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**Isolation and characterization of antifungal and antibacterial
compounds from *Combretum molle* (Combretaceae) leaf extracts**

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DECLARATION

I **Motanti Mary Mogashoa** declare that the thesis hereby submitted to the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria for the degree of Magister Scientiae has never been submitted by me for a degree at this or any other university. The results obtained from this study are my own investigation, except where the work of others is specifically acknowledged.

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Prof. J.N. Eloff (Promoter)

Date:.....

DEDICATION

I dedicate this work to everyone who contributed in one way or another to the completion of the study and those who are passionate and committed to indigenous knowledge systems (IKS).

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INSPIRATIONAL QUOTES:

“For to be free is not merely to cast off one’s chains, but to live in a way that respects and enhances the freedom of others.” **Nelson Rolihlahla Mandela**

“You have to know what sparks the light in you so that you, in your own way, can illuminate the world.” **Oprah Winfrey**

LIST OF ABBREVIATIONS

A	Acetone
AIDS	Acquired immune deficiency syndrome
AmB	Amphotericin B
AS	anisaldehyde/sulphuric acid
ATTC	American Type Culture Collection
AV	Average
B	Butanol
B.C.	Before Christ
BEA	Benzene/Ethanol/Ammonium hydroxide (90:10:1)
°C	Degree Celsius
C	Chloroform
CEF	Chloroform/Ethyl acetate/Formic acid (5:4:1)
Cm	centimetre
<i>C. molle</i>	<i>Combretum molle</i>
CNS	Central nervous system
COX	Cyclooxygenases
DCM	Dichloromethane
DEPT	Distortionless enhanced by polarization transfer
DNA	Deoxyribose nucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl radical
E	ethanol
EA	ethyl acetate
EMW	Ethyl acetate/Methanol/Water (40:5:4)
ERG3	ergosterol pathway
ERGII	ergosterol pathway
EtOAc	ethyl acetate
F	Formic acid
FDA	Food and Drug Administration
g	gram
gCOSY	gradient-selected Correlation Spectroscopy
GIT	Gastrointestinal tract
HIV	Human immunodeficiency virus

HMBC	Heteronuclear multiple bond correlation spectroscopy
HSQC	Heteronuclear single quantum correlation spectroscopy
Hx	Hexane
IDSA	Infectious Disease Society of America
IFN-A	Interferon gamma
INT	<i>p</i> -Iodonitrotetrazolium violet
IR	Infrared
Kg/ml	Kilogram per millilitre
L	Liter
LPS	Lipopolysaccharide
Ltd	Limited
M or MeOH	Methanol
MAG	Mollic acid glucoside
MAPKs	Mitogen-activated protein kinases
MDR1	Multi-drug resistant
Mg/kg	Milligram per kilogram
Mg/ml	Milligram per millilitre
MIC	Minimum inhibitory concentration
ml	Milliliter
ml/g	Millilitre per gram
MLS	macrolides, lincosamides and streptogramin
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MW	Molecular weight
MS	Mass Spectroscopy
nm	nanometer
NMR	Nuclear Magnetic Resonance
PPARs	Peroxisome proliferator-activated receptors
Rec A	recA bacterial DNA recombination protein
R _f	Retardation factor
RNA	Ribose nucleic acid
rRNA	ribonucleic acid
Spp	species
<i>T. arjuna</i>	<i>Terminalia arjuna</i>
TB	Tuberculosis

TLC	Thin layer chromatography
TM	Traditional medicine
TNF	Tumour necrosis factor
UK	United Kingdom
UNESCO	United Nations Educational Scientific Cultural Organization
US	United States
US\$ or USD	United States Dollar
USDA	United States Dept of Agriculture
UV	Ultraviolet
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-Resistant <i>Staphylococcus aureus</i>
WHO	World Health Organisation
XDR	Extremely resistant

ABSTRACT

The main aim of this study was to isolate and characterise antifungal and antibacterial compounds from leaf extracts of *Combretum molle* which belonging to the Combretaceae family. *C. molle* is one of the commonly used medicinal plants in southern Africa for numerous ailments.

Three animal fungal pathogens, namely, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and five plant fungal pathogens, namely, *Aspergillus niger*, *Aspergillus parasiticus*, *Fusarium oxysporum*, *Penicillium janthinellum*, *Rhizoctonia solani* and four nosocomial bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* were used as test microorganisms for bioactive compounds in leaf extracts of *C. molle*.

Experiments for phytochemical analysis were done using different *C. molle* leaf extracts which were made using acetone, methanol, ethanol, ethyl acetate, chloroform, butanol and hexane as extractants. Thin Layer Chromatography (TLC) fingerprints of different leaf extracts were developed in three mobile phase systems, EMW, CEF and BEA and detected with vanillin-sulphuric acid spraying agent. The different extracts of *C. molle* showed the presence of many different compounds with distinct retardation factors (R_f), separated according to their polarities.

Bioautography was carried out to determine the number of active compounds and their R_f values. The TLC plates were developed in three mobile systems, each sprayed with either fungal or bacterial strains. In BEA bioautograms of *A. fumigatus*, clear zones of inhibition were observed at R_f values of 0.12, 0.23, and 0.40. In EMW bioautogram of *C. albicans*, clear zones of inhibition were observed at R_f value of 0.73, 0.81, 0.87. *C. neoformans* had weak growth inhibition. Most of the fungal and bacterial strains tested in the bioautography displayed susceptibility to the active compounds, with *P. janthinellum* and *P. aeruginosa* showing exceptional sensitivity.

The minimum inhibitory concentrations (MIC) values ranged from 0.02 to 2.5 mg/ml against the tested pathogens. The acetone and ethyl acetate extracts had the best inhibitory activity against *P. janthinellum* with an MIC value of 0.02 mg/ml. The acetone extract of *C. molle* gave the highest total activity (775 ml/g) against *P. janthinellum*. *C. albicans* was the most resistant pathogen with an average MIC

value of 0.56 mg/ml compared with the other tested strains. Extracts were active against both Gram-positive and Gram-negative strains. *P. aeruginosa* extracts had the highest average MIC value (0.24 mg/ml) among the tested bacterial strains. In general, there was good overall inhibitory activity by different extracts of *C. molle*.

Bioassay-guided fractionation of DCM extract of the leaves of *C. molle* yielded 32 fractions. Further fractionation led to the isolation of five compounds (C1, C2, C3, C4 and C5). Compound C1 was selected for structure elucidation due a larger quantity isolated and higher antimicrobial activity compared with the other isolated compounds. Nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS) was used to show that compound C1 was taraxerol, belonging to the taraxerane group. Antimicrobial activity of the isolated compound against *P. janthinellum* had an MIC value of 0.08 ug/ml. Although the compound taraxerol have been discovered in other plant species, it is reported for the first time from *C. molle* in the study. The results illustrate that crude extracts and compound taraxerol from *C. molle* can be used as either an antibacterial or antifungal, and warrants further investigation.

Keywords: *Combretum molle*, antibacterial activity, antifungal activity, taraxerol.



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CHAPTER 1

Introduction

1.1 Introduction

“All nature is at the disposal of humankind. We are to work with it. For without it, we cannot survive.” These words were mentioned by Saint Hildegarde of Bingen during the 12th century, who was one of the most famous women in the herbal tradition and wrote a medical text called *Causes and Cures*. These important words indicated the essential usefulness of plants and the relationship between plants, humans and animals (Hozeski, 2001). Traditional or natural medicine existed in one way or another in different cultures/civilizations, such as Egyptian, Chinese, Western, Kampo (Japan) and Greco-Arab or Unani/Tibb (South Asia) (Gilani and Atta-ur-Rahman, 2005). The search for new molecules, nowadays, has taken a slightly different route where science of ethnobotany and ethnopharmacognosy are used to lead the chemist towards different sources and classes of compounds (Gurib-Fakim, 2006).

The traditional use of medicinal plants in southern Africa is widespread with over 80% of Black South Africans households using medicinal plants (Holdstock, 1978; Mander, 1998). Southern Africa is one of the richest temperate centres of plant diversity in the world. The area does not only has unique flora, but it is extremely rich, diverse (30,000 higher plant taxa) (Goldblatt and Manning 2000), and largely endemic in character (Arnold and De Wet, 1993). The indigenous people of Southern Africa have a long history of traditional plant usage for medicinal purposes, with c. 3000 taxa being used for this purpose (van Wyk *et al.*, 1997). The trade in medicinal plants is an important part of the regional economy with over 700 plants species being reported as traded and the value of trade in the province of KwaZulu-Natal alone being of the order of US\$10 million (Mander, 1998). The global market for herbal drugs is lucrative and the world herbal trade is expected to reach USD 7 trillion by 2050 (Sen *et al.*, 2011).

Medicinal plants have become the focus of intense study recently in terms of conservation and their pharmacological use (Rabe and van Staden, 1997). Traditional herbal medicines form an important part of the healthcare of South Africans, and rely heavily on the use of indigenous plants. In South Africa alone it is

estimated that there are 27 million indigenous medicine consumers (Mander, 1998). In Africa, a large number of people are using traditional medicines and trade in medicinal plants is an important part in the internal economies and providing employment to many in these countries.

Although, South Africa possesses a rich tradition in the use of medicinal plants and an outstanding floral diversity, not that much research has been done on phytochemical leads for therapeutic use. Lately, there is a renewed interest in various alternatives to standard medical treatment and the issue of drug resistance and HIV-AIDS, among other problems, which is swiftly changing the landscape of research. New areas of research are sought after or old ones revisited, including search for antifungal compound in medicinal plants, personalised medical diagnoses. Although, antibacterial and antifungal activities have already been found in several plant species (Baba-Moussa *et al.*, 1999; Masoko *et al.*, 2005; Eloff *et al.*, 2008; Ahmed *et al.*, 2013), but further research needs to be done before some of the plants become extinct. There is an urgent need in South Africa for on-going scientific documentation of indigenous knowledge. It is also important that this be used for the application and benefit of sustainable development in South African communities (van Wyk and Geriecke, 2000).

The use of medicinal plant in their crude form, without scientific evaluation of their efficacy and safety could be harmful. Therefore, there is also the need to continue scientific evaluation of these plants. Through scientific validation of effective remedies, coupled with training of healthcare practitioners, it may be possible to bring traditional medicine to western healthcare systems (Light *et al.*, 2005). Because of the increasing global threat of multi drug resistance, new approaches and strategies need to be investigated in order to combat infectious diseases. The strategies may include text mining for new drug leads, modelling molecular pathways, predicting the efficacy of drug cocktails, analysing genetic overlap between diseases and predicting alternative drug use and “personalised medicine” (Yao *et al.*, 2009). Drug discovery strategies based on natural products and traditional medicines are re-emerging as attractive options. With this background knowledge of southern Africa having one of the richest biodiversity on earth, the study focuses on utilization of plant products as resource for finding new antimicrobial products. The tree, *Combretum molle*, belonging to the family of

Combretaceae, was dselected for this study. In a preliminary study (Eloff, 1999) *C. molle* had the highest antibacterial activity of 27 Combretaceae species investigated. Based on bioautography (Masoko and Eloff, 2006) *C. molle* leaf extracts contained at least three antimicrobial compounds. At the start of this study no antimicrobial compounds was isolated from *C. molle*.

1.2 AIMS and OBJECTIVES

1.2.1 AIMS

The aim of this study is to investigate the antifungal and antibacterial activities of *C. molle* leaf extracts and try to isolate and characterise the antimicrobial compounds.

1.2.2 OBJECTIVES

1. To screen *C. molle* extracts for antifungal and antibacterial activities.
2. To select one or two *C. molle* extracts to study further.
3. To isolate and characterise the antifungal and antibacterial bioactive compounds from *C. molle*.

1.3 BENEFITS OF THE STUDY

Although the antibacterial and antifungal activities of various medicinal plants have been investigated before, the antifungal activity of *C. molle* has not been studied in detail yet. Our aim in this work is to investigate the antifungal activity of *C. molle* extracts and fractions and to try to isolate and characterise the bioactive compounds. The results of this research will contribute towards the growing database of knowledge, which will eventually contribute to the rational use of safe and effective herbal remedies. This may help in understanding the mechanism and efficacy of medicinal plants.

CHAPTER 2

Literature Review

2.1 Medicinal plants

Many medicinal plants used in traditional medicine are readily available in rural areas at a much lower cost than modern medicine (Mann *et al.*, 2008). Since time immemorial, man has used plants to treat common diseases and some of these traditional medicine are still included as part of the common treatment of various diseases. Medicinal plants act as both potential antimicrobial crude drugs as well as a source for natural compounds that act as new anti-infective agents (Rios and Recio, 2005). Plants produce a variety of small-molecules, named “phytoalexins”, which defend plants against infections. The use of, and search for, drugs and dietary supplements derived from plants have accelerated in recent years. The World Health Organization (WHO) estimates that 80 percent of the population of the developing countries presently use herbal medicine for some aspect of primary health care.

2.2 Ancient and Modern History of Medicinal Plants

Material presented in this section was obtained mainly from the following publications:

(Solecki, 1975; Yesilada, 2005; Halberstein, 2005; Castleman, 2001)

Medicinal plants have been used long before the written human history. The Middle Palaeolithic age (60,000 years ago) is the approximate date which is indicated by fossil records as the documented period of medicinal plant use by humans. The discovery was made by palaeontologists when pollen clusters of different kinds of flowers were found in the grave of one of Neanderthals people, No IV, at the Shanidar cave, Iraq (Solecki, 1975; Yesilada, 2005). Five thousand years ago Sumerians wrote a list of hundreds of medicinal plants on clay tables from Nagpur during ancient Mesopotamian civilizations and during 1500 B.C. the Ancient Egyptians wrote the Ebers Papyrus, which contains information on over 850 plant medicines (Sumner, 2000). In India, as early as 1900 B.C., Ayurveda medicine was already practiced. The Sushruta Samhita attributed to Shushruta, the Indian herbalist, in the 6th century BC describes 700 medicinal plants, 64 preparations from mineral sources, and 57 preparations based on animal sources (<http://en.wikipedia.org/wiki/Herbalism>).

The Chinese emperor Shen Nung (of the Han Dynasty) is said to have written the first Chinese herbal, the *Pen Tsao*, which list 365 medicinal plants and their uses. The healers in the Aztec and Maya Indian culture of Mexico and Central America were experimenting with natural curing substances, developing large and effective pharmacopoeia, formulae for medicines concocted from animals, minerals and plants about a 1000 years ago (Halberstein, 2005).

The earliest known Greek herbals were those of Diocles of Carytus, written during the third century B.C., and one by Krateuas from the 1st century B.C. There was a large amount of overlap between the documented herbals of the early Greek and Egyptian herbalists. The Greek physicians, Hippocrates (460-377 B.C.) from the Island of Kos, and Dioscorides (1 A.D.) from Anarzabos have also described several plants remedies (Yesilada, 2005). Greek and Roman medical practices, as preserved in the writings of Hippocrates and Galen, provided the pattern for later western medicine (<http://en.wikipedia.org/wiki/Herbalism>).

During the Middle Ages the Benedictine monasteries were the primary source of medical knowledge in Europe and England. Medical schools known as Bimaristan began to appear from the 9th century in the medieval Islamic world among Persian and Arabs. Baghdad was an important centre for Arab herbalism, as was Al-Andalus (936-1013) between 800 and 1400. Abulcasis of Cordoba authored *The Book of Simples*, an important source of later European herbals, while Ibn al-Baitar (1197-1248) of Malaga authored the *Corpus of Simples*, the most complete Arab herbal which introduced 200 new healing herbs, including tamarind, *Aconitum*, and *nux vomica* (Castleman, 2001). Avicenna's *The Cannon of Medicine* (1025) lists 800 tested drugs, plants and minerals (Jacquart, 2008). He laid the foundation of Greco-Arab system of herbal medicine (Unani Tibb), based on the philosophy of individualised treatment considering the genetic variation amongst the individuals, similar to the concept of Pharmacogenetics in current conventional medicine. Aviceinna, (also known as al-Qanun fi al-Tibb), contributed to the sciences of pharmacy and medicine to such a degree that his works in Cannon Medicinæ is regarded as "the final codification of all Greco-Roman medicine" (Castleman, 2001).

The fifteenth, sixteenth, and seventeenth centuries were the great age of herbals, many of them available for the first time in English and other languages. Some

people refer to this period as the “Golden Age of Herbalism”. The two best-known herbals in English were *The Herbal or General History of Plants* (1597) by John Gerard and *The English Physician Enlarged* (1653) by Nicholas Culpeper. The Age of Exploration and the Columbia Exchange introduced new medicinal plants to Europe. The *Badianus Manuscript* was an illustrated Mexican herbal translated into Nahuatl and Latin in the 16th century (Gimmel, 2008).

Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80% show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived. At least 7000 compounds in the modern pharmacopoeia are derived from plants. It can be concluded that, there are many factors that played important roles in influencing the translations, writings and illustrations of these herbals (http://en.wikipedia.org/wiki/History_of_herbalism).

2.3 Traditional importance of medicinal plants

Medicinal plants play an important role in the development of human culture (religions and different ceremonies) and in the primary health care as drugs. Over the past 20 years, there has been a resurgence of worldwide scientific research in the field of ethnopharmacology. With the western world acknowledging the continued use of traditional medicines by the majority of third world countries and the need for novel developments, much of the pharmaceutical research in recent years has focused on ethnobotanical approach to drug discovery (Light *et al.*, 2005).

Medicinal plants have been used all over the world for the treatment and prevention of different diseases. Plant derived medicines have made large contributions to human health and they play an important role in the promotion and maintenance of good health (UNESCO, 1996). There have been many new developments in recent years in therapy. Many potent drugs have been purified from medicinal plants including anti-malarial, anti-cancer and anti-diabetic. In Africa, traditional medicine plays an important role and traditional healers are regularly consulted (Kamanzy *et al.*, 2002). Compounds derived from plants may become the base for the development of a medicine, a natural blue print for the development of a new drug or a phytomedicine to be used for the treatment of diseases.

In contrast to the two well-developed systems of traditional medicine, which have been extensively documented over centuries (Cragg and Newman, 2002), there is very little recorded documentation of most of Africa traditional medicine. The fact that African traditional knowledge systems are largely oral, and not written, it is passed from generation orally to the next. Traditional healers do not easily give information on traditional medicine because they are afraid of losing their ancestral-given healing powers (Personal discussion with traditional healers). As a result of urbanisation and strong cultural influences from other regions in the world, there is an ever-increasing loss of traditional knowledge in Southern Africa (van Wyk and Wink, 2004). The indigenous cultures are under threat from the patterns of social interaction and less knowledge is being handed down. Some of the modern younger generation are not interested in ancient indigenous knowledge systems. Consequently, some effort has been made towards investigating and documenting the traditional medicinal practices of a number of southern African cultural groups in more recent years. To date, only the tiny surface of these complex health systems has been exposed (Light *et al.*, 2005). The shift in research emphasis to the study of plants used in traditional medicine has been very noticeable over the last decade. There is an urgent need in South Africa for ongoing scientific documentation of indigenous knowledge systems.

The African concept of healing differs completely from the Western way of healing. For most indigenous South Africans, good health requires not only a healthy body, but also a healthy environment, because it is possible to absorb harmful elements from the environment that can cause misfortune and ill-health (Ngubane, 1977; Cocks and Dold, 2006). Therefore, ceremonies that involve ritual purification and communication with one's ancestors (badimo or izinyanya) are of foremost importance and using medicines, remedies and wearing protective necklaces are further good health strategies (du Pisani, 1988). Melmed (2001) discusses the lack of the integrative system by mentioning that considering the enormous scientific advances over the last century, it is ironic that the established medical system in the Western world has failed to develop a style of medical practice that is sufficiently sensitive to patients' feelings and needs.

The use of medicinal plant in their crude form, without scientific evaluation of their efficacy and safety could be harmful. Therefore, there is also the need to continue scientific evaluation of these plants for their safe use. Through scientific validation of

effective remedies, coupled with training of healthcare practitioners, it may be possible to bring traditional medicine to western healthcare systems (Light *et al.*, 2005). Traditional medicine-based bio-prospecting may offer promising new leads and numerous advantages in drug development that include reduction in dose, toxicity and treatment cost. It is also important that this be used for the application and benefit of sustainable development in South African communities where principles of access to benefit sharing can be practiced (van Wyk and Gericke, 2000).

Pharmacologically active compounds belong to the secondary metabolites of plants. Plants have developed some defence mechanisms in the form of antimicrobial metabolites, namely, prohibitins and phytoalexins. Plants use an intricate defense system against pests and pathogens, including the production of low molecular mass secondary metabolites with biological activity, which are synthesized *de novo* after stress and are collectively known as phytoalexins. These constituents are part of the arsenal of constitutive antifungal compounds produced by plants, also called pre-infectious metabolites, prohibitins or phytoanticipins (Grayer and Kokubun, 2001). Some may have developed because of interplay competition or an attractant role such as pollinators or symbionts (Wink and Schimmer, 2000). Chemical constituents of the bark and leaves may be similar and show identical biological activity (Zaschocke *et al.*, 2000b). The efficacy and safety of different medicinal plants can be tested for both human and animal consumption. The biodiversity of different plants can be protected through cultivation and sustainable harvesting.

2.4 Use of medicinal plants

For many thousands of years, nature has been a source of medicinal agents and an impressive number of modern drugs have been isolated from natural sources (Cragg and Newman, 2002). Led by instinct, taste, and experience, primitive people treated illness by using plants, animal parts, and minerals that were not part of their diet. All cultures have long folk medicine histories that include the use of plants. Even in ancient cultures, people methodically and scientifically collected information on herbs and developed well-defined herbal pharmacopeias. Much of the pharmacopeia of scientific medicine was derived from the herbal lore of native peoples. Due to inaccessibility, unavailability, cultural inclination and high cost of western drugs, most people in developing countries opt to use medicinal plants in their primary health

care. In Africa, traditional medicine plays an important role and it is estimated that up to 60% of the population consult traditional healers (Iwu, 1993; Van Wyk *et al.*, 1997; Kamanzy *et al.*, 2002).

Modern pharmacopoeias contain at least 25% of drugs derived from plants and many others, which are synthetic analogues (Farnsworth *et al.*, 1985; De Silva, 2005). Medicinal plants and poisonous plants have always played an important role in African society. Some of the most important and useful plants have been documented and an African Herbal Pharmacopoeia (AfrHP) (Brendler *et al.*, 2010) has been published by the Association of African Medicinal Plants Standards (AAMPS)[www.aamps.org]. The published book propose the establishment of a living database to update information on the selected species as well as phytochemical, pharmacological, toxicological and other regulatory standards. An updated document of African herbal plants was recently published with high quality monographs that go above and beyond those prepared in the original African Herbal Pharmacopoeia (Abegaz *et al.*, 2013).

There have been many new developments in recent years in disease therapy. Many potent drugs have been purified from medicinal plants including anti-malarial, anti-cancer, and anti-diabetic. None of the existing systemic antifungals satisfies the medical need for completely, there are weaknesses in spectrum, potency, safety, and pharmacological properties (Georgopapadakou, 1998).

A great number of screening programs have been initiated world-wide by pharmaceutical industries, universities and national research institutions with the aim of searching for new bioactive natural products in plants and other natural organisms that are promising enough to be developed into new drugs (Yaniv and Bachrach, 2005). The study requires the interaction of researchers with indigenous communities, the study of the chemical composition of the extracts and the pharmacological activities of the compound present (Mulholland, 2005). Indigenous knowledge is about the use of plants and other related uses (eg. food, building materials, curing of ailments, etc.). It has been developing for a long time through trial and error, and still is passed from generation by oral tradition (de Boer *et al.*, 2005). Samuelsson (1999) concludes that traditional medicine has provided western medicine with more than 40% of all pharmaceuticals. Plants may become the base

for the development of a medicine, a natural blue print for the development of a new drug or a phytomedicine to be used for the treatment of diseases. Nanotechnology addresses these challenges by using novel nanosized platforms for efficient drug delivery as illustrated in Fig.1 (Huh and Kwon, 2011).

