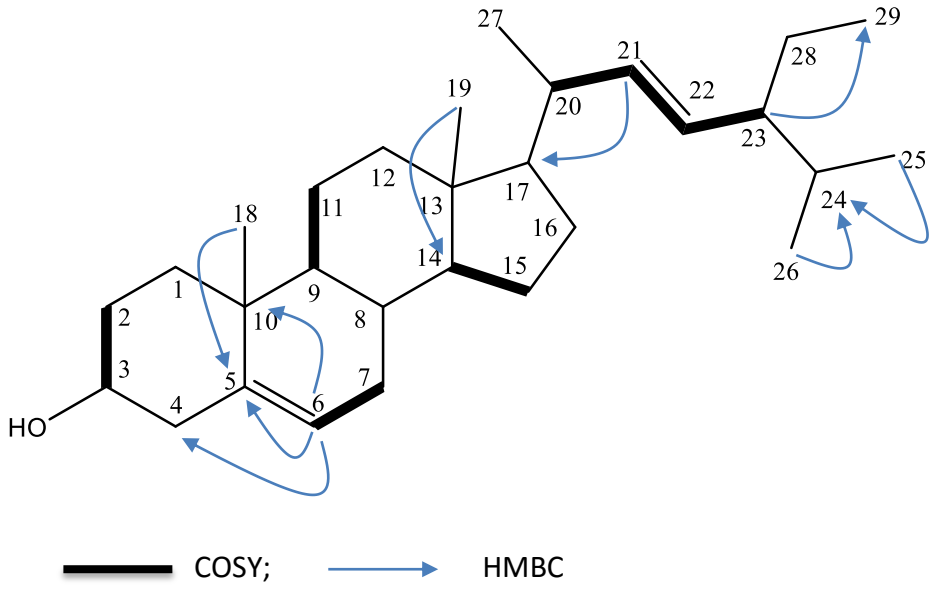


Figure 2.11: Key COSY and HMBC correlations used in the elucidation of AM3C structure

Data from the ^{1}H NMR spectrum (Appendix, Figure 4.17) showed the presence of six methyl groups, two of them singlets at δ_H= 0.68 ppm 1.00 ppm; three methyl doublets that appeared at δ_H = 0.79 ppm (J = 6.6 Hz), 0.84 ppm (J = 6.6 Hz) and 0.92 ppm (J = 6.2 Hz) corresponding to protons on C-25, C-26 and C-27.

The spectrum also showed a methyl triplet corresponding to C-29 appearing at δ_H = 0.80 ppm with a typical coupling constant of 7.1 Hz. The ^{1}H NMR spectrum for AM3C also showed three protons corresponding to that of di-substituted and tri-substituted olefinic bonds at δ_H = 5.02 ppm, 5.14 ppm and 5.34 ppm. These corresponded to the protons on C-22, C-21 and C-6 respectively.

The appearance of the doublet of doublets at δ_H = 3.52 ppm for H-3 confirmed the position of the hydroxyl group in the sterol moiety. The key correlations in **AM3C** are shown in figure 2.11. The spectral and physical data are consistent with that recorded in literature (Habib et al., 2007; Moghaddam et al., 2007; Jamal et al., 2009) for stigmasterol the only difference was in the melting point.

The melting point obtained from **AM3C** was lower than that recorded in literature. This may be due to the fact that the sample used in the melting point was not of a high purity. The structure of **AM3C** was therefore confirmed as the known compound stigmasterol. **AM3C,** stigmasterol has been reported in literature as a metabolite from various plant species (Habib et al., 2007; Jamal et al., 2009), but according to our knowledge it is the first time it has been isolated in *A. mannii.*

Table 2.4: **^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), COSY, HMBC and NOESY data of AM3C in $CDCl_3$**

Position	δC ppm	δH ppm [m, J(Hz)]	COSY	HMBC	NOESY
1	37.2	1.06m, 1.31m			
2	31.7	1.50m, 1.28m	3.52		
3	72.1	3.52tdd (4.5, 4.2, 3.8)	1.5		
4	42.3	2.24m, 1.95m	3.52		
5	141.1				
6	121.8	5.34t (6.1)	2.17	141, 42.3	36.1,
7	33.5	2.17	5.34		
8	31.9	1.53m			
9	50.2	1.17m	1.15		
10	36.1				
11	23.1	1.15m	1.17		
12	39.7	1.67m			
13	42.3				
14	56.8	1.04m	1.65		
15	24.3	1.65m	1.04		
16	29.7	1.65m			
17	56.2	1.25m			
18	18.8	0.68s		141	
19	12.1	1.00s		56.8	
20	40.5	1.99m	5.14		
21	138	5.14m	1.99, 5.02	56.2	
22	129	5.02m	5.14, 1.83		
23	46.1	1.83dd	5.02	11.9	
24	29.2	1.66m			
25	19.8	0.84d (6.6)		29.2	
26	19.4	0.79d (6.6)		29.2	
27	21.2	0.92d (6.2)			
28	25.4	1.66m			
29	11.9	0.80t (7.1)			

2.4 Quantitative estimation of AM1C, AM2C in crude using ^{1}HNMR

The estimation of the quantities of the two pure cholestanes in the crude obtained where carried out using ^{1}H NMR as described in Chapter 3. The peaks that were used are the ones for the methine protons and the Carbon bonded to the hydroxyl group. As the experiments were run under the same conditions the peak length was used as a measure of the concentration. Five different peaks were used and the average concentration from these taken as a good estimation of the concentration of the cholestanes. Table 2.5 shows the

results obtained from the spectra (Appendix Fig 4.26, 4.27 and 4.28) that were acquired specifically for quantitative purposes.

Table 2.5 Peaks heights used in quantitative ^{1}HNMR for estimation of metabolites

	Peaks/ (ppm)	**5.83**	**5.29**	**5.08**	**4.26**	**3.25**
	Concentration/ (mg/mL)	**Height of peak/ (mm)**				
AM1C	1.64	8.0	9.0	7.0	7.0	7.0
AM2C	1.82	6.0	8.0	5.0	5.0	7.0
AMC	3.50	3.0	4.0	2.5	2.0	2.0

Since the cholestanes had the same peaks in the region of interest in the ^{1}HNMR, estimated masses of the metabolites were calculated as follows:

Estimated mass from peak = (CPH/PHPM)*CPM.

Where CPH is crude peak height, PHPM is peak height of pure metabolite and CPM is concentration of pure metabolite.

For example for **AM1C** using the peak at 5.83 ppm, estimated mass of cholestane is:

(3.0/8.0)*1.64 mg = 0.62 mg/mL.

This implies that in 3.50 mg/mL of the crude extract about 0.62 mg/mL were cholestanes. The average concentration of cholestanes in the 3.5 mg/mL of the crude estimated using **AM1C** was 0.58 mg/mL, while that calculated using **AM2C** was 0.80 mg/mL. Using these estimates shows that the percentage of cholestanes in *A. mannii* extract was between 16 % and 23 %.

2.5 Bioassays

As has been mentioned in the introduction *A. mannii* is used traditionally in the treatment of a variety of ailments such as malaria, diarrhoea, snake bites and gastroenteritis (Djeuss., 2013). These diseases are caused by micro-organisms.

2.5.1 Antimalarial Results

From the results (table2.6; graph 2.1) it can be seen that *A. mannii* has moderate activity against *P. falciparum* as the crude extract at a single concentration of 50 µg/mL reduce the

viability of the parasite to less than half (45.5 ± 3.4 %). Of the pure metabolites tested **AM1C** gave the best activity albeit being a moderate viability of 40.4 ± 5.5 % at a single concentration dose of 20 µg/mL.

Table 2.6: Percentage viability of the malaria parasite for fractions and pure metabolites from *A. mannii*

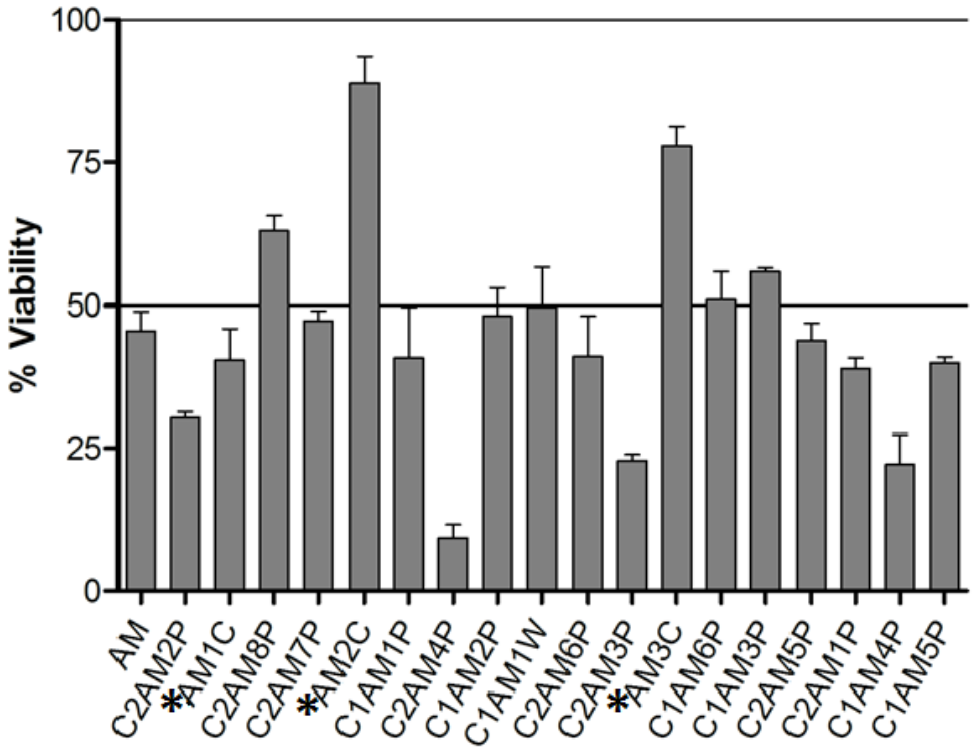
sample	% viability	deviation
AM	45.49	3.44
C2AM2P	30.28	1.04
*__AM1C__	40.36	5.53
C2AM8P	63.09	2.71
C2AM7P	47.33	1.77
*__AM2C__	89.02	4.72
C1AM1P	40.73	8.91
C2AM4P	9.12	2.34
C1AM2P	48.16	4.86
C1AM1W	49.62	7.11
C2AM6P	41.10	7.01
C2AM3P	22.86	1.04
*__AM3C__	77.85	3.44
C1AM6P	50.90	4.95
C1AM3P	55.95	0.55
C2AM5P	43.85	3.06
C2AM1P	38.90	1.90
C1AM4P	22.13	5.22
C1AM5P	39.91	1.04

*Refers to pure secondary metabolites

Considering the three pure metabolites **AM1C**, **AM2C** and **AM3C** which are all very structurally similar it appears as if increasing the side chain length at C-17 (figure 2.6) increases the activity of the metabolite, this can be inferred from comparing **AM1C** and **AM2C**. Also reducing the number of C – C double bonds and the hydroxyl groups reduces the activity as inferred from **AM1C** and **AM2C**. Considering **AM2C** and **AM3C** whose difference in activity is small it could be deduced that chain length on C – 17 has a great effect on activity. The greater the chain length the more bioactive the metabolite, this may be due to the arrangement in space which could favour hydrogen bonding of the hydroxyl moieties as the chain length increases thus producing the endoperoxy group similar to the one in artemisinin (Sarker et al., 2012).

Steroids have been reported in literature, to show biological activity and various pharmacological actions. These include antimalarial and anticancer activity (Sarker et al., 2012). Some of the leading antimalarial drugs on the market are based on the artemesinin structure with the endoperoxy group being the active moiety.

Graph 2.1: Percentage viability of the malaria parasite at 40 µg/mL of fraction



*Refers to pure secondary metabolites

Sarker and Nahar (2012) reported that steroids can dimerise through formation of an endoperoxy group similar to that in artemesinin giving them the ability to act as antimalarials. They contend that this dimerisation can enhance, retain, or reduce the bioactivity. They also noted that new bioactive compounds with potent and reduced toxicity maybe produced as a result of dimerisation. In this instance 5α and 5β cholestane gem dihydroperoxides and tetraoxanes were synthesized and their antimalarial activity was studied (Todorovic et al, 1996; Sarker et al, 2012). The tetraoxane dimer shown in figure 2.12 alongside artemisinin with the endoperoxy groups highlighted, gave an IC_{50} value of 155nM compared to 8.6nM for artemesinin (Sarker et al., 2012).

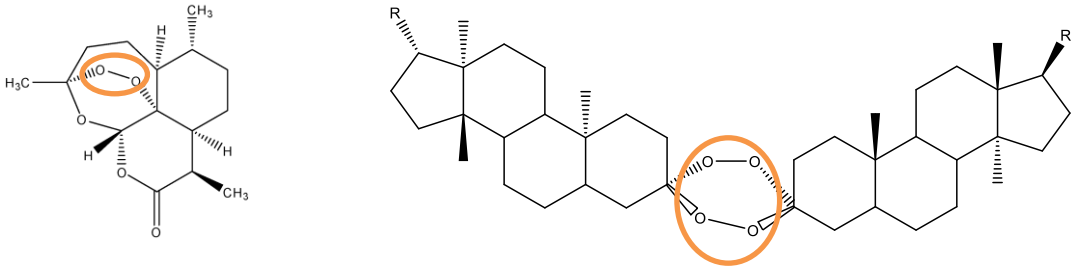
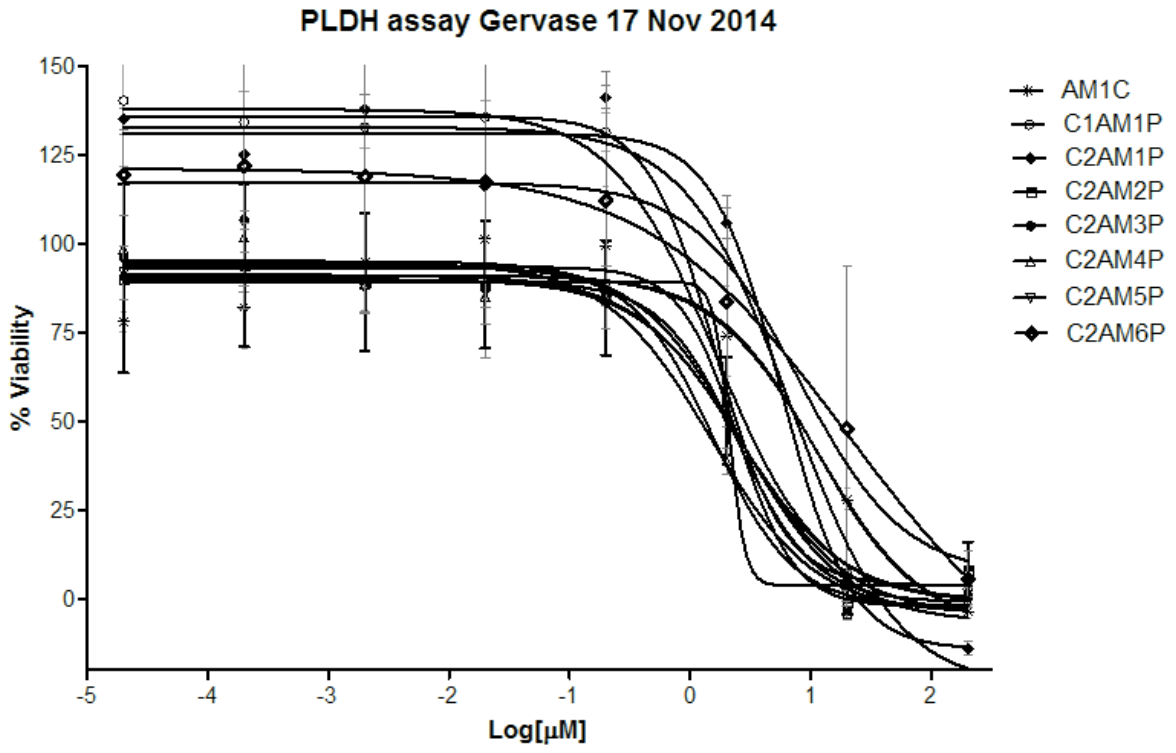


Figure 2.12: Artemesinin and a dimer of a steroid with endoperoxy groups shown

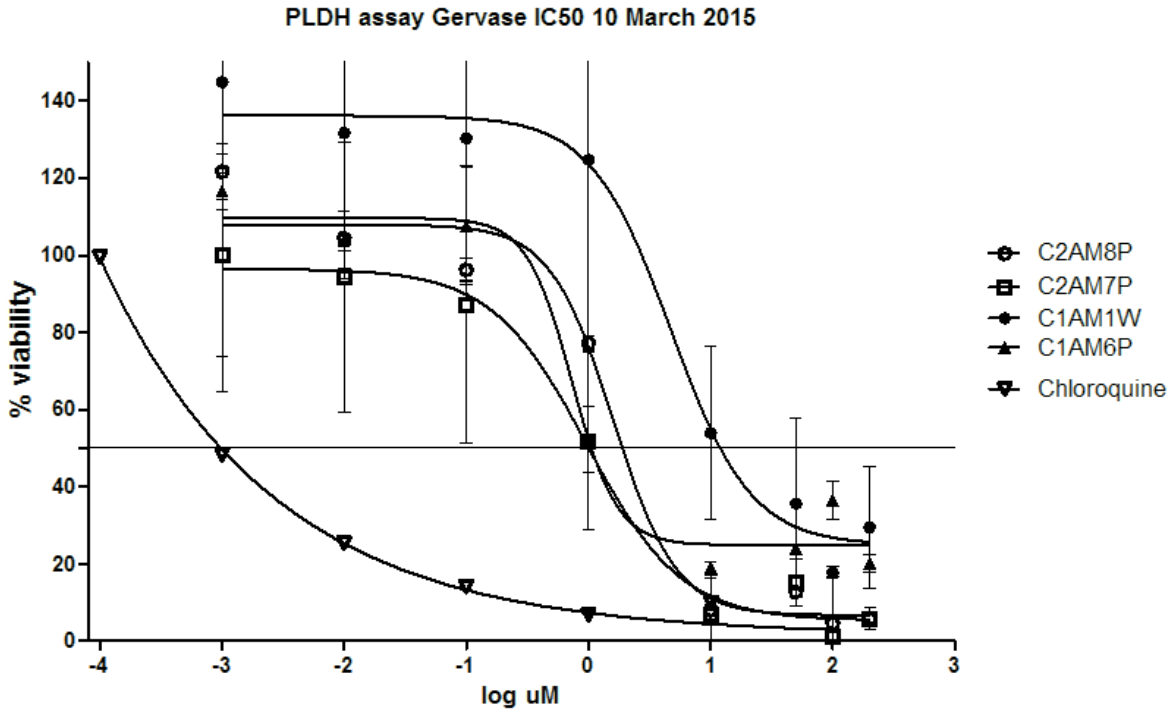
It is therefore not surprising that the secondary metabolites extracted from *A. mannii* showed some mild activity against malaria caused by *P. falciparum* 3D7 strains as they showed a positive result for the steroids test.

The best activity was from fraction **C2AM4P** giving a viability of 9.12 ± 2.33 % at a single concentration of 50 µg/mL. This fraction was obtained using a mobile phase varying between 3:1 and 3:2 of Hex/EtOAc (v/v). The fractions that showed promising viability were then used in an IC_{50} assay and the results are show in graph 2.2 (a, b). The standard used was chloroquine which is plotted in the graph 2.2 (b).

Graph 2.2: IC_{50} for the most active fractions and pure metabolites from *A. mannii*



(a)



(b)

The IC_{50} values ranged between 0.73 µg/mL and 20.23 µg/mL for the samples that were screened. Chloroquine has antiplasmodial IC_{50} values ranging between 10 – 50 nM against both chloroquine sensitive and chloroquine resistant strains while the IC_{50} range for artemesinin is between 5 – 15 nM for both chloroquine sensitive and and chloroquine resistant strains of *P. falciparum* (Sebisubi et al., 2010). According to literature, in order for an extract to be considered promising its IC_{50} value should be less than 10 µg/mL and less than 1 µg/mL for it to be notable. For pure substances to be promising the IC_{50} should be less than 1 µM (Sebisubi et al., 2010). From these literature values there is potential in further purifying and identifying the compounds responsible for antimalarial activity in the following fractions **C2AM7P**, **C1AM6P** as they had IC_{50} values below 1 µg/mL. The IC_{50} values of the different fractions are shown in table 2.7. **AM1C** had a moderate IC_{50} value of 11.21 µg/mL which is outside the promising range. However, it may be worth using it as a scaffold and introduce some hydroxyl groups on the side chain on C – 17 to produce compounds similar to the marine extracts shown in figure 2.4 to improve its activity (Sarker et al., 2012).

Table 2.7: IC_{50} values of fractions that showed promising antimalarial activity from *A. mannii* extracts

Sample code	IC_{50} (µg/mL)
AM1C	11.21
C1AM1P	1.62
C1AM1W	4.73
C1AM6P	0.73
C2AM1P	5.71
C2AM2P	~2.11
C2AM3P	2.48
C2AM4P	1.55
C2AM5P	2.49
C2AM6P	20.23
C2AM7P	0.97
C2AM8P	1.59
Chloroquine	0.002

2.5.2: Cytotoxicity

The data in table 2.8 shows that *A. mannii* crude extract was not cytotoxic, however several of the fractions obtained from the crude and some of the pure metabolites are cytotoxic. From the pure metabolites **AM1C** and **AM2C** recorded considerable cytotoxicity. A comparison of the pLDH and cytotoxicity assay shown in table 2.8 shows that the active fractions for the pLDH assays were also the most cytotoxic, which might imply that the metabolites are simply destroying all the cells. In the case of the fractions we decided to further purify the fractions so as to isolate the antimalarial active metabolite, hoping that activity was not due to synergy between the metabolites in the fraction, and then reassess the activity and cytotoxicity. Work in that regard is still on-going on fraction C2AM4P. The cytotoxic fractions have also been earmarked for cancer cell line screening as they may well have some anti-cancer activity since the cytotoxicity assay was run using cancerous HeLa cells. The cytotoxicity was measured against emetine as the standard.