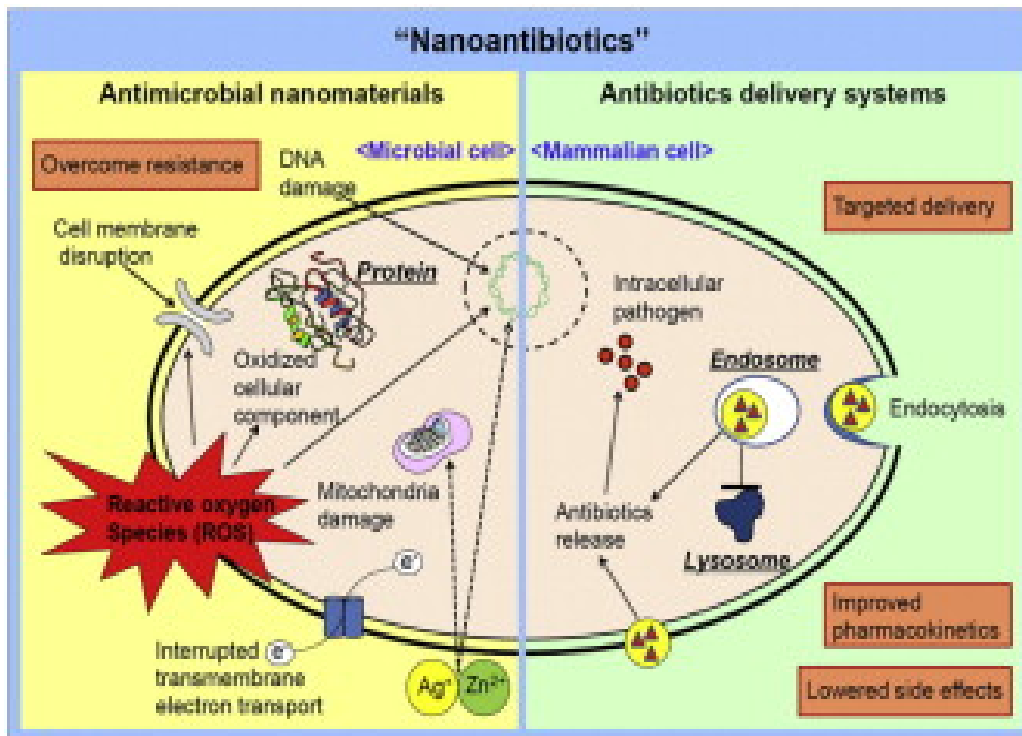


Figure 2. 1. Various antimicrobial mechanisms involving the use nanomaterials for improved drug delivery. (from Huh and Kwon, 2011).

2.5 Approach of the Phytomedicine Programme

Because traditional healers have mainly water as extractant and water does not extract antimicrobial compounds from plants (Eloff, 1998b, Kotze and Eloff, 2002) using traditional use as a guide for isolating antimicrobial compounds is not effective (Eloff and McGaw, 2014). Another approach was followed in the Phytomedicine Programme, i.e. the random screening of acetone leaf extracts of trees against 8 important human pathogens to select plant species to work on (Pauw and Eloff, 2014). This approach has led to many publications, discovery of highly active extracts, active compounds and patents.

2.5 Antibiotic resistance

Unwise use of antibiotics have led to microbes adapting genetically to changes in the environment, this has led to the search for new drugs to combat antibiotic resistance. As a result new challenging diseases are on the rise. The Infectious Disease Society of America (IDSA) recently reported that in US hospitals alone, approximately 2 million people acquire bacterial infections each year, and in 90,000 cases these infections have fatal outcomes (http://www.idsociety.org/pa/IDSA_Paper4_final_web.pdf). Infectious diseases are the world's leading cause of premature deaths, killing almost 50000 people every day (Ahmad and Beg, 2001). The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in immunocompromised, AIDS and cancer patients (Rinaldi, 1991; Diamond, 1993).

Research is being done on plant extracts showing targets sites other than those used by antibiotics which are already resistant to certain pathogens (Ahmad and Beg, 2001; Hasegawa *et al.*, 1995; Lee *et al.*, 1998). The type and frequency of resistance mechanisms varies in environments and they are related to qualitative and quantitative differences in the antibiotic used and, subsequently, the evolution of different resistance mechanisms may influence future use of antibiotics in the hospital and in the communities (Speldooren *et al.*, 1998; Essack, 2006).

When microorganisms are exposed to antimicrobial agents, severe consequences occur in their metabolism, and cells try to overcome the growth inhibitory action by development of various resistance mechanisms. The emergence of resistance has brought the industry to the point of requiring a severe paradigm shift in how antimicrobials are developed and brought to the market place. Numerous factors have impacted on the value of antibiotics in the market place: increase in the antibacterial sales, generics, segmentation, increase in the regulatory handle, total Research and Development cost versus return on investment and the competition of resources. There are considerable reports on the progress of resistance to the last line of antibiotic defences, which has led to the search for reliable methods to control vancomycin-resistant *Enterococci* (VRE) and vancomycin-resistant *Staphylococcus aureus* (VRSA), and methicillin resistant *Staphylococcus aureus* (MRSA). In addition, the synergy between tuberculosis and the AIDS epidemic, along with the surge of

multidrug-resistant isolates of *Mycobacterium tuberculosis*, has reaffirmed it as a primary health threat (Chambers, 2001; Hemaiswarya *et al.*, 2008).

2.5.1 Mechanisms of antimicrobial resistance

The information from this section was compiled from the following publications: (Heinemann, 1999; Heinemann *et al.*, 2000; McDonnell and Russell, 1999; Jin and Gross, 1989; Kristiansen, 1990)

Biochemical, phenotypic and genetic cross-activities of resistance mechanisms and genes can maintain resistance to medicinal antibiotics and they can be illustrated in several descriptive categories:

- a) DNA damage repair: *recA* is an example of a single gene that simultaneously neutralizes two antimicrobial agents, namely UV radiation and mitomycin-C. UV radiation is an example of a single antimicrobial agent that can select multiple genes with the commonability to detoxify a single antibiotic (Heinemann, 1999; Heinemann *et al.*, 2000)
- b) Permeability and efflux: reductions in membrane permeability and the multidrug efflux pathways illustrate the ability of a single mechanism to neutralize chemically different drugs. Efflux permeases pump out a remarkable variety of compounds with a few or no common chemical properties, ranging from antibiotics to amino acids (Heinemann, 1999; McDonnell and Russell, 1999)
- c) rRNA mutations: Multidrug resistance can be caused by modifications to, or mutations in, a single rRNA gene. Methylation of a base in the 23S rRNA results in complete cross-resistance to macrolides, lincosamides and streptogramin Type B (MLS), three structurally unrelated antibiotics (Saves and Masson, 1998)
- d) Multi-generational resistance: Single genes can detoxify drugs of different generations. The class A β -lactamases that hydrolyze both cephalosporins and penicillins are potent examples.
- e) Aminoglycoside modification: Resistance to aminoglycosides arising from various phosphorylating, adenylating or acetylating enzymatic activities is caused by both the ability of single enzymes to modify multiple drugs and multiple enzymes with overlapping activities acting on the same drug.

- f) Viruses and antibiotics: Single genes can confer resistance to both drugs and viruses or bacteriocins. Rifampicin-resistant *E. coli* are cross resistant to phages T7, T4 and λ and streptomycin resistant can also confer resistance to λ and f2 (Jin and Gross, 1989).
- g) Medicinal toxins. Single genes can simultaneously detoxify antibiotics prescribed to treat infections as well as other drugs that have unintended antimicrobial activities. The latter, which includes psychotherapeutics, anaesthetics, antihypertensives, diuretics and antihistaminics, might not have been developed to treat infectious diseases but they are, nevertheless, toxic to microbes (Kristiansen, 1990).

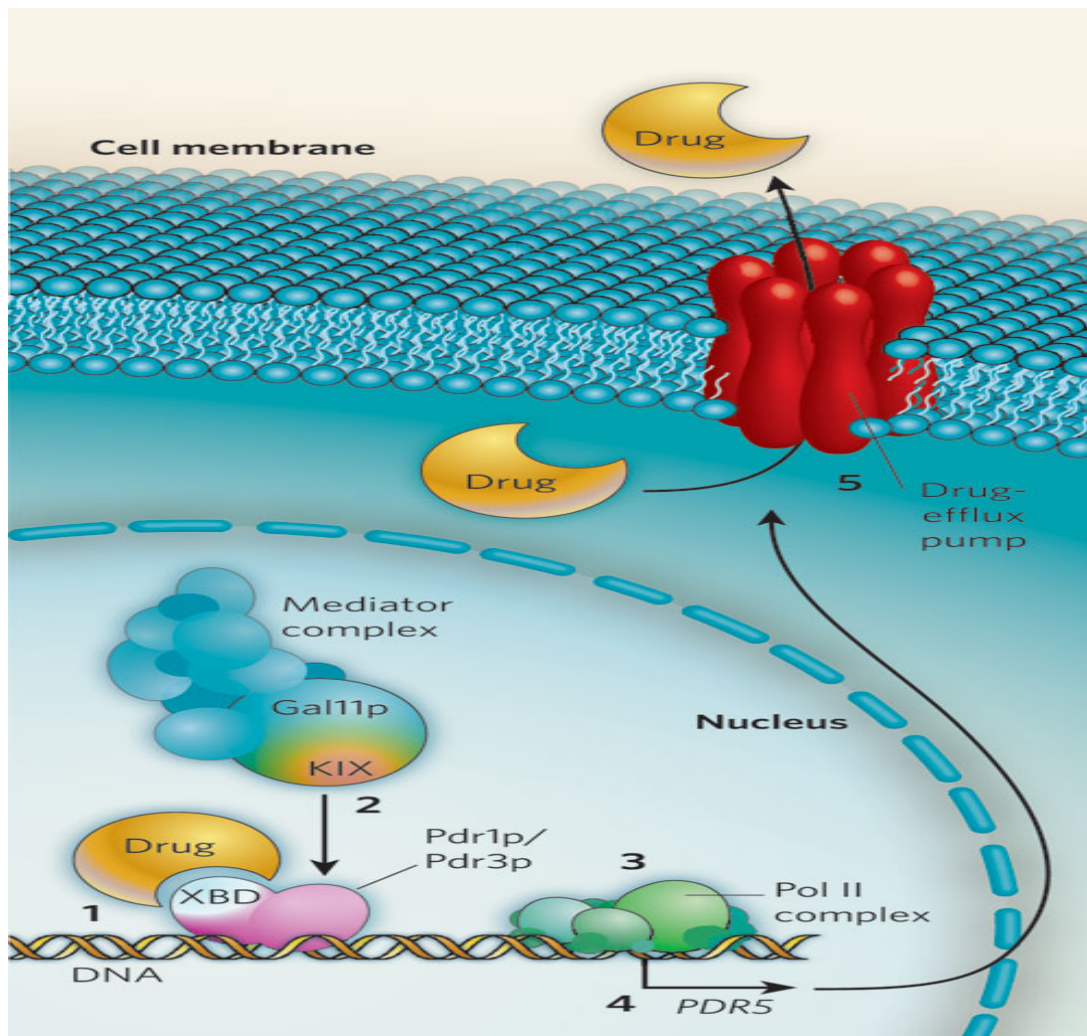


Figure 2. 2 Different mechanisms of antimicrobial resistance.

(<http://www.aspergillusblog.blogspot.com>)

Despite the overuse or underuse of antibiotics, there are other factors that have led to the resistance of drugs by certain infectious pathogens, namely; shifting priorities by business, complacency, incorrect diagnosis, shifting the blame, “seduction” of genomics, underappreciation of resistance, lack of integrated-consensus solution plan and industrial shift from natural product sources for novel chemotypes (Overbye and Barrett, 2005).

Candida albicans remains the most prevalent encountered human fungal pathogen, although other “non-albicans species have been reported which exhibit naturally occurring resistance to antifungal drugs already in use (Sobel, 1998; Pfaller *et al.*,1998). Antifungal resistance is a broad concept describing a failure of a fungal infection to response to antifungal therapy. Antifungal resistance has been traditionally classified as either primary (intrinsic) or secondary (acquired). The third type is described as “clinical resistance”, which encompasses progression or the relapse of an infection by a fungal isolate that seems, in the laboratory testing, to be fully susceptible to the antifungal used for the treatment of infection. Clinical resistance of fungi is typically seen in patients with persistent, profound immune defects or infected prosthetic material such as central venous catheters. In some cases, suboptimum drug concentration in the blood caused by drug interaction might contribute to clinical resistance (Alexander and Perfect, 1997; Kontoyiannis and Lewis, 2002).

Various resistance testing procedures have been proposed, including broth macrodilution and microdilution, agar diffusion, disk diffusion, and E-TEST. These techniques vary in cost, concordance, between methods, reproducibility, and interpretation. Methods further validated, antifungal susceptibility testing, possibly only in reference mycology laboratories, will probably become a useful tool for identifying optimum antifungal treatment strategies (Kontoyiannis and Lewis, 2002).

From epidemiological studies, antibiotic resistance may emerge by antibiotic selection pressure, but is mostly perpetuated by diverse risk factors and maintained within environments as a result of poor infection control. The WHO and a number of different countries have initiated strategies for the containment of resistance, with surveillance and delineation of the cause(s) cited as essential. Surveillance of antibiotic efficacy should be disease-based, establishing sensitivity profiles of

common causative agents to inform the development of or amendment to standard treatment guidelines and essential drugs lists adopted within the national drug policy. The manner of antimicrobial use associated with resistance should be established for appropriate intervention in terms of rational drug use, a reduction in use and dosing regimens based on population-specific pharmacokinetics and pharmacodynamics. Risk factors unique to South African communities (poverty, HIV) and hospitals must be determined and due vigilance exercised in patients exhibiting classical risk factors for the acquisition of or colonisation with resistant pathogens (Essack, 2006).

The pace of drug resistance development has outstripped the discovery of new antimicrobial agents and there is an urgent need for new antibiotic drugs with novel mechanisms of action. The question is how we tackle the problem more efficiently in the future, particularly given the fact that since 1970, only three new classes of antibiotics have been marketed (Graul and Prous, 2005; Butler and Buss, 2006).

2.6 Drug discovery

The introduction of the antibiotics in the 1930s revolutionised medical practice in combating bacterial infections, these discoveries led to a concerted search for new antibacterial drugs. Given this success, it is surprising to note that only three new antibacterial classes, the topical antibiotic mupirocin in 1985, the oxazolidinone linezolid in 2000 and the lipopeptide daptomycin in 2003, have entered the market since 1970. Over the past 20 years, there has been a 56% decline in the number of antibiotics approved annually by the Food and Drug Administration (FDA) and over the last decade, only 22 new antibacterial drugs based on a modification of the classes listed above have been launched. The 12 natural product (not plant) derived drugs belong to five different structure classes (β -lactam, streptogramin, macrolide, tetracycline and daptomycin), while the 10 synthetic drugs launched belong to only two antibacterial classes, with the quinolone class accounting for nine of the drugs (Newman *et al.*, 2000; Graul and Prous, 2005; Butler and Buss, 2006; Wright, 2010).

One of the most exciting anticancer drugs to be discovered recently is paclitaxel (Taxol[®]). It occurs, along with several key precursors (the baccatins), in the leaves of various *Taxus* species (commonly known as the yew). The discovery of taxol as an antitumor product started as early as 1962, when USDA botanist Arthur Barkley

collected *Taxus brevifolia* and submitted that biomass to the NCI's anticancer evaluation effort (Kingston *et al.*, 1993; Suffness, 1995; McChesney *et al.*, 2007). The chemical structure of paclitaxel (Taxol[®]) was reported and identified as the cytotoxic active constituent of extracts of *Taxus brevifolia* in 1971 (Wani *et al.*, 1971). Taxol[®] was approved for marketing as a cancer therapeutic agent in 1992 (McChesney *et al.*, 2007). It was found to act by promoting the assembly of tubulin into microtubules, and the discovery of this activity in 1979 by Schiff and others was an important milestone in the development of paclitaxel as a drug. After an extended period of development it was finally approved for clinical use against ovarian cancer in 1992 and against breast cancer in 1994. Since then it has become a blockbuster drug, with annual sales of over \$1 billion (Newman *et al.*, 2008).

It is estimated that 74% of pharmacologically active plant-derived compounds were discovered after the ethnomedical uses of the plants started to be investigated (Farnworth and Soejarto, 1991). Nowadays, few drugs are developed and approved since it is so costly and time consuming to develop a drug up to distribution level as the figure 3 below illustrate.

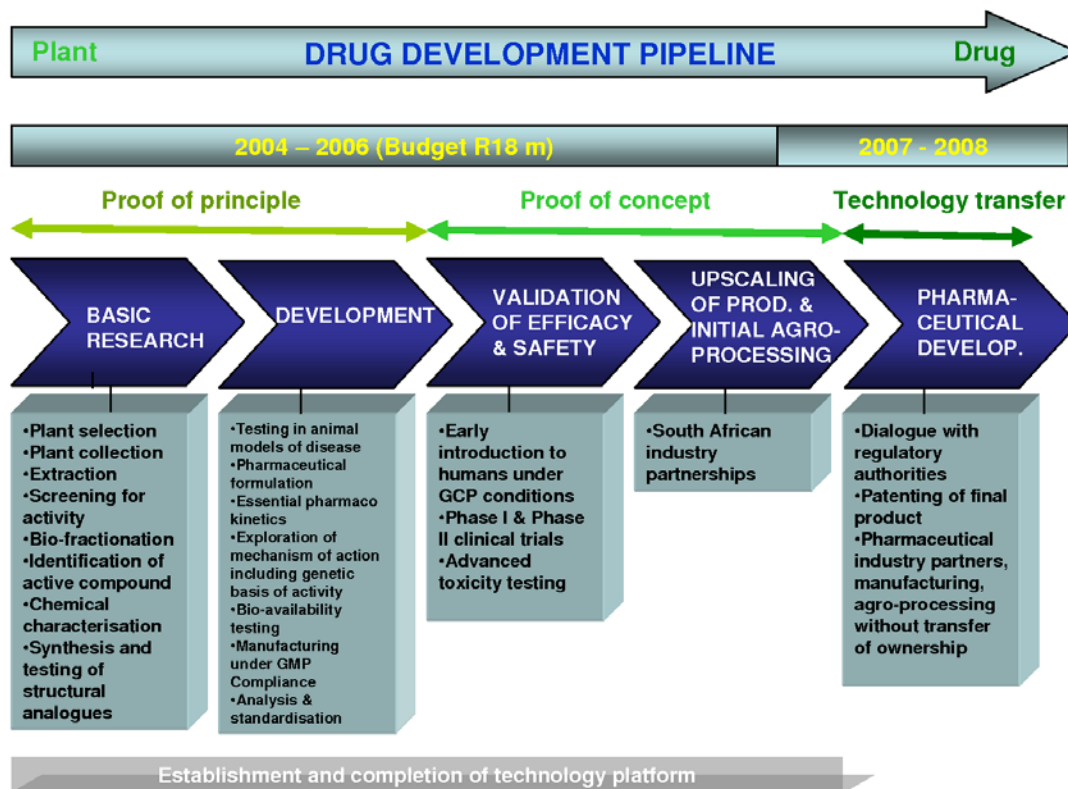


Figure 2. 3 Drug Discovery pipeline .(<http://www.docstoc.com>)

Drug discovery and development is frequently based on traditional and local knowledge (Heinrich, 2010). The urgency to develop new drugs stems from the therapeutic limitations of the two current class of polyenes (amphotericin B) and azoles (fluconazole) and itraconazole which display toxicity and a limited spectrum (Jiang *et al.*, 2002). Compounding this need is an increased incidence in life-threatening fungal infections associated with immunocompromised and neutropenic individuals.

Over the past 20 years, there has been a resurgence of worldwide scientific research in the field of ethnopharmacology. With the western world acknowledging the continued use of traditional medicines by the majority of third world countries and the need for novel developments, much of the pharmaceutical research in recent years has focused on ethnobotanical approach to drug discovery (Light *et al.*, 2005).

2.7 Microbial diseases

Many diseases are caused either by fungal, viral, bacterial, helminthic or protozoan pathogens. Pathogenic fungi have two forms – filamentous (moulds) and cellular (yeasts). Fungal infections (mycoses) can be classified into four categories, namely, superficial mycoses, cutaneous mycoses, subcutaneous mycoses and systemic mycoses. With the rise of HIV and organ transplantation, opportunistic fungal pathogens have become a common cause of morbidity and mortality (Fishman and Rubin, 1998; Garbino *et al.*, 2001).

Due to antibiotic resistance and other challenging infections, diseases of microbial origin have become increasingly prevalent, and the rapid increase in systemic and life-threatening mycoses due to the immune-compromised patient population is of great concern. The increase is due largely as a result of the progress in medical science that has led to treatment of cancer, transplants, corticosteroid use postsurgical intensive care, broad-spectrum antibiotic use, greater longevity, and increase survival rates in neonates and trauma patients (Dorr, 2007). Invasive fungal infections have an enormous impact on morbidity and mortality in immune-compromised patients. Affordable, effective and safe alternative drugs are needed to combat these diseases. Recently, a number of microorganisms have developed drug resistance to currently used drugs and they cause a number of therapeutic limitations (Fishman and Rubin, 1998; Garbino *et al.*, 2001). Currently therapy is generally limited to amphotericin B in its parent and lipid formulations, 5-flucytosine, fluconazole, and itraconazole (Neely and Ghannoum, 2000). As a result of these new challenges, new drugs have to be constantly developed to counteract the development of resistance and to possibly reduce the cost of controlling the disease (Cowan, 1999; Suleiman *et al.*, 2010). Antimicrobials are used in food for two main reasons: (1) to control natural spoilage processes, and (2) to prevent/or control growth of micro-organisms (Tajkarimi *et al.*, 2010).

2.7.1 Animal fungal pathogens

Fungi are eukaryotes and play an important role in the health and livelihood of people worldwide. They have numerous beneficial and harmful functions, for example, degrade organic waste material, cause diseases, spoil food and fabric. (Odds, 1987; Walsh and Dixon, 1996).

2.7.1.1 Candidiasis

Candidiasis (candidosis, moniliasis, iodiomycosis, yeast, or thrush) is a fungal infection (mycosis) of any of the *Candida* species, of which *Candida. albicans* is most common (James *et al.*, 2006). *Candida* is the predominant commensal fungus inhabiting the human oral cavity, genito-urinary tract. *C. albicans* is one of the many oral *Candida* species and is responsible for most oral candida infections. Systemic and local factors may reduce host resistance and promote the transition of *Candida* from commensal, to parasitic forms. *Candida* spp. are recognised as the fourth most common causes of nosocomial bloodstream infections in the USA (Edmond *et al.*, 1999). The aforementioned factors include medication, malnutrition, malignancies, immunopathologies, endocrinological disorder, xerostomia, trauma and poor denture hygiene. Medication associated with the emergence of clinical candidiasis are anticholinergic agents, antibiotics, corticosteroids, and immunosuppressives (Budtz-Jorgensen, 1990; Muzyka and Glick, 1995).

Antifungal-drug resistance in *Candida* spp. results from alterations in the membrane ergosterol biosynthesis pathway seems to be the most characterised mechanism of polyene resistance of *Candida* and the genetically similar non-pathogenic model yeast *Saccharomyces cerevisiae* (Dick *et al.*, 1980). Three mechanisms of secondary azole resistance have been described in *C. albicans*: reduced azole accumulation through active efflux, alteration or overexpression of the binding site 14 α -sterol-demethylase, encoded by ERG11), and a loss-of-function downstream mutation in the ergosterol pathway (defective Δ -5,6-desaturase, encoded by ERG3), allowing the accumulation of less toxic sterols in the presence of azoles (Karyotakis *et al.*, 1993). Fungal genomics is directly applicable to target identification and validation, to target prioritization and assay development, and when compounds are discovered, in confirming their mechanism of action and in toxicity-related issues, and it will still take some time until the process is completed (Jiang *et al.*, 2002). Alternative therapeutic agents, like drugs from natural products, must be sought in the meantime.

2.7.1.2 Cryptococcosis

Cryptococcus neoformans has become a major opportunistic fungal pathogen worldwide. *C. neoformans* is a yeast-like fungus which causes a progressive meningoencephalitis in 5 to 13% of all patients with AIDS (Zuger *et al.*, 1986). Most

HIV-related cases are caused by *C. neoformans* var *grubii* (serotype A), while var. *neoformans* (serotype D) is responsible for a proportion, and there are a small number of *Cryptococcus gattii* infections (Morgan *et al.*, 2006). Cryptococcosis is believed to be acquired by inhalation of the infectious propagule from the environment. The rare resistance of *Cryptococcus* spp. to amphotericin B is due to mutations in the ergosterol biosynthesis pathway but other mechanisms might also exist (Perfect and Cox, 1999). The mechanism of azole resistance involve

- (i) the alteration of the cytochrome P450 Erg11 protein: Erg11p is responsible for the demethylation of lanosterol and is an important enzyme of ergosterol biosynthesis (Sanglard *et al.*, 1998)
- (ii) Alteration of antifungal transport by enhanced efflux.
- (iii) Alteration in specific steps of the ergosterol biosynthesis pathway, where mutations of specific genes can occur (Wirsching *et al.*, 2000).