Table 2:8: Percentage cell viability for the cytotoxicity and pLDH assay from *A. mannii* extracts

Sample	Cell toxicity		PLDH	
	Mean	SD	Mean	SD
AM	77.31	4.49	45.49	3.44
C2AM2P	123.00	4.88	30.28	1.04
AM1C	20.20	1.21	40.36	5.52
C2AM8P	109.20	9.78	63.09	2.70
C2AM7P	107.48	16.50	47.33	1.76
AM2C	30.35	6.46	89.02	4.71
C1AM1P	26.27	5.74	40.73	8.90
C2AM4P	20.00	2.67	9.11	2.33
C1AM2P	25.76	1.74	48.15	4.85
C1AM1W	91.52	5.89	49.62	7.11
C2AM6P	97.94	8.03	41.09	7.01
C2AM3P	7.14	0.16	22.86	1.04
AM3C	88.85	13.10	77.84	3.43
C1AM6P	93.51	15.42	50.90	4.95
C1AM3P	41.23	2.11	55.94	0.54
C2AM5P	54.12	3.77	43.84	3.06
C2AM1P	103.41	8.51	38.90	1.90
C1AM4P	60.19	6.58	22.13	5.22
C1AM5P	89.64	4.12	39.90	1.04

2.5.3: Microbial results

As mentioned earlier *A. mannii* is used traditionally for the treatment of some bacterial and fungal infections which include some sexually transmitted infections and skin inflammation (Djeuss et al., 2013). *A. mannii* was therefore, screened against some gram - negative and gram – positive bacteria as well as *Candida albicans* a fungus. The results from the screening are shown in table 2.9.

Unfortunately due to time constraints screening was not done using some of the fractions, except for the crude extract and the pure metabolites. Work is still in progress in that regard and also using other bacterial strains. The results obtained showed that some of the metabolites (**AM2C, AM7C, AM9C, AM2CP** and **AM3CP**) had notable activity against *S. typhi* and *C. albican*. The lowest activity was recorded against *S. aureus*. These results justify the use of *A. mannii* in treatment of some bacterial infections.

Table 2.9: MIC (in mg/mL) results from the microbial bioassays from *A. mannii* extracts

Samples	***E. coli***		***E faecalis***		***S. thyphimurium***		***S. aureus***		***C. albicans***		
	ATCC 8739		**ATCC 6230434**		**ATCC 1403**		**ATCC 25923**		**ATCC 10231**		
AM crude	4	4	4	4	2	2	4	4	>8	>8	
AM1C	0.25	0.25	0.13	0.13			>0.25	>0.25	0.25	0.25	0.06
AM2C	0.25	0.13	0.25	0.25	0.06	0.06	>0.25	>0.25	0.06	0.06	0.06
AM3C	0.13	0.13	0.25	0.25			>0.25	>0.25	0.06	0.06	0.06
AM4C	0.13	0.13	0.13	0.13			>0.25	>0.25	0.06	0.06	0.13
AM5C	0.13	0.25					>0.25	>0.25	0.06	0.25	0.06
AM6C	0.25	0.25	0.13	0.25			>0.25	>0.25	0.06	0.06	
AM7C	0.25	0.25	0.25	0.13	0.06	0.06	>0.25	>0.25	0.06	0.06	
AM9C	0.25	0.25	0.13	0.13	0.06	0.03	>0.25	>0.25	0.06	0.06	
AM9P	0.25	0.25	0.25	0.25			>0.25	>0.25	0.06	0.06	
AM2CP	0.25	0.25	0.06	0.13	0.03	0.03	>0.25	>0.25	0.06	0.06	
AM3CP	0.25	0.25	0.13	0.13	0.03	0.03	>0.25	>0.25	0.06	0.25	
3C5	0.25	0.25	0.25	0.25	0.25	0.25	>0.25	>0.25	0.03	0.06	
5C5	0.25	0.25	0.25	0.25			>0.25	>0.25	0.06	0.06	

2.54: Anti-mycobacterium assay

There has been no report of the use of *A. mannii* in the treatment of tuberculosis (TB) and no references have been found of its use in the ethno-medical treatment of TB. The results of the screens against TB are shown in Table 2.10.

Although some notable MIC_{90} values were obtained for **AM1C** and **AM7C** the values were much higher than those of the commercial drugs that are available on the market like isoniazid, rifampin and ethambutol which have MIC_{90} values in the range of 0.1 µg/mL to 10 µg/mL (Sullivan et al., 2006; Khare et al., 2013). There may be value in pursuing these metabolites further as one of the disadvantages of the available drugs is the length and quantity of drugs that are required for treatment (Yew et al., 2010). Since most treatments are greater than six months and a combination of drugs are used it may be worth checking whether these metabolites could treat TB in a shorter time and using only one drug.

Table 2.10: MIC (in µg/mL) results from the anti-mycobacterium assay of extracts from *A. mannii*

Sample	10pt DR: MIC_{90}/(µg/mL)	10pt DR: MIC_{99}/(µg/mL)
AM1C	39.4	47.6
AM2C	46.6	107
AM3C	96.5	> 125
AM4C	56.3	93
AM5C	91.9	> 125
AM6C	> 125	> 125
AM7C	40.7	51.5
AM1W	53.1	68.5
AM2W	54.1	82
AM3W	36.5	83.9
AM4W	> 125	> 125
1C42	> 125	> 125
1C54	> 125	> 125
1C69	72.3	83.8
2C26	74.5	105
2C38	88.6	> 125
2C59	43.6	55.7
3C5	48.1	70.8
3C31	> 125	> 125
3C102	> 125	> 125
C4AM3P	> 125	> 125
C4AM7P	> 125	> 125
4C31	> 125	> 125
14C5	> 125	> 125
20C5	> 125	> 125
AM9P	> 125	> 125
AMC crude	> 125	> 125

2.6 Conclusion and Future Work

The results that have been obtained this far show that *A. mannii* produces metabolites that could be of value in the treatment of malaria, bacterial and fungal infections. Although there was some *M. tuberculosis* activity the MIC_{90} values were above those of commercially available drugs. Only a few of the pure metabolites' structures were elucidated as the purity of the other metabolites needed further confirmation. Other metabolites are almost pure according to TLC although the NMR of the metabolites suggested that there were impurities and also the quantities obtained were too small to use open column chromatography.

Therefore, further purification needs to be done using LC – MS before these structures can be confirmed.

For the structures that have been elucidated, further confirmation needs to be done using high resolution mass spectrometry and also XRD to deduce the absolute stereochemistry of the stereogenic centres. For these structures there may be a need to carry out structure activity relationship studies to gain a full understanding of the mode of action of the metabolites. From then on semi-synthesis and introducing structural changes could be done to try and study the effect of these changes on the bioactivity of the new compounds.

As is evident this work is still in progress, a lot has been done but also more needs to be done. Further bioassays need to be carried out to assess the effect on these metabolites as potential anti – cancer drugs. A study of the mode of action of the cytotoxic fractions and metabolites to assess whether they are destroying the cells by apoptosis or necrosis as this would give insights on whether the metabolites are potential anti – cancer drugs. Also screening the metabolites against different types of cancer cell lines would be useful as previous studies have reported anti – cancer activity from fractions from *A. mannii.* Also studies on drug delivery would be ideal should some of the metabolites prove to have both *in – vivo* and *in – vitro* activity.

2.7 References

1. Habib, M. R., Nikkon. F., Rahman, M., Haque, M. E., Karim, M. R. **2007**. Isolation of Stigmasterol and β- sitosterol from methanolic extract of root bark of *Calotropis gigantean* (Linn). *Pakistan Journal of Biological Sciences. 10: 4174-4176*.
2. Jamal, A. K., Yaacob, W. A., Din, L. B. **2009**. A Chemical study on *Phyllanthus columnaris. European Journal of Scientific Research. 28: 76-81*.
3. Kandati, V., Govardhan, P., Reddy, C. S., Nath, A. R.,Reddy, R. R. **2012**. In-vitro and In-vivo anti-inflammatory activity of *Andrographis serpyllifolia. International Current Pharmaceutical Journal. 1: 199-204.*
4. Chemspider. http://www.chemspider.com/Chemical-Structure. Accessed 25/01/2016.

5. Moghaddam, F. M., Farimani, M. M., Salahvarzi, S., Amin, G. **2006**. Chemical constituents of dichloromethane extract of cultivated *Satureja khuzistanica*. Evidence-based complementary and alternative medicine. 4: 95-98.
6. Raju, V. H., Ganapaty, S., Prasanna, S. S., Vijaya, G. J., Kishore, P. S., Asif, A. K. **2012**. Phytochemical and pharmacological evaluation of *Tragia cannabina* for anti-inflammatory activity. *International Current Pharmaceutical Journal. 1: 213-216.*
7. Sarker, S.D., Nahar, L. **2012.** Steroid Dimers: Chemistry and Applications in Drug Design and Delivery. John Wiley and Sons. 623-626 online.
8. Díaz-Marrero, A. R., Dorta, E., Cueto, M., Rovirosa, J., San-Martín, A., Loyola, A and Darias, J. **2003(x).** New Polyhydroxylated Steroid from Marine Pulmonate *Trismusculus peruvianus.* ARKIVOC. 107-117.
9. Djeuss. D. E., Noumedem. J. A. K., Seukep. J. A., Fankman. A. G., Voukeng. I. K., Tankeo. S. B., Nkuete. A. H. L and Kuete. V. **2013.** Antibacterial activities of selected edible plant extracts against multi-drug resistant Gram-negative bacteria. *BMC Complementary Alternative Medicine.* 13. 164-169.
10. The LIPID MAPS Lipidomics Gateway, http://www.lipidmaps.org/ accessed 03/01/2016.
11. http://www.anabolicsteroids.net/types-of-steroids.php. **2013.** Types of Steroids. Accessed 08/01/2016.
12. Lupus News. **2010.** Researchers Uncover Biological Rationale for Why Intensive Lupus Treatment Works. http://www.lupusresearch.org/news-and-events/lupus-news/researchers-uncover.htmL. Accessed 11/01/2016.
13. http://www.chemspider.com/Chemical-Structure.2971022.htmL. Accessed 17/01/2016.
14. Sullivan. T. J., Truglio. J. J., Boyne. M. E., Novichenok. P and others. **2006.** High Affinity inhA Inhibitors with Activity against Drug – Resistant Strains of *Mycobacterium Tuberculosis*. *American Chemical Society Chemical Biology* 1 (1). 43-53.
15. Khare. G., Kumar. P., Tyagi. A. K. **2013.** High Affinity InhA Inhibitors with Activity against Drug-Resistant Strains of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 57 (12). 6372-6377.

16. Yew. W. W., Lange. C., Leung. C. C. **2011.** Treatment of Tuberculosis: update 2010. *European Respiratory Journal* 37 (2). 441 – 462.

CHAPTER 3: EXPERIMENTAL

3.1 General Information

Materials: The plant leaves were collected in bulk in Cameroon in identification was done by a botanist from the Herbier National da Cameroun in Yaounde. The voucher specimen, 45582 HNC, was also deposited at the same herbarium. Reagents for extraction and fractionation were used as supplied by Sigma-Aldrich without further purification. Thin layer chromatography was carried out using pre-coated Merck silica gel F254 plates and viewed under UV light (Spectroline ENF – 260C/FE) (254/365 nm)and at times following exposure to iodine. In other cases the TLC plates were sprayed with vanillin to view spots which were not visible under UV light. Column chromatography was carried out using Merck silica gel 60 (particle size 0.040-0.063 mm).

Instrumental: Melting points were determined using a Reichert 281313 hot-stage apparatus and were uncorrected. IR spectrum was recorded using a Perkin-Elmer FT-IR Spectrum 100 spectrometer operated in an attenuated total reflectance (ATR) mode. Mass spectra were recorded using an Advion mass expressions/CMC (CMS-4614-0269) coupled with a TLC – MS interface/CAMAG (201599), while high resolution mass spectra was recorded on a Waters API Q-TOF Ultima spectrometer (University of Stellenbosch, South Africa). NMR spectra were recorded using Biospin 600 MHz spectrometer using signal frequency of 150.9 MHz for C-13 and DEPT 135 while all the other experiments used a signal of 600 MHz and were referenced using residual protonated solvent signals (δ_H: 7.260 ppm for $CDCl_3$, 3.31 ppm for CD_3OD and 2.50 ppm for DMSO-d_6; δ_C: 77.000 ppm for $CDCl_3$, 49.00 ppm for CD_3OD and 39.50 ppm for DMSO-d_6). Elementary analysis was done using an Elementar Vario Microcube with a TCD detector, a combustion tube at 1150 ^oC, a reduction tube at 850 ^oC and gas setting pressures of Helium between 1200 – 1350 millibars and Oxygen at about 17 millibars.

Screening: The antimalarial screens for IC_{50} and pLDH were all carried out following standard procedures at Rhodes University. Cell cytotoxicity was also carried out under standard procedure using HeLa cells at Rhodes University. These assays were carried out using microdilution using the following concentration of metabolites: for crude sample and fractions 32 mg/mL and from 1 mg/mL for pure samples. Anti – tuberculosis screens were carried out under standard procedure at the University of Western Cape. Antimicrobial and

antifungal screens were carried out under standard procedure using 96 well plates and serial dilution method at University of Witwatersrand (Eloff., 1998). The standards used were water for the negative control, ciprofloxacin for the positive control and broth for the culture control. These were of concentrations of 32, 0.01 and 30 mg/mL respectively. The culture control (used to check microbe growth in culture), was prepared by mixing 30 g of Tryptone soya broth with one litre of purified water. The mixture was put in an autoclave at 121^0C for 45 minutes to sterilize it. It was then cooled down to room temperature. The presence of bacteria was checked using Iodonitrotetrozaluim chloride (INT) indicator.

3.2 Extraction of crude

The *A. mannii* sample that was used for the research was obtained from Cameroon and the details recorded at Herbier National da Cameroun (HNC) under voucher specimen 45582 HNC. The leaves were taken and treated air dried then stored.

A 274.6 g portion of the dried leaves was weighed and soaked at room temperature and pressure 1.6 litres of a 1:1 mixture of DCM/MeOH (v/v) in a closed vessel for 72 hours. After the 72 hours the plant material was filtered off using the normal Whatman filter paper under gravity. The filtrate which contained the extract was concentrated using a rotary evaporator. The residual plant material was treated again to a 1:1, DCM/MeOH extraction using a total volume of 1.1 litres and the filtration and concentration repeated. This was repeated for a third time and the crude extracts obtained were pooled together. A mixture of 1:1 DCM/MeOH was used to enable a range of metabolites of different polarities to be extracted.

The extraction protocol that is described in detail below is summarised in in figure 3.1 (a) and (b). After extracting, concentrating the extract and allowing all the solvent to evaporate and the crude extract to dry, the mass of crude extract was determined and found to be 13.514 g. Several TLCs were run with varying polarities for the crude extract dissolved in three different solvents to determine the metabolites in the extract. The crude extract was dissolved in:

1. Methanol
2. 1:1 DCM/MeOH and
3. DCM

The chromatograms that were obtained are shown in the figure 3.2.

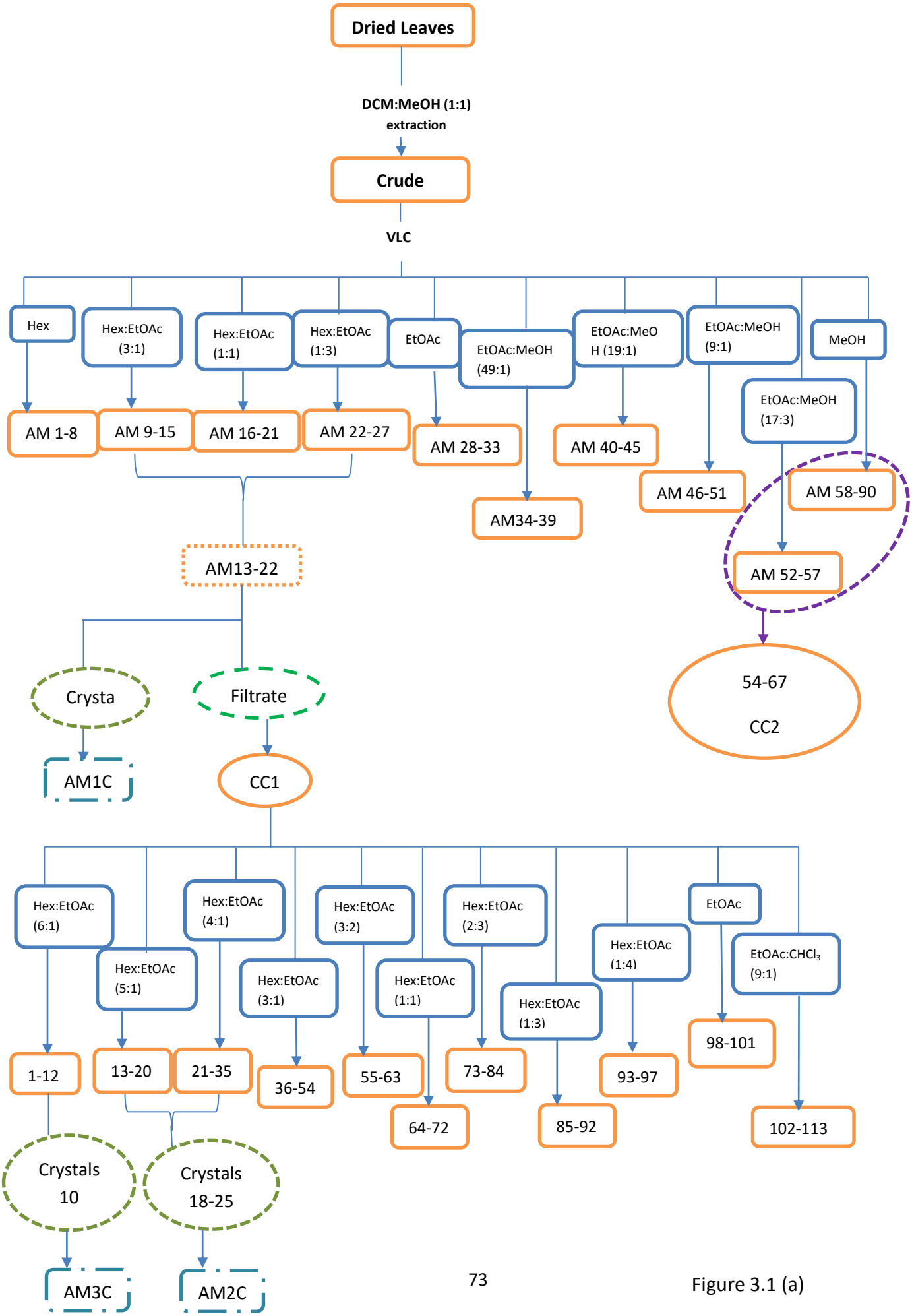

Dried Leaves
DCM:MeOH (1:1) extraction
Crude
VLC
Hex
Hex:EtOAc (3:1)
Hex:EtOAc (1:1)
Hex:EtOAc (1:3)
EtOAc
EtOAc:MeOH (49:1)
EtOAc:MeOH (19:1)
EtOAc:MeOH (9:1)
MeOH
EtOAc:MeOH (17:3)
AM 1-8
AM 9-15
AM 16-21
AM 22-27
AM 28-33
AM 40-45
AM 46-51
AM 58-90
AM34-39
AM 52-57
AM13-22
54-67
CC2
Crysta
Filtrate
AM1C
CC1
Hex:EtOAc (6:1)
Hex:EtOAc (5:1)
Hex:EtOAc (4:1)
Hex:EtOAc (3:1)
Hex:EtOAc (3:2)
Hex:EtOAc (2:3)
Hex:EtOAc (1:1)
Hex:EtOAc (1:3)
Hex:EtOAc (1:4)
EtOAc
EtOAc:CHCl3 (9:1)
1-12
13-20
21-35
36-54
55-63
64-72
73-84
85-92
93-97
98-101
102-113
Crystals 10
Crystals 18-25
AM3C
AM2C

Figure 3.1 (a)

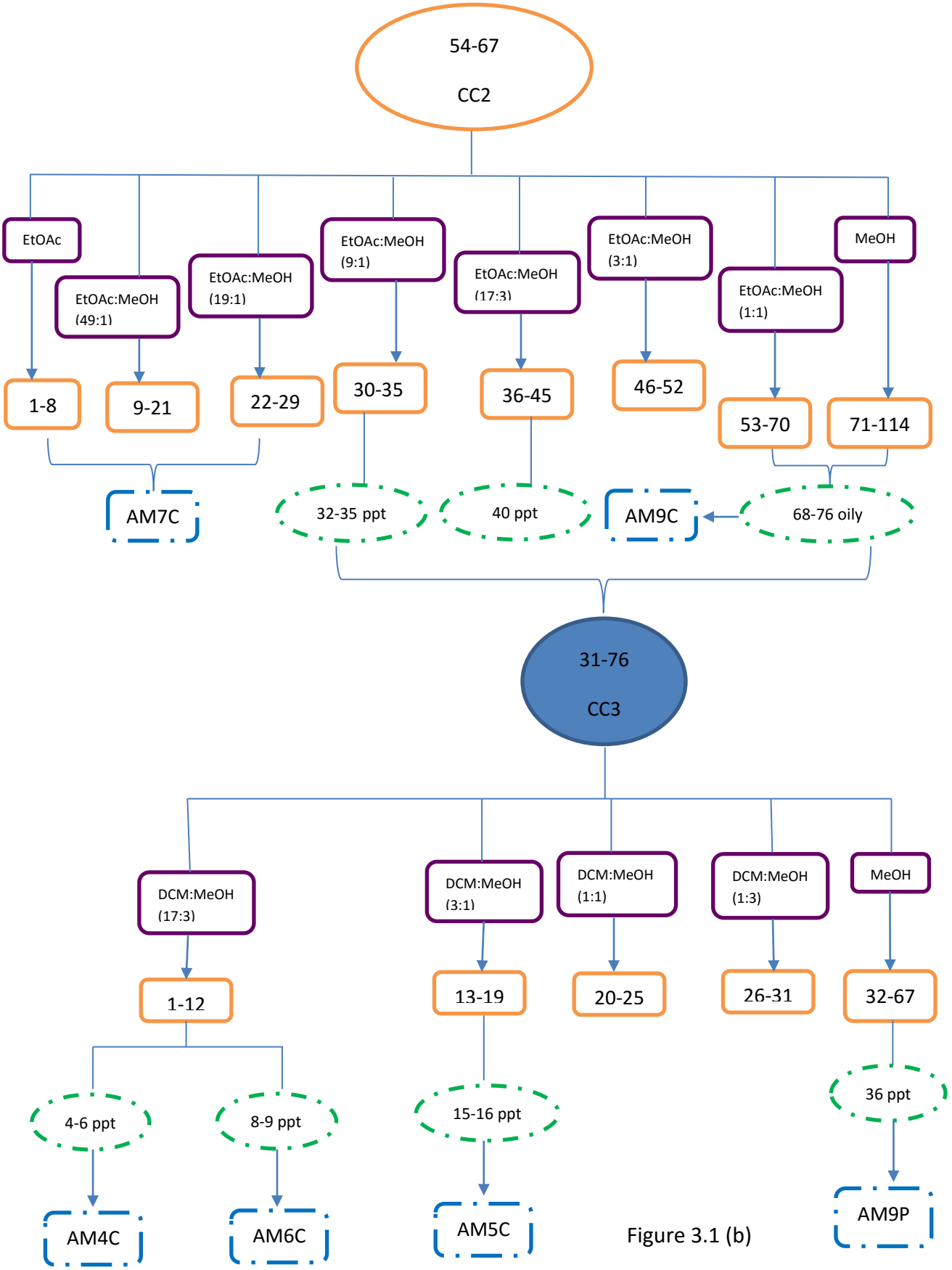


Figure 3.1 (b)

Figure 3.1 (a) and (b): Schematic diagram showing the extraction protocol and fractions

Figure 3.2: Chromatograms of crude extract dissolved in three different solvents using different mobile phase polarities

The TLC showed that there were several fractions of varying polarity which could be separated or fractionated by column chromatography. A portion of 12.92 grams of the crude extract was adsorbed on silica and the dry mixture loaded onto a vacuum liquid chromatography (VLC) column for fractionation. The remainder was kept for biological assays and for use as a standard. The picture in Figure 3.3 (a) shows the setup used for VLC, while (b) and (c) show some of the fractions obtained. In this setup a filter paper was placed at the bottom of the Buchner funnel and the funnel loaded with silica. Another filter paper was placed at the top of the silica with care being taken to ensure that the surface of the silica was level (horizontally flat) so that the height of silica was uniform at each level. The adsorbed crude extract was then loaded onto the VLC column and elution down beginning with 100% hexane and collecting fractions of 200mL until a colour change was observed.

After obtaining 8 fractions labelled AM1 to AM8, the polarity was changed to 75:25 hexane (Hex)/ethyl acetate (EtOAc) (v/v) and fractions AM9 to AM15 were collected. For this polarity the first 5 fractions were obtained after every 200 mL of mobile phase was collected and the rest were obtained after 300 mL this was to reduce the spreading of the same compounds in several fractions. After this the polarity was adjusted to 50:50 Hex/EtOAc and fractions collected every 300 mL of mobile phase, this led to fractions AM16 to AM21. The polarity was changed to 25:75 Hex/EtOAc and fractions AM22 to AM27 were collected. This was repeated at other mobile phase compositions as shown in figure 3.1. Each of the fractions obtained was concentrated on a rotary vapor and left to stand with observations

for formation of crystals being noted. AM 10, AM 12 to AM 23 formed white crystals. The different fractions were also checked using TLC to find out the level of purity of the fractions. The TLC were observed under UV light and where only one spot was obtained the chromatograms were sprayed with vanillin (a locating agent) to check for any spots that were not visible under UV light.

a b c

Figure 3.3: Images showing the VLC setup (a) and some of the fractions collected during VLC (b,c)

3.2.1: Separation and treatment of crystals

Since AM 12 to AM 23 formed crystals these fractions were taken and re-crystallisation done. The dry crystals were taken and dissolved in a small volume of 4:1 Hex/EtOAc (v/v) in sample viles and the top of each vile sealed with parafilm which had many small needle holes and left in the refrigerator to recrystallize. After re-crystallisation the excess solvent was carefully pipetted off using pasteur pipettes. After removing the solvent the crystals were left to dry and their solubility in different solvents was tested. It was found that the crystals were sparingly soluble in MeOH. MeOH was then used to wash the crystals several times to remove any other impurities that were in the crystals. The MeOH used to wash was carefully removed from each fraction using pasteur pipettes and collected in vials labelled with fraction name and MeOH wash. Once satisfied that the crystals were pure as shown by TLC, the proton NMR (^{1}HNMR) and carbon - 13 NMR were run for the different fractions to ascertain whether the crystals were of the same compound. The crystals which gave the same HNMR and C-13 NMR were pooled together if they also gave a spot on TLC with the same retention factor (R_f) value. The crystals were soluble in $CHCl_3$ and so the TLC was developed with samples dissolved in $CHCl_3$ and the NMR was also run in $CDCl_3$. The TLC of

AM13 to AM22 gave a spot with the same R_f value and the NMR spectra were also the same so these crystals were pooled together and gave the secondary metabolite **AM1C**. **AM1C** was left to dry, weighed, melting point determined, IR spectra run and a full set of NMR data including 1D and 2D obtained using $CDCl_3$ as solvent. The mass of **AM1C** was found to be 0.2619g. Also elementary analysis was carried out for **AM1C**. The pictures of the TLC in figure 3.4 were used as justification for pooling the samples together.

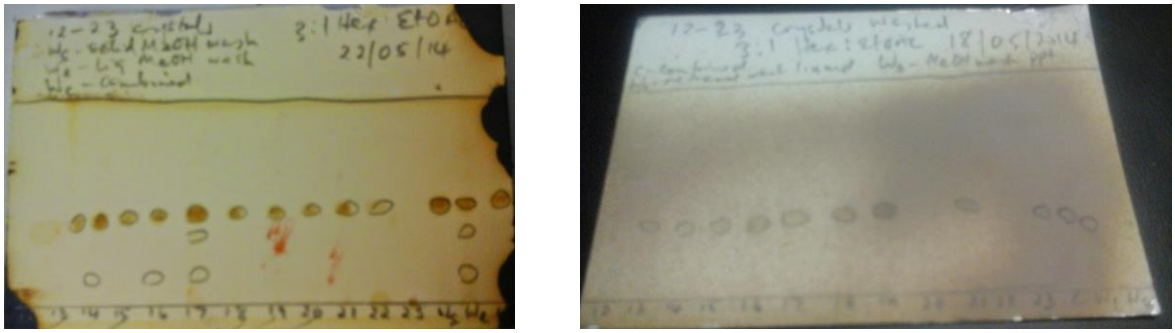
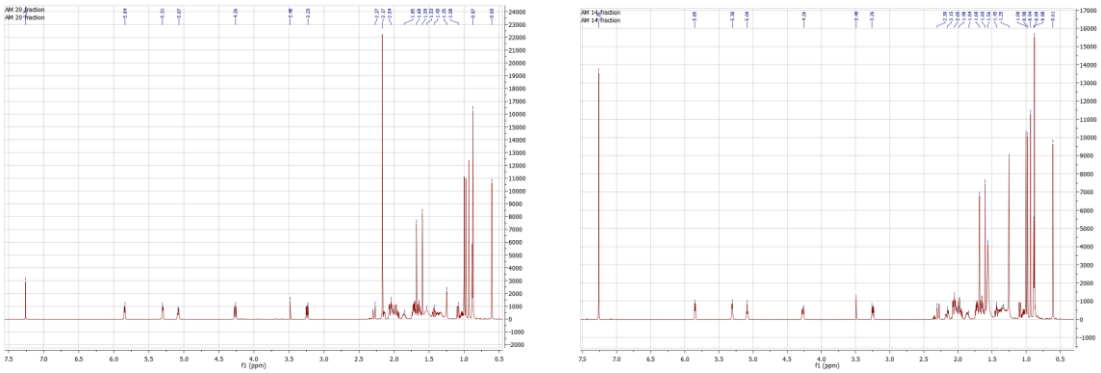


Figure 3.4: Pictures showing the TLCs for the crystals from AM13 - AM23 labelled as 13-23 and the solvents recovered during the washing of the crystals

The ^{1}HNMR in figure 3.5 were also used as justification for pooling the samples AM13-AM23 crystals together.



a b

Figure 3.5: ^{1}HNMR spectra for fractions AM20 (a) and AM14 (b) showing the similar peaks at similar chemical shifts

After removing the crystals TLC were run for the other fractions and the supernatant of the fractions that had crystals to ascertain which fractions could be pooled together. The bioactivity of the pooled fractions was also determined using the pLDH assay described in

section 3.3. The TLC in figure 3.6 was used as justification for pooling AM9 - AM23 after removing crystals and then using this mixture for fractionation in an open column chromatography. This mixture was also further fractionated as the pLDH assay showed activity against malaria.

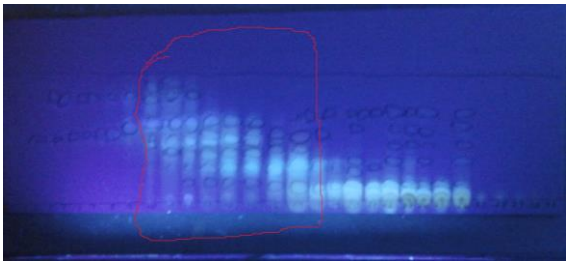


Figure 3.6: TLC for fractions from VLC with crystals removed as viewed under UV light

The mass of the pooled fraction was 2.2976 g. A small amount of this was kept for reference and for biological assays; the rest was dissolved in $CHCl_3$ and adsorbed on silica before loading on to the column. Before elution was started several TLCs with different solvent polarities were run to determine the mobile phase mixture to use initially. An initial mobile phase of 5:1 Hex/EtOAc (v/v) was selected from the TLC analysis.

Since the fraction being processed had fewer spots the mobile phase was collected at 100 cm^3 per portion. After the colour of the extracted portion cleared a molar polar mobile phase was used. The fractions obtained for each mobile phase were as shown in Figure 3.1 (a). Figure 3.7 shows the open column that was used and some of the fractions that were collected. Fraction 10 produced white crystals while fractions 18 -25 also produced white crystals.

Figure 3.7: Pictures of the column used and some of the fractions obtained

The crystals obtained from fraction 10 were washed with methanol, while those obtained from fractions 18 - 25 were washed with acetone as they were not very soluble in these solvents. After drying and considering the TLC for the crystals dissolved in $CHCl_3$ it was seen that fractions 18 - 25 gave one spot with same R_f value so these were pooled together and gave **AM2C** a pure metabolite. The mass of dried white powder of **AM2C** was found to be 0.1427 g. A set of 1 D and 2D NMR using $CDCl_3$ as solvent, IR, elementary analysis and mass spectra were run for **AM2C** for use in structure elucidation. Some samples were used for elementary analysis and biological assays. The dried mass of fraction 10 gave a different R_f value from that of **AM2C** and **AM1C** and also gave one spot on TLC as viewed both under UV light and after spraying with vanillin. This was named **AM3C** another pure metabolite. The mass of **AM3C** was determined and found to be 0.045 g. A set of 1 D and 2D NMR using $CDCl_3$ as solvent, IR, elementary analysis and mass spectra were run for **AM3C** for use in structure elucidation. Some samples were used for elementary analysis and biological assays. Figure 3.8 shows the TLC for fractions 18 to 24 which also show the rationale of why they were pooled together after washing them with acetone.

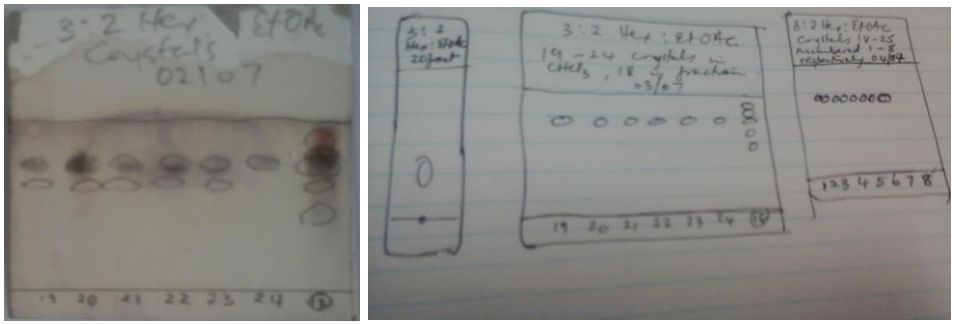


Figure 3.8: TLCs for crystals obtained from various fractions

TLC was run for the fractions from this column and for the fractions 10, and 18-24 after the crystals were removed. From the fractions of the VLC fractions 54 to 67 which gave only one spot visible under UV light were pooled and adsorbed on silica gel for further purification in an open CC as shown in figure 3.1 (b). These fractions gave a combined mass of 3.6719 g. Since these fractions were eluted using 85:15 EtOAc/MeOH (v/v) and 100 % MeOH, the initial eluting solvent was 100 % EtOAc and fractions were collected after every 100 mL of mobile phase was produced. The fractions collected for each mobile phase were as shown in figure 3.1 (b). Fractions 32 to 35 and fraction 40 produced red-brown precipitates.

The fractions obtained were monitored by TLC. The precipitates produced between fractions 32-35 and 40 produced several spots on TLC. Several TLCs were also run for all the fractions to monitor the purity. After a careful study of the TLCs some fractions were combined as shown in table 3.1 and comments made on the mixtures and TLCs run for the mixtures. When the fractions were mixed they were dissolved in $CHCl_3$ to ensure homogenous mixing. As the solvent evaporated observations were made and recorded.

The oily solid formed from combined fractions 61-68 and 69-76 were filtered off and allowed to dry. The dry solid was checked using TLC and found to give only one spot and this was labelled **AM9C**. TLCs for the other combined fractions where precipitation occurred showed that the precipitates were almost pure. These precipitates were taken and washed with hexane first then ethyl acetate to remove any impurities. The precipitates were dissolved in MeOH and TLCs run to check purity.

Table 3.1: Fractions from the column that were combined and observations that were made

Fractions combined	Comments
1-8	
9-19	
20-30	
31-33	precipitation occurred
34-37	
38-39	precipitation occurred
40-41	precipitation occurred
42-48	
49-50	precipitation occurred
51-53	
54-55	precipitation occurred
56-60	precipitation occurred
61-68	oily solid formed
69-76	oily solid formed
77-93	precipitation occurred
94-101	
102-114	

The purity of some of these mixed fractions was checked using HPLC. The fractions were labeled with AM1Pp in front of the fraction that had precipitated. 10 µg were dissolved in 1 mL of HPLC grade MeOH as samples for the reverse phase HPLC and the chromatogram run using a kinetex C18 analytical column under isocratic conditions. The mobile phase was 80:20 acetonitrile/methanol (v/v) with 0.1 % trifluoroacetic acid. The flow rate of the mobile phase was 1 mL/min. Each time 20 µL sample was injected. A diode array detector (DAD) single wavelength acquisition was used. It acquired the signal at a wavelength of 210 nm

and used a reference at a wavelength of 360nm. Some of the chromatograms obtained are shown in figure 3.9; these showed that the precipitates were not pure.

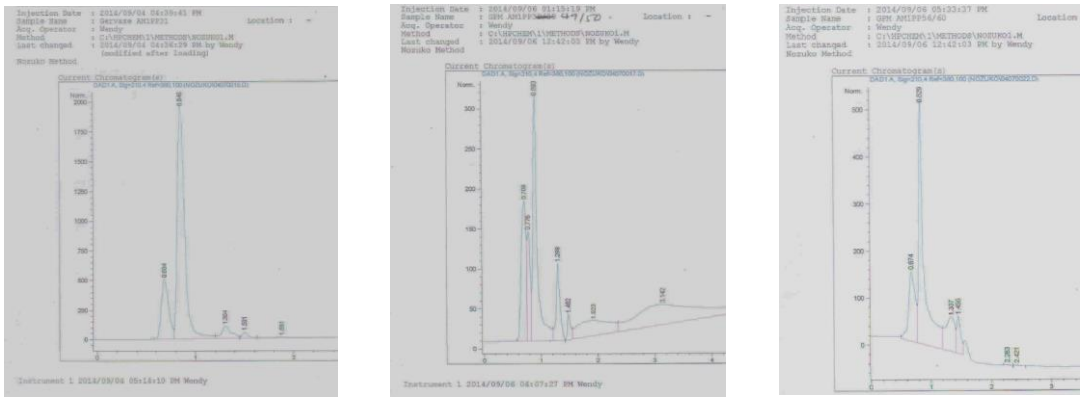


Figure 3.9: HPLC chromatograms obtained from samples.

All the fractions from 31 to 76 were combined and adsorbed on silica using MeOH as solvent to try and separate the two major fractions that showed in the HPLC chromatograms, the one with a retention time of 0.85 minutes and the other with a retention time of 0.68 minutes. The other pure compound expected to be isolated had a retention time of 1.30 minutes. The mass of the combined fractions was 1.8490 g. The TLC showed that beginning from a mobile phase of 15:1 DCM/MeOH (v/v) would be ideal and so that was the initial mobile phase used. Fractions were collected in 150 mL portions for this column. The fractions collected for the different mobile phases are as shown in figure 3.1 (b). Fractions 4 to 6 produced a yellow precipitate, fractions 8 to 9 a red – brown precipitate while fraction 36 produced a brown precipitate.

The precipitates/crystals were separated from solvent, allowed to dry and their purity checked using TLC. The crystals were washed with EtOAc to remove impurities. The TLC for 4-5 showed that the fractions had a pure compound with same R_f value and this was labelled compound **AM4C**, the TLC for 8 and 9 showed another pure compound with same R_f and this was called compound **AM6C** while fractions from 15 and 16 gave a single spot with same R_f value and that gave compound **AM5C**. **AM4C**, **AM5C** and **AM6C** were dissolved in MeOH and NMR run. Elementary analysis, IR and melting points were determined for the sample. After removing the precipitates/crystals and using TLC for guidance fractions were

combined. Table 3.2 shows the codes given to all the combined fractions as they were used in biossays under these codes.

Table 3.2: Codes for combined fractions.

Code	Fractions	Original column
C1AM1W	1-7	VLC
C1AM1P	9-23	VLC
C1AM2P	24-31	VLC
C1AM3P	32-41	VLC
C1AM4P	42-53	VLC
C1AM5P	54-68	VLC
C2AM1P	4-9	1st open CC
C2AM2P	11-17	1st open CC
C2AM3P	26-37	1st open CC
C2AM4P	38-57	1st open CC
C2AM5P	59-67	1st open CC
C2AM6P	69-83	1st open CC
C2AM7P	84-95	1st open CC
C2AM8P	96-108	1st open CC
C2AM9P	113-114	1st open CC
C3AM1P	1-8	2nd open CC
C3AM2P	9-19	2nd open CC
C3AM3P	21-30	2nd open CC
C3AM4P	77-93	2nd open CC
C3AM5P	94-101	2nd open CC
C3AM6P	102-114	2nd open CC
C3AM7P	EtOAc wash of 54-55	2nd open CC
C3AM8P	EtOAc wash 38-39	2nd open CC
C4AM1P	1-10	3rd open CC

C4AM2P	14-19	3rd open CC
C4AM3P	21-25	3rd open CC
C4AM4P	26-31	3rd open CC
C4AM5P	32-39	3rd open CC
C4AM6P	40-49	3rd open CC
C4AM7P	50-60	3rd open CC
C4AM8P	61-67	3rd open CC
$^{4}C_{5}$	4	4th open CC
$^{5}C_{5}$	5	4th open CC
$^{6}C_{5}$	6	4th open CC

The antimalarial screening (pLDH assay) results (Table 2.6) showed that C2AM4P had the best activity and was also cytotoxic. A TLC of the fraction was run to check the purity so that this could be further purified. 3.461 g of C2AM4P were adsorbed on silica using $CHCl_3$ as the solvent and loaded onto an open column and this was eluted originally with 15:1 DCM/MeOH (v/v) as shown by the TLC. The column broke during the elutions and so only a few fractions were obtained and the rest of the sample recovered by washing broken column using MeOH. The fractions obtained are shown in figure 3.1 (a).

The crystals from 4-6 were washed with EtOAc and so were the other solids obtained. The purity of crystals was checked using TLC. The precipitate fraction 4 gave **AM8C** a pure compound. **AM7C** was a mystery as these white pure crystals were obtained in the vials which had fractions and had been emptied and ready for washing, so they could not quite be placed when they were formed.

3.3 Bioassays

3.3.1 Antimalarial assays and cell cytotoxicity

Anonidium mannii has been used traditionally for treatment of malaria, cancer and skin inflammation among other uses (Djeuss., 2013). Table 3.3 shows the fractions and pure

compounds that were screened for antimalarial properties. The codes for the fractions and pure metabolites are identified in Table 3.2.

Table 3.3: Fractions and compounds that were used in antimalarial, pLDH, IC_{50}, and cytotoxicity screening

Sample name	**Formula**	**concentration**	**Volume prepared (μL)**
AM1C	$C_{30}H_{48}O_2$	20mM	90
AM2C	$C_{27}H_{42}O_2$	20mM	100
AM3C	$C_{29}H_{48}O$	20mM	40
AM	crude	0.5mg/10 μL	70
C1AM1W	fraction	0.5mg/10 μL	30
C1AM1P	fraction	0.5mg/10 μL	86
C1AM2P	fraction	0.5mg/10 μL	26
C1AM3P	fraction	0.5mg/10 μL	22
C1AM4P	fraction	0.5mg/10 μL	32
C1AM5P	fraction	0.5mg/10 μL	36
C1AM6P	fraction	0.5mg/10 μL	86
C2AM1P	fraction	0.5mg/10 μL	54
C2AM2P	fraction	0.5mg/10 μL	22
C2AM3P	fraction	0.5mg/10 μL	60
C2AM4P	fraction	0.5mg/10 μL	44
C2AM5P	fraction	0.5mg/10 μL	32
C2AM6P	fraction	0.5mg/10 μL	22
C2AM7P	fraction	0.5mg/10 μL	46
C2AM8P	fraction	0.5mg/10 μL	56

In the pLDH assay the extracted compounds, crude, fractions and pure were added to parasite cultures in a 96 - well plates and incubated for 48 hours in a 37 ^{o}C carbon dioxide incubator. For these wells 20 µL of culture were removed from each well and mixed with 125 µL of a mixture of Malstat solution and Nitro blue tetrazolium (NBT)/phenazine ethosulphate(PES) in a fresh 96 - well plate. NBT/PES solution was prepared by dissolving 160 mg NBT and 8 mg PES in 100 mL of water and the solution covered with foil as it is light sensitive. This solution measures the activity of pLDH enzymes in culture, when pLDH is present the colour will be purple. Quantification was done using a 96 - well plate reader by measuring absorbance at 620 nm. The absorbance obtained is related to the activity of the pLDH and to the number of parasites that are in the well.

Preparing culture medium.