The figure 4 below shows the action of different antifungal drugs, including azole, in a eukaryotic cells.

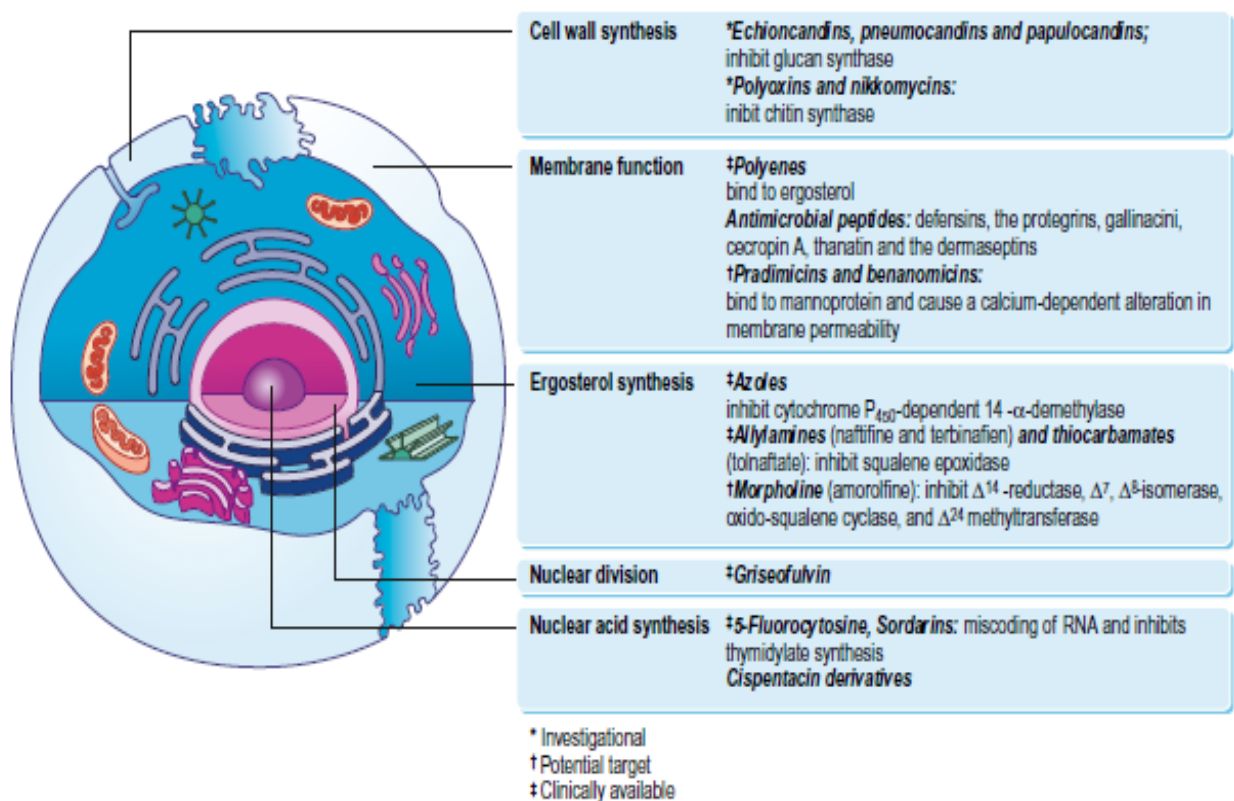


Figure 2. 4 Site of action of antifungals.

2.7.1.3 Aspergillosis

Aspergillosis is the name given to a wide variety of diseases caused by fungal infection of the genus *Aspergillus*. *Aspergillus fumigatus* is the most pathogenic among the causative agents of aspergillosis with widespread resistant spores present in the natural environment (Richard, 1997; Suleiman, 2009). Invasive mould infections, especially aspergillosis and candidiasis are currently a major cause of death in patients with haematological malignancies and in transplant recipients, and the cost of their treatment is staggering (Dasbach *et al.*, 2000; Kontonyiannis and Lewis, 2002). *Aspergillus fumigatus* also causes mycotoxicoses in poultry, which is a toxic syndrome resulting from the intake of mould contaminated feed, which has toxic metabolites of mould called mycotoxins (Suleiman *et al.*, 2012).

Aspergillus fumigatus is the species causing the majority of cases in aspergillosis (Kontoyiannis and Bodey, 2002), although less susceptible to antifungal agents non-fumigatus aspergillii are increasingly reported in severely immunocompromised individuals (Balajee *et al.*, 2004a). Failure of Amphotericin B treatment against invasive aspergillosis is common. Correlating that failure to Amphotericin B resistance of Aspergillosis spp. *in vivo* is difficult to prove. Therefore, few attempts have been made to correlate clinical outcome and Amphotericin B resistance in *Aspergillus* spp. either *in vitro* or in animal models. This is further complicated by the fact that individuals who develop *Aspergillus* infections are often severely immunocompromised (Moore *et al.*, 2000).

2.7.2 Plant fungal pathogens

A large part of agricultural plant production is lost yearly because of fungal pathogens. This can be by a direct effect of the fungi on plant growth or the production of toxins in plant products by fungi. It appears that mycotoxins have plagued mankind since the beginning of organized crop production. Analysis of weather records, grain production and mortality patterns provided evidence for a role for *Fusarium* mycotoxins in disease from medieval Europe through to American colonial times (Matossian, 1989; Miller, 1995). Fungal pathogens produce toxic substances in the form of mycotoxins or aflatoxins which lead to food spoilage or food poisoning. Four types of toxigenic fungi can be identified:

1. Direct plant pathogens such as *Fusarium graminearum* and *Fusarium oxysporium*.

2. Fungi that grow and produce mycotoxins on senescent or stressed plants, such as *Fusarium moniliforme*.
3. Fungi that initially colonize the plant and predispose the commodity to mycotoxin contamination after harvest, e.g. *Aspergillus flavus*.
4. Fungi that is found in the soil or decaying plant material that occur on the developing kernels in the field and later proliferate in storage if conditions permit, e.g. *Penicillium verrucosum*.

2.7.2.1 *Fusarium* species

Fusarium oxysporium appears to be largely cosmopolitan, with higher concentrations of the various *Formae speciales* in different areas across the globe http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html.

F. oxysporium causes vascular wilt disease in tomato crops. *F. oxysporium* plays the role of a silent assassin – the pathogenic strains of this fungus can be dormant for 30 years before resuming virulence and infecting a plant. *F. oxysporum* is infamous for causing a condition called Fusarium wilt, which is lethal to plants and swift – by the time a plant shows any outward signs of infection, it is already too late, and the plant will die (Katan, 1971; http://en.wikipedia.org/wiki/Fusarium_oxysporum). *Fusarium* head blight (FHB) causes huge economic losses to wheat and barley farmers worldwide.

2.7.2.2 *Penicillium* species

Penicillium chrysogenum is a widely studied species of *Penicillium* and is sometimes known as *P. notatum*, *P. meleagrinum*, or *P. cyaneofulvum* though occasionally they are not synonymous. It plays a significant role in the medical community as an antibiotic because it can create penicillin which inhibits the biosynthesis of bacterial cell walls affecting lysis of the cell (Fleming, 1929). *P. chrysogenum* is a common fungus that can inhabit a wide variety of habitats including the soils of degraded forests, on the pollen and provisions of alfalfa leafcutter bees, and in Arctic subglacial ice where they feed on sediment-rich basal ice shelves (Sonjak *et al.*, 2006). Pitt (1991) has pointed out that as early as 1881, extracts were made of rice infected with *Penicillium islandicum* that induced mortality in various animals. Penicilic acid from *P. puberulum* from corn was discovered in 1913. *Penicillium expansum* (agent of blue mould disease) is one of the main causes of spoilage of pears and apples after harvest and is frequently isolated from a wide range of other

fruit, including stone fruit, soft fruit and berry fruit (Snowdon, 1990). *Penicillium chrysogenum* is rarely pathogenic except in certain circumstances such as people with severely compromised immune systems, like those with human immunodeficiency virus (HIV) (Adrian *et al.*, 2005).

2.7.2.3 *Aspergillus* species

Aspergillus niger is a saprophyte and it causes black mould in onion, garlic and shallot. *Aspergillus* spp. cause considerable crop losses by producing highly toxic aflatoxins that can contaminate cotton seed, corn, peanuts, and tree nuts during harvesting or storage (Wilson and Payne, 1994). *Aspergillus* species belonging to the section *Nigri* can cause considerable damage on the yield and the quality of the wine grape harvest. Despite the expenditure of a large research effort worldwide, aflatoxins contamination continues to be a major problem, especially in maize (Miller, 1995). Aflatoxin B₁, the most toxic of the aflatoxins, causes a variety of adverse effects in different animal species, especially chickens. It has been observed that tannins isolated from medicinal plants possess remarkable toxic activity against bacteria and fungi and it may assume pharmacological importance (Bobbarala *et al.*, 2009; Bansa and Adeyemo, 2007).

2.7.3 Bacterial Diseases

The ability of pathogen to attach to fresh produce depends on intrinsic and extrinsic factors including motility of the pathogen, their interaction with the other organisms and leaching of nutrients from the plant (Aruscavage *et al.*, 2006)

Bacteria play a larger role in animal and human infection than plant infection. Due to antibiotic resistance and toxic effects associated with the use of antibiotics such as vancomycins, tetracyclines and penicillin, alternative modes of treatment must be sought. Side effects of vancomycin include nephrotoxicity, ototoxicity, anaphylactic reactions and phlebitis (Rybak *et al.*, 1990).

2.7.3.1 *Escherichia coli* and related species

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. They are facultative anaerobic non-sporulating bacteria. The genera *Escherichia* and *Salmonella* diverged around 102 million years ago. Most *E. coli* strains are harmless, but some serotypes can cause

serious food poisoning in humans, and are occasionally responsible for product recalls due to food poisoning

One of the most significant foodborne pathogens that has gained increase attention in recent years is *E. coli* O157:h7. Mead *et al.* (1999) estimate that this organism is responsible for approximately 73,000 cases of human illness and 61 deaths per year in the United States.

2.7.3.2 *Pseudomonas* species

Pseudomonas aeruginosa is a Gram-negative, oxidase-positive, aerobic rod that belongs to the family Pseudomonadaceae. The organism is an opportunistic pathogen that causes nosocomial infections, but it can also be found in other environments throughout the world. An opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds and also causes blood infections. Pyocyanin is a virulence factor of the bacteria and has been known to cause death in *Caenorhabditis elegans* by oxidative stress. However, research indicates that salicylic acid can inhibit pyocyanin production (Prithiviraj *et al.*, 2005). *P. aeruginosa* uses the virulence factor exotoxin A to ADP-ribosylate eukaryotic elongation factor 2 in the host.

2.7.3.3 *Staphylococcus* species

Staphylococcus aureus is a Gram-positive bacterium that is a member of the Staphylococcaceae. *S. aureus* cause diseases such as bacteremia, endocarditis, toxic shock syndrome, sepsis and other metastatic infections. The virulent pathogenesis of *S. aureus* infection is due to a combination of nasal carriage and bacterial immune-evasive strategies (Kluytmans *et al.*, 1997). The virulence of *S. aureus* infection is remarkable, given that the organism is a commensal that colonizes the nares, axillae, vagina, pharynx, or damaged skin surfaces (Lowy, 1998). *S. aureus* produces numerous toxins that are grouped on the basis of their mechanisms of action. Cytotoxins, such as the 33-kd protein-alpha toxin, cause pore formation and induce proinflammatory changes in mammalian cells which eventually lead to sepsis syndrome. Different domains of the enterotoxin molecule are responsible for the two diseases caused by superantigens, the toxic shock syndrome and food poisoning. *Staphylococci* produce various enzymes, such as proteases, coagulases, lipases and hyaluronidases, that cause tissue destruction. Resistance to

methicillin confers resistance to all penicillinase-resistant penicillins and cephalosporins, which requires the presence of the *mec* gene that encodes penicillin-binding protein 2a (Carter *et al.*, 2000).

2.7.3.4 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive, non-motile, facultatively anaerobic bacterium inhabiting the gastrointestinal tracts of humans and other mammals. *E. faecalis* can cause endocarditis and bacteremia, urinary tract infections, meningitis, and other infections in humans. Several virulence factors are thought to contribute to *E. faecalis* infections. A plasmid-encoded hemolysin, called the cytolysin, is important for pathogenesis in animal models of infection, and the cytolysin in combination with high-level gentamicin resistance is associated with a five-fold increase in risk of death in human bacteremia patients (Huycke *et al.*, 1991; Chow *et al.*, 1993).

Useful strategies for the management of aflatoxin-producing fungi have come from studies of their natural history (Wicklow, 1994). Natural products may be able to play a role in combating the fungal related pathogens. Neely and Ghannoum (2000) discussed novel treatment strategies such as drug combination therapy, pharmacological reformulations to improve the efficacy or reduce toxicity of current antifungal drugs, immune function augmentation, nanomedicine and vaccine development. All of these strategies, although in their infancy, will enhance the clinician's ability to care for patients with invasive fungal infections.

2.8 Southern African Biodiversity

The continent of Africa, and especially southern Africa, has a rich diversity of plants, and statistics show that about 25% of the total number of higher plants in the world is found in Africa south of Sahara (van Wyk, 2008). With this background knowledge of southern Africa having one of the richest biodiversity on earth, the study focuses on utilization of plant products as resource for finding new antimicrobial products (Fyhrquist, 2007). The study was also based the progress made on random screening of leaf extracts of trees against bacteria and fungi of medical importance by our group, the Phytomedicine Programme at the University of Pretoria (www.up.ac.za/phyto). The tree plant, *Combretum molle*, belonging to the family of Combretaceae, was a plant of choice.

2.9 Family of Combretaceae

The Combretaceae family (Table 1.1) consists of nineteen genera and it belongs to the order Myrtales. The six genera in “our area” Southern Africa include *Combretum*, *Lumnitzera*, *Meiostemon*, *Qiusqualis* and *Terminalia*. It consist of a total of some six hundred species distributed throughout the tropics and subtropics (Klopper *et al.*, 2006) considering the first checklist of flowering plants in sub-Saharan Africa. The family may include trees, shrubs, small shrubs, climbers and mangroves. *Combretum molle* (R. Br. Ex G. Don) Engl. Diels (*Combretum*), is a small to medium-sized perennial, erect, terrestrial, semi-deciduous tree of about 6-10 metres in height, with spreading crown.

Table 2. 1 The Combretaceae family (Carr, 1988)

THE COMBRETACEAE		
Combretum L.	Section <i>Spathulipetala</i> Engl. & Diels	Section <i>Oxystachya</i> Excell
Subgenus <i>Combretum</i>	<i>C. Zeyheri</i> Sond	<i>C. Oxystachyum</i> Welw. Ex Laws.
Section <i>Hypocrateropsis</i> Engl. & Diels	Section <i>Ciliatipetala</i> Engl. & Diels	Section <i>Poivrea</i> (Comm. Ex DC)
<i>C. celastroides</i> Welw. Ex Laws	<i>C. albopunctatum</i> Suesseng.	<i>C. bracteosum</i> (Hochst)
<i>C. imberbe</i> Wawra	<i>C. apiculatum</i> Sond.	<i>C. mossambicense</i> (Klotzsch)
<i>C. padoides</i> Eng. & Diels	<i>C. edwardsii</i> Exell. (provisional)	Section <i>Megalantherum</i> Excell
Section <i>Combretastrum</i> Eichl	<i>C. moggii</i> Excell.	<i>C. wattii</i> Excell.
<i>C. umbricola</i> Engl.	<i>C. molle</i> R. Br.	
Section <i>Angustimarginata</i> Engl. & Diels	<i>C. petrophilum</i> Retief	Terminalia L
<i>C. caffrum</i> (Eckl. & Zeyh.) Kuntze	<i>C. psidoides</i> Welw.	

<i>C. erythrophyllum</i> (Burch.)	Section <i>Fusca</i> Engl. & Diels	Section <i>Abbreviatae</i> Excell
<i>C. kraussii</i> Hochst (incorporating <i>C. nelsonii</i> Duemmer)	<i>C. coriifolium</i> Engl. & Diels	<i>T. prunioides</i> Excell.
<i>C. vendae</i> Van Wyk	Section <i>Breviramea</i> Engl. & Diels.	<i>T. randii</i> Bak. F.
<i>C. woodii</i> Duemmer	<i>C. hereroense</i> Schinz.	<i>T. stuhlmannii</i>
Section <i>Macrostigma</i>tea	Section <i>Elaegnoida</i>	Section <i>Psidoides</i>
Engl. & Diels	Engl. & Diels	Excell
<i>C. engleri</i> Schinz	<i>C. elaegnoides</i> Klotzsch	<i>T. brachstemma</i> Welw. Ex Hierr
<i>C. kirkii</i> Laws		<i>T. sericea</i> Burch. Ex DC
<i>Combretum</i> sp. Nov. (provisional)	Subgenus <i>Cacoucia</i> (AUBL.)	<i>T. trichopa</i> Diels
Section <i>Metallicum</i> Excell & Stace	Section <i>Lasiopetala</i> Engl. & Diels	Section <i>Platycarpae</i> Engl. & Diels
<i>C. collinum</i> Fresen	<i>C. obovatum</i> F. Hoffm	<i>T. gazensis</i> Bak. f.
Section <i>Glabripetala</i> Engl. & Diels	Section <i>Conniventia</i> Engl. & Diels	<i>T. phanerophlebia</i> Engl. & Diels
	<i>C. microphyllum</i> Klotzsch	<i>T. mollis</i> Laws
	<i>C. paniculatum</i> Vent.	<i>T. sambesiaca</i> Engl. & Diels
	<i>C. platypetalum</i> Welw. Ex Laws	<i>T. stenostachya</i> Engl. & Diels

The leaves usually opposite or verticillate, having scales or stalked glands, dense with velvety hairs, particularly below, with tapering apex, rounded to shallowly lobed base, and netting conspicuously raised below. Inflorescences auxiliary or terminal, spicate, paniculate. Flowers sessile, 4- to 5-merous, bisexual, the receptacle having 2 parts,

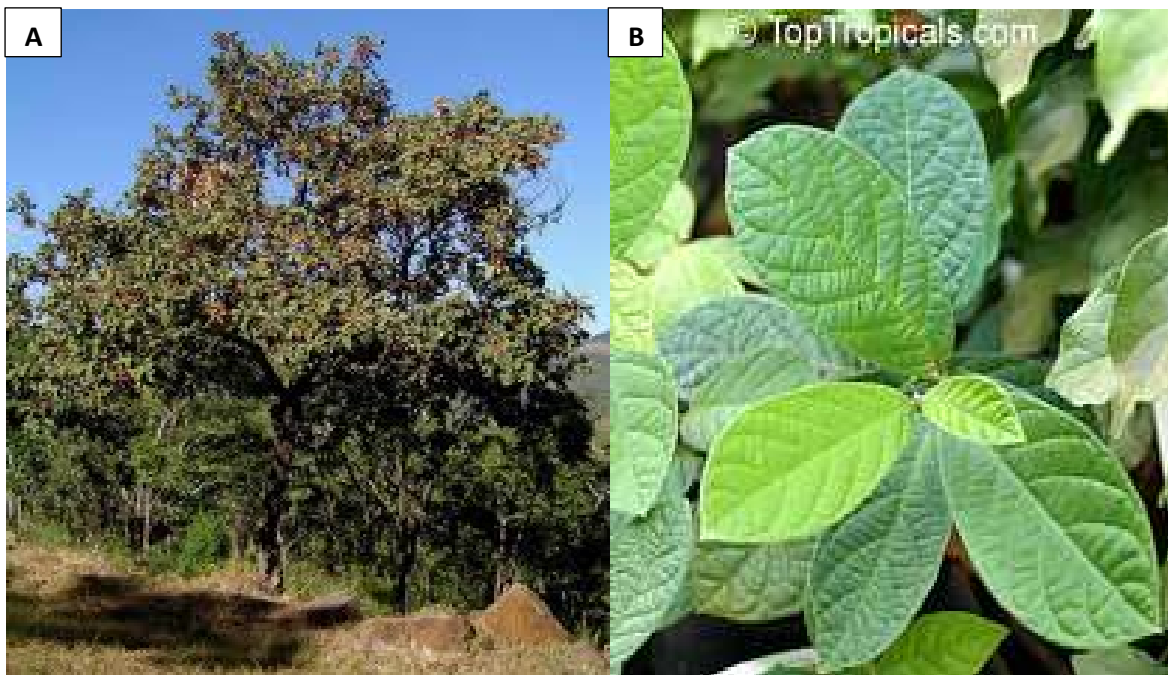




Figure 2.5 **A.** *Combretum molle* full-grown tree. **B.** *C. molle* twig with leaves. **C.** Flowers and fruits of *C. molle*. **D.** The stem bark of *C. molle*.

(<http://www.google.co.za/images>)

lower and upper, petals 4 or 5, stamens 8 or 10. The fruit (with one exception) has 4- or-5-winged samara, which is a unique feature of the group. The yellowish-green fruit flushed with red, drying to golden reddish-brown, are borne abundantly with, some old fruit remaining on the tree into the next flowering season (van Wyk and van Wyk, 1997). The chemical composition and antimicrobial activity varies greatly among different families and genera of Combretaceae (Eloff *et al.*, 2008).

2.10 Traditional uses of Combretaceae

Different species of the genera of Combretaceae have been used in traditional for many years. This include treating bilharziasis, burns, headache, earache, toothache, hookworm, abdominal disorders, abdominal pains, diarrhoea, dysentery, gallstones, gastric ulcers, urinary infections, nosebleeds, sore throats, colds, pneumonia, chest cough, conjunctivitis, dysmenorrhoea, infertility in women, venereal diseases, fattening babies, kidney pains, haemoptysis, mumps, convulsion, leprosy, caput medusae, jaundice, heart disease, mumps, weak body, blennorrhagia, dysmenorrhea, stomach and gastric problem, constipation, scorpion and snake bite,

aphrodisiac and general weakness. Leaves, bark and roots are normally used but not the fruits due to their toxicity to humans (Rogers and Verotta, 1996). Alcoholic extracts of leaves with water extracts of twigs have shown capacity to reduce sarcoma tumors in animals. Aqueous extracts of stem bark of *C. molle* have a longstanding reputation for the treatment of liver diseases, malaria and tuberculosis (Asres *et al.*, 2006; Fyhrquist *et al.*, 2002; Koné *et al.*, 2004).

2.11 Biological activities of Combretaceae

The Phytomedicine Programme have studied several aspects of the Combretaceae since the early seventies and the work is continuing. Combretaceae has the a number of biological activities including: antibacterial, antifungal, antitumor, antiplasmodial, antihelmintic, analgesic, anti-inflammatory, etc. Martini and Eloff (1998) demonstrated 14 unidentified antibacterial compounds of different polarities from members of the Combretaceae. Eloff (1999) found that all the leaf extracts from 27 southern African members of Combretaceae exhibited antibacterial activity against *Staphylococcus aureus*, *E. coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. McGaw *et al.* (2001) reported both anti-inflammatory and antischistosomal activity. Asres *et al.* (2001) reported that acetone extracts of leaves from *C. molle* has antiprotozoal activity. Analgesic, anti-inflammatory, and cardiovascular effects of mollic acid glucoside were isolated from *C. molle* leaves (Ojewole, 2008; Asres *et al.*, 2001). Baba-Moussa *et al.*, (1999), tested antifungal activities of seven West African Combretaceae used in traditional medicine. Masoko *et al.* (2007) investigated the antifungal activity of twenty-four southern African *Combretum* species (Combretaceae) species against five fungal animal pathogens (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigates*, *Microsporum canis* and *Sporothrix schenkii*) pathogens. The methanolic extracts of *C. moggii* and *C. petrophilum* were most effective against all pathogen. All extracts of *C. nelsonii* were also very effective against all pathogens and based on these results and work done earlier, *C. nelsonii* was selected for fractionation and bioassay-guided isolation of the antifungal compounds followed by *C. albopunctatum* and *C. imberbe*. Bacterial and antifungal activities as already indicated has been investigated by several authors but the antifungal activities of *C. molle* have not been studied in depth.