Medium composition:

500 mL RPMI 1640 with Hepes and glutamine

2 g Glucose

2.5 g Albumax II

0.044 g Hypoxanthine

0.5 mL Gentamicin solution

A 2.5 g Albumax II and 2 g glucose portion were added to a 50 mL plastic centrifuge tube. In the laminar flow hood, 50 mL of the RPMI 1640 medium was added into the tube and the Albumax and glucose dissolved. Then 44 mg hypoxanthine were weighed out separately into a microfuge tube and dissolved by adding 1 mL of 0.1 N NaOH. The hypoxanthine solution was then added to the Albumax/glucose medium solution in the 50 mL tube. A 0.3 mL portion of the Gentamicin solution was then added to the 50 mL tube. The solution in the 50 mL tube was then sterilized by filtration – in the laminar flow hood, the solution was pushed through a 0.2 µm syringe filter (using a 50 mL syringe) into a fresh sterile 50 mL tube. The filtered solution is then added back into the bottle containing the 500 mL RPMI 1640 medium. The medium was stored in a fridge at 4 ˚C.

Preparing human red blood cells.

The human blood was drawn into 4 – 5 blood collection vials (with EDTA), after it was obtained from a blood bank. The blood group was irrelevant; however, care was taken to ensure that the blood donor was not on antibiotics or antimalarial medication at all.

In the laminar flow hood, the blood was poured into a sterile 50 mL centrifuge tube and centrifuged in a swing-out rotor at 2500 rpm for 4 - 5 minutes (the brake on the centrifuge was set to low) to pellet the red blood cells. Using a glass Pasteur pipette attached to a vacuum pump and liquid trap, the serum supernatant from the pelleted red blood cells, as well as a small amount of the top layer of the pelleted cells were aspirated off (this included the "buffy coat" of white blood cells). The tube was filled with cold culture medium and the red blood cells were re-suspended in the medium by pipetting up-and-down. The centrifugation and aspiration of the supernatant and buffy coat was repeated. The pelleted red blood cells were stored at 4 ^{o}C.

Summarised culturing and transfection procedure

Culturing malaria parasites

The malaria parasites, *P. falciparum* strain 3D7, that were used in the assay were maintained in RPMI 1640 medium that contained 2 mM L-glutamine and 25 mM Hepes (Lonza). The medium was further supplemented with 5 % Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 µg/mL gentamycin and 2-4 % hematocrit human red blood cells. The parasites were cultured at 37 ^{o}C under an atmosphere of 5 % CO_2, 5 % O_2, 90 % N_2 in sealed T75 culture flasks. Parasitaemia which is the concentration of parasites in the culture was assessed by light microscopy of Giemsa-stained thin blood smears. Giemsa-stained thin-blood smears were prepared as follows: a small amount of red blood cells from the culture was smeared onto a glass microscope slide, stained with a Giemsa solution and viewed with a light microscope. Figure 3.10 shows different stages of parasite development as they would appear under a microscope (Shahinas., 2013).

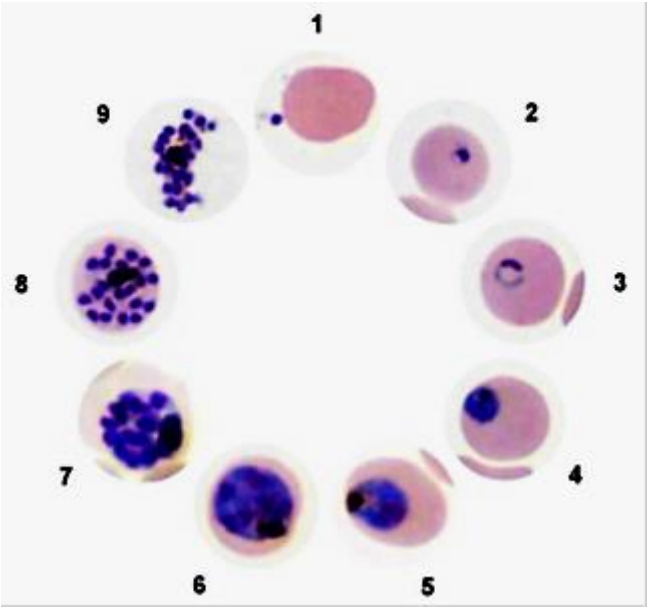


Figure 3.10: Micrographs of the various developmental stages of blood-stage P. falciparum parasites

Giemsa-stained thin-blood smears were prepared from an *in vitro* culture over 48 hours and photographed at 1000 x magnification. Sequential parasite developmental stages are presented as numbered from 1-8: 1 – merozoite invasion of a RBC, 2 – newly invaded RBC, 3 – ring, 4 – early trophozoite, 5 – late trophozoite, 6 – early schizont, 7 – schizont, 8 – mature schizont, 9 – ruptured schizont. The parasites are stained purple. The parasites are inside red blood cells, which are stained a light purple-brown. Stage 3 (the ring stage) is predominantly seen in the first 24 h of culturing, and stages 4-7 (trophozoites and schizonts) in the next 24 h.

When cultures were maintained at relatively high parasitaemia (>1 %), the culture medium was replaced every day, and red blood cells were replenished every second day. The life-cycles of the parasites were regularly synchronized by treatment with 5 % sorbitol.

Transfecting malaria parasites

This was achieved by a red blood cell (originally uninfected) preloading electroporation protocol. Approximately 75 µg of purified malaria expression plasmid DNA (previously obtained using a Maxiprep kit) was precipitated with ethanol and re-dissolved in 25 mM Hepes buffer solution. Red blood cells infected with trophozoite/schizont-stage parasites were enriched from a malaria culture by Percoll density centrifugation and placed back into

culture medium. Fresh uninfected red blood cells were mixed with cytomix buffer and the purified plasmid in a 0.2 cm gap electroporation cuvette and subjected to two consecutive electroporations at 310 V, 950 µF. The electroporated red blood cells were combined with the culture medium that contained the enriched parasite-infected red blood cells and incubated for 3 days with daily medium changes and replenished with fresh red blood cells. The medium was replaced with medium containing 2.5 nM WR99210 (selection drug) and incubation was continued in a shaking incubator. The medium was replaced regularly in the first week, and then less regularly until transfected (WR99210-resistant) parasites appeared.

Culturing mammalian cells

Mammalian cells were cultured in a 5 % CO_2 incubator at 37 ^{o}C in DMEM medium supplemented with 10 % foetal bovine serum and antibiotics (penicillin/streptomycin/fungizone). For routine culturing, the cells were split every 3 to 5 days (when the cells had reached close to full confluency): the cells were detached from the culture flask surface using trypsin/EDTA, and the majority aspirated off. Medium was added to the flask and the remainder of the cells, and the flask returned to incubation. The cells in the medium were diluted to a parasitaemia of 6.7 x 10^4 cells/mL for use in the 96 – well plates. A parasite density of 1 x 10^4 cells/well was used for the assay. The confluency and state of the cells was regularly assessed using an inverted light microscope. Cells were cryopreserved by detaching the cells from the culture flask in trypsin/EDTA, pelleting the cells, transferring them to cryotubes in 10 % DMSO in foetal bovine serum, and placing the tubes in a -80 °C freezer.

Transfecting mammalian cells

Mammalian cells were plated into 96 - well plates, and incubated overnight. Plasmid DNA and FuGENE 6 transfection reagent was mixed in medium without serum/antibiotics in a 3:1 ratio (volume FuGENE:amount of DNA), and added to the cells in the plates, 0.15 µg plasmid DNA/well for 96 - well plates. After an overnight incubation, the cells were ready to be analysed.

Antimalarial assay

The extracts were first added as a single concentration in the 96 - well plate and incubated for 48 hours to determine whether they had any antimalarial activity at all. The most promising extracts from this assay were then used in a dose- response assay so as to determine the IC_{50} concentration of the compounds. This is the concentration of compound that is required to kill 50% of the parasite, or to inhibit 50% of the parasite.

For the single concentration assay the samples were prepared as follows: for extracts stock solutions of 20 mg/mL were used whereas for pure compounds 20 mM/mL were used. These were dissolved in DMSO and stored at -20 ^{o}C. Chloroquine was used as a reference and its concentration was 20 mM/mL. The pure compounds were then diluted in a ratio of 1:1000 in culture medium to give a concentration of 20 µM. This was achieved by adding 1 mL medium to 1 µL compound from stock solution followed with mixing by vortex. For extracts the dilution was 1:200 to obtain solutions of concentration 100 µg/mL, this was achieved by adding 5 µL from extract stock to 1 mL culture medium in a sterile microfuge tube. The dilutions were then mixed in a ratio of 50:50 with parasite cultures to give final concentrations of 10 µM and 50 µg/mL for use in the screens. The chloroquine reference was diluted 1:10000 by adding 1 µL of the stock solution to 10 mL of medium to produce a 2 µM solution.

In the 96 - well tissue culture plate 100 µL of the diluted compound were pipetted into wells of the plate and the lid of the plate replaced. Each compound was tested in duplicate wells. The first column in the 96 - well plate was dedicated for controls and these were filled with 100 µL medium only. Wells A2 and B2 were used for reference. The plate appeared as shown in figure 3.11.

	1	2	3	4	5	etc.						
A	Positive	CQ	AM									
B	Positive	CQ	AM									
C	Positive	AM1C	C1AM1P									
D	Positive	AM1C	C1AM1P									
E	Backgrnd	AM2C	etc									
F	Backgrnd	AM2C										
G	Backgrnd	AM3C										
H	Backgrnd	AM3C										

Figure 3.11: Sample 96 well - plate showing different metabolites

Positive refers to the positive control wells which only had 100 µL medium with no drug, backgrnd refers to background control wells which had 100 µL medium alone and CQ represents chloroquine and the numbers refer to wells with test compounds or extracts. The parasite was prepared using the following protocol: Parasite was removed from the T75 flask and the parasite infected red - blood cells (pRBC) were made into pellets by centrifugation at 2000 rpm for 5 minutes. The supernatant was removed and the Giemsa-stained smear was prepared from the pellet. The percentage parasitaemia (% P) was determined. A 2 % haematocrit and a 2 % parasitaemia (2 % H, 2 % P) suspension were prepared in the medium solution. This was achieved by adding 10 mL medium to 50 mL sterile centrifuge tube, then adding pRBC and fresh red-blood cells (RBC) to the medium using the following volumes as calculated from % P: volume of pRBC (V_{pRBC}) added = 2/% P*200 µL and volume of fresh RBC added = 200 µL - V_{pRBC}. To each well which already contained test compound or extract dilution except wells E1 to H1 (the background wells) in the 96 - well plate 100 µL of 2 % H, 2 % P culture were added. A 2 % H fresh RBC suspension was prepared and 0.5 mL of this added per well. To a sterile microfuge tube 1 mL of culture medium and 20 µL fresh RBC were added. A 100 µL aliquot of this was added to each of the wells from E1 to H1. The plate was then incubated in a CO_2 incubator for 48 hours. In order to reduce evaporation of the medium the plate was placed in a tupperware container that had a beaker of water and a hole in the side to allow for gas exchange.

The following procedure was then followed to run the pLDH assay: After a 48 hour incubation, the 96 - well plate containing pLDH reagent was was prepared by mixing a 10 mL portion of Malstat solution and 2.5 mL NBT/PES solution in a tube. A 125 µL aliquot of this mixture was then transferred to each well in the 96 - well plates. The plate with parasite culture and test compounds was removed from the incubator. The RBC was re-suspended in each well by pipetting up and down with a multichannel pipette to ensure thorough mixing. Using a multichannel pipette 20 µL of the RBC was transferred to the plate containing pLDH reagent. Air bubbles were removed by blowing over the plate with a hair dryer. The plate was then placed in the dark for between 10 minutes to 1 hour to allow the purple colour to develop.

The absorbance of each well was measured at a wavelength of 620 nm in a multi-well plate reader. The expected or ideal absorbance for the control wells A1 to D1 was between 0.6

and 0.9, and these readings are meant to be 3 fold higher than those for the background control wells E1 to H1. From these Abs_{620} the pLDH assay calculation and processing was carried out as follows:

1. The average Abs_{620} of the background wells (E1-H1) was determined = $Abs_{backgrnd}$.
2. The $Abs_{backgrnd}$ was subtracted from the absorbance of the wells with test compounds/extracts = $Abs_{test\ compound}$.
3. The average absorbance of the positive control wells (A1-D1) was obtained = $Abs_{positive}$.
4. The percentage viability of the parasite was calculated using the formula: $Abs_{test\ compound}/Abs_{positive}*100$.
5. The average percentage parasite viability and standard deviation for each test compound were obtained.

The expected percentage viability of chloroquine from wells A2 and B2 should be less than 10 %. For pure compounds if the percentage parasite viability is less than 20 % then the compound is worth considering while a parasite viability of less than 40 % for fractions or impure extracts may be worth considering.

The compounds and fractions that reduced parasite viability significantly from the single concentration assay were then used in the dose response assay to determine IC_{50} values. The approach used was similar to the one used for single concentration screening but with three-fold dilutions of test compounds. The plate lay out and compound preparations were therefore different and as outlined below.

A 1:100 dilution of each test compound solution in culture was prepared in sterile microfuge to obtain either 200 µM or 200 µg/mL solutions from pure compounds or fractions. A 100 µL aliquot of culture medium was pipetted into all the wells in a laminar flow hood, row 2 was left out. In row 2, 150 µL of test compounds prepared above were pipetted. Each test compound was pipetted into 2 wells e.g **AM1C** in well A2 and B2; **C1AM1P** in well C2 and D2 and so on. A multichannel pipette was then used to pipette 50 µL from wells in column 2 and transfer these into column 3 and mixed thoroughly by pipetting up and down in wells in column 3. A 50 µL aliquot is pipetted from wells in column 3 and transferred to column 4 and thoroughly mixed. These serial dilutions were repeated up to wells in column 12. This

lay out meant only 4 test compounds could be assayed per plate. The plate layout looked like figure 3.12.

The concentration of compounds in wells in column 2 was 200 µM while those in column 3 were 66.7 µM and that in column 4 being 22.2 µM. These concentrations were halved when the parasite culture was added to the wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive	AM1C										
B	Positive	AM1C										
C	Positive	C1AM1P										
D	Positive	C1AM1P										
E	Backgrnd	C2AM1P										
F	Backgrnd	C2AM1P										
G	Backgrnd	C2AM2P										
H	Backgrnd	C2AM2P										

Figure 3.12: Appearance of 96 – well plate

Chloroquine was used as a standard reference compound. This was prepared by a 1: 10 000 dilution of the compound stock to obtain a 2 µM dilution. A 150 µL aliquot was pipetted into 2 wells in column 2 and similar serial dilutions as those used for test compounds used to fill the other columns. The parasite culture was prepared as described for the single concentration screen.

3.3.2 Cytotoxicity assay

All the samples that were used in the antimalarial assay were also used in the cytotoxicity assay. For the cytotoxicity assay we used the HeLa cells (Keusch., 1972). The cells were cultured in 10 mL of Dulbecco's modified Eagle's medium (DMEM) which had 5 mM L-glutamine supplemented with 10% fetal bovine serum (FBS) and antibiotics e.g. penicillin or streptomycin. The cells were incubated overnight in a 5% CO_2 incubator with the temperature set at 37°C. On the next day, they were ready for the toxicity assay.

A 150 µL aliquot of suspension with 6.7 x 10^4 cells / mL was transferred into each well of a 96 well plate, giving an approximate concentration of 1 x 10^4 cells per well. After incubating the plate overnight, the cells were treated with a 100 µL of crude extract and fractions. The

control in the assay was DMSO. The crude extract and fractions were prepared by dissolving 20 mg of sample in 1 mL of DMSO followed by a 1:200 dilution (1 mL culture medium : 1 µL extract stock). The mixture produced was mixed thoroughly to ensure a homogeneous solution. The plate was incubated for another two days. 125 µL of Malstat and NBT / PES solutions (made from 10 mL Malstat and 2.5 mL NBT / PES) were added to the wells and mixed by pipetting up and down. The plate was then covered (for 10-20 minutes) with foil to develop. The absorbance at 620 nm was then read.

3.3.3 Antimicrobial and antifungal assay

Since ethno-botanical and ethno-medicinal surveys and studies of the use of *A. mannii* point to the plant being used to treat the following ailments: snake and spider bites, syphilis, bronchitis, dysentery, gastroenteritis, diarrhoea, gonorrhoea, inflammations, cancer and malaria we ran antibacterial and antifungal screening of the samples we managed to collect in analytical quantities (Djeuss., 2013). The pure metabolites obtained and some of the fractions were prepared and microbial and anti-fungal assays obtained for them. The types of bacteria chosen were guided by the ethno-medicinal uses and information reported in literature on what assays have been carried out by other researchers. The information from literature is shown in table 3.4 (Kuete., 2013).

Table 3.4: Some literature results of microbial tests that have been carried on methanolic crude extracts of *Anonidium mannii*

	Bacterial culture	Gram positive	Gram negative	Diseases caused	ATCC	MIC (µg/mL)
1	*Escherichia Coli*		✓	Urinary tract infection; diarrhoea; abdominal cramp	ATCC8739	-
					ATCC10536	-
					AG100	-
					AG100A	-
					$AG100A_{TET}$	1024

					AG102	-
					MC4100	512
					W3110	-
2	*Klebsiella pneumonia*		✓	Bronchopneumonia; urinary tract infection; meningitis; diarrhoea	ATCC11296	-
					KP55	-
					KP63	-
					K24	-
					K2	1024
3	*Pseudomonas aeruginosa*		✓	Respiratory infection; urinary tract infection; dermatitis; gastrointestinal infection	PA01	-
					PA124	-
4	*E. aerogenes*		✓		ATCC130481	-
					CM64	-
					EA27	1024
					EA289	1024
					EA298	-
					EA294	-
5	*E.cloacae*		✓		ECC169	1024
					BM47	-
					BM67	1024

6	*P. stuartii*		✓		ATCC2914	-
					PS2636	-
					PS299645	-
7	*Bacillus Subtilis*	✓		Benign, not a threat		
8	*Staphylococcus aureus*	✓		Skin infections; mastitis; pneumonia; osteomyllitis and diarrhoea		
9	*Enterococcus faecalis*	✓		Meningitis; urinary tract infection; endocarditis		
10	*Lactobacillus casei*	✓		Usually benign but can cause cavity and tooth decay.		

Bioassays were done on other types of bacteria. Also the extracts and fractions under review were obtained from DCM/Methanol (1:1) (v/v) mixture which was significantly different from what has been reported in literature.

The method used to run the antibacterial and antifungal screens was very similar to that used to run the antimalarial IC_{50} screens. Table 3.5 shows the bacteria and fungi that were used in the screens.

Preparing samples for the bacteria bioassay

The crude sample as well as the fractions that were used had a starting concentration of 32 mg/mL while the starting concentration of the pure samples was 1 mg/mL. The starting concentrations of the ciprofloxacin (the positive control), water (the negative control) and the broth (culture control) were 0.01, 32 and 30 mg/mL respectively. The culture control (used to check microbe growth in culture), was prepared by mixing 30 g of tryptone soya broth with one litre of purified water. The mixture was put in an autoclave at 121 ^{0}C for 45 minutes to sterilize it. It was then cooled down to room temperature before use.

Table 3.5: Bacteria and Fungi used for microbial assays

Bacteria/fungi	ATCC number	Diseases caused
E. Coli	8739	Urinary tract infection, diarrhoea, abdominal cramp
E. faecalis	6230434	Meningitis; urinary tract infection; endocarditis
S. typhi	1403	
S. aureus	25923	Skin infections; mastitis; pneumonia; osteomyllitis and diarrhoea
C. albicans	10231	Inflammation of skin

Preparation of well plates

The 96 - well plates were prepared in a sterile room in laminar flow cabinets. They were labelled with the name of researcher, name of pathogen, date and number of plate. Each plate had columns 1 to 12 and rows A to H just as was done for the antimalarial assay. All wells from A1 to H12 were filled with 100 µL of broth (prepared in the same way as the culture control) using a 20 – 200 µL eight tipped micropipette fitted with yellow tips. After filling all wells with broth, well A of each column was then filled with 100 µL of sample using a micropipette. The letters G1 to G14 were the sample codes. The same concentration and amount of each sample was filled in two consecutive wells of row A. 100 µL of the positive control, an antibiotic, ciprofloxacin, with a code of +C, the negative control, water, with a code of –C and culture control, broth, with a code of CC, were added in different wells across row A. Several 96 - well plates were used to ensure that all the samples from G1 to G14 were screened. These were labeled as Plate 1, Plate 2 and so on up to the last plate.

Serial dilution of the samples, positive, negative and culture controls were done by collecting 100 µL of mixture from wells A1 to A12 and depositing in wells B1 to B12. Then 100 µL of mixture was collected from B1 to B12 and deposited in wells C1 to C12, this was repeated until well H was reached after thorough mixing before transferring into next well. The 100 µL collected from well H1 to H12 was discarded. Table 3.6 shows the different concentrations of mixtures in each well for the different starting concentrations.

Table 3.6: The concentrations of mixtures in the wells for starting concentrations

	Crude, Fractions and Water (32 mg/mL)	**Pure samples (1 mg/mL)**	**Antibiotic (+Control) 0.01 mg/mL**	**Broth (Culture control) (30 mg/mL)**
A	8	0.25	2.500	7.5
B	4	0.125	1.250	3.75
C	2	0.0625	0.625	1.875
D	1	0.0313	0.313	0.9375
E	0.5	0.0156	0.156	0.4688
F	0.25	0.0076	0.078	0.2344
G	0.125	0.0036	0.039	0.1172
H	0.0625	0.0020	0.020	0.0586

Adding the bacteria pathogens

After preparing the plates with samples and controls, the plates were ready for the bacteria pathogens. In this experiment, a total of five pathogens were used. These are listed in Table 3.5 together with their American Type Culture Collection (ATCC) numbers.

A 1 mg aliquot of the culture was diluted to 0.01 mg/mL by mixing it with 100 mL of broth. The culture solutions were prepared in test tubes. Before use, the culture solution had to

be stirred using a vortex mixer, while holding the lid on top so as not to spill the solution. The top of the test tube containing the culture solution was sterilised on a flame before and after use. 100 µL of this mixture of broth and culture was added to the sample and control wells on the plates using the multi-headed pipette. The plates were sealed with sterile adhesive sealer and placed in an incubator set at 37 ^{0}C for 24 hours. Culture in Petri dishes

Apart from adding the culture to the well plates, it was also added to agar filled Petri dishes to check for pathogen vitality. The agar used in the Petri dishes was prepared by mixing 40 g of tryptone soya agar with 1 litre of purified water. The mixture was then put in the autoclave set at 121 ^{0}C for 45 minutes. It was then poured into the Petri dishes while hot and placed in the sterile room in the laminar flow cabinet overnight to cool down. When cool, the prepared Petri dishes were stored in the cold room set at 4 ^{o}C. The same culture solution used for the plates was used for the Petri dish. An inoculation loop was sterilised on a flame then cooled on the edge of the agar filled Petri dish then dipped into culture solution. This was then used to mark the agar in Petri dish as shown in Figure 3.13. After drawing the first three lines, the loop was sterilised, then three more lines were drawn from the first (without dipping the loop in culture again), in a way spreading the pathogens from the first to the second triplet of lines, from the second to the third and then to the s-shape, without touching the Petri dish edge. The Petri dish was then incubated together with the prepared plates.

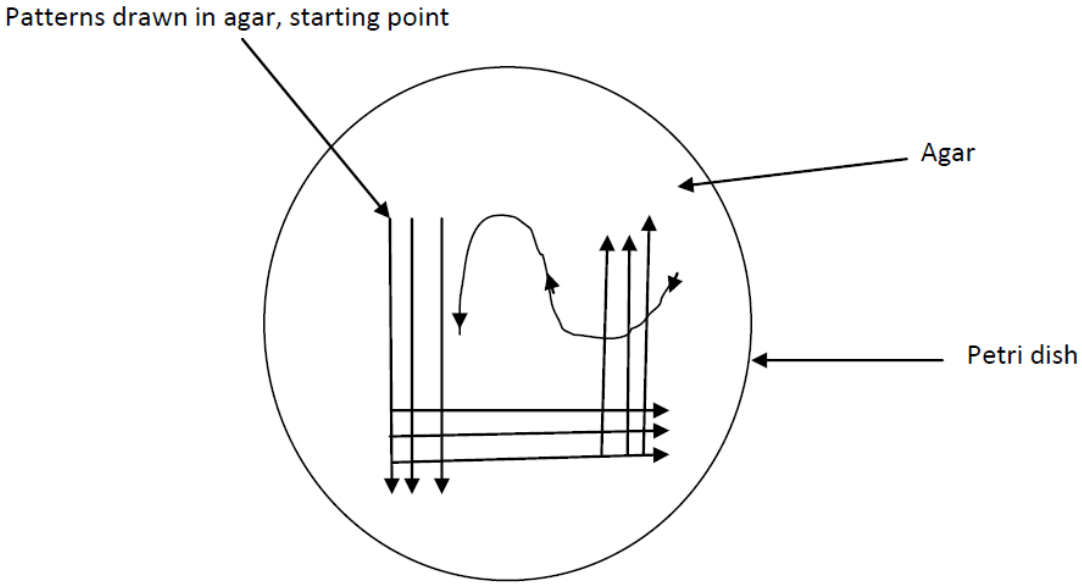


Figure 3.13: Petri dish showing patterns made with culture solution

Assessment of antimicrobial activity of samples

The activity of the sample was shown by a colour change. Colourless wells after 4 to 6 hours of adding Iodonitrotetrozaluim chloride (INT) indicates the absence of bacteria and the sample is considered to be active against that particular pathogen that was introduced in the wells. When the INT changes to pink, then it shows that the sample is inactive, that the bacteria is still alive and oxidising the INT. To add the INT, the sealer was removed from the plates and 40 mL of the INT was added to all wells on the plate. The plates were then covered and left to stand for at least 4 hours. *The reading of results was done under a lamp*. The minimum inhibitory concentrations (MIC) for active samples were recorded. After 24 hours, the Petri dishes were checked for growth of pathogen. Growth in the Petri dishes was an indication that the plates were also ready for INT solution to be added to them.

INT is an indicator used to check the presence or absence of bacteria in the plates. The solution of INT is made by dissolving 0.08 mg of INT in 200 mL of purified and sterilised water. The mixture is placed in a shaker incubator (platform shaker) set at 30 ^{o}C for 1 hour to completely dissolve the INT.

INT solution is colourless but changes to a pink colour when it is oxidised. When the INT was added to the plates after the 24 hours, the colour change was an indication of the

activity of the sample. Clear wells after 4 to 6 hours of adding INT indicates the absence of bacteria and the sample is considered to be active against that particular pathogen that was introduced in the wells. When the INT changes to pink, then it shows that the sample is inactive so that the bacteria is still alive and oxidising the INT.

The INT was added by removing the sealer from the plates and adding 40 µL of the INT to all wells on the plate. The plates were then covered and left to stand for at least 4 hours. The reading of results was done under a lamp. The minimum inhibitory concentrations (MIC) for active samples were recorded.

3.3.4 Anti – tuberculosis assay

A preliminary anti- TB screen was carried out using an approach similar to that carried out for the IC_{50} for anti – malarial screens. The assay was carried out using the strain of H3DTB of *Mycobacterium tuberculosis (M. tuberculosis).* The chromosomal DNA of *M. smegmetis* mc^2 155 was purified and the acetamidase promoter amplified by polymerase chain reaction (PCR) with primers AmiP1 and AmiP2 annealing to sequences 0.6 and 2.9 kb. These were then subcloned into sites of pFPV2 to remove Hsp 60 promoter. Colonies were selected on the basis of kanamycin resistance and detection of the acetamidase promoter using primers AmiP1 and AmiP2. The plasmids were purified and digested. The plasmids which contained the expected fragment of 2.3 kb were selected for confirmatory sequencing. Plasmid DNA were isolated from *E. Coli* by miniprep extraction and cut with KpnI (one site for pFPV2). The presence of linear plasmid was confirmed on a 1.0 % agarose gel. H3DTB was then cultivated in 7H9GC broth containing 0.05 % (v/v) Tween 80, it was then pelleted and suspended in 10 % sterile glycerol. Electroporation and selection of transformants were performed as described by Cooksey et al (Cooksey., 1993). The selected transformants were cultured in 200 mL of 7H9GTw with kanamycin. The bacterial suspension was washed once then suspended in 20 mL of phosphate buffered saline (PBS) before passing it through an 8 mm pore size filter. Aliquots were then stored at – 80 ^{o}C. Antimicrobial agents stock solutions were prepared in DMSO, filter sterilized and stored at – 70 ^{o}C.

Green Fluorescent Protein Mycobacterial Assay

Anti – mycobacterial susceptibility testing was performed in black, clear – bottomed 96 – well microplates so as to minimise background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in the experimental wells. Initial drug dilutions were prepared in DMSO and subsequent two – fold dilutions were prepared in 0.1 mL of 7H9GC without Tween 80 in the microplates. Frozen H3DTB were thawed, sonicated for 15 seconds and cultured at 37 ^{o}C with shaking in 200 mL of 7H9GTw with kanamycin until optical density at 550 nm reached 0.4 – 0.5. Cultures were diluted in 7H9GC and 10^5

CFU (colony – forming unit) was added to each test well in a volume of 0.1 mL. The final volume was 200 µL and the final bacteria density was 5 x 10^5 CFU/mL. Wells that had drugs only were used to detect autoflourescence of compounds. Additional wells consisted of bacteria only and others of medium only. Plates were incubated at 37 ^oC and fluorescence measured daily for eight days in a Cytofluor II microplate fluorometer in the bottom reading mode with excitation at 485 nm and emission at 508 nm. The average fluorescence from triplicate medium only cells was used as background and subtracted from the test wells and bacteria only wells. By definition percentage inhibition was used as (test well fluorescence/average fluorescence of triplicate bacteria wells) multiplied by 100 on day seven incubation. The lowest drug concentration effecting inhibition of 90 % and 99 % were considered as the MICs.

Table 3.7: Some of the *A. mannii* fractions and theirs labels for the 96 – well plates that were screened for TB

Column	Sample Name	Purity	Quantity supplied
A	**AM7C**	Pure	1 mg/mL in DMSO
B	**AM1W**	Partially fractionated	1.9 mg
C	**AM2W**	Partially fractionated	1.1 mg
D	**AM3W**	Partially fractionated	1.3 mg
E	**AM4W**	Partially fractionated	2.2 mg
F	**1C24**	Partially fractionated	2.0 mg
G	**1C42**	Partially fractionated	2.7 mg

3.4 Estimation of metabolites in the crude using ^{1}HNMR

The pure metabolites that were obtained and whose structures had been elucidated were used in quantitative ^{1}HNMR as external standards to estimate the amount of metabolites in the crude extract. This was done under the same conditions. The NMR's tuning and matching was fixed throughout the experiments. The same solvent $CDCl_3$ was used and the temperature of the probe kept constant. The NMR tubes used were of the same specification. The spectral acquisition conditions were kept constant and the same pulse sequence used. Some of the conditions used during the experiments were:

PULPROG – zg30

SW – 20.0309 ppm

SWH – 12019.230 Hz

FW – 125 000 Hz

Probe temperature – 298 K

VPU temperature – 291.5 K

8.2 mg of AM1C were weighed and dissolved in 0.5 mL of $CDCl_3$ and kept for use as the first external standard. 9.1 mg of AM2C were also weighed and dissolved into 0.5 mi of $CDCl_3$ as the second standard. 17.5 mg of the crude sample labelled AMC were also dissolved in 0.5 mL $CDCl_3$ as the unknown. The solutions were loaded into identical NMR tubes and ^{1}HNMR spectrum acquired for them. The peaks which were used for estimation purposes were the ones at the following chemical shifts: 5.84 ppm, 5.29 ppm, 5.08 ppm, 4.26 ppm and 3.25 ppm. Only the heights of the peaks were considered as the peak area is proportional to the concentration but for estimation purposes we decided to use the heights only and take an average.

3.5 Phytochemical tests.

The pure metabolites extracted were tested using the Liebermann Burchard tests (Kandati et al., 2012; Raju et al., 2012). The pure compound was dissolved in $CHCl_3$ and acetic anhydride followed by 1mL of concentrated sulphuric acid to test for steroids and triterpenes.

3.6 References

1. Djeuss. D. E., Noumedem. J. A. K., Seukep. J. A., Fankman. A. G., Voukeng. I. K., Tankeo. S. B., Nkuete. A. H. L and Kuete. V. **2013.** Antibacterial activities of selected edible plant extracts against multi-drug resistant Gram-negative bacteria. *BMC Complementary Alternative Medicine.* 13. 164-169.

2 Eloff. J. N. **1998.** A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica.* 64. 711-713.

3. Keusch. G. T., Jacewicz. M., Hirschman. S. Z. **1972.** Quantitative microassay in cell culture for enterotoxin of *Shigella dysenteriae. Journal of Infectious Diseases.* 125(5). 539-541.

4. Kandati, V., Govardhan, P., Reddy, C. S., Nath, A. R.,Reddy, R. R. **2012**. In-vitro and In-vivo anti-inflammatory activity of *Andrographis serpyllifolia. International Current Pharmaceutical Journal, 1. 199-204.*

5. Raju, V. H., Ganapaty, S., Prasanna, S. S., Vijaya, G. J., Kishore, P. S., Asif, A. K. **2012**. Phytochemical and pharmacological evaluation of *Tragia cannabina* for anti-inflammatory activity. *International Current Pharmaceutical Journal, 1. 213-216.*

6. Shahinas, D., Folefoc, A., Pillai, D R. **2013.** Targetting *P. falciparum* Hsp90: Towards Reversing Antimalarial Resistance. *Pathogens,* 2(1). 33 -54.

7. Cooksey, R C., Crawford, J T., Jacobs, W R., Shinnick, T M. **1993.** A Rapid Method for Screening Antimicrobial Agents for Activities Against a Strain of *M. tuberculosis* expressing firefly luciferase. *Antimicrobial Agents Chemotherapy,* 37. 1348 – 1352.