2.12 Phytochemistry of Combretaceae

The Combretaceae is the source of a wide range of tannins, flavonoids, terpenoids and stilbenoids (Eloff *et al.*, 2008; Dawe *et al.* 2013). Pettit *et al.* (1982) isolated combretastatins from the South African *Combretum caffrum* and discovered potent anti-tubulin activity. Pegel and Rogers (1985) observed that the major constituent of the acetone extract of *C. molle* is a colourless, sparingly water-soluble, crystalline triterpene acid saponin which has been named mollic acid glucoside. The dihydrophenanthrenes and phenanthrenes from *C. caffrum* showed good activity against murine P388 lymphocytic leukaemia cell lines (Pettit *et al.*, 1982). Rogers and Thevan (1986), have shown that mollic acid glucoside is a mixture of mollic β -D-xyloside and α -L-arabinoside. Martini *et al.*, (2004), investigated biological activity of five antibacterial flavonoids from *C. erythrophyllum*. Two related triterpenes, asiatic acid and arjunolic acid were identified for the first time, as the main antifungal compounds from the leaves of *C. nelsonii* (Masoko *et al.*, 2008). It was suggested that, tannins and saponins may have been responsible for the antifungal activity. Since then, several compounds have been isolated from the Combretaceae family, including flavonoids, phenanthrenes, stilbenes and cyclobutanes. Five species of Combretaceae, namely, *Anogeissus leiocarpus*, *Combretum fragrans*, *Terminalia glaucescens*, *T. laxiflora* and *T. macroptera*, growing in Togo were investigated for their antifungal activity against 20 pathogenic fungi (Batawila *et al.*, 2005). The five hydroethanolic extracts of *T. glaucescens* and *A. leiocarpus* appear to be the most active. The motivation for investigating tree leaves is that it can become a sustainable resource if a product is developed (Eloff, 2001).

Asres *et al.* (2006) determined minimum inhibitory concentration of *C. molle* using the checker board technique and found that the highest antibacterial action of the acetone extract was against *E. coli* and *Shigella* spp with an MIC value of 50 mg/ml. The antimicrobial activity was attributed to the high amount of hydrolysable tannins present in the bark of the plant. For antiprotozoal activity, two tannins and two oleanane-type pentacyclic triterpene glycosides were isolated (Asres *et al.*, 2001). The specific compounds identified were ellagitannin, punicalagin, arjunglucoside sericoside and the structure of the other (CM-A) which was not elucidated. Punicalagin and CM-A had IC₅₀ values of 1.75 and 1.50 μ M, respectively, against *Trypanosome brucei rhodesiense*.

In 2008, Ojewole reported that *C. molle* leaf extractive mollic acid glucoside (MAG) possesses analgesic and anti-inflammatory properties. The extractive (MAG, 5-80 mg/kg i.p) also significantly reduced ($p < 0.05-0.001$) rat paw oedema induced by subplantar injections of fresh egg albumin in a dose-related fashion. Ojewole and Adewole (2009) further investigated the hypoglycaemic and antidiabetic properties of mollic acid glucoside (MAG), an alpha-hydroxycloarteniod extractive from *C. molle* leaf. These findings lend a pharmacological credence to the folkloric, ethnomedicinal uses of the plant's leaf in the management of diabetes mellitus in some rural communities of southern Africa. Antimycobacterial, antibacterial and antifungal activities of the methanol extract from the stem bark of *Terminalia superba* as well as as the fractions and compounds were evaluated (Kueté *et al.*, 2010). The antimicrobial activity of 50 medicinal plants in Cote-d'Ivoire were also screened (Kone *et al.*, 2004). Of the 14 plants from western Canada analysed for antifungal activity, three demonstrated strong antifungal activity potential (Webster *et al.*, 2008). Seventeen plants from Tanzania have been evaluated for antifungal activity before and found to be active against various fungi including *Candida* species and *Cryptococcus neoformans* (Cavert, 1997). The Combretaceae is the source of a wide range of tannins, flavonoids, terpenoids and stillbenoids (Eloff *et al.*, 2008). The dihydrophenanthrenes and phenanthrenes from *C. caffrum* showed good activity against murine P388 lymphocytic leukaemia cell lines (Pettit *et al.*, 1982). Arjunolic acid and glycosides have been isolated from *C. molle* and *Terminalia arjuna* (Kumar and Prabhakar, 1987; Panzini *et al.*, 1993). Co-occurrence of tetracyclic and pentacyclic triterpenoids is unusual but *C. molle* contains both (Panzini *et al.*, 1993).

Recently, researchers have extensively engaged themselves in the area of antibacterial and antifungal activities in Combretaceae (Baba-Moussa *et al.*, 1999; Masoko *et al.*, 2005; 2007; Eloff *et al.*, 2008; Shai *et al.*, 2008), more research needs to be done on these genera because of their importance in traditional medicine and plant conservation. Angeh and co-workers (2007) isolated a new oleanene-type triterpenoid glycoside from *C. padoides* identified as 1 α ,23 β -dihydroxy-12-oleanen-29-oic-acid-23 β -O- α -4-acetylramnopyranoside. A new oleanane-type triterpene saponin, β -D-glucopyranosyl 2 α , 3 β , 6 β -trihydroxy-23-galloylean-12-en-28-oate was isolated by Ponou and others in 1993. Katerere *et al.* 2003, isolated four pentacyclic triterpenes from *C. imberbe* of which two are novel glycosidic derivatives 1 α , 3 β ,

trihydroxyolean-12-en-29-oic acid. Structures of some of the isolated compounds from the Combretaceae are illustrated below.

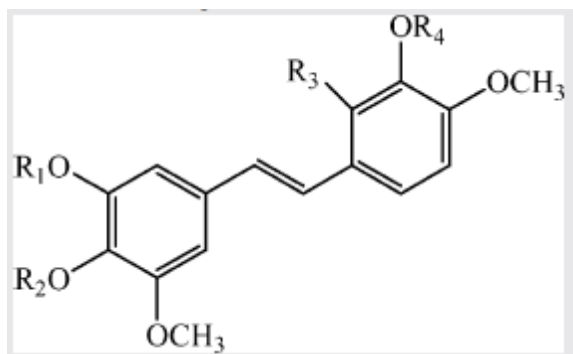


Figure 2. 6 Primary structure of Combretastin A isolated from *C. caffrum*, *C. kraussi* (Pettit *et al.*, 1982)

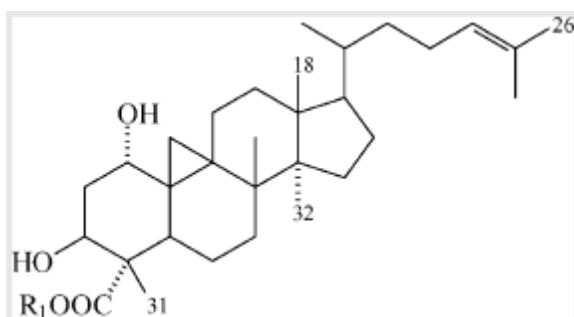


Figure 2. 7 Mollic acid and derivatives from *C. molle* (Panzini *et al.*, 1993)

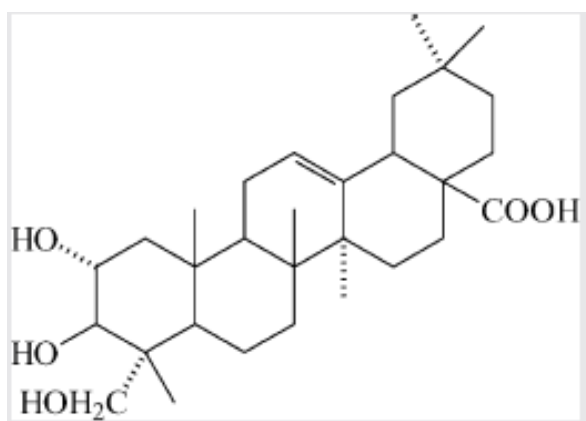


Figure 2. 8 Arjunolic acid from *C. molle* and *T. arjuna* (Panzini *et al.*, 1993., Kumar and Prabhakar, 1987).

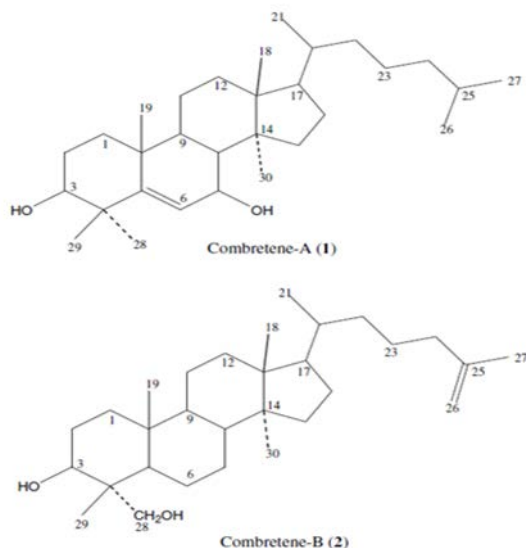


Figure 2. 9 Structure of combretene-A and B: Two new triterpenes from *Combretum molle* (Ahmed *et al.*, 2004).

CHAPTER 3

Extraction and phytochemical analysis

3.1 Introduction

Medicinal plants have become the focus of intense study recently in terms of conservation and their pharmacological use (Rabe and van Staden, 1997). The World Health Organization (WHO) estimates that 80% of the population of the developing countries presently use herbal medicine for some aspect of primary health care. In South Africa alone it is estimated that there are 27 million indigenous medicine consumers (Mander, 1998). Medicinal plants have been used all over the world to treat and prevent different diseases. Plant-derived medicines have made a large contribution to human health and they play an important role in the promotion and maintenance of good health (UNESCO, 1996). There have not been many new developments in recent years in antimicrobial therapy. Many potent drugs have

been purified from medicinal plants including anti-malarial, anti-cancer, anti-diabetic products. The use of medicinal plants in their crude form, without scientific evaluation of their efficacy and safety could, however be harmful. Therefore, there is also the need to continue scientific evaluation of these plants.

In a paper investigating the antimicrobial activities of different members of the Combretaceae (Eloff, 1999) *Combretum molle* had the highest activity of the 27 species examined. Although there has been done some work on the phytochemistry (Rogers, 1989), the antimicrobial compounds present in *C. molle* has not been examined in detail, motivating this study.

3.2 Materials and Methods

3.3 Plant collection

Leaves of *Combretum molle* were collected from the Onderstepoort Campus of the University of Pretoria. Voucher samples were made and kept in the Medicinal Plant Herbarium of the Phytomedicine Programme , Onderstepoort, University of Pretoria, South Africa.

3.4 Plant treatment

Leaves of *C. molle* were dried at room temperature in the dark. They were milled to a fine powder using a Macasalab mill (Model 200 Lab) and stored at room temperature in closed containers in the dark until use.

3.5 Extraction procedure

3.5.1 Phytomedicine laboratory extraction method

Leaf powder of the *C. molle* (1 g) was extracted with 10 ml of different solvents (Eloff, 1998a). The following extractants were chosen according to their polarity range: methanol (polar), acetone, ethyl acetate and chloroform (intermediate polarity), butanol, dichloromethane and hexane (non-polar). The samples were shaken on a Labotec (model 20.2) shaking machine at a moderate speed for 30 min at room temperature. The mixture was centrifuged at 3500 xg for 10 min decanted and filtered through Whatman No. 1 filter paper using a Büchner funnel. The extract was stored into pre-weighed, labelled glass vials. The extraction procedure was

repeated twice on the marc to exhaustively extract the plant material. The solvent was allowed to dry in front of stream of cold air. The percentage (%) yield of extract was calculated after weighing the vials with the dried extract.

3.6 Phytochemical analysis

Thin Layer Chromatography (TLC) is a technique that allows for the separation of compounds based on their polarities and other interactions and is frequently used for the analysis of herbal medicines. Chemical constituents of the extracts were analysed using aluminium-backed TLC plates (ALUGRAM-Silica gel 60 /UV 254 – MACHEREY-NAGEL), and developed with one of the three eluent systems developed in the Phytomedicine Programme (Kotze and Eloff, 2002):

- EMW [ethyl acetate: methanol: water: 40:5:4] (polar)
- CEF [chloroform: ethyl acetate: formic acid: 5:4:1] (intermediate polarity/acidic)
- BEA [benzene: ethanol: ammonia hydroxide: 90:10:1](non-polar/basic)

Development of the chromatograms was done in closed tanks saturated with the solvent system. The separated compounds were visualized under visible and ultra violet light (254 and 360 nm). For detection of chemical compounds extracted, vanillin in sulphuric acid spray reagent (0.1 g vanillin (Sigma®): 28 ml methanol: 1 ml sulphuric acid) was used. The developed TLC plates were carefully heated at 110°C until optimum colour developed.

3.7 Test organisms

3.7.1 Fungal test organisms

3.7.1.1 Animal pathogenic fungi

Candida albicans, *Cryptococcus neoformans*, and *Aspergillus fumigatus* were used. The animal fungal species were obtained from the culture collection in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science at the University of Pretoria. Fungal cultures were maintained in Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK).

3.7.1.2 Plant fungi

Plant fungal species used were *Penicillium janthinellum*, *Aspergillus niger*, *Aspergillus parasiticus*, *Fusarium oxysporum*, and *Rhizoctonia solani*. The plant fungal test species were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria. Fungal cultures were maintained in Potato dextrose starch (Oxoid, Basingstoke, UK) for plant fungi.

3.7.2 Bacterial cultures

Bacterial cultures used were *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 27853), *Pseudomonas aeruginosa* (ATCC 25922). The bacterial cultures were maintained on Müller Hilton agar at 4°C and in M-H broth at 37°C before use.

3.8 Bioautographic assay

A bioautography method developed by Masoko and Eloff (2005) was used to determine active compounds for fungi and bacterial strains (Begue and Kline, 1972). TLC plates (20 X 10 cm) were loaded with 100 µg of each of the extracts using a 10 µl micropipette. The prepared plates were developed at different mobile systems. The chromatograms were dried at room temperature under a stream of air overnight to remove the remaining solvent. The chromatograms were inoculated with a fine spray of the concentrated suspension of actively growing fungal conidia or bacterial cells. The plates were sprayed until wet, and incubated overnight at 25 - 37°C. The following day plates were sprayed with a 2 mg/ml solution of [*p*-iodonitrotetrazolium violet (INT)] (SIGMA). Plates were incubated at 25 - 35°C for several hours or overnight in the dark. Clear zones on the chromatograms indicated inhibition of growth. The chromatograms were scanned to produce a record of the results.

3.9 Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) value of different plant extracts were determined using the serial microplate dilution method developed by Eloff (1998a) with slight modification by Masoko *et al.* (2005) for the fungi. The plant extracts were dissolved in acetone to a concentration of 10 mg/ml and 100 µl of the plant extracts were serially diluted two-fold with water and 100 µl of the prepared culture of the test organisms was added to each well. Acetone was used as a negative control and gentamicin and amphotericin B were used as positive controls for bacteria and fungi,

respectively. The plates were incubated overnight at their required temperature of microbial growth. After incubation, 40 μ l of 0.2 mg/ml of INT was added to each well as a growth indicator and incubated further until colour change occurred. The plates were checked at 30 minutes intervals. Microorganism growth led to the emergence of purple-red colour resulting from the reduction of INT into the formazan.

The total activity of extracts was calculated by dividing the mass (in mg) of the extract obtained from 1 g of plant material by the MIC value (mg/ml). Total activity indicates the volume to which the extract derived from 1 g of plant material can be diluted and still inhibit the growth of microbial cells.

3.10 Results and Discussions

3.10.1 Extraction

The solvents used for extracting leaves were as follows: methanol, butanol, acetone, ethyl acetate, dichloromethane, chloroform and hexane. Butanol extracted a much higher quantity of plant material (41 mg) (24%) than any other solvent (Table 3.1, Figure 3.1). The masses extracted by ethanol (34 mg) and acetone (31 mg) were second and third highest, respectively. Hexane and dichloromethane extracted the lowest masses, 8 and 9 mg/ml, respectively.

The percentage yield of acetone extract (18%) falls within the range 2.6 to 22.6% observed in a study of selection of the best extractant done by Eloff (1998b). Hexane and dichloromethane extracted the lowest mass of plant material extracted, as also observed by Masoko (2006). In the study of Eloff (1998b), the rate of extraction of

Combretum erythrophyllum and *Anthocleista grandiflora* was the highest for acetone, but in the case of *A. grandiflora* methanol (MeOH) and methanol:chloroform:water (MCW) had the highest rate of extraction. MCW extracted a total of 45% of dry weight of *A. grandiflora* and 35% of the dry weight of *C. erythrophyllum* in 24 h, respectively. According to Masoko *et al.* (2007) on a study of the activity of 24 southern African *Combretum* species, methanol was the quantitatively the best extractant. Total percentages extracted with methanol of *C. apiculatum* subspecies *apiculatum*, *C. petrophilum*, *C. hereroense* and *C. microphyllum* varied between 25 and 41%. Acetone was chosen as the extractant of choice because of its volatility, miscibility with polar and non-polar solvents and its relatively low toxicity to the test organisms (Eloff, 1998) and also it extracted the most antimicrobial compounds from *Combretum microphyllum* (Kotze and Eloff, 2002).

Table 3. 1 Mass in mg extracted from 1 g of *C. molle* leaves by different extractants and % yield of extracts

Extract	Mass	Percentage (%) yield
Acetone	31	3.1
ethanol	34	3.4
methanol	17	1.7
chloroform	14	1.4
DCM	8	0.08
Hexane	9	0.09
Ethyl acetate	15	1.5
Butanol	41	4.1

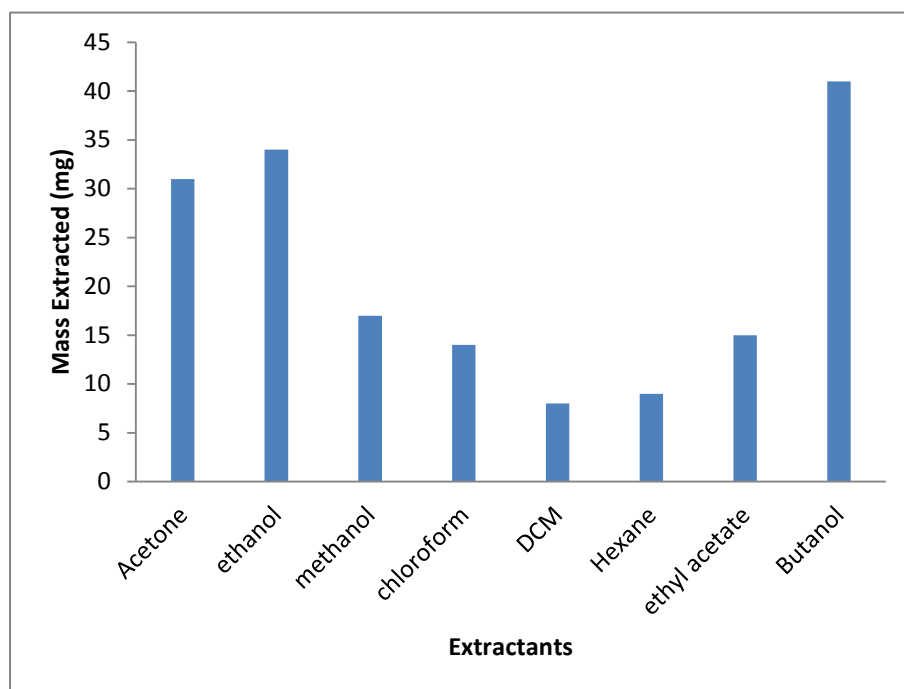


Figure 3. 1 Mass of *C. molle* dried ground leaves extracted with different extractants from 1.0 g of leaves.

(DCM – dichloromethane)

3.10.2 Phytochemical analysis

Thin Layer Chromatography (TLC) fingerprints of different extracts of *C. molle* were developed in three mobile phase systems, EMW, CEF and BEA. Acetone, methanol, ethanol, ethyl acetate, butanol and hexane were used as extractants. The separated compounds were detected with vanillin sulphuric acid spraying agent. The compounds separated according to their polarities as illustrated in the Figure 3.2. The extracts of acetone, methanol and dichloromethane eluted in the EMW mobile system, had a higher number of bands than that of butanol and chloroform. Hexane extracted fewer compounds, as expected in the non-polar region of the bioautogram as observed in the study on *Combretum imberbe* and *Combretum padoides* (Angeh, 2006). This concurs with the findings of Masoko *et al.* (2007), where it was observed

that acetone and methanol extracted more compounds in different species of *Combretaceae*.

The EMW chromatogram in Figure 3.2 was used to separate the different compounds according to their polarity. EMW separated compounds which are mostly polar. R_f value for each compound was calculated to analyse their corresponding positions and their similarity. The R_f values (EMW) of most prominent bands of acetone, methanol and ethanol extracts were almost similar to that of chloroform at R_f values of 0.62, 0.66, and 0.73, respectively. Separated bands on butanol extracts were four (R_f values 0.24, 0.26, 0.33, 0.37) compared with those of acetone which were nine.

In the non-polar mobile phase system BEA, most of the compounds from all extracts separated well and seemed to be of intermediate polarity since most of them appeared at the lower part of the chromatogram, as expected. The chloroform and DCM extracts displayed intense bands as compared with the other extracts. Most intense bands on the BEA mobile system had the following R_f values of 0.12, 0.20, 0.24, 0.34. indicating the intermediate polarity of compounds separated on BEA mobile system.

In the CEF chromatogram, most of the compounds separated very well. They show even distribution of bands throughout the TLC chromatogram, with the exception of hexane. The most intense bands appear at these R_f values 0.20, 0.30, 0.50, 0.56, 0.78 and 0.83. The good separation observed illustrate that the right choice of extractant and mobile system is very important in the extraction of plant material observed by Angeh *et al.* (2006).

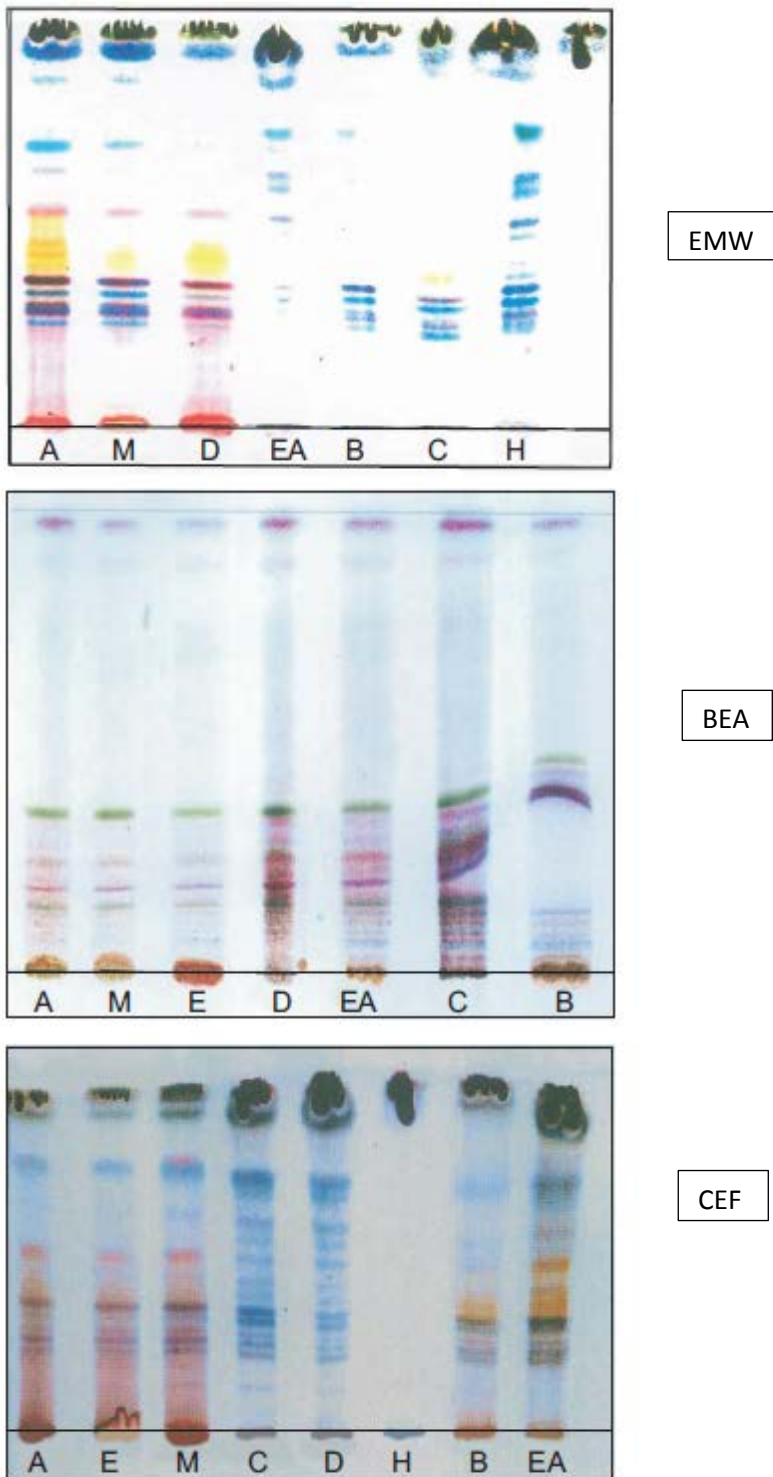


Figure 3. 2 TLC chromatograms of *C. molle* extracted with different extractants and developed in EMW (top), BEA (middle) and CEF (bottom) mobile systems and sprayed with vanillin-sulphuric acid reagent.