4. APPENDIX

4.1 Spectra for AM1C

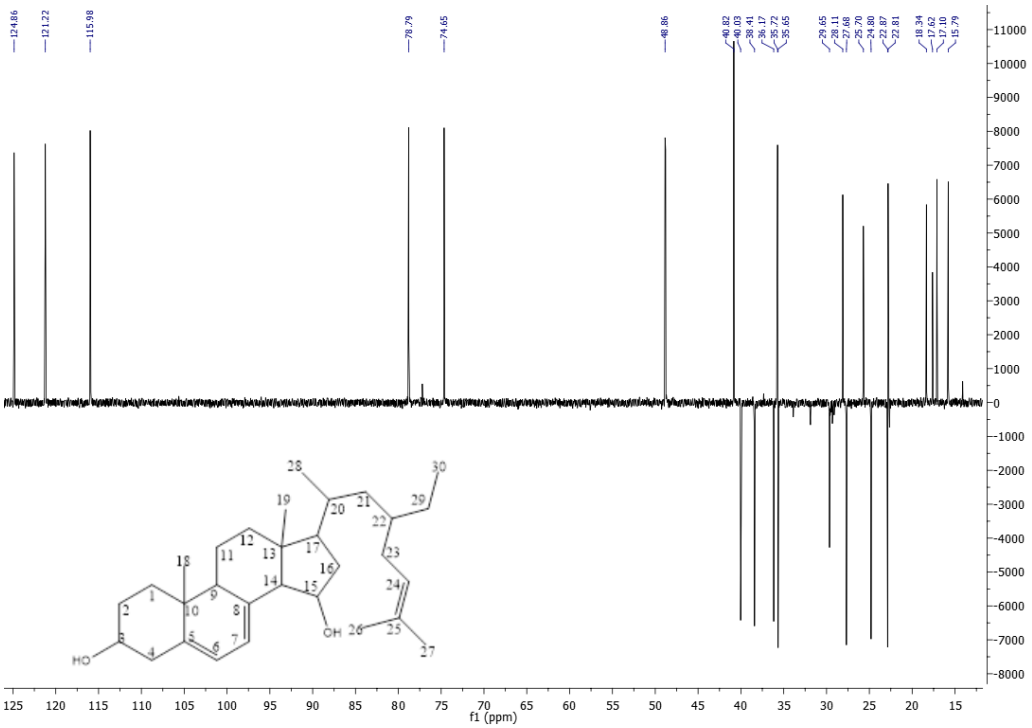


Figure 4.1: C13DEPT135 spectra of AM1C in $CDCl_3$

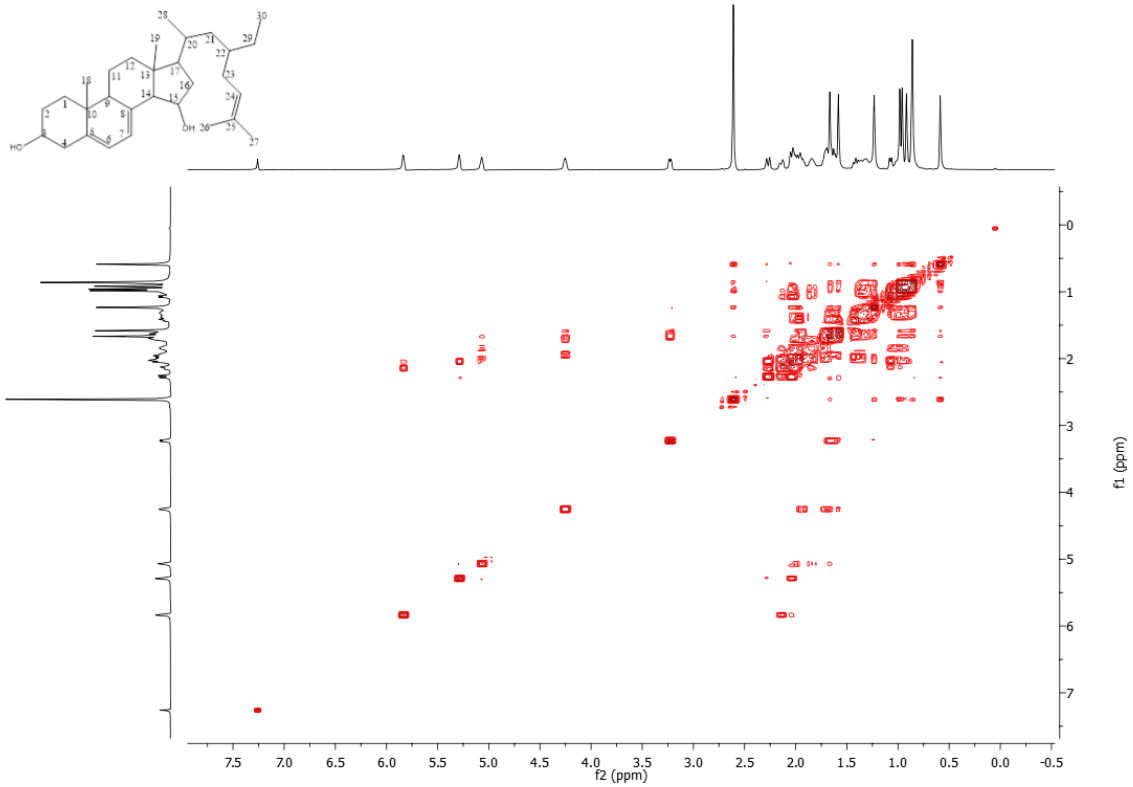


Figure 4.2: H-H COSY spectra of AM1C in $CDCl_3$

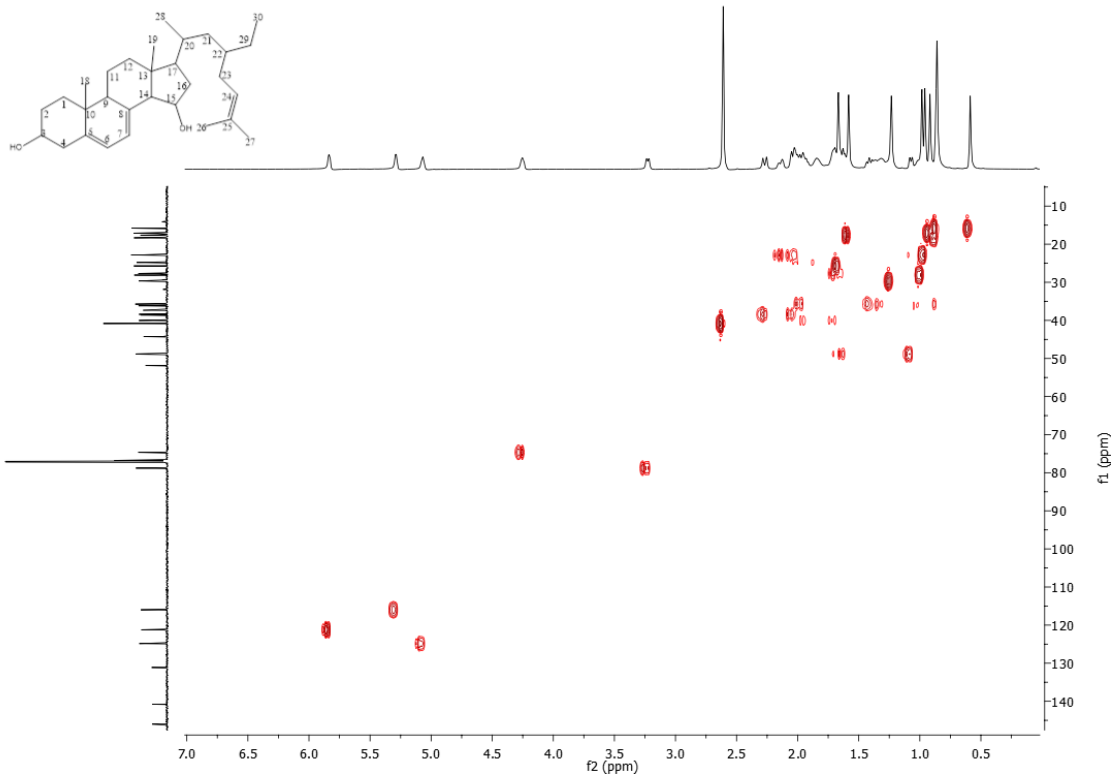


Figure 4.3: HSQC spectra of AM1C in $CDCl_3$

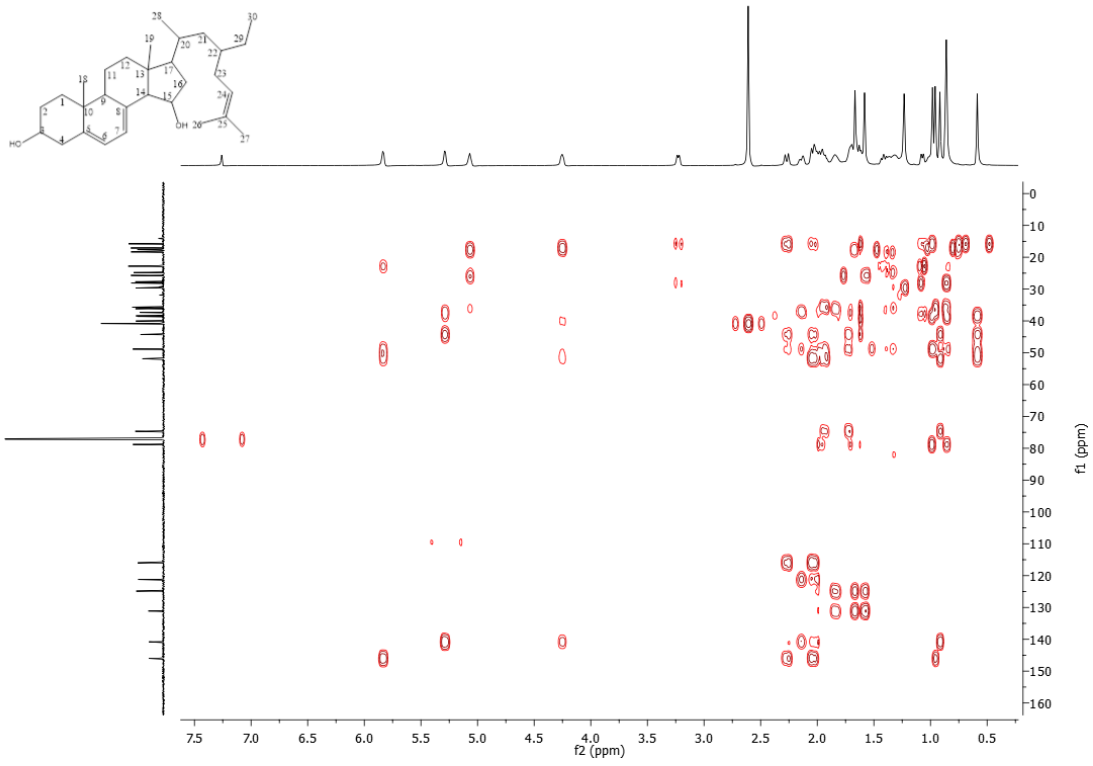


Figure 4.4: HMBC spectra of AM1C in $CDCl_3$

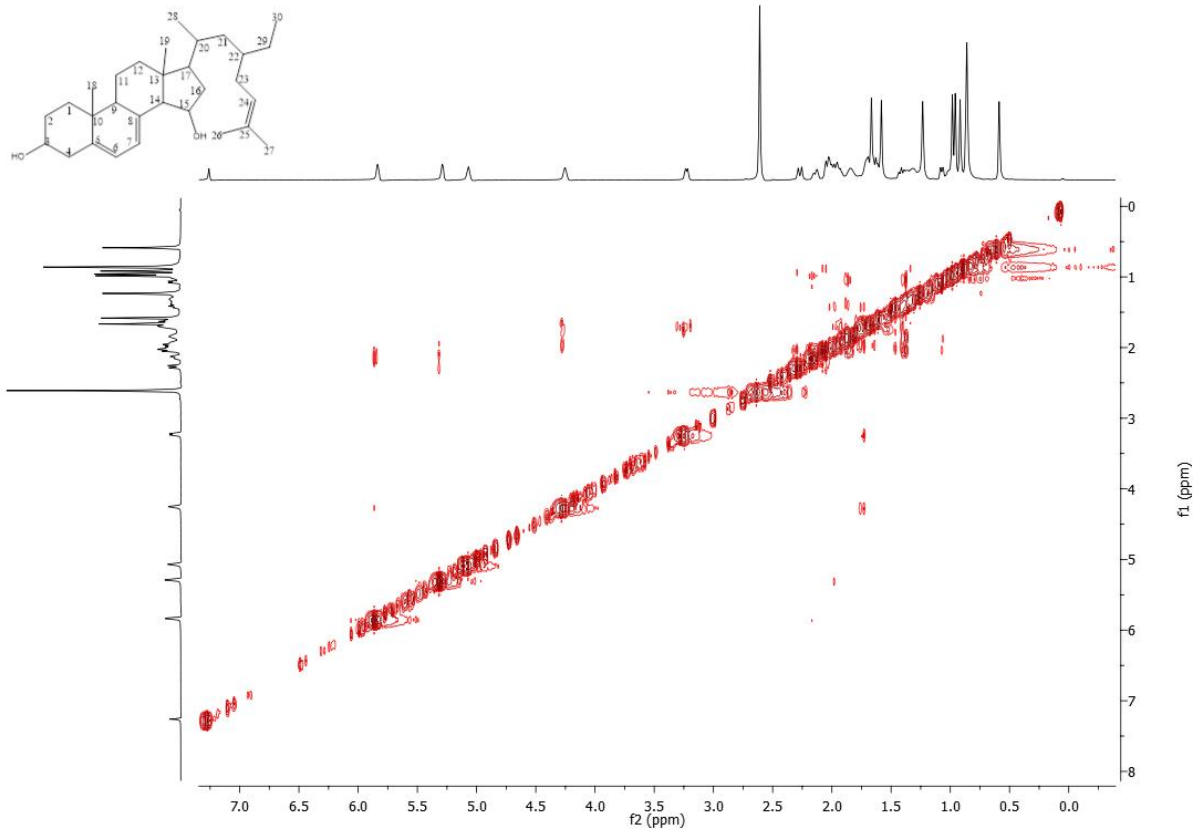


Figure 4.5: NOESY spectra of AM1C in $CDCl_3$

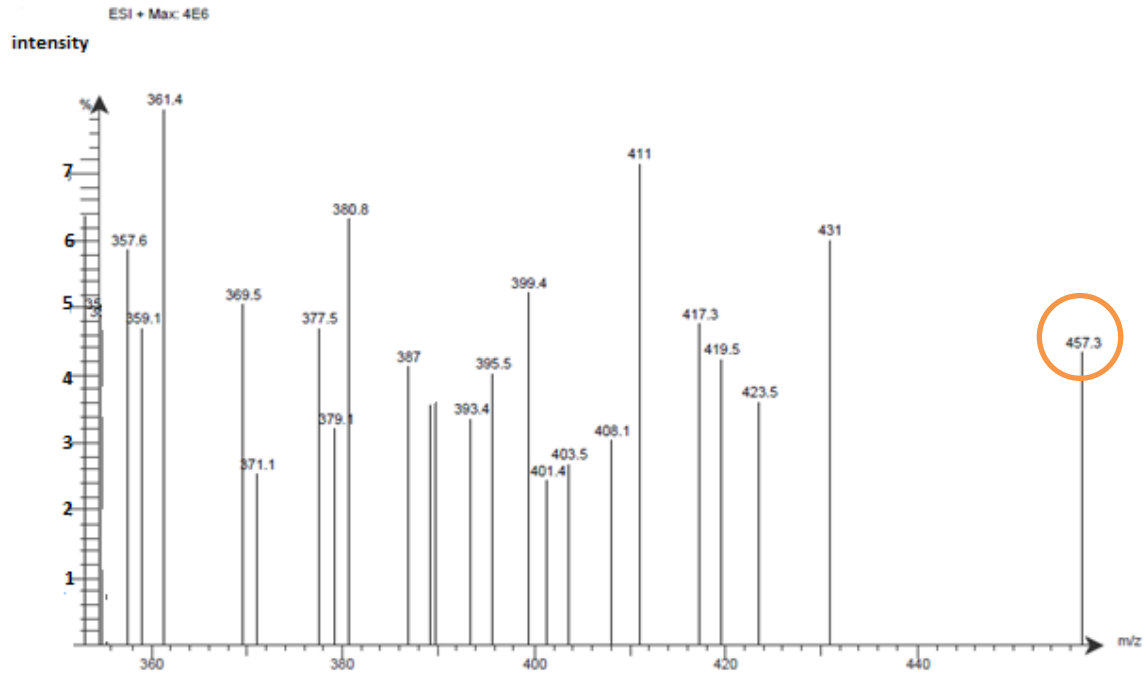


Figure 4.6: ESI-MS mass spectra of AM1C using Advion mass spectrometer with TLC interface