(A – acetone, E - ethanol, M - methanol, C - chloroform, D – dichloromethane, H – hexane, B - butanol, EA - Ethyl acetate).

3.10.3 Bioautography

The bioautography was used to determine the number of active compounds in the different extracts and their retardation factors (R_f). The TLC plates were developed in three mobile systems (EMW, CEF and BEA), all sprayed with animal and plant fungi and bacteria. After an overnight growth, the bioautograms were sprayed with INT as an indicator of growth. Zones of inhibition are shown by the clear white bands where growth was inhibited on the bioautogram indicating high levels of antifungal activity.

In BEA bioautograms sprayed with *Aspergillus fumigatus* as illustrated in Figure 3.3A, all the extracts had clear zones of inhibition at R_f values of 0.12, 0.23, and 0.40. The clear zones of inhibition correspond with some of the intense bands displayed on the lower base of the TLC chromatogram (Figure 3.3b).

The EMW bioautograms of *Candida albicans* also had clear zones of inhibition indicating antifungal activity. For *C. albicans*, zones of inhibition were observed at R_f values of 0.73, 0.81, 0.87 (Figure 3.4). With all the extracts, the compounds separated very well. The butanol extract however had only two bands at 0.81 and 0.87. *Cryptococcus neoformans* had weak lines of growth inhibition, but within 24 h of incubation, the zones of inhibition disappeared, suggesting that there might have been fungistatic and not fungicidal activity.

BEA, EMW, CEF bioautograms sprayed with *A. niger* had clear zones of inhibition and corresponding TLC fingerprints showing distinct bands (Figure 3.5). The best resolution of bioactive compounds was obtained in most of the bioautograms. *Pseudomonas aeruginosa* was more susceptible to the ethyl acetate extract than other test strains (Figure 3.7). Antibacterial activity of ethyl acetate extract on *P. aeruginosa* (EMW bioautogram), had five (5) bands of zones of inhibition than acetone extract (2 bands) and dichloromethane (3) extracts (Figure 3.7). *P. aeruginosa* was more susceptible to the ethyl acetate extract than acetone and dichloromethane extracts. The R_f values were indicated as follows 0.43, 0.50, 0.73, 0.79, 0.97 in Figure 3.7. The acetone, ethyl acetate and DCM extracts eluted in CEF exhibited antifungal activity at the following R_f values 0.37, 0.56, 0.76, respectively.

The acetone, ethyl acetate and DCM extracts (BEA bioautogram) indicated zones of inhibition of growth of *P. aeruginosa* at the following R_f values, 0.09, 0.19, 0.27

(Figure 3.7). All bacterial strains tested in the bioautography, namely, *S. aureus*, *E.coli* and *E. faecalis* displayed similar antibacterial activity except *P. aeruginosa* which was most sensitive to all the extracts.

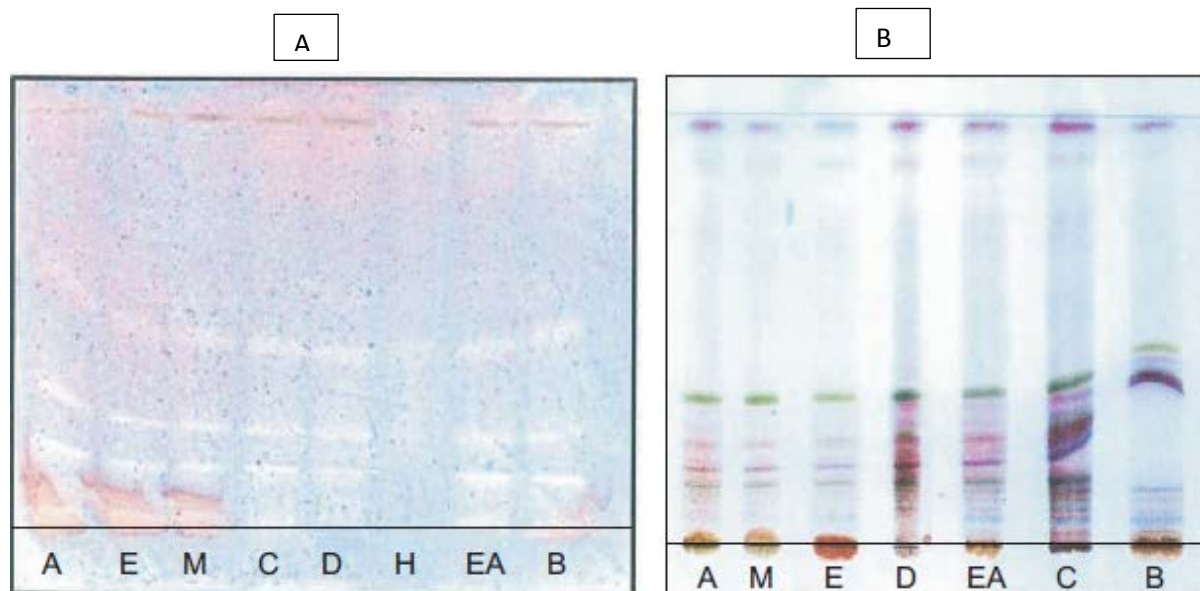


Figure 3. 3 TLC Bioautogram sprayed with *Aspergillus fumigatus* (A) developed in BEA, and sprayed with INT and TLC fingerprints of *C. molle* extracted with different extractants (B), developed in BEA, sprayed with vanillin sulphuric acid reagent . Clear zones of the bioautograph indicate fungal growth inhibition.

(A – acetone, E- ethanol, M- methanol, C- chloroform, D – dichloromethane, H – hexane, B- butanol, EA- Ethyl acetate.)

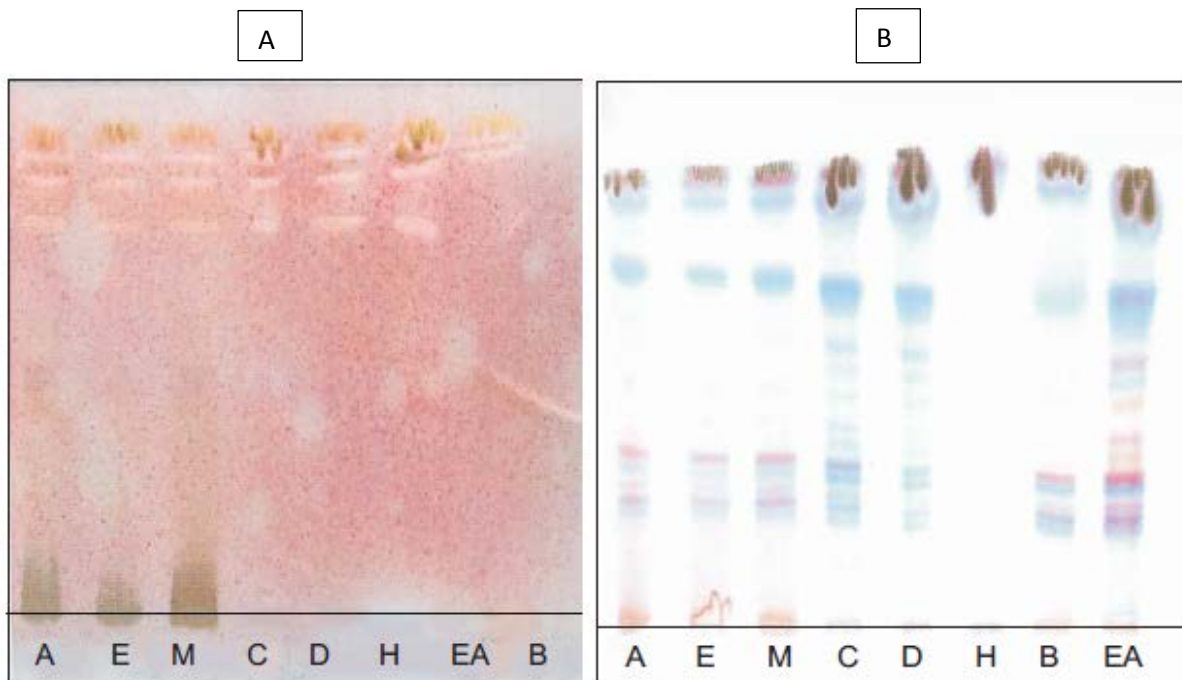


Figure 3. 4 TLC Bioautogram sprayed with *Candida albicans* (A) developed in EMW, and sprayed with INT and TLC fingerprints of *C. molle* extracted with different extractants (B), developed in EMW, sprayed with vanillin sulphuric acid reagent. Clear zones of the bioautograph indicate fungal growth inhibition.

(A – acetone, E- ethanol, M- methanol, C- chloroform, D – dichloromethane, H – hexane, B- butanol, EA- Ethyl acetate).

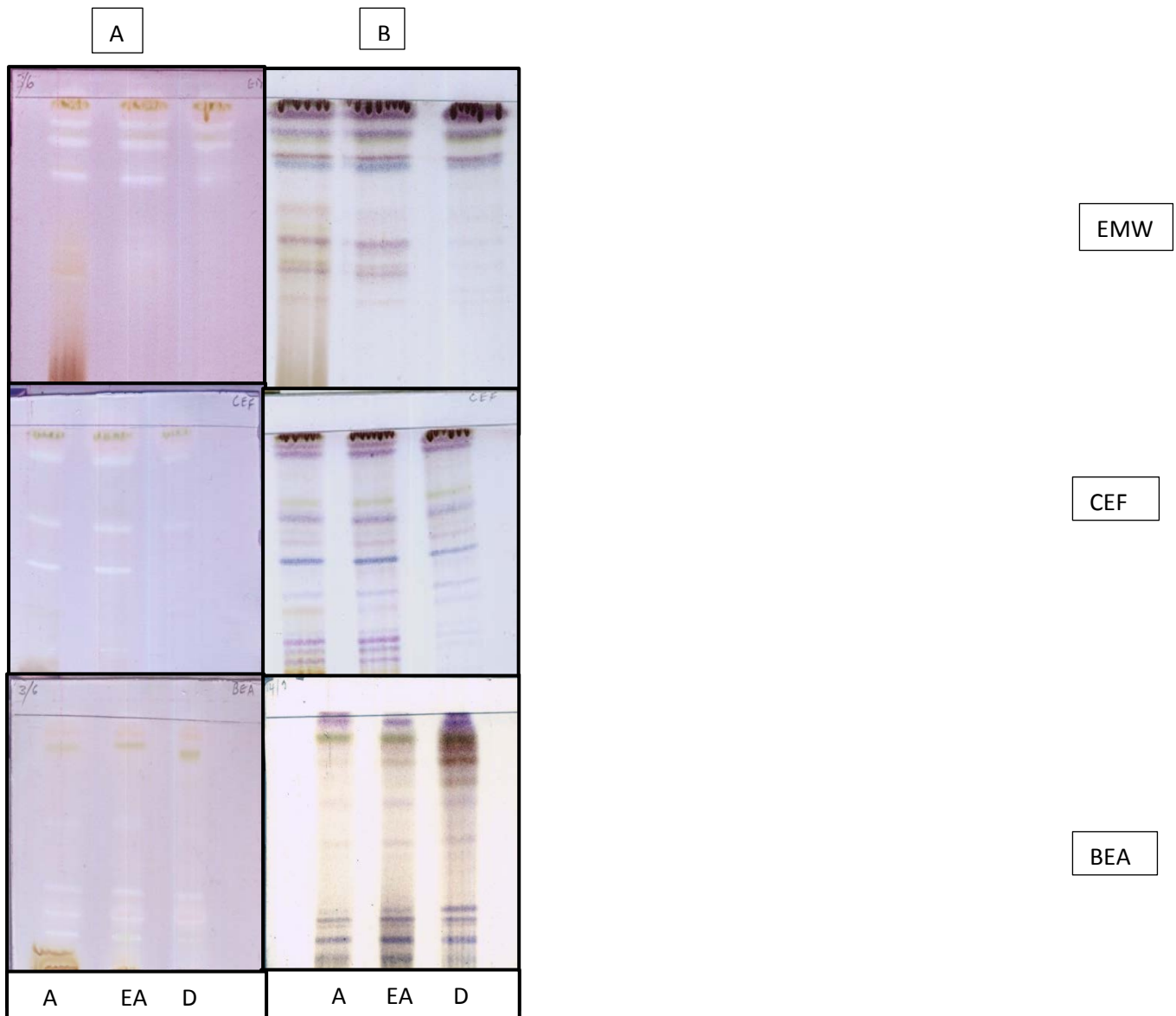


Figure 3. 5 TLC bioautograms sprayed with *Aspergillus niger* (A) developed in EMW, CEF, BEA, and sprayed with INT; TLC fingerprints of *C. molle* extracted with different extractants (B), developed in EMW, CEF, and BEA, sprayed with vanillin sulphuric acid reagent. Clear zones on the bioautogram indicate fungal growth inhibition.

(A - acetone, EA - ethylacetate, D – dichloromethane).

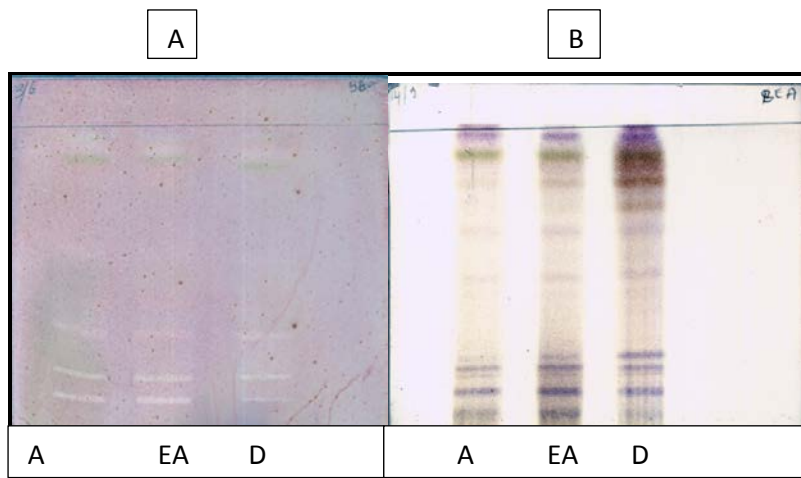


Figure 3. 6 TLC bioautogram sprayed with *P. janthinellum* (A) developed in BEA, and sprayed with INT and TLC fingerprints of *C. molle* extracted with different extractants (B), developed in BEA, sprayed with vanillin sulphuric acid reagent . Clear zones of the bioautogram indicate fungal growth inhibition.

(A - acetone, EA - ethylacetate, D – dichloromethane).

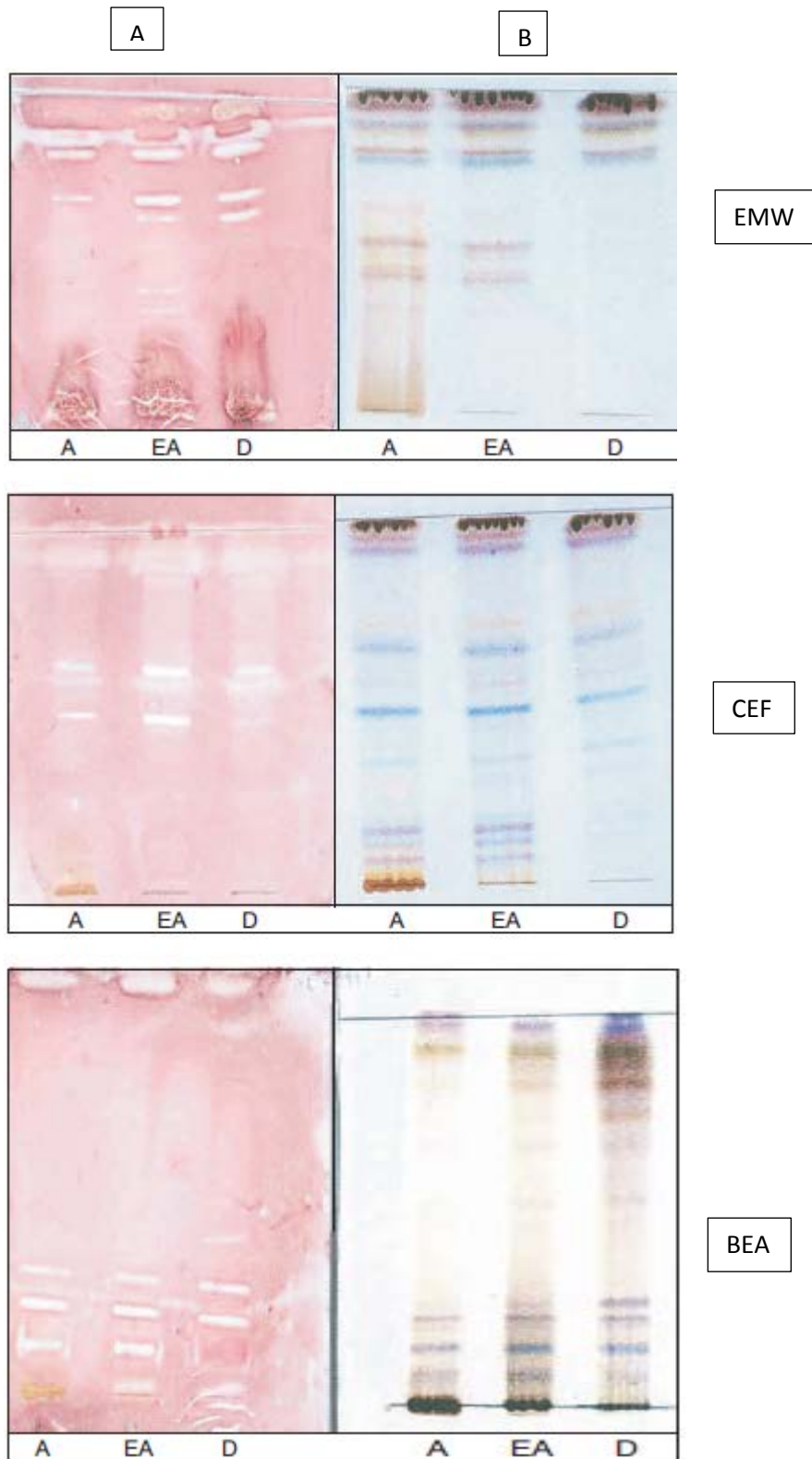


Figure 3. 7 TLC Bioautogram sprayed with *P. aeruginosa* (A) developed in EMW, CEF and BEA, and sprayed with INT and TLC fingerprints of *C. molle* extracted with different

extractants (B), developed in EMW, CEF and BEA, sprayed with vanillin sulphuric acid reagent. Clear zones of the bioautogram indicate bacterial growth inhibition.

(A - acetone, EA - ethyl acetate, D – dichloromethane).

3.10.4 Minimum Inhibitory Concentration (MIC)

3.10.4.1 Introduction

The MIC values of the *C. molle* extracts were determined by the serial microplate dilution plate method (Eloff, 1998a), and the values in this study ranged from 0.04 to 0.77 mg/ml after 12 hours of incubation (Table 3.2 – 3.4). MICs are considered the “gold standard” for determining the susceptibility of organisms to antimicrobials (Andrews, 2001). Extracts with low MIC values could be a good source of compounds with high antimicrobial activity.

Table 3. 2 Minimum Inhibitory Concentration (MIC) of *C. molle* extracts tested against bacterial strains after 24 hours

Bacterial Strains	MIC values (mg/ml)			
	A	EA	D	AV
<i>P. aeruginosa</i>	0.16	0.23	0.33	0.24
<i>S. aureus</i>	0.17	0.31	0.35	0.28
<i>E. coli</i>	0.26	0.42	0.49	0.45
<i>E. faecalis</i>	0.22	0.25	0.32	0.26
Average	0.20	0.30	0.37	

(A - acetone, EA - ethyl acetate, D – dichloromethane).

Table 3. 3 Minimum Inhibitory Concentration (MIC) of *C. molle* extracts tested against animal fungi after 24 hours

Animal Fungi	MIC values (mg/ml)			
	A	EA	D	AV
<i>C. albicans</i>	0.45	0.47	0.77	0.56
<i>C. neoformans</i>	0.37	0.51	0.61	0.50
<i>A. fumigatus</i>	0.46	0.28	0.58	0.44
Average	0.43	0.42	0.65	

(A - acetone, EA - ethyl acetate, D – dichloromethane).

Table 3. 4 Minimum Inhibitory Concentration (MIC) of *C. molle* extracts tested against plant fungi after 24 hours

Plant Fungi	MIC values (mg/ml)			
	A	EA	D	AV
<i>P. janthenillium</i>	0.04	0.04	0.09	0.06
<i>A. parasiticus</i>	0.21	0.22	0.32	0.32
<i>A.niger</i>	0.24	0.20	0.40	0.40
<i>F. oxysporium</i>	0.27	0.19	0.21	0.22
<i>R. solani</i>	0.16	0.19	0.18	0.18
Average	0.18	0.17	0.24	

(A - acetone, EA - ethyl acetate, D – dichloromethane).

In general, there was good overall inhibitory activity by different extracts of *C. molle* (Table 3.2 – 3.4). The best inhibitory activity was found with the acetone and ethyl acetate extracts against *P. janthinellum* with an MIC value of 0.04 mg/ml. Inhibitory activity of *R. solani* was the second best with an average MIC value of 0.18 mg/ml. The results correspond with those found by Mahlo (2009) were fungal pathogens were more susceptible to the plant extracts. The acetone extract had the best overall antibacterial (0.20 mg/ml) and antifungal (0.18 mg/ml) activity compared with the other extractants. The inhibitory activity of extracts was not exclusive to specifically Gram positive or Gram negative strains. *C. albicans* was the most resistant pathogen with an average MIC value of 0.56 mg/ml compared with the other tested strains. The low average MIC values obtained in this study ranging from 0.16 – 0.49 mg/ml indicate high antibacterial activity compared with the findings obtained by Eloff (1999) which ranged 0.6 – 3.0 mg/ml. Buwa and van Staden (2006) observed that plants extracts with MIC value of 0.78 mg/ml against *C. albicans* are regarded to have a good antifungal activity. The fungistatic action was observed on the CEF bioautograms on *C. neoformans*. Most of the extracts had high antibacterial and antifungal activity, which warrants further investigation on *C. molle* for antibacterial or antifungal activities.

3.10.5 Total Activity

3.10.5.1 Introduction

Total activity indicates the volume of which the bioactive compound present in 1 g of dried plant material can be diluted and still inhibit growth of the test organism (Eloff, 2004). Total activity in ml/g was calculated as the total mass extracted from 1 g of plant material divided by MIC value. Table 3.5 – 3.7 illustrate the total activity of different extracts of *C. molle* on the test organisms.

Table 3. 5 Total activity (ml/g) of *C. molle* leaves extracts on bacterial strains.

Bacterial Strains	Total Activity (ml/g)			
	A	EA	D	AV
<i>P. aeruginosa</i>	194	65	24	94
<i>S. aureus</i>	183	48	22	84
<i>E. coli</i>	119	35	16	57
<i>E. faecalis</i>	141	60	25	75
Average	159	52	22	

(A - acetone, EA - ethyl acetate, D – dichloromethane).

Table 3. 6 Total activity (ml/g) of *C. molle* leaves extracts on animal fungi.