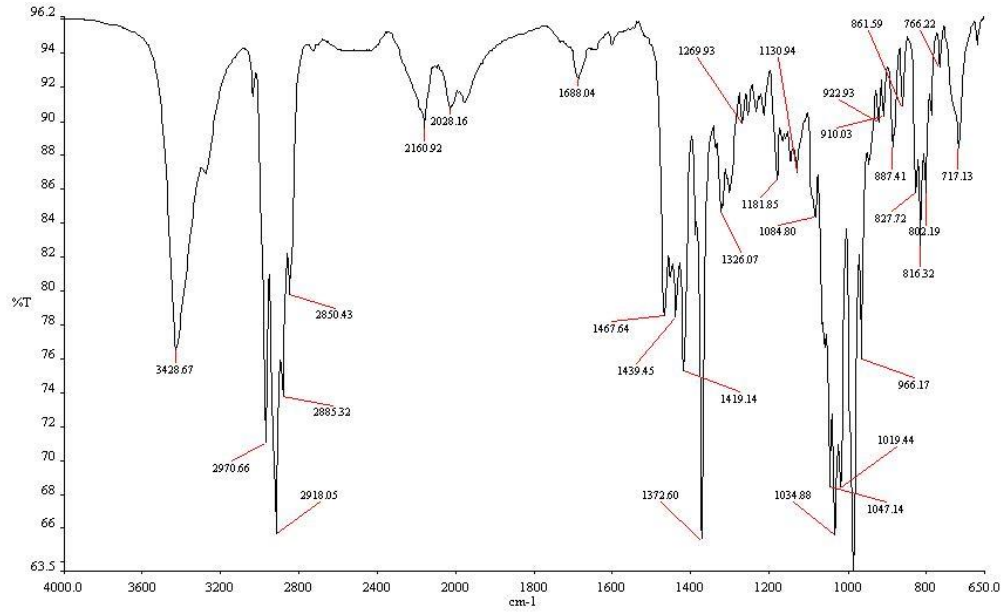


Figure 4.7: FT-IR spectrum of AM1C

4.2 Spectra for AM2C

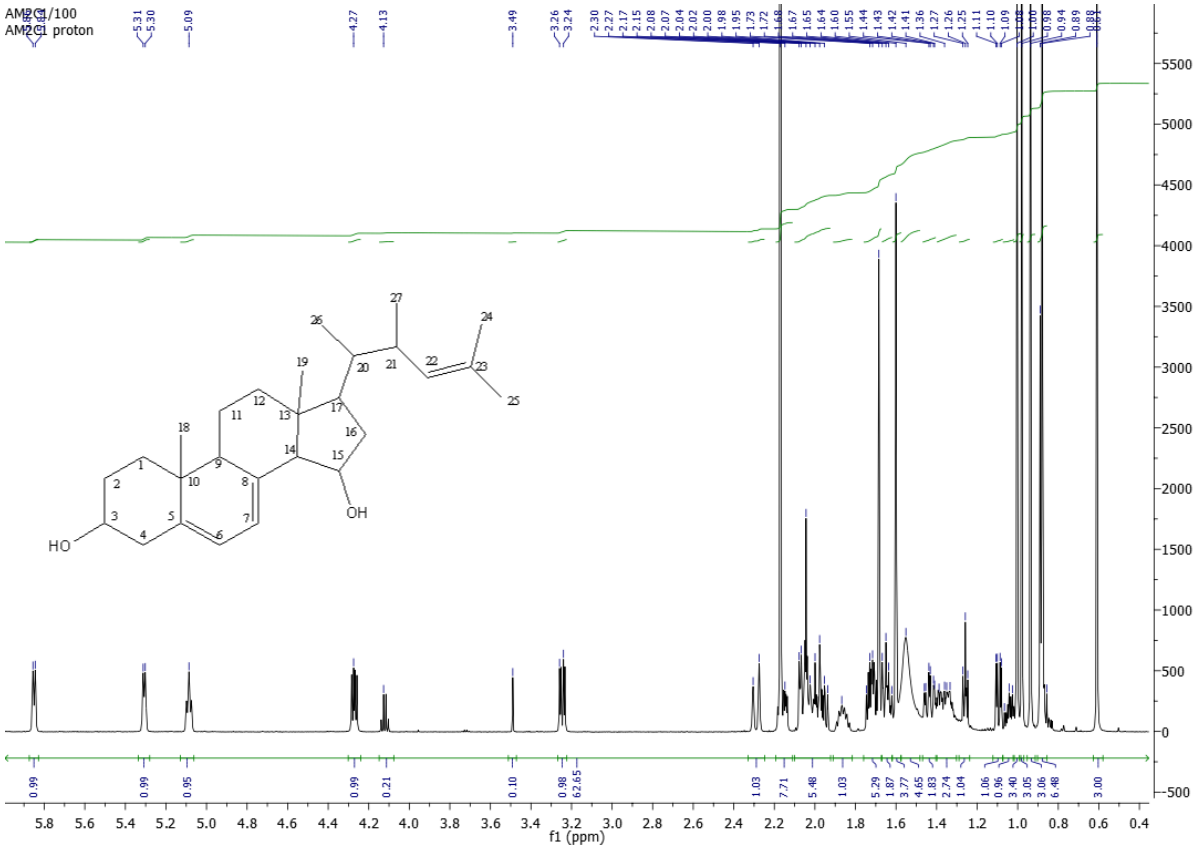


Figure 4.8: ^{1}H NMR of AM2C in $CDCl_3$

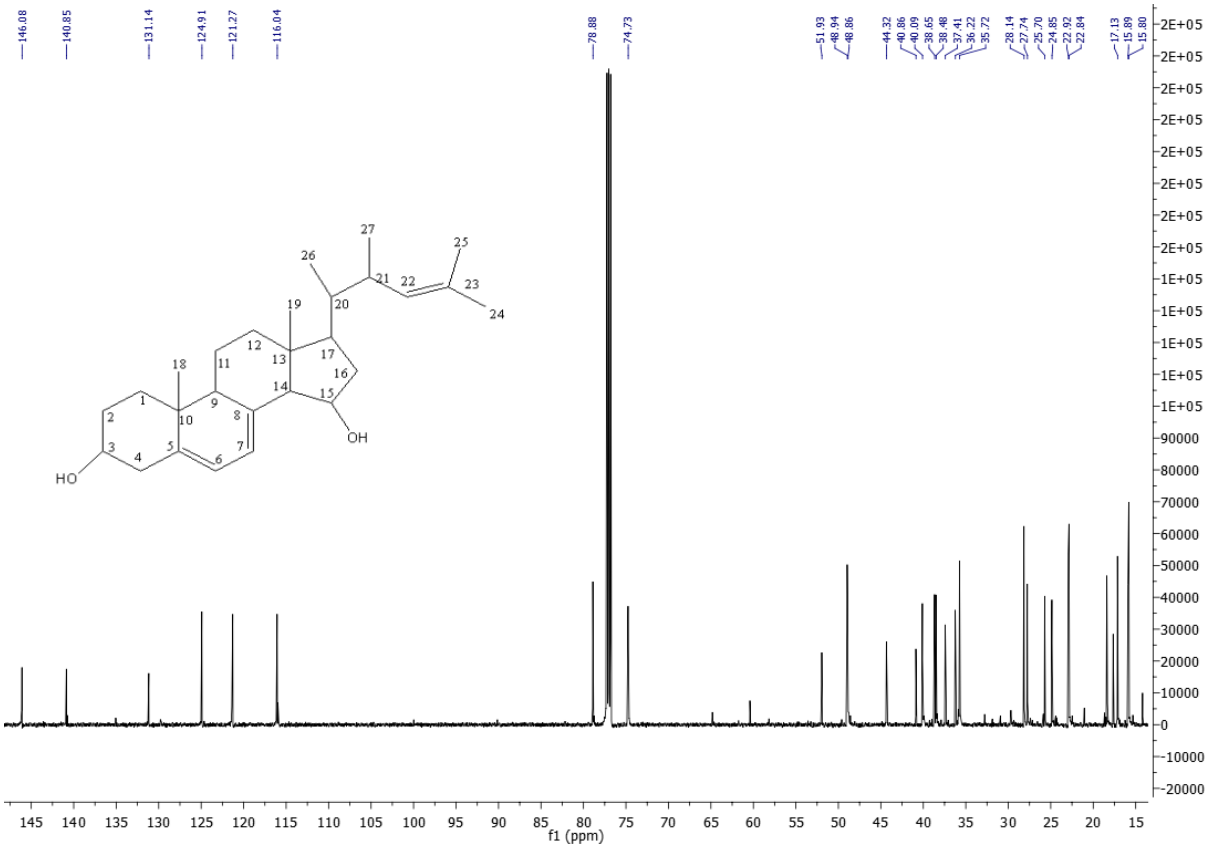


Figure 4.9: ^{13}C NMR of AM2C in $CDCl_3$

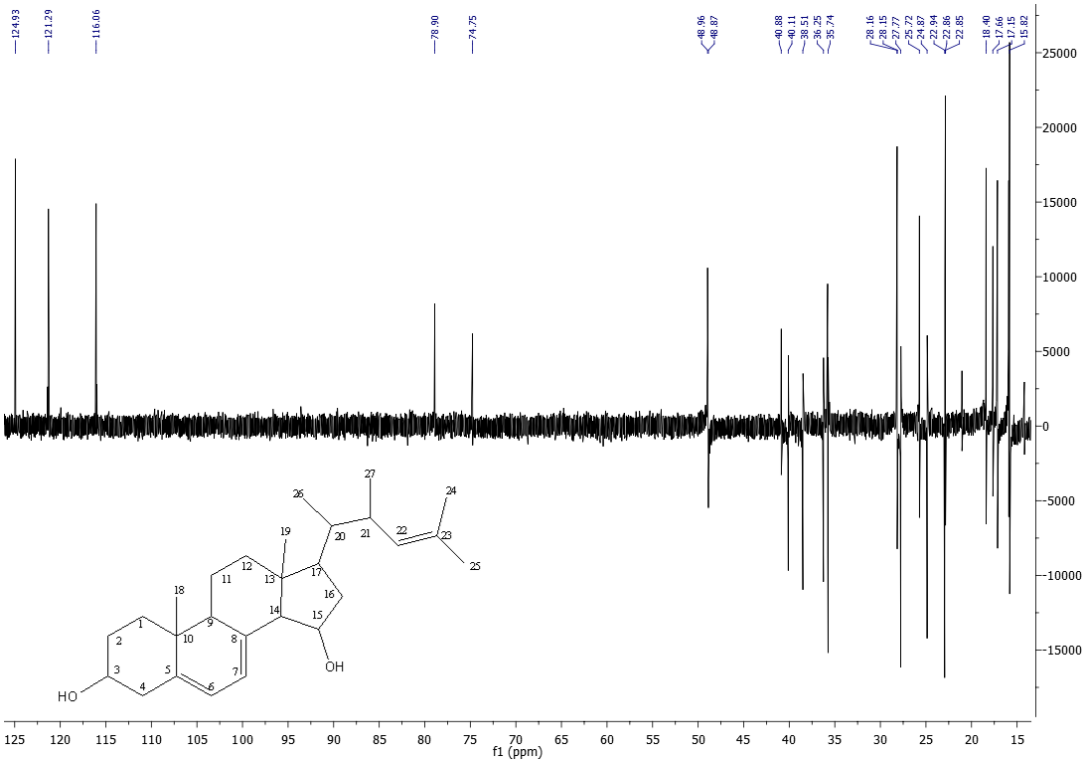


Figure 4.10: DEPT 135 NMR spectrum of AM2C in $CDCl_3$

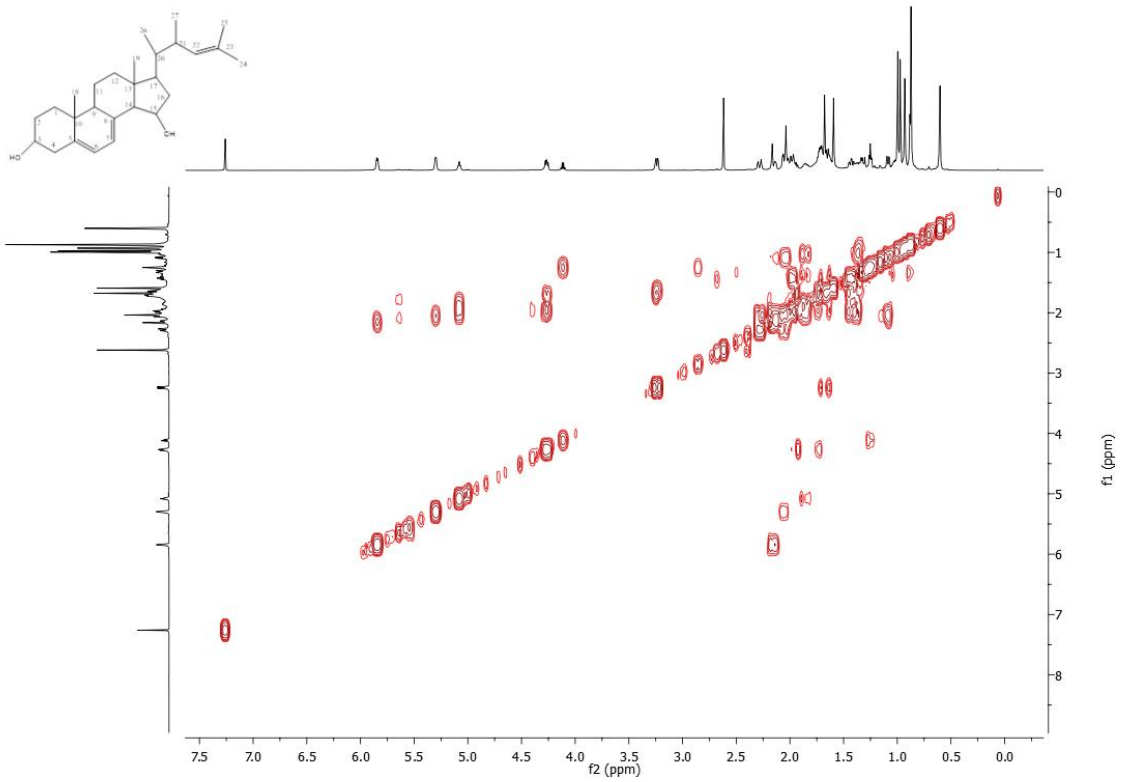


Figure 4.11: H-H COSY spectrum of AM2C in $CDCl_3$

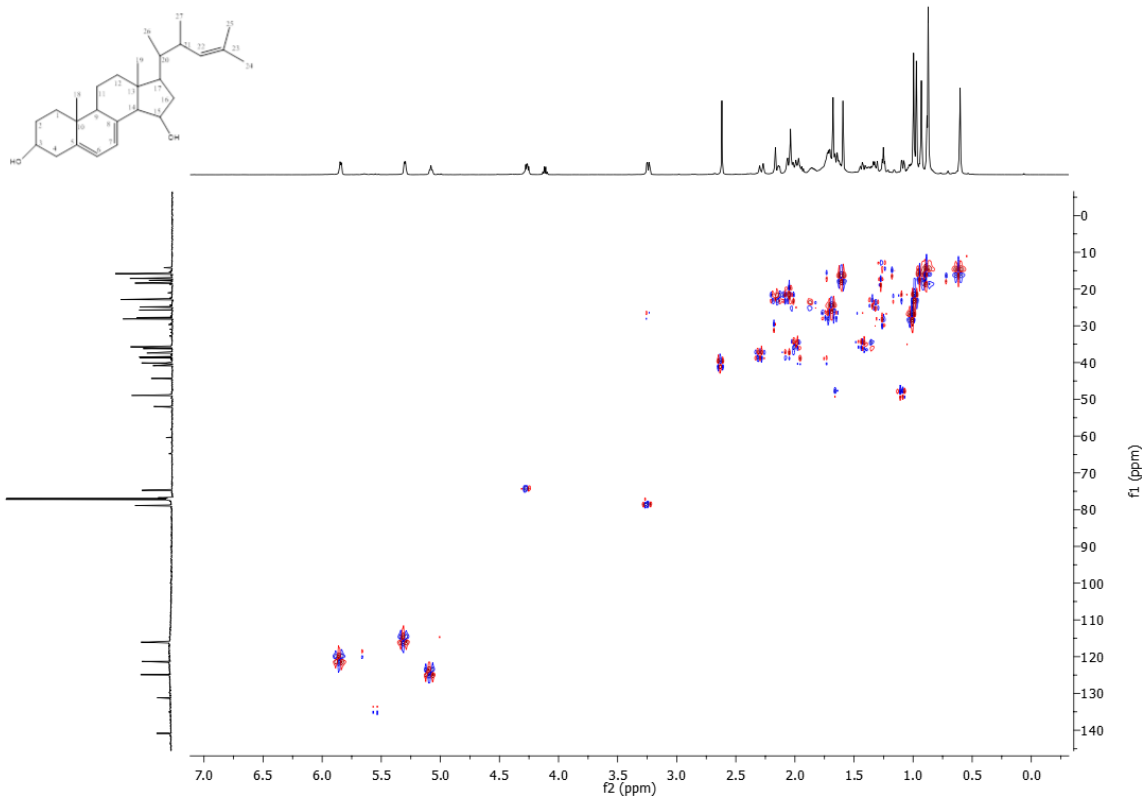


Figure 4.12: HSQC spectrum of AM2C in $CDCl_3$

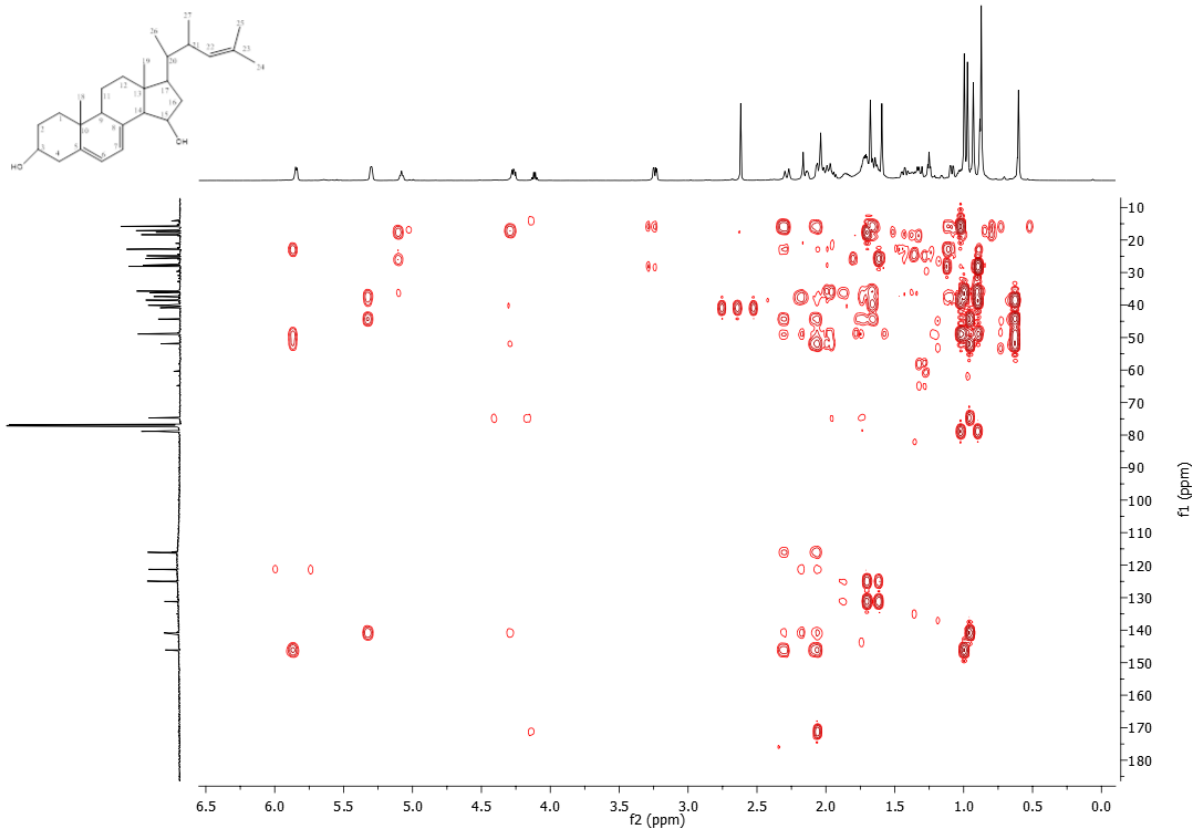


Figure 4.13: HMBC spectrum of AM2C in $CDCl_3$

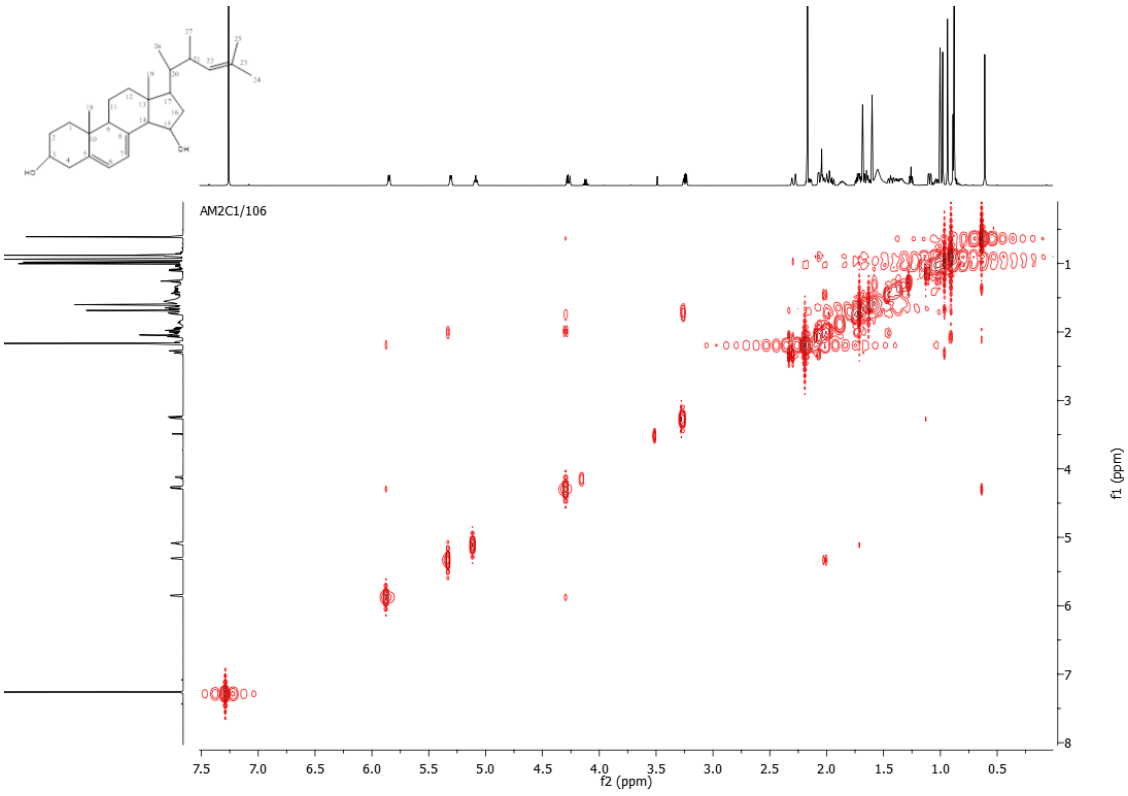


Figure 4.14: NOESY spectrum of AM2C in $CDCl_3$

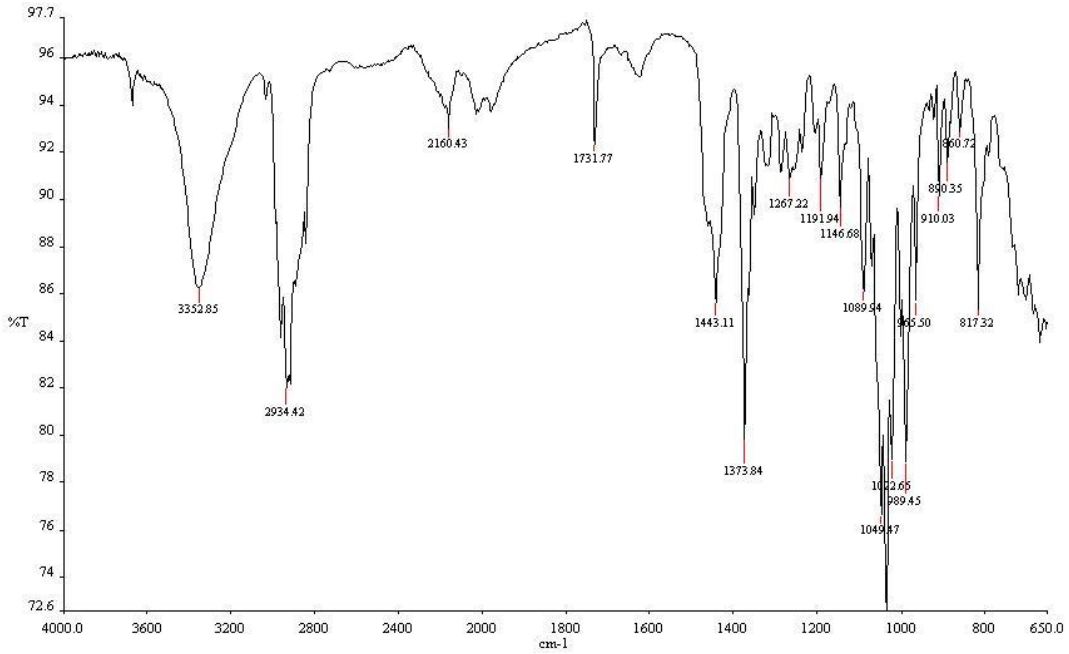


Figure 4.15 FT-IR spectrum of AM2C

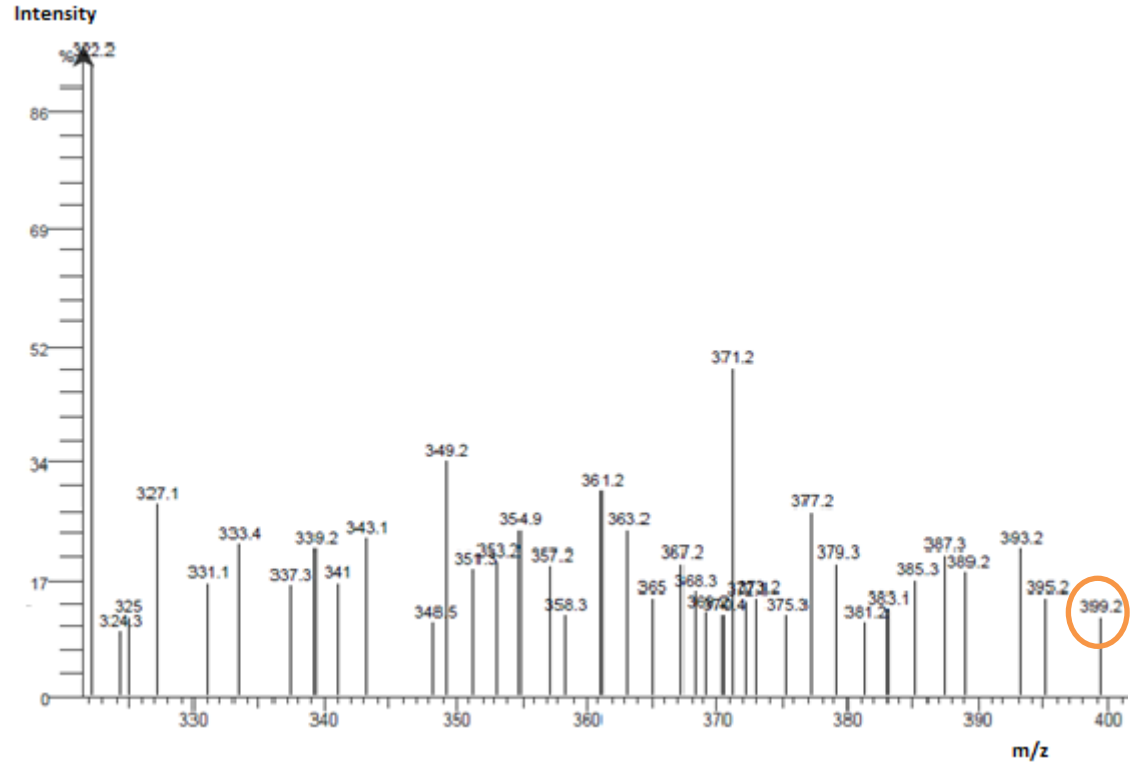


Figure 4.16: ESI-MS spectrum of AM2C from the Advion(TLC) interface mass spectrometer.