Animal Fungi	Total Activity (ml/g)			
	A	EA	D	AV
<i>C. albicans</i>	68	31	10	36
<i>C. neoformans</i>	83	29	13	42
<i>A. fumigatus</i>	67	53	14	45
Average	73	38	12	

(A - acetone, EA - ethyl acetate, D – dichloromethane).

Table 3. 7 Total activity (ml/g) of *C. molle* leaves extracts on plant fungi.

Plant Fungi	Total Activity (ml/g)			
	A	EA	D	AV
<i>P. janthenillium</i>	775	375	89	413
<i>A. parasiticus</i>	147	68	25	80
<i>A.niger</i>	129	75	20	75
<i>F. oxysporium</i>	115	79	31	75
<i>R. solani</i>	194	79	44	106
Average	272	135	42	

(A - acetone, EA - ethyl acetate, D – dichloromethane).

The acetone extract of *C. molle* had the highest total activity (775 ml/g) against *P. janthinellum*. This means that 1 gram of *C. molle* acetone leaf extract can be diluted in 775 ml of solvent and will still be able to inhibit the growth of the organism. On plant fungi, the extracts on *P. janthinellum* gave the highest total activity compared with the other pathogens. The second best was the extract of ethyl acetate on *P. janthinellum* which gave the total activity (375 ml/g). Based on total activities the extracts were between 3.5 and 3.7 times more active against plant fungal pathogens than against animal fungal pathogens. This may have some evolutionary significance.

The acetone extracts of all the bacterial test pathogens had the highest total activity as compared with those of ethyl acetate and dichloromethane. The dichloromethane extract of *C. molle* against *C. albicans* gave the lowest total activity value (10 ml/g), confirming the less intense inhibition zones on the bioautographs. Study done by Mokoka, (2008) on *Maytenus undata* DCM and MeOH extracts had a moderate total activity of 636 ml/g and 128 ml/g respectively. In general, extracts of *C. molle* on plant fungal strains displayed better total activity than on animal fungal and bacteria. Good antimicrobial activities were obtained from plant extracts by other authors which validate the traditional use of medicinal plants (Masoko *et al.*, 2008; Shahid, 2009; Regassa and Araya, 2012; Mangoyi *et al.*, 2012; Masevhe *et al.*, 2015)

3.10.6 Conclusion

The acetone extract generally had an overall high antibacterial and antifungal activity in both bioautograms and MIC bioassays (0.04 mg/ml) against *P. janthinellum*. The activity of ethyl acetate extract against *P. aeruginosa* had five prominent bands of inhibition instead of the three bands that are usually observed. *C. albicans* was less susceptible to the plants extracts with an average MIC value of 0.56 mg/ml. Some of the plant extracts had high to low activity fungal or bacterial activity, indicating that the activity is not based on general metabolic toxicity. Some of the compounds had a broad spectrum against both fungal and bacterial strains, which warrants further investigation of *C. molle* plant extracts for potential antifungal and antibacterial activity.

In the next chapter attempt will be made to isolate the antimicrobial compounds.

CHAPTER 4

Isolation and characterizing of an antimicrobial compound from *Combretum molle*

4.1 Introduction

Natural products offer unmatched chemical diversity with structural complexity and are gaining international popularity as drug candidates of choice. Chemical screening of crude plant extracts therefore, constitutes an efficient complementary approach, allowing localization and target isolation of new type constituents with potential activities (Wolfender *et al.*, 1998).

Purification of different extracts can be achieved by preliminary removal of inactive substances such as fatty acids, chlorophyll, highly polar and non-polar substances during isolation. This approach has to be handled with care because active compounds may also be removed during this process. When extracting plant material, it is important to consider the polarity of solvents, in terms of targeting specific plant compounds (Eloff, 1998b).

Different separation techniques can be employed to unravel the complexity of numerous compounds found in plants extracts. Each assay needs to be thoroughly examined in order to evaluate the probability of discovering a novel lead compound and the likelihood that a compound derived from this lead will enter a preclinical program (Butler, 2004). Bioassay-guided isolation should lead to development of novel agents for various disorders.

4.2 Materials and Methods

4.2.1 Bulk extraction of leaves of *C. molle*

Powdered leaf material of *C. molle* (300 g) was extracted with 4L of acetone for several hours. The unextracted residue was allowed to settle and the extract was decanted and filtered through Whatman #1 paper in a Buchner funnel. The extract was concentrated using a Büchi rotavapor R114 (Labotec) and dried under a stream of air at room temperature. Phytochemical analysis of the acetone, dichloromethane (DCM), ethyl acetate (EtOAc) using TLC plates and bioautography to determine bioactivity (Figure 4.2). For bioautography of the various fractions *Pseudomonas aeruginosa* was used as the test organism. DCM fraction was selected as the best active fraction and gravitational chromatography was done as illustrated in the flow chart shown in Figure 4.1.

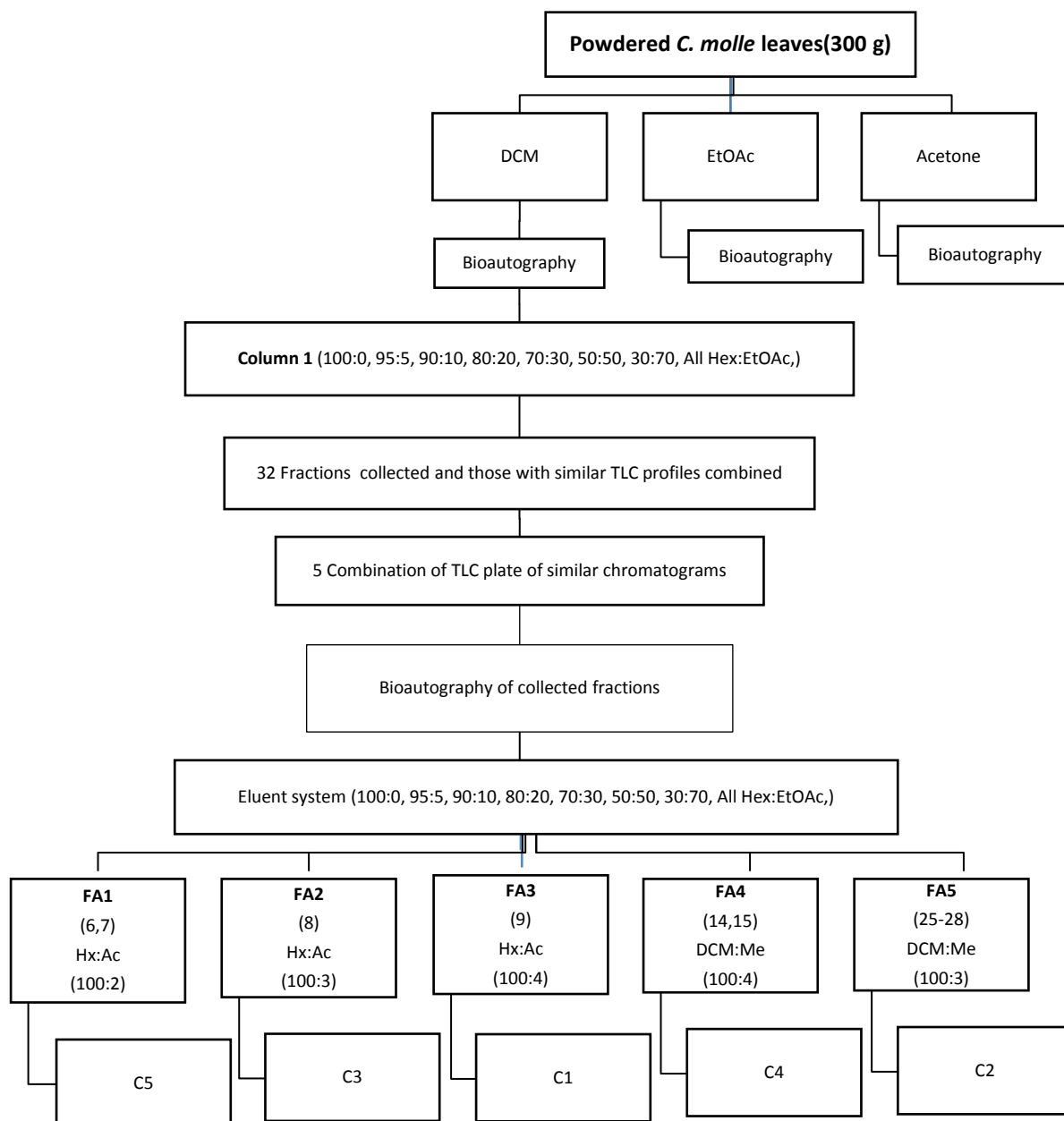


Figure 4. 1 Schematic representation of bioassay-guided isolation pathway of compounds from the leaf extract of *C. molle*.

(Ac – acetone; DCM – dichloromethane; EtOAc – ethyl acetate; Hx – hexane; Me – methanol)

4.2.2 Initial Group fractionation (Column 1)

The DCM fraction was subjected to column chromatography. Silica gel 60 Merck (300 g) was mixed with DCM to form a slurry and packed into a glass column (2.5 x 73 cm). The solvent was allowed to flow out of the column opening to allow the gel to settle. DCM extract (22.75 g) was dissolved in a small volume of DCM, mixed with 20 g of silica gel 60 (Merck), allowed to dry under a stream of cold air and then thinly spread on the packed column. A litre of 100% hexane was initially used to elute the column and subsequently, the polarity of the eluting solvent was increased using a gradient solvent mixture of hexane:ethyl acetate (100:0, 98:2, 96:4, 94:6, 90:10, 85:15) as a mobile phase.

Thirty two fractions were collected and concentrated under a stream of cold air. TLC plates were used to analyse fractions and the ones with similar chemical components were combined. Combined fractions were reconstituted to 10 mg/ml in DCM, and 10 μ l was loaded onto TLC chromatograms. The fractions were eventually developed on TLC plates and sprayed with vanillin-sulphuric acid reagent spray. The antimicrobial activities of the fractions were assayed as described in section 3.8, using bioautography as the method of choice with *P. aeruginosa* as the bacterial test organism. Active compounds were found in most of the fractions obtained.

4.2.3 Combination of Fractions from Initial Column 1

The five fractions combined from column 1 were designated as FA1 to FA5 using the category below:

Fraction [6, 7] were combined to form major fraction FA1, as they contained similar components when analysed with TLC; Fraction [8] was designated as fraction FA2. Fraction [9] was designated as fraction FA3. Fractions [14, 15] were combined to form major fraction FA4. Fractions [25 – 28] were combined and the resultant mixture was called FA5, according to TLC chromatogram fingerprinting.

4.2.4 Isolation of Compound (C1) from FA3 fraction

Silica gel 60 (Merck) (300 g) was mixed with hexane and packed in a column. The solvent was allowed to flow out of the column opening to allow the gel to settle. The sample was prepared by mixing fraction FA3 with 4 g silica gel. The mixture was dried and loaded carefully on top of the gel in the packed column. The fraction was subjected to column chromatography on silica gel using a gradient solvent system of

100:0 98:2, 96:4, 94:6, 90:10, 85:15 (hexane:acetone) as a mobile phase. Fractions of 20 ml were collected in test tubes. Similar fractions were monitored by TLC fingerprinting and combined resulting in compound C1.

4.2.5 Isolation of Compound (C3) from FA2 fraction

Silica gel 60 (Merck)(200 g) was mixed with hexane:acetone (2:1) and packed in a column. FA2 (8) fraction mixed with silica gel 60, dried and loaded on the packed column. The column was eluted with gradient solvent system of 100:0, 98:2, 96:4, 94:6, 90:10, 85:15 (hexane:acetone). Fractions of 20 ml were collected in test tubes. Similar fractions were monitored by TLC fingerprinting and combined for further analysis to obtain compound C3.

4.2.6 Isolation of Compound (C5) from FA1 fraction

A glass column was packed with silica gel 60 (Merck) (200 g) for fractionation of FA2 fraction (6,7). The sample was prepared by mixing FA2 fraction mixed with 3 g of silica. The column was eluted gradient solvent system of 100:0, 98:2, 96:4, 94:6, 90:10, 85:15 (hexane:acetone). Test tubes were used to collect 20 ml fractions (17 - 33) which was purified further, resulting in compound C5.

4.2.7 Compound (C4) from FA4 fraction

Silica gel 60 (Merck) (150 g) was mixed with 100% hexane and packed in column. The solvent was allowed to flow out of the column to allow the gel to settle. The sample was prepared by mixing the fraction FA4 (14, 15) with 1 g silica gel. The mixture was dried, layered carefully on top of the packed column. The column was eluted with gradient solvent system of 100:0, 98:2, 96:4, 94:6, 90:10, 85:15 (hexane:actone). Fractions of 20 ml were collected, and similar components were combined to yield compound C4 which was kept for further analysis.

4.2.8 Isolation of Compound (C2) from FA5 fraction

Silica gel 60 (Merck) (150 g) was mixed with 100% hexane and packed in column. The sample was prepared by mixing the fraction FA5 (25-28) with 1 g silica gel. A mixture was formed which was packed in the column and eluted with gradient solvent system of 100:0, 98:2, 96:4, 94:6, 90:10, 85:15, hexane:acetone). Fractions of 20 ml were each collected in test tubes, and similar fractions were monitored by TLC fingerprinting and bioautography. Further column chromatography on silica gel resulted in obtaining compound C2.

4.3 Results and Discussion

Bioautograms and TLC analysis (Figure 4.2) were performed to visualise the active compounds in preparation for the fractionation process. Separation processes were done using EMW as mobile systems. Fractions were tested for their antifungal and antibacterial activity on TLC plates. Based on these findings, the dichloromethane extract was chosen for further fractionation in order to isolate active compounds.

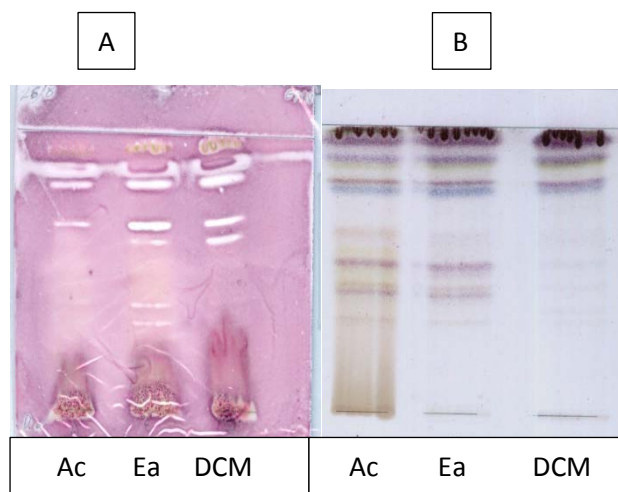


Figure 4. 2 Bioautograms of *Pseudomonas aeruginosa* (A) and TLC chromatograms (B) developed in EMW and sprayed with vanillin-sulphuric reagent spray.

(Ac – acetone; Ea – ethyl acetate; DCM – dichloromethane).

After developing, the plates were sprayed with vanillin-sulphuric acid reagent spray, and heated at 110°C until maximum colour development. Bands separated well according to their different polarities. The acetone extract had two zones of inhibition at R_f values 0.43, and 0.50. The ethyl acetate had five zones of inhibition at R_f values 0.43, 0.50, 0.73, 0.79, and 0.97. The dichloromethane (DCM) fraction had three zones of inhibition which showed intense bacterial activity at R_f values 0.73, 0.79, and 0.97 respectively.

The DCM extract was selected because it had more active compounds against *Pseudomonas aeruginosa* which was used as the test organisms (Figure 4.2). Chromatographic analysis was performed using silica gel chromatography and the fractionation process resulted in 32 fractions. Chromatograms of different fractions are demonstrated on Figure 4.3 below.

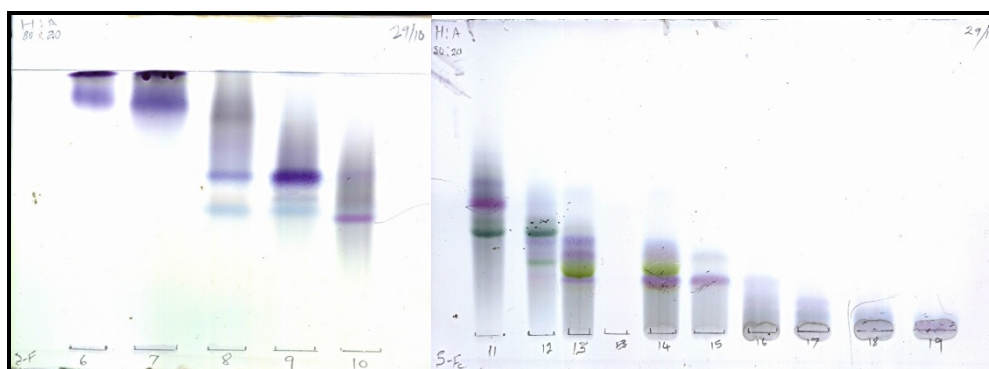


Figure 4. 3 Fractionation of *C. molle* DCM extract developed in Hexane:Acetone (80:20) and sprayed with vanillin-sulphuric acid spray.

The collected fractions were analysed by TLC chromatography as in Figure 4.3 and those with similar constituents were pooled together. Chromatograms of *C. molle* DCM extract (Fractions 6 – 19) developed and sprayed with vanillin-sulphuric reagent spray as shown in Fig 4.3. Several intense bands on the TLC (fractions 6-19) indicate the presence of plant constituents which can be further fractionated and analysed for activity.

TLC chromatograms of subfractions 6 – 20 were combined and purified further, and developed in dichloromethane:methanol:formic acid (DCM:M:F) (95:5:100 μ l) and sprayed with vanillin-sulphuric reagent spray as illustrated in Figure 4.4.

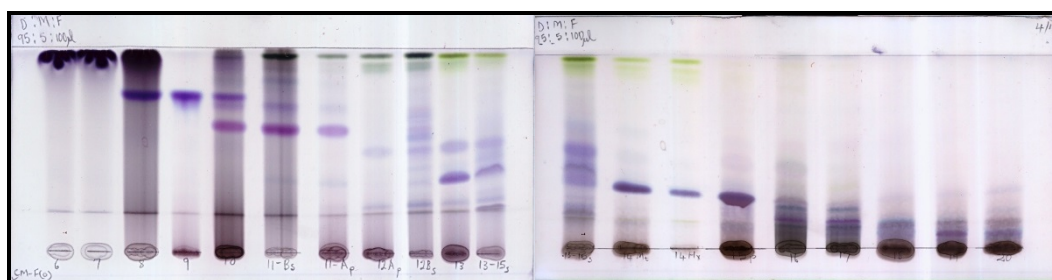


Figure 4. 4 TLC chromatograms of subfractions 6 – 20 purified further and developed in DCM:M:F (95:5:100 μ l).

Some of the plant constituents were not eluted when the polarity of the eluting solvent was increased. The majority of the fractions showed a purplish colour when sprayed with vanillin-sulphuric reagent spray.

The bioautograms had zones of inhibition of *Pseudomonas aeruginosa* growth (Figure 4.5). White areas on the bioautograms indicate zones where reduction of INT to the coloured formazan did not take place due to the presence of compounds that

inhibited the growth of *P. aeruginosa*. A total number of eight (8) fractions had active compounds as indicated by zones of inhibition (Figure 4.5).

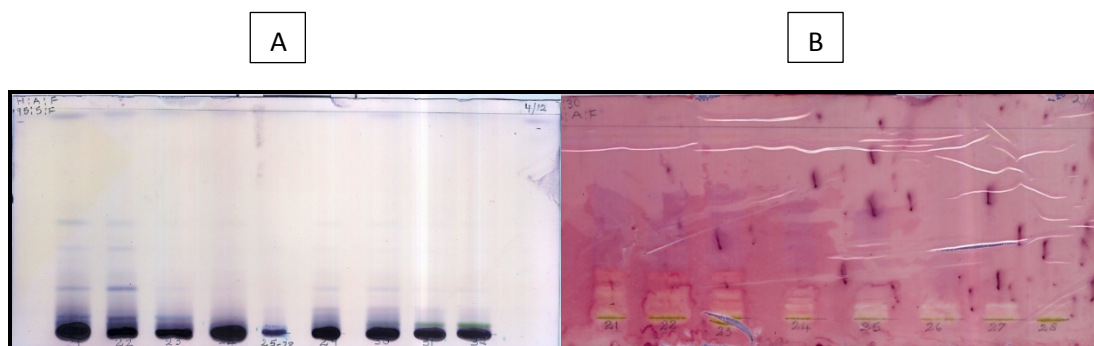


Figure 4. 5 TLC analysis of the chromatograms (A) and bioautograms (B) of FA4 fractions developed in Hexane:Acetone:Formic acid, using *P. aeruginosa* as the test organism.

Bioautography results reveal that *P. aeruginosa* was most susceptible to inhibitory properties of *C. molle* plant extract and the highest number of active compounds was present in subfractions (21-27). The bioautographs show zones of inhibition of *P. aeruginosa* growth as an indication of antibacterial activity.

Fraction of FA1 loaded onto 10 cm x 20 cm TLC plate and developed in Hexane:Acetone (60:40) as shown in Figure 4.6. Fractions (44-71) were collected and aliquots loaded on TLC plates and developed in DCM:M:F (95:5:100 μ l). Fractions (58 -66) showed a sequence of single purple spots (Figure 4.6). They were combined together to be further purified.

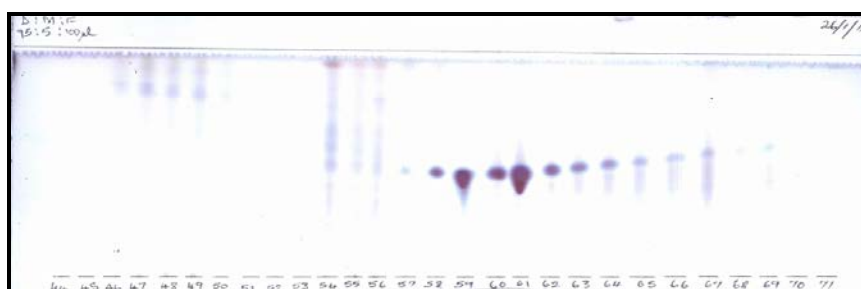


Figure 4. 6 Chromatograms of fractions FA1 developed in DCM:M:F (95:5:100 μ l) and visualised using vanillin-sulphuric acid spray.

Subsequent purification of fractions was performed as illustrated in the Figure 4.7 below. Chromatograms of fractions FA2 developed in DCM:M:F (95:5:100 μ l) and visualised using vanillin-sulphuric acid spray. Fractions (32-39) were collected and

aliquots loaded on TLC plates for further analysis. The collected fractions were analysed by TLC chromatography and those with similar constituents were pooled together.

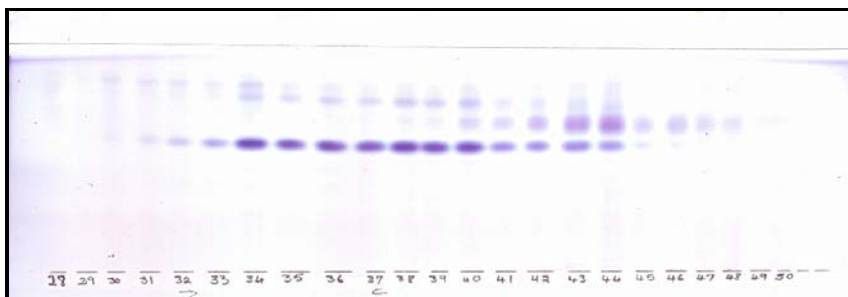


Figure 4. 7 Chromatograms of fractions FA2 developed in DCM:M:F (95:5:100 μ l) and visualised using vanillin spray.

Fractions (143 -178) as shown below were collected and developed on TLC plates using the eluent system Hexane: Acetone (60:40) and sprayed with vanillin-sulphuric acid reagent spray (Figure 4.8). Fractions containing similar components were combined for further analysis and to remove impurities.

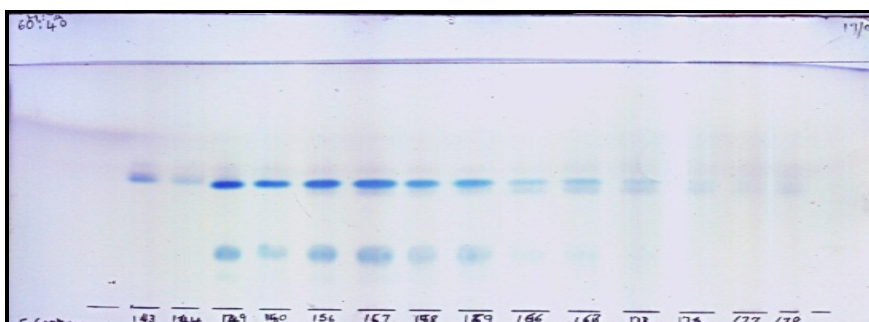


Figure 4. 8 Chromatograms of fractions (F15) developed in Hx:A (60:40).