4.3 Spectra for AM3C

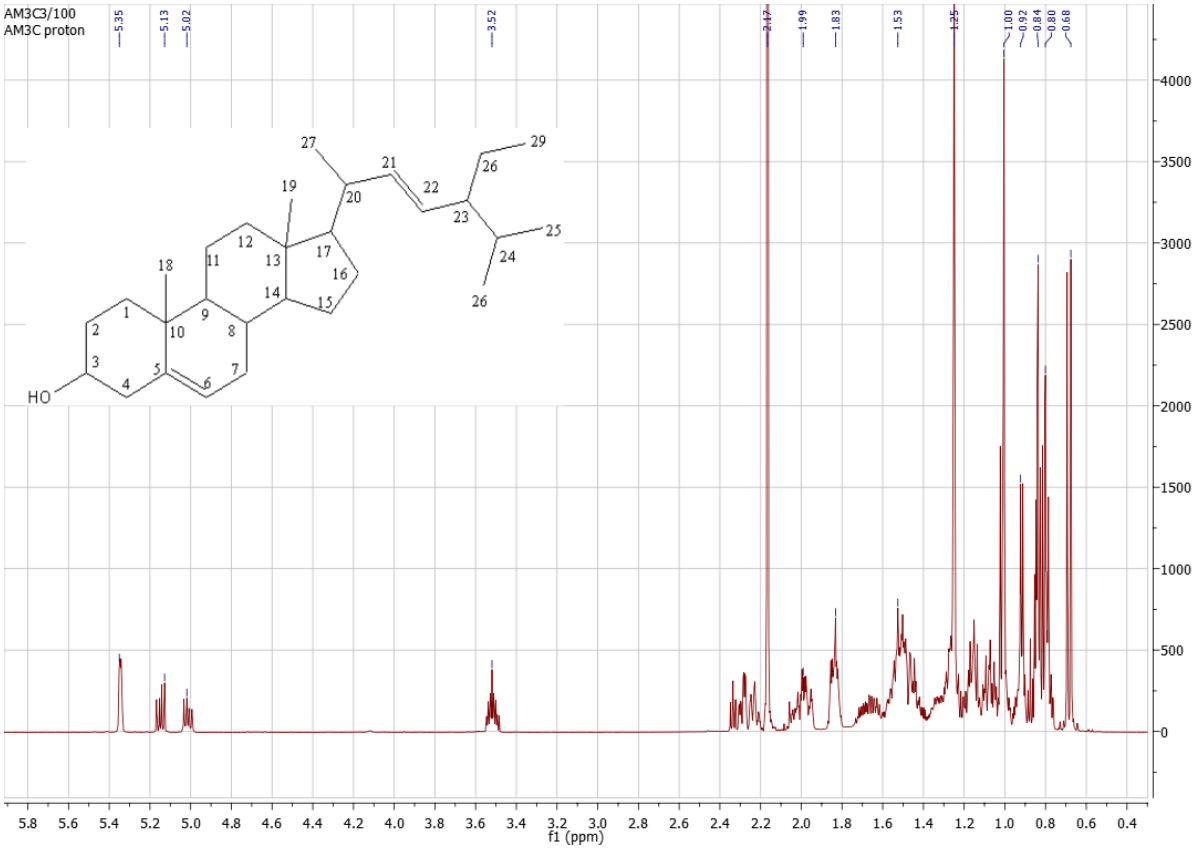


Figure 4.17: ^{1}H NMR spectrum of AM3C in $CDCl_3$

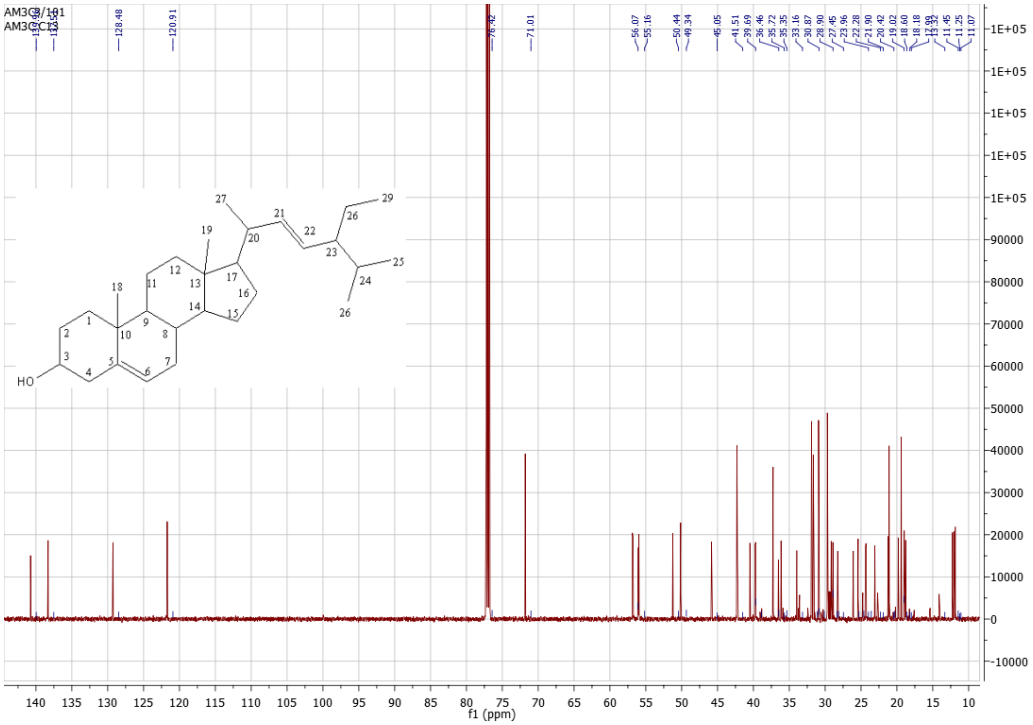


Figure 4.18: ^{13}C NMR spectrum of AM3C in $CDCl_3$

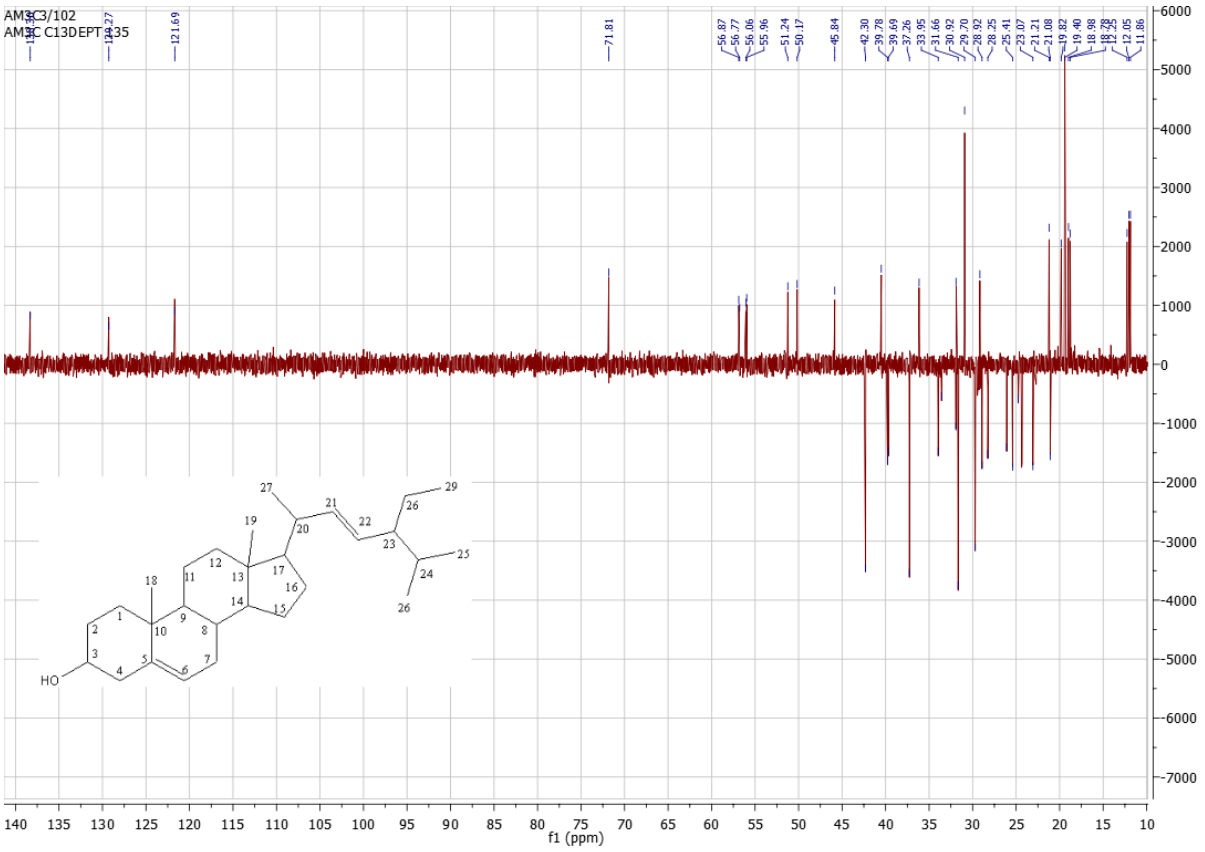


Figure 4.19: DEPT 135 NMR spectrum of AM3C in $CDCl_3$

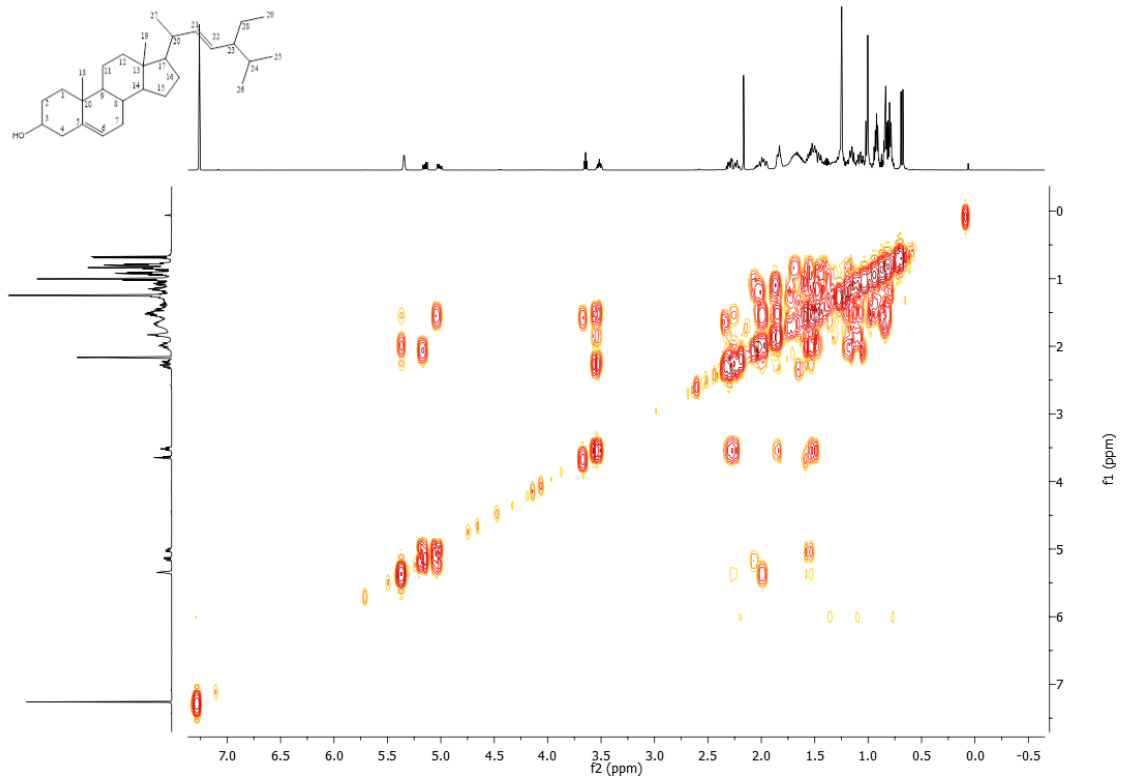


Figure 4.20: H-H COSY NMR spectrum of AM3C in $CDCl_3$

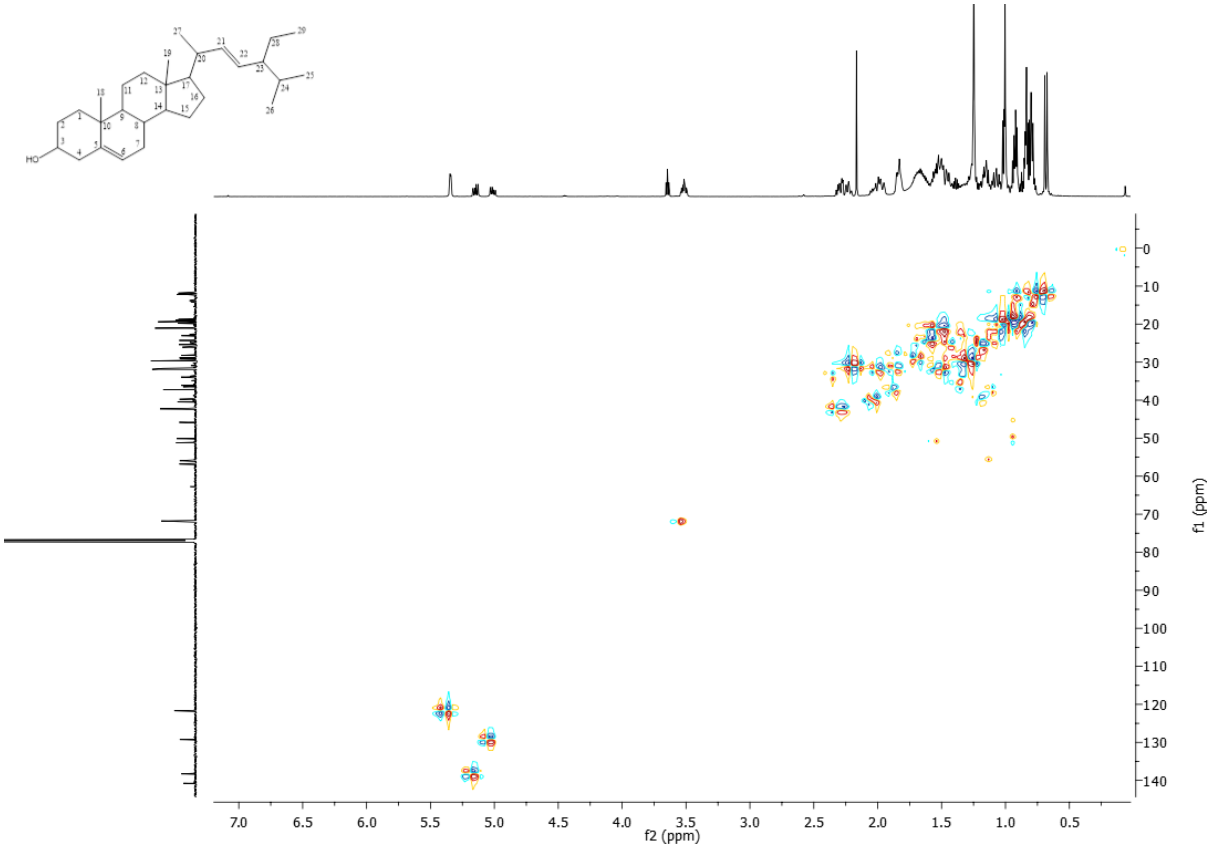


Figure 4.21: HSQC NMR spectrum of AM3C in $CDCl_3$

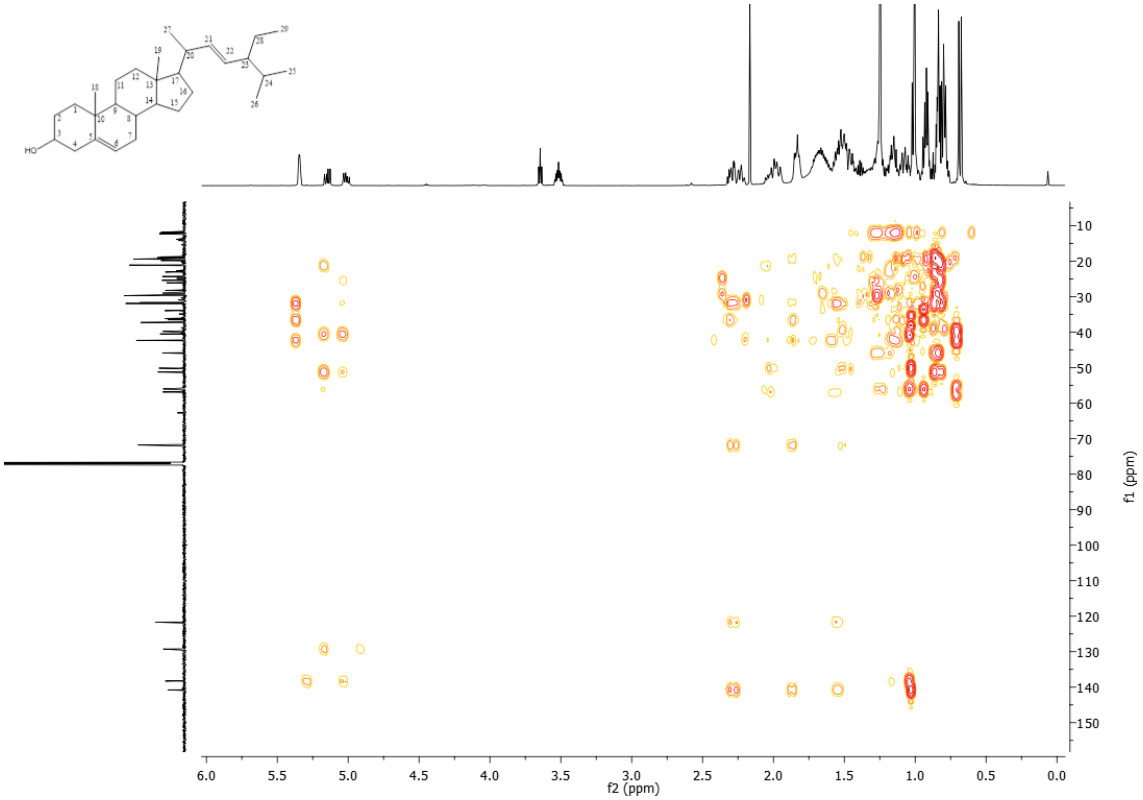


Figure 4.22: HMBC NMR spectrum of AM3C in $CDCl_3$.

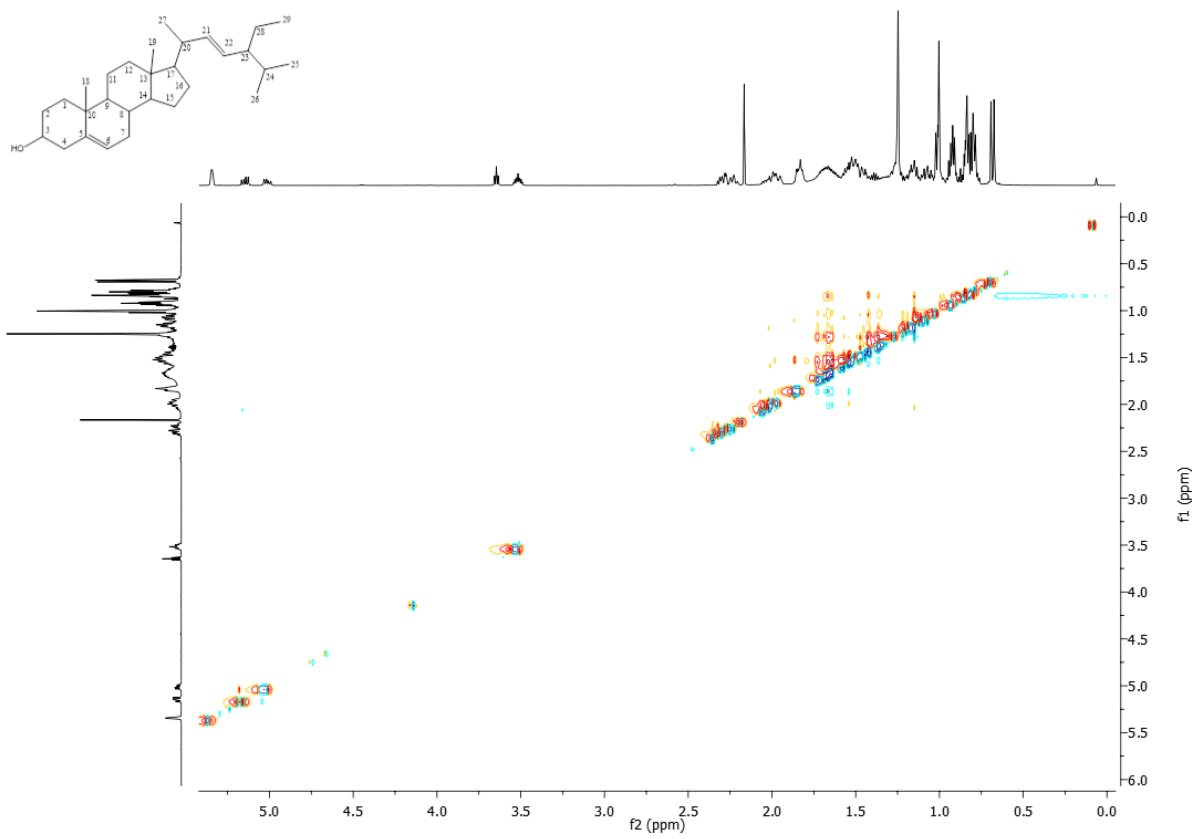


Figure 4.23: NOESY NMR spectrum of AM3C in $CDCl_3$.

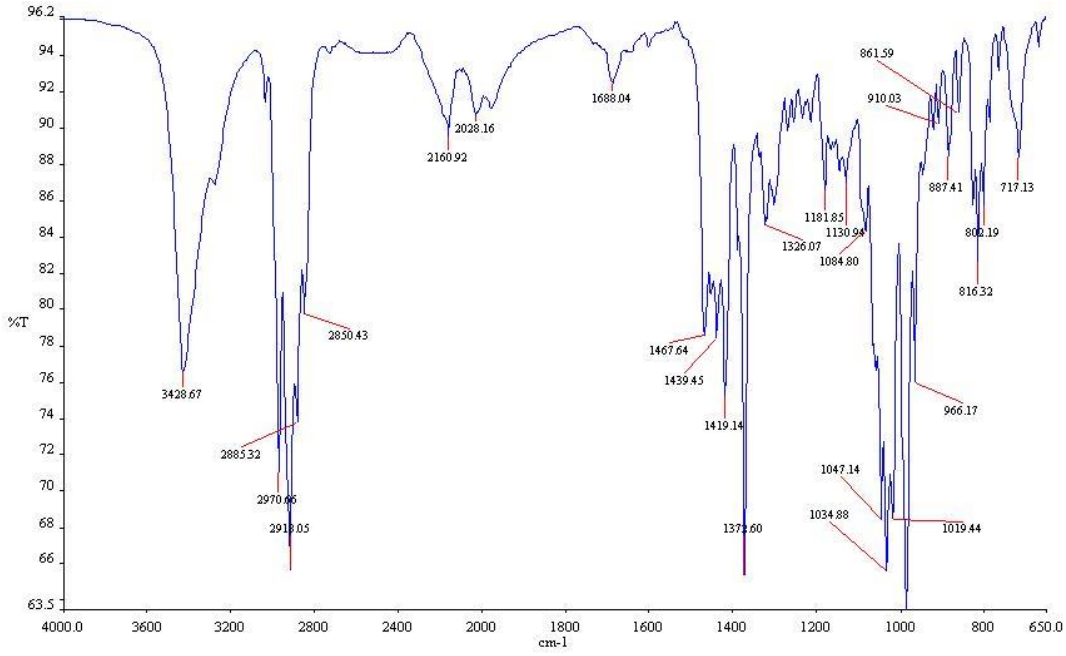


Figure 4.24: FT-IR spectrum of AM3C.

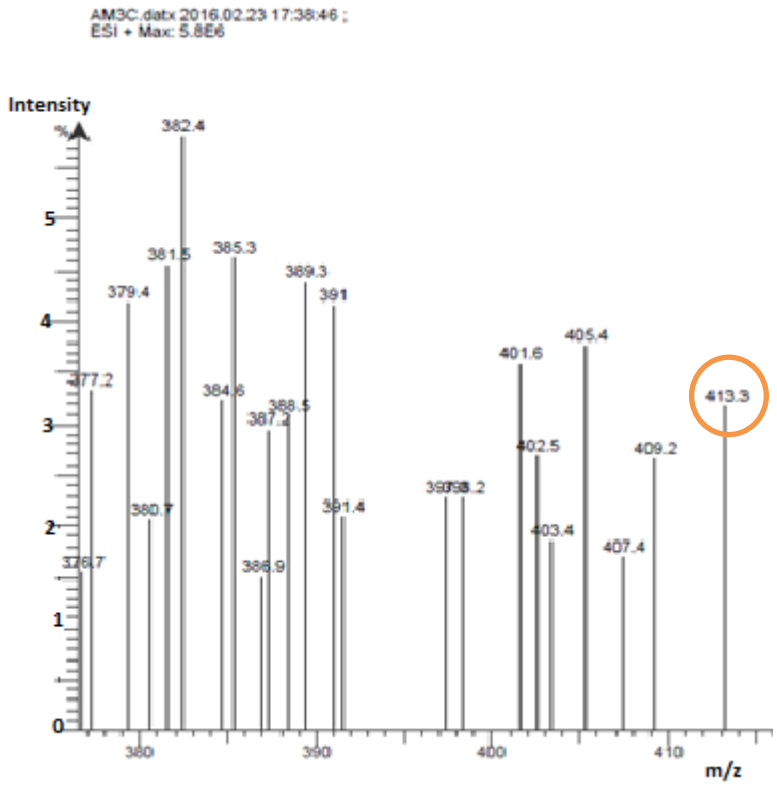


Figure 4.25: ESI-MS spectrum of AM3C from the Advion(TLC) interface mass spectrometer.

4.4 Spectra for quantitative ^{1}HNMR

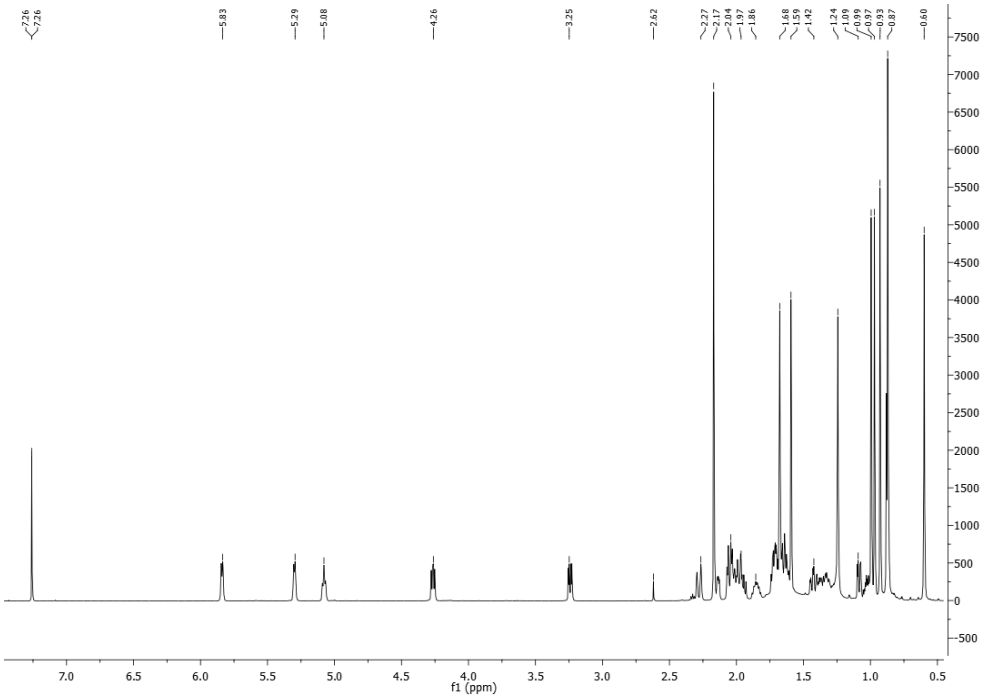


Fig 4.26: ^{1}HNMR spectrum of 8.2 mg AM1C in 5 mL of $CDCl_3$ used for estimation of cholestanes in crude.

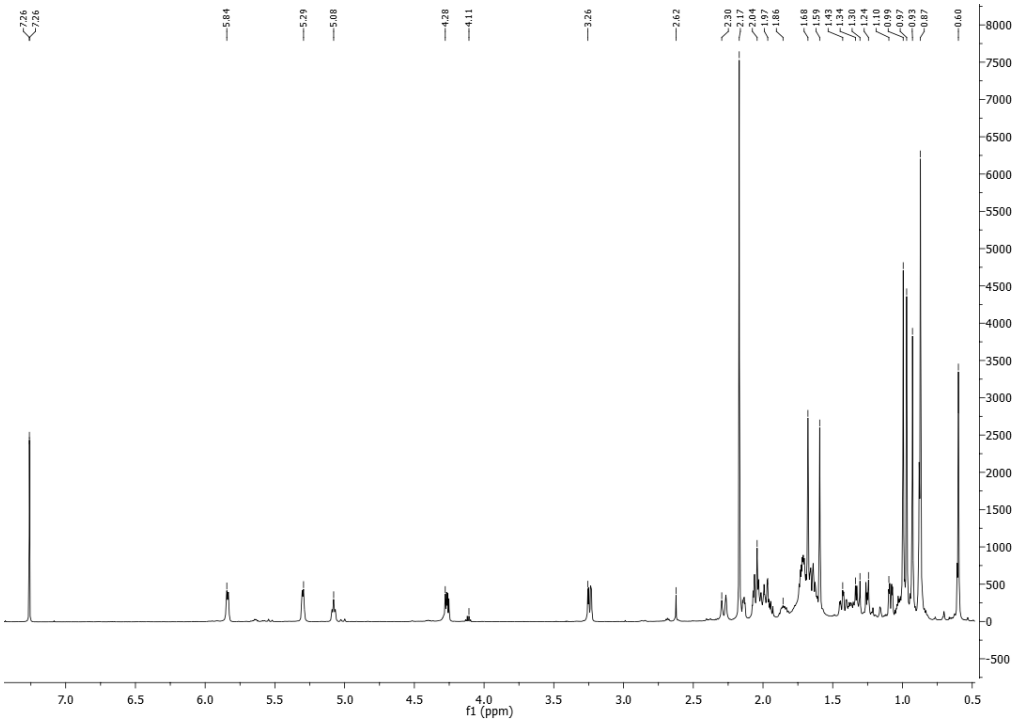


Fig 4.27: ^{1}HNMR spectrum of 9.1 mg AM2C in 5 mL of $CDCl_3$ used for estimation of cholestanes in crude.

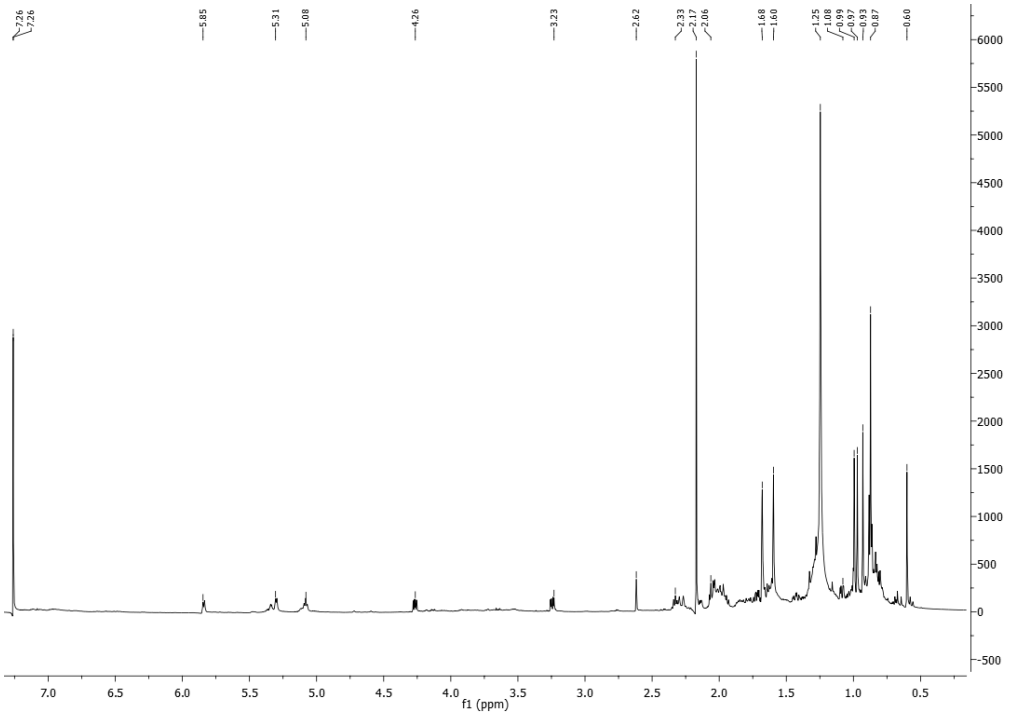


Fig 4.28: ^{1}HNMR spectrum of 17.5 mg AMC (crude) in 5 mL of $CDCl_3$ used for estimation of cholestanes in crude.