Fractions (149 -159) has shown upper single line with intense blue colour and a lower single spot line which indicated there were some impurities that needed further purification. Fractions F (22- 28) were developed in DCM:M:F (95:5:100 μ l) as shown in Figure 4.9 below. The developed fractions were sprayed with vanillin- sulphuric acid spray reagent and heated at 110^o C in an oven until optimal colour development.

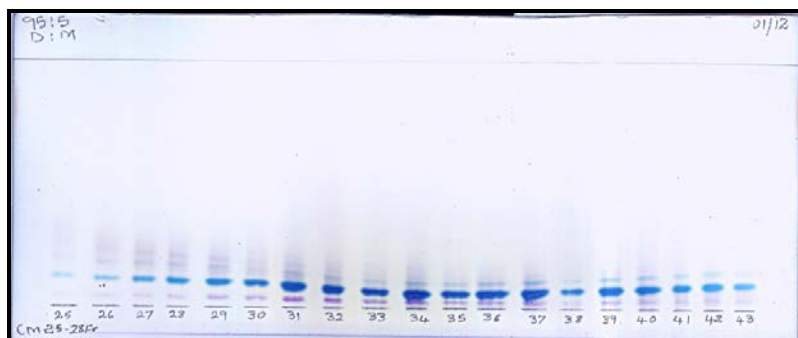


Figure 4. 9 Chromatograms of fractions F (25 – 43) developed in DCM:M:F (95:5:100 μ l) and visualised using vanillin spray.

The fractions contained a series of a single blue spot on the chromatograms after spraying with vanillin-sulphuric acid reagent spray. Some contained minor impurities and were combined and evaporated before being purified further. Fractions were combined based on similarity of chromatograms and pooled together further analysis.

For further purification of fraction FB3, a total of three sub-fractions (9A), (9B) and (9C) were purified further into fraction FA-12 and FB-13. Fraction FA and FB were combined and upon further purification resulted in compound 1 (C1) precipitated out as white powder.

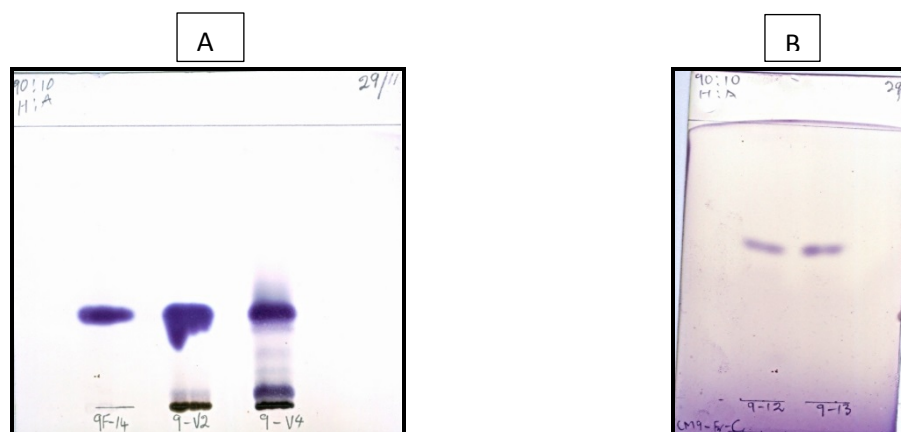


Figure 4. 10 TLC fingerprints of the purified compounds of C1 (A and B) from fraction FB3 sprayed with vanillin-sulphuric acid.

The isolated compounds C1 - C5, showed different colours on the TLC plate as illustrated in Table 4.1, showing that they might have different chemical structures.

Table 4. 1 Characteristics of the isolated compounds C1 to C5 and final purified amounts

Compound	Colour on TLC	Colour of Powder	R _f values	Final Quantity (mg)
C1	Purple	White	0.83	8.4
C2	Bright Blue	Cream	0.75	4.8
C3	Reddish	Cream	0.64	6.3
C4	Blue	White	0.42	3.5
C5	Yellow	White	0.35	4.7

Compound C1 had a higher final mass followed by C3, C2, C5 and C4. Their initial weight decreased during the process of compounds isolation as consequences of purifying. The compound C1 was also much purer and present in a higher quantity than the other isolated compounds and it was selected for further analysis.

Microdilution antimicrobial assays of compound C1 was performed to determine MIC values against different microbial strains as presented in Table 4.2.

Table 4. 2 MIC values (mg/ml) of isolated compound C1 from leaf extracts of *C. molle* against the selected microbial test organisms.

MIC (mg/ml)					MIC (mg/ml)		
Fungi					Bacteria		
	<i>C. a</i>	<i>C. n</i>	<i>P. j</i>	<i>A. p</i>		<i>P. a</i>	<i>E. f</i>
Compound C1	0.64	1.25	0.08	0.16	Compound C1	0.08	0.32
Amphotericin	0.64	1.25	0.16	0.62	Gentamicin	0.32	0.32

Candida albicans (*C. a*), *Cryptococcus neoformans* (*C.n*), *Penicillium janthinellum* (*P.j*), *Pseudomonas aeruginosa* (*P.a*) and *Enterococcus faecalis* (*E.f*).

Compound C1 had a similar or higher activity than the positive controls in all cases with MIC values of 0.08 mg/ml on *P. janthinellum*. The compound showed the least activity on the *C. neoformans* with MIC value of 1.25 mg/ml. The bioactivity of C1 was more potent on *P. aeruginosa* than *E. faecalis* with MIC values of 0.08 mg/ml and 0.32 mg/ml respectively. Comparing the microbial susceptibility of other test microorganisms with that of *P. janthinellum*, *P. janthinellum* is more susceptible to the inhibitory properties of compound C1 of the leaf extract of *C. molle*. Mahlo *et al.*, 2010 observed that among the fungal test strains used, *P. janthinellum* had significant sensitivity to the plant extract with the lowest MIC values of 0.28 mg/ml.

4.4 Conclusion

Isolation of active compounds from the leaves of *C. molle* using column chromatography yielded five compounds namely C1, C2, C3, C4 and C5. One bioactive compound (C1) was finally analysed in details. Attempts to isolate other bioactive compounds from other active fractions were unsuccessful due to the final small amounts and some impurities present. A larger initial amount of plant material is recommended to enable the isolation of more compounds present in small quantities. Structure elucidation of the isolated compound C1 will be determined in the next chapter.

CHAPTER 5

Structure elucidation of isolated compounds

5.1 Introduction

The advances in extraction technology, separation science and analytical and spectroscopic techniques have greatly increased the prospects of discovery of complex natural products of novel carbon frameworks possessing useful biochemical profiles (Mahato, 2000). Modern standardised analytical methodologies are used routinely in phytochemistry to isolate, characterise and identify compounds. Current analytic platforms employed include: gas chromatography (GC), high performance liquid chromatography (HPLC), mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). The ^1H - and ^{13}C -NMR spectra exhibits signals of in part per million (ppm), determining protons and carbons present in the molecule. Sophisticated equipments and technologies facilitate rapid, convenient, cheaper and shorter analysis time in the procedure of isolation of compounds. Li *et al.*, (2011) stated that HPLC/MS combine the separation of components with quantitative analysis or qualitative identification provides an effective means of analysing complex samples, and it has been the one of the most significant chromatographic technologies of the 21st century. Furthermore, some of the ingredients not identified by traditional methods have been found and their structures have been rapidly identified by HPLC/MS. These modern equipments and procedures give advantages such as simplicity, specificity, sensitivity and speed of sample preparation and measurement procedures. The instrumental stability and the ease with which spectra can be interpreted have contributed to the growing popularities of the techniques (Ignat *et al.*, 2011). Barrett (2002) suggested that when selecting compounds for further investigation, the following points should be taken into consideration: structural novelty of the compound, novel mechanism of action, intrinsic biological activities, clinical proof of concept, and the possibility of chemical modification or optimization of the chemical structure.

5.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR typically only observes the most abundant metabolites, but requires minimal sample preparation technique because of the low MW diversity of metabolites. NMR can provide information of both a qualitative and quantitative nature concerning purity and molecular constitution and it can be used to determine molecular

conformation in solution as well as in studying. The coupling of HPLC with NMR represents one of the most powerful methods for performing the structure elucidation of compounds in a mixture. Despite the wide range of disciplines with which NMR can interact, however, the applications themselves have always fit into three distinct groups: solution-state NMR; solid-state NMR; and NMR imaging (MRI) (Keifer, 1999).

5.1.2 Mass spectrometry

Mass spectrometry, had and still has, a very important role for research and its analytic power is relevant for structural studies on numerous compounds. The MS principle consists of ionising chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios (Sparkman, 2000). Mass spectrometry is significantly more sensitive than NMR, but requires the inclusion of separation techniques because of low MW diversity of metabolites and is limited to detecting metabolites that readily ionize.

5.2 Materials and Methods

5.2.1 Structure elucidation of isolated compounds from *Combretum molle*

The structure of the antimicrobial isolated compounds in this study were elucidated by extensive 1D NMR system (^1H , ^{13}C NMR, DEPT), 2D NMR (COSY, HSQC and HMBC) respectively, and by comparison with published literature data. 8.4 mg of the sample was subjected to NMR analysis under specified conditions.

5.3 Results

The following set of spectra were used to elucidate the structure ie. ^1H NMR (Figure 5.1), ^1H NMR (Figure 5.2), ^{13}C NMR (Figure 5.3), ^{13}C DEPT-NMR (Figure 5.4), gCOSY (Figure 5.5), HSQC (Figure 5.6), HMBC (Figure 5.7). The detailed spectra below is for the compound C1 and for the impure compounds C2, C3 and C4 are presented in the Appendix

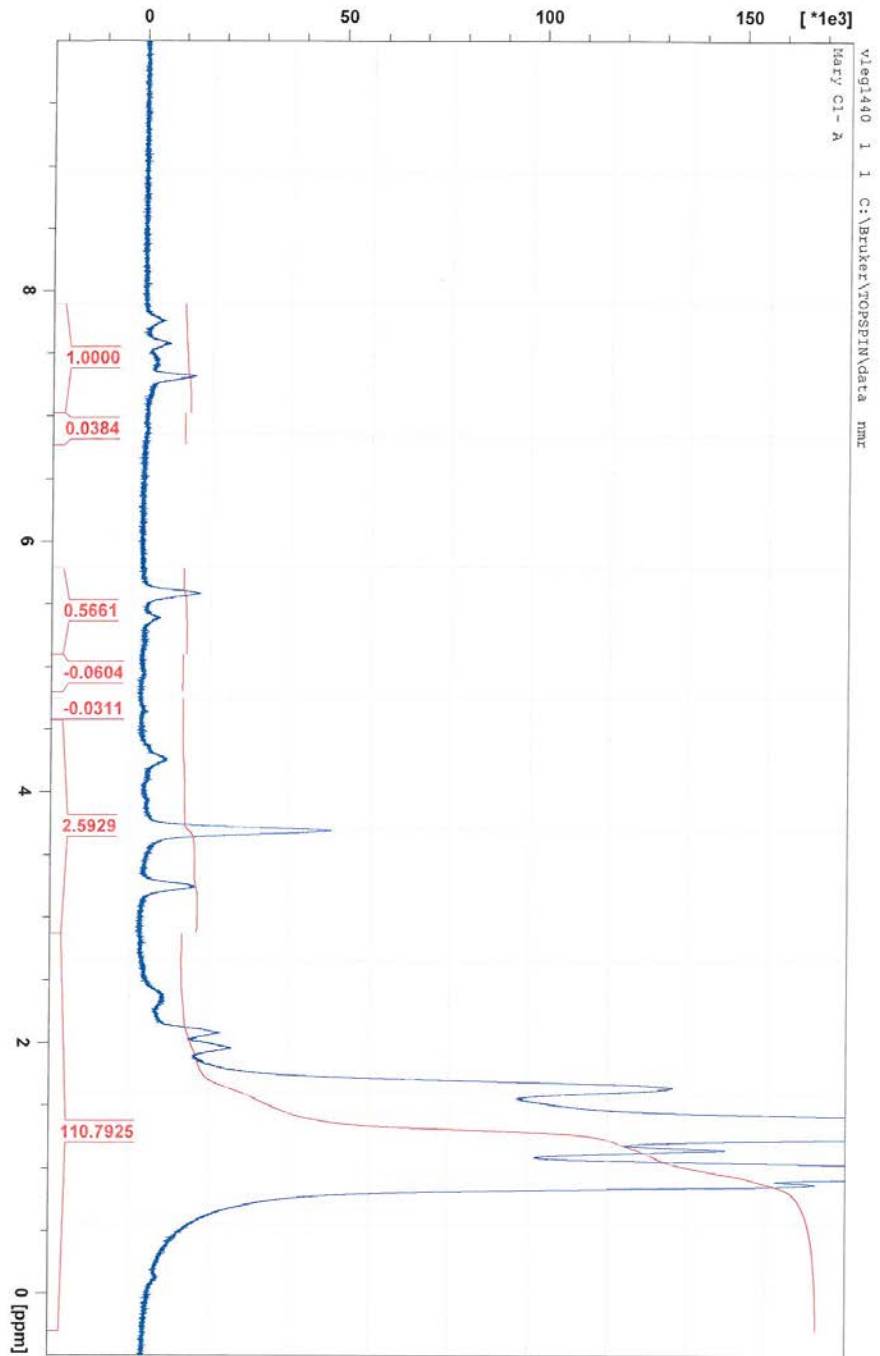


Figure 5. ^2H NMR spectrum of compound C1

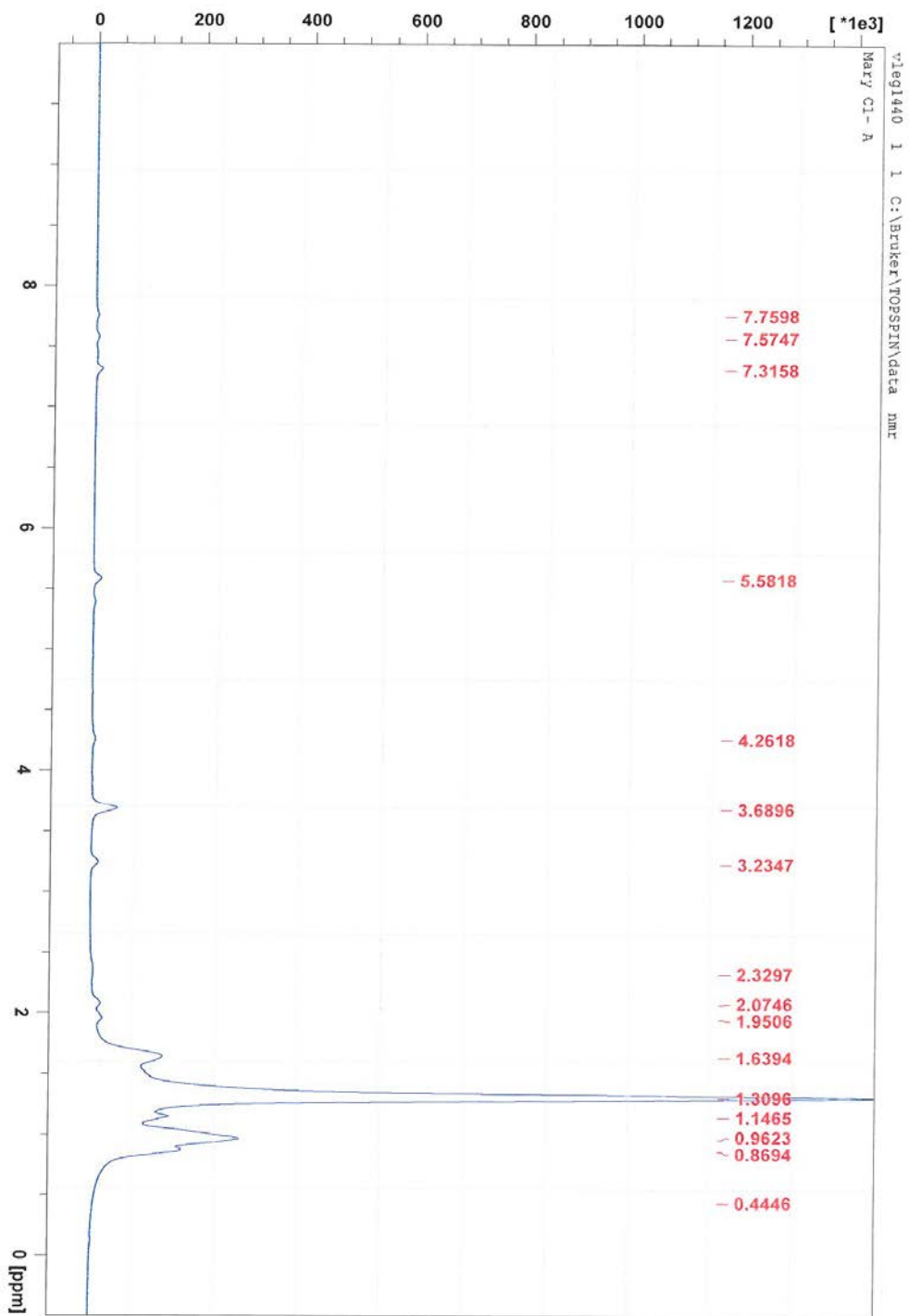
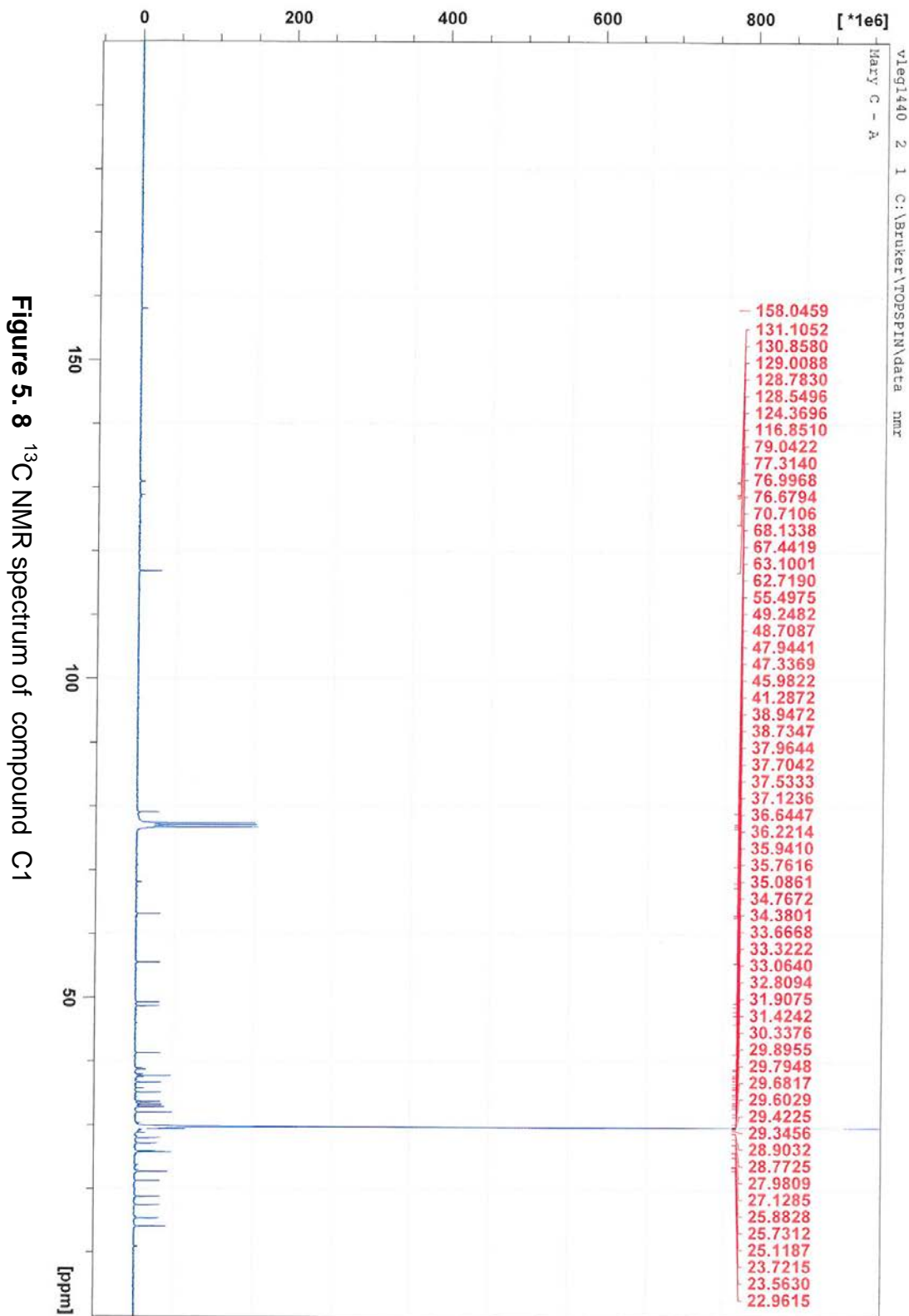
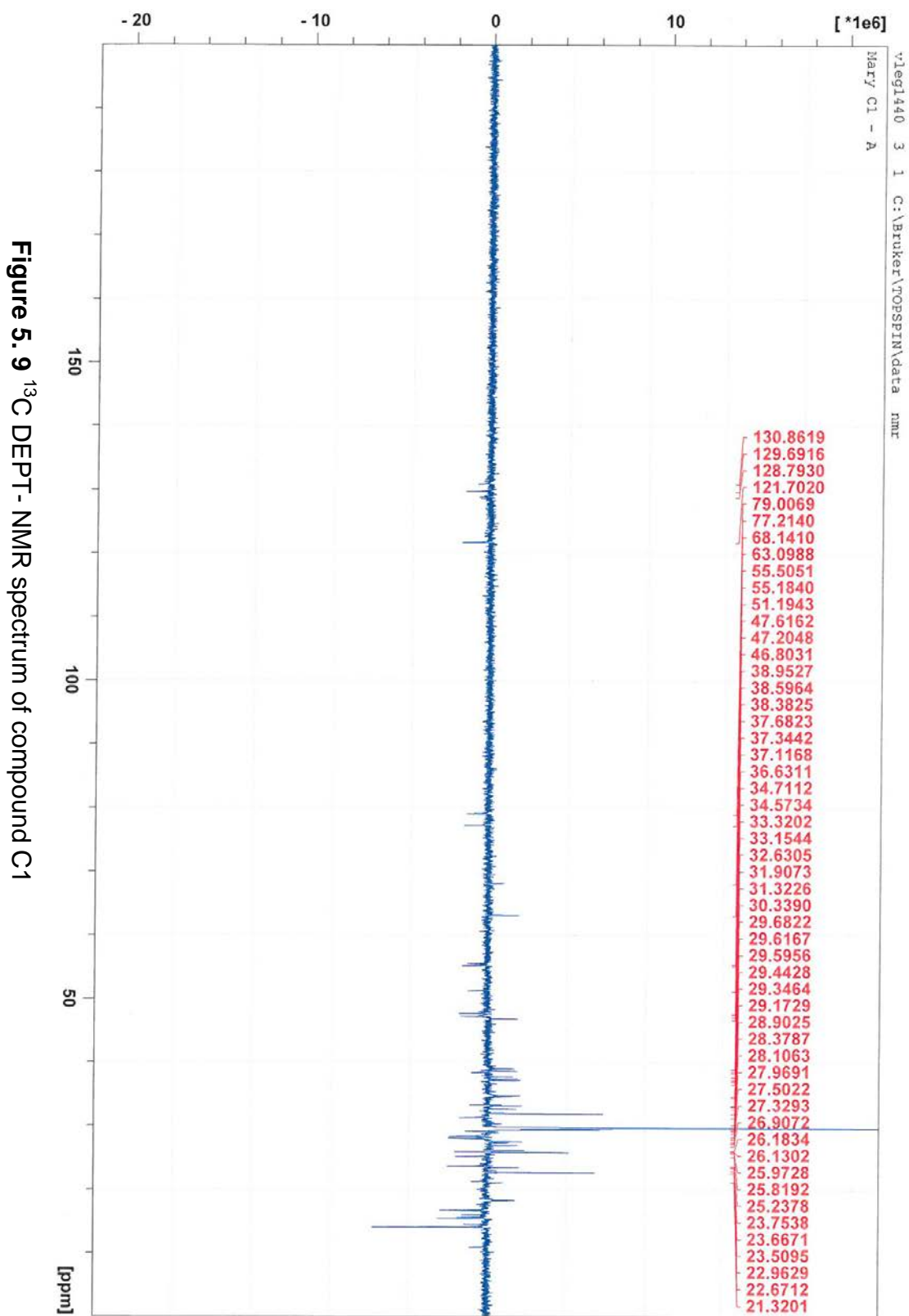


Figure 5. 7 ¹H NMR spectrum of compound C1





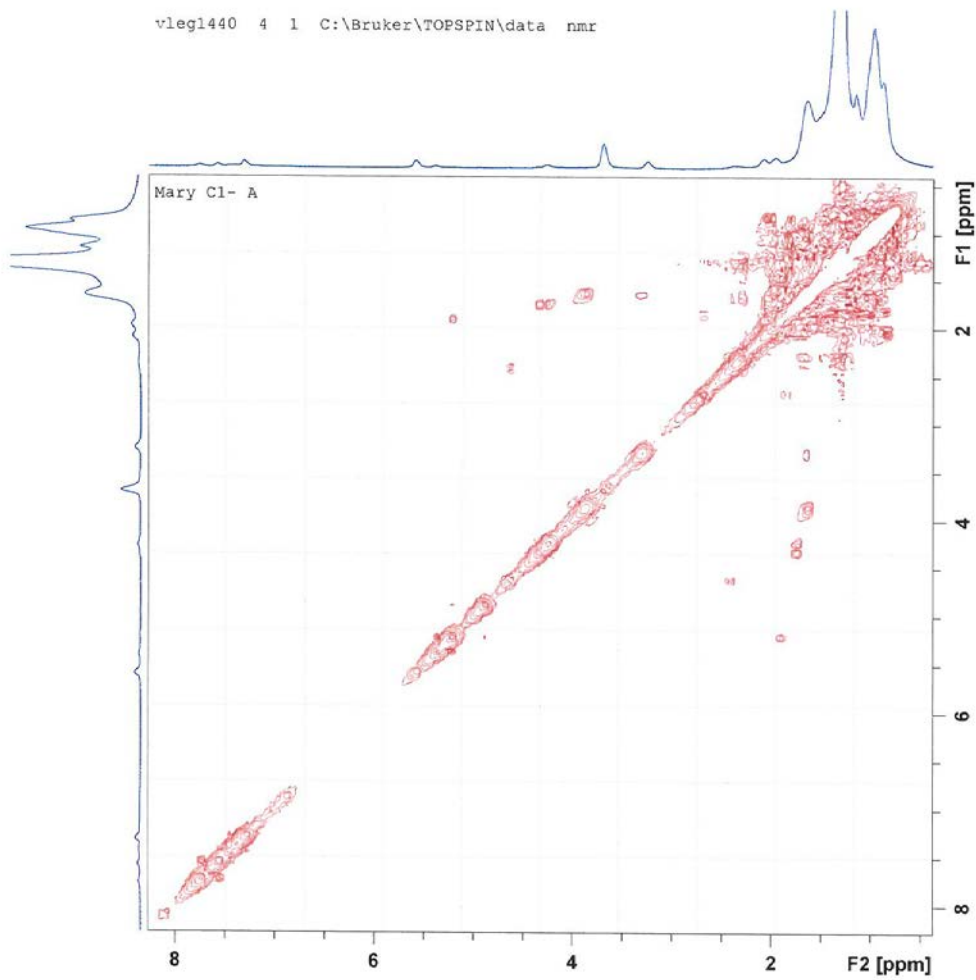


Figure 5. 10 gCOSY spectrum of compound C1

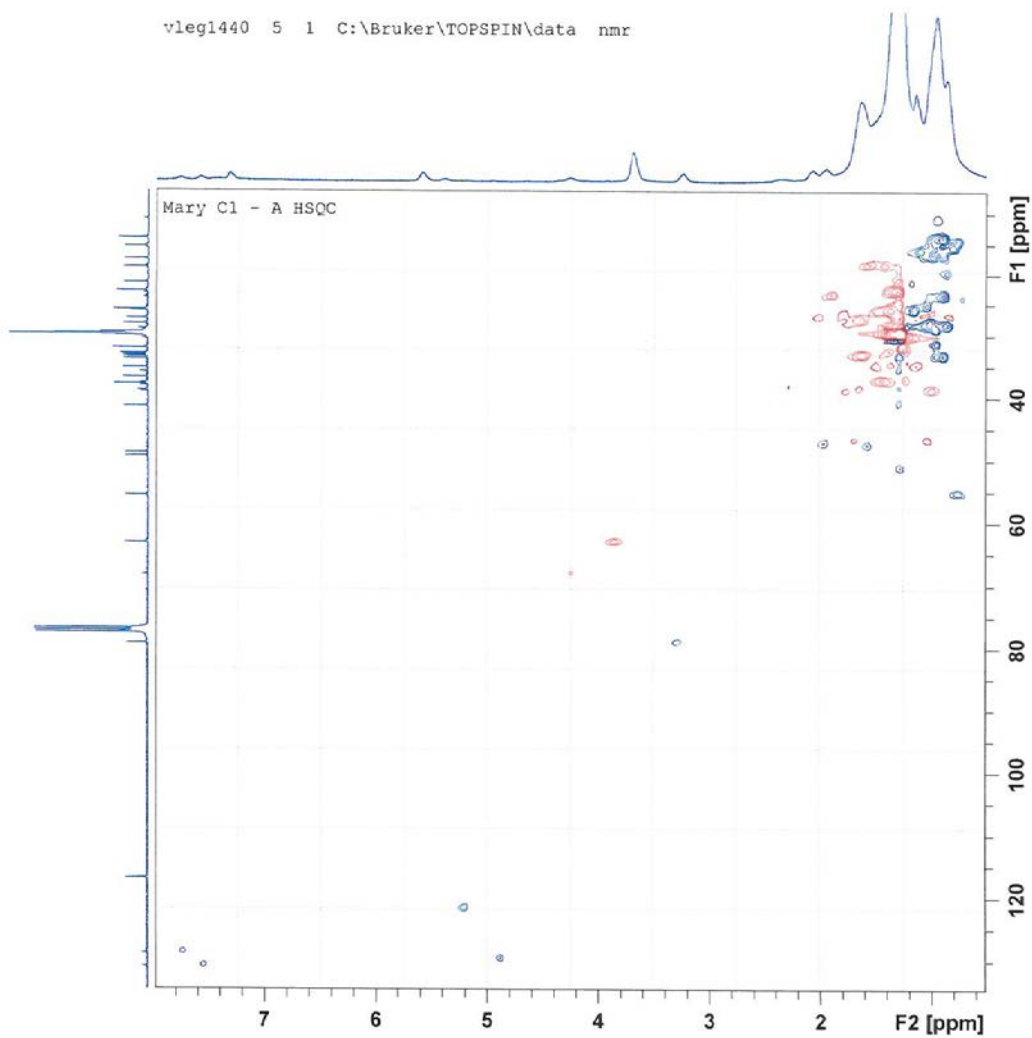


Figure 5. 11 HSQC spectrum of compound C1

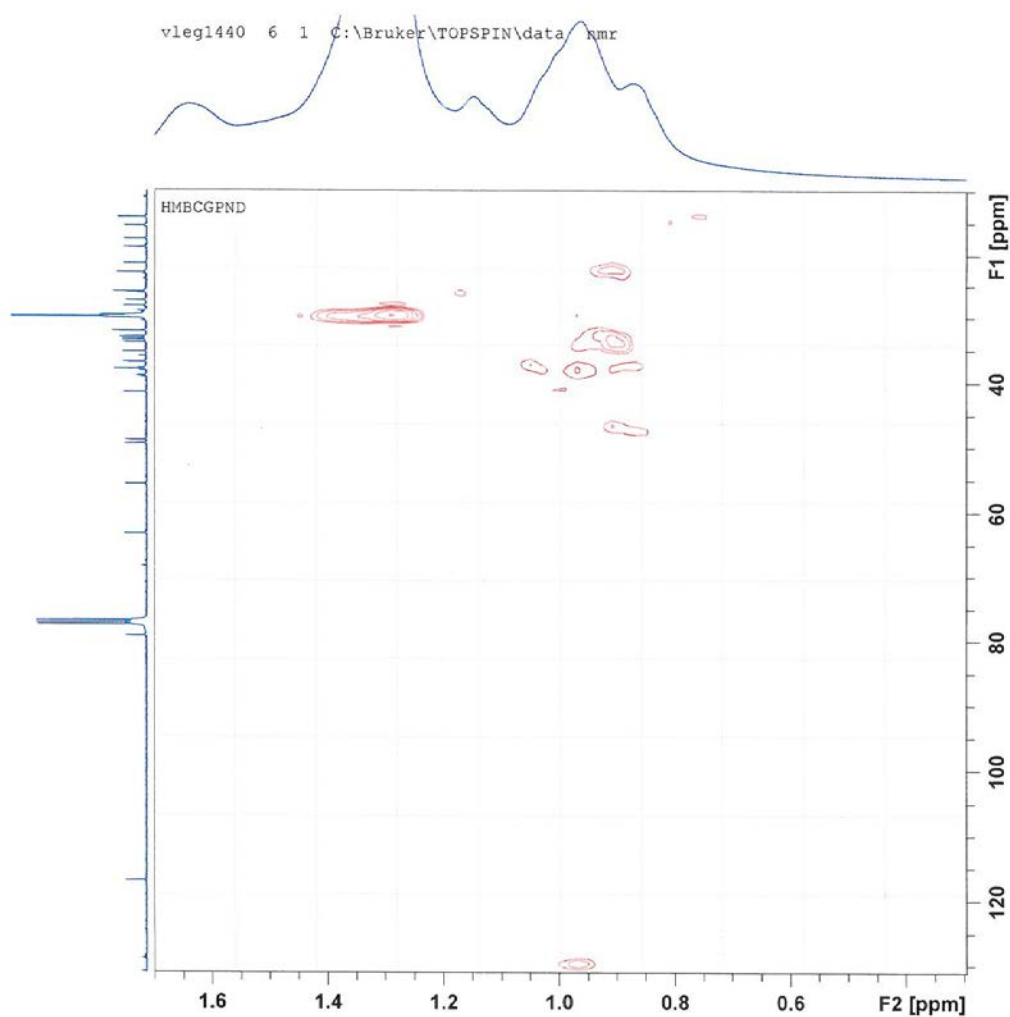


Figure 5. 12 HMBC spectrum of compound C1

From the figures stated above the table (Table 5.1) indicating the carbon positions were prepared and compared with the known compound taraxerol isolated by Viswanadh *et al.*, 2006.

Table 5. 1 NMR (500 Hz) data for compound C1 (taraxerol) in CDCl₃

Atom Position	¹³ C	Published data (Viswanadh <i>et al.</i> , 2006)
1	37.70 (CH ₂)	37.97
2	27.12 (CH ₂)	27.11
3	79.04 (CH)	79.09
4	38.94 (C)	38.96
5	55.49 (CH)	55.49
6	18.77 (CH ₂)	18.77
7	35.08 (CH ₂)	35.07
8	38.94 (C)	38.96
9	48.70 (CH)	48.70
10	37.70 (C)	37.97
11	17.48 (CH ₂)	17.49
12	35.08 (CH ₂)	35.78
13	38.73 (C)	38.74
14	158.04 (C)	158.05
15	116.85 (CH)	116.85
16	36.64 (CH ₂)	36.65
17	37.70 (C)	37.70
18	49.24 (CH)	49.25
19	41.28 (CH ₂)	41.29
20	29.60 (C)	29.36
21	33.66 (CH ₂)	33.63
22	33.06 (CH ₂)	33.07
23	27.98 (CH ₃)	27.98
24	15.44 (CH ₃)	15.44
25	15.44 (CH ₃)	15.44
26	29.89 (CH ₃)	29.91
27	25.88 (CH ₃)	25.87
28	29.78 (CH ₃)	29.81
29	33.32 (CH ₃)	33.34
30	21.29 (CH ₃)	21.30

From all the analysis done above, it was concluded that it belonged to the compound taraxerol (Figure 5.8).

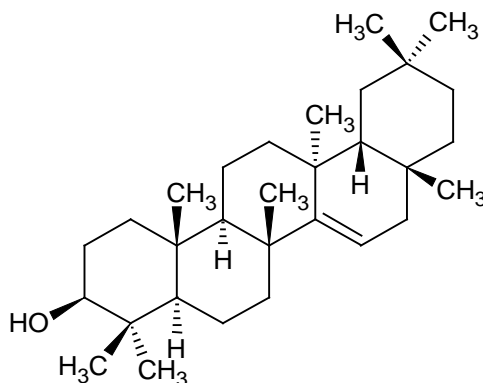


Figure 5. 13 Structure of taraxerol (C1) isolated from *Combretum molle* leaves

5.4 Discussions

Compound C1 was obtained as a white precipitate indicating a triterpene-like compound. For NMR procedures, it was crystallised from chloroform-methanol as crystalline solid. m.p. 279-280°C; C₃₀H₅₀O; ¹H NMR (500 MHz, CDCl₃): δ 0.81(3H,s, 24-Me), 0.84(3H,s,24-Me), 92(6H,s, 25, 29-Me), 0.95(3H,s, 30-Me), 0.98(3H,s, 23-Me), 1.07(6H,s, 26, 27-Me), 3.18(1H,m,H-3), 5.44(1H, dd, J=8.1 and 4.0 Hz, 15-H); ¹³C NMR (125.70 MHz, CDCl₃); EIMS m/z (%): 426.4 (5) [M⁺], 411 (11), 393 (9), 302 (36), 287 (35), 269 (27), 204 (100), 189 (38). All these NMR data were similar to those published for taraxerol (C1, Figure 5.7), belonging to the taraxerane group.

Although the compound taraxerol have been discovered in other plant species including Combretaceae (Mokoka, 2008; Rabie, 2008), it is to the best of our knowledge, the first report of the isolation of taraxerol from the leaf extract of *C. molle*.

The antimicrobial activities studies done showed that most activity appeared in the dichloromethane fraction with MIC values of 0.08 mg/ml on the *P. janthinellum*. The compound showed the least activity on the *C. neoformans* with MIC value of 1.25 mg/ml. The bioactivity of C1 was more potent on *P. aeruginosa* than *E. faecalis* with MIC values of 0.08 mg/ml and 0.32 mg/ml respectively. The compound C1 generally had good activity in both antifungal and antibacterial activity. In a study done by

Rabie (2008), found that the MIC values of the bioactivity of taraxerol isolated from the leaf extracts of *Pteleopsis myrtifolia* were 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* respectively.

Taraxerol is a member of the triterpenoid family, and has been isolated from a variety of plants including dandelion. It has been reported to exhibit a number of biological and pharmaceutical activities including anti-diabetic potential (Sangeetha *et al.*, 2010) and potent anti-inflammatory (Tsao *et al.*, 2008) effects. In a study by Yao *et al.*, 2013, aimed to determine the anti-inflammatory effects of taraxerol on LPS-activated RAW264.7 macrophages, a number of observations were made. It was observed that, taraxerol apparently suppressed LPS-induced release of the pro-inflammatory mediators. Taraxerol did not affect cell viability under the experimental conditions described. Taraxerol significantly inhibited LPS-stimulated iNOS and COX-2 upregulations in dose-dependent manner. Taraxerol was found to decrease the levels of iNOS and COX-2 mRNA significantly, but taraxerol did not affect the expression of the housekeeping genes GAPDH.

CHAPTER 6

Summary of results, general discussion and conclusion

Natural products offer unmatched chemical diversity with structural complexity and biological potency especially for numerous diseases are caused either by fungal, bacterial, viral, helminthic or protozoan pathogens. Emerging diseases, drug resistance, HIV-AIDS, TB, among other health problems, are swiftly changing the landscape of research. New areas of research are sought after or old ones revisited, including search for antifungal and antibacterial compounds from indigenous medicinal plants since it is difficult in finding new antimicrobials (Eloff and McGaw, 2014). Traditional herbal medicines form an important part of the healthcare of South Africans and rely heavily on the use of indigenous medicinal plants.

The main aim of this study was to isolate and characterise antifungal and antibacterial compounds from leaf extracts from an indigenous medicinal plant *Combretum molle*. The motivation to choose *C. molle* in Combretaceae family was based on our Phytomedicine research group's long interest in the chemistry, antimicrobial properties and ethnomedicinal use of the Combretaceae family (Martini *et al.*, 2004; Eloff *et al.*, 2008; Dawe *et al.*, 2013). The family consisting of nineteen genera and it belongs to the order Myrtales. *C. molle* is one of the commonly used medicinal plants in southern Africa for numerous ailments. Though medicinal products from the Combretaceae family are extensively used in Africa by traditional healers, limited scientific data has been reported on the antimicrobial activity of components isolated from some members of the Combretaceae. Based on this information, phytochemical analysis of different leaf extracts of *C. molle* were carried through bio-assay guided tests to investigate and isolate antimicrobial compounds.

Plant fungal pathogens or pathogenic bacteria cause devastating damage to either crops, animals or human beings. Three animal fungal species, namely, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and four plant fungal species, namely, *Aspergillus niger*, *Aspergillus parasiticus*, *Fusarium oxysporum*, *Penicillium janthinellum*, *Rhizoctonia solani* and bacterial strains *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* were used as test microorganisms for bioactive compounds in leaf extracts of *C. molle*.

The phytochemical analysis of different leaf extracts of *C. molle* were used to select the best extractant. The extracts of acetone, methanol and dichloromethane eluted in the EMW mobile system, had a higher number of bands than that of butanol and chloroform. Hexane extracted fewer compounds, usually found in the non-polar region of the bioautogram. TLC was used to compare the chemical composition of the crude extracts of the plants. Different chemical compounds separated very well displaying different R_f values and a variety of colours. The R_f values (EMW) of most prominent bands of acetone, methanol and ethanol extracts were almost similar to that of chloroform at R_f values of 0.62, 0.66, and 0.73, respectively. Most intense bands on the BEA mobile system had the following R_f values 0.12, 0.20, 0.24, 0.34. In the CEF chromatogram, most of the compounds show even distribution of bands throughout the chromatogram, with the exception of hexane. The most intense bands appear at these R_f values, 0.20, 0.30, 0.50, 0.56, 0.78 and 0.83.

Most of the active compounds on bioautographs of *C. molle*, show diverse antifungal or antibacterial activity. Bioautography was used to determine the number of active compounds and their retardation factors (R_f) against different microorganisms. The chromatograms developed in three mobile systems (EMW, CEF and BEA) were sprayed with animal or plant fungi or nosocomial bacteria. The highest number of active compounds were present in ethyl acetate and acetone extracts. In BEA bioautograms of *Aspergillus fumigatus*, all the extracts exhibited clear zones of inhibition at R_f values of 0.12, 0.23, and 0.40. The clear zones of inhibition correspond with some of the intense bands displayed on chromatograms sprayed with vanillin sulphuric acid.

The EMW bioautograms of *Candida albicans* had several clear zones of inhibition which indicated antifungal activity. In EMW bioautogram of *C. albicans*, zones of inhibition were observed at R_f values of 0.73, 0.81, 0.87. For all the extractants, the compounds separated very well, except butanol which had only two bands at 0.81 and 0.87. *Cryptococcus neoformans* had weak bands of growth inhibition, while *A. niger* on eluent system BEA, EMW, CEF had some bands of growth inhibition. The best resolution of bioactive compounds was obtained in most of the bioautograms, with *Pseudomonas aeruginosa* being more susceptible to the ethyl acetate extract

than other test strains. The R_f values of active compounds were 0.43, 0.50, 0.73, 0.79, 0.97. The acetone, ethyl acetate and DCM extracts eluted in CEF had antifungal activity at the following R_f values 0.37, 0.56, 0.76,. The BEA bioautography of acetone, ethyl acetate and DCM extracts had zones of inhibition of growth of *P. aeruginosa* at the following R_f values, 0.09, 0.19, 0.27.

Two-fold serial microdilution was used to determine the minimum inhibitory concentrations of extracts against the test microorganisms. In general, there was good overall inhibitory activity by different extracts of *C. molle*. The best inhibitory activity was demonstrated by acetone and ethyl acetate extracts against *P. janthinellum* with an MIC value of 0.02 mg/ml. Inhibitory activity of *R. solani* was the second best with an average MIC value of 0.18 mg/ml. *C. albicans* was the most resistant pathogen with an average MIC value of 0.56 mg/ml compared with the other tested strains. All extracts of *C. molle* had strong antifungal activities against *A. fumigatus* with MIC value as low as 0.08 mg/ml, which correlated with plant and animal antifungal study done by Mahlo *et al.* (2013). Suleiman *et al.* (2010) evaluated several tree species for activity against several fungal and microbial species and found that *Microsporum canis* had the highest susceptibility to the extracts at concentrations as low as 0.05 – 0.08 mg/ml, while *Candida albicans*, had the lowest sensitivity to the plant extracts and the acetone extract of *Loxostylis alata* had the highest antifungal activity with lowest MIC against *Sporothrix schenckii*.

The four nosocomial bacteria tested in the microdilution assay, namely, *S. aureus*, *E. coli* and *E. faecalis* had similar antibacterial activities but *P. aeruginosa* was most sensitive, with the lowest MIC value of 0.16 mg/ml. Studies done by Eloff (1999) it was observed that *C. molle* have shown to possess good antibacterial activity. The antibacterial and antifungal activities of the leaf extracts of *C. molle* against different bacteria and fungi used here agree with results of other authors using different plant species (Fyhrquist *et al.*, 2002; Asres *et al.*, 2006; Masoko and Eloff, 2007a; Masoko and Eloff, 2007b; Eloff *et al.*, 2008; de Marias Lima *et al.*, 2012)

Column chromatography and bioassay-guided fractionation of dichloromethane extract of the leaves of *C. molle* yielded 32 fractions. Further fractionation led to the isolation of a five bioactive compounds with varying purity. The R_f values of the compounds ranged from 0.35 – 0.83. The most active compound originated from the

dichloromethane fraction with MIC values of 0.08 mg/ml on the *P. janthinellum*. The compound showed the least activity on the *C. neoformans* with MIC value of 1.25 mg/ml. The bioactivity of C1 was more potent on *P. aeruginosa* than *E. faecalis* with MIC values of 0.08 mg/ml and 0.32 mg/ml respectively. The compound C1 generally had good activity in both antifungal and antibacterial activity. Insufficient material was isolated for compounds 2 - 5 to elucidate the structure.

Structure elucidation was performed using 1D NMR, 2D NMR, ¹H, ¹³C, COSY, HMBC, HMQC. It was concluded that compound C1 present in the leaves of *C. molle* was taraxerol (C1), belonging to the taraxerane group. Taraxerol is a member of the triterpenoids family, and has been isolated from a variety of plants. It has been reported to exhibit a number of biological and pharmaceutical activities including anti-diabetic potential (Sangeetha *et al.*, 2010) and potent anti-inflammatory (Tsao *et al.*, 2008) effects. In these study, the MIC values for both antibacterial and antifungal activities ranged from 0.08 to 1.25 mg/ml. Rabie (2008) in an unpublished thesis found that the MIC values of the bioactivity of taraxerol isolated from the leaf extracts of *Pteleopsis myrtifolia* were 0.04, 0.016, 0.63 and 0.31 mg/ml against *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* respectively. These results indicate that taraxerol has a broad spectrum of antifungal and antibacterial activities.

In a study by Yao *et al.*, 2013, aimed to determine the anti-inflammatory effects of taraxerol on LPS-activated RAW264.7 macrophages, anti-inflammatory and immunomodulatory activities were observed. Seven flavonoids with antibacterial activity were isolated from *Combretum erythrophyllum* using bioassay-guided fractionation (Martini *et al.*, 2004). Interestingly, tetracyclic and pentacyclic classes co-occurrence of these triterpenoids is unusual but *C. molle* contains both (Panzini *et al.*, 1993). Although the compound taraxerol have been discovered in other plant species Mokoka (2008), it is to the best of our knowledge the first report of the isolation of taraxerol from the leaf extract of *C. molle*.

A study done by U.S. National Cancer Institute, found that an extract of *C. molle* possessed significant activity against murine P-388 lymphocytic leukemia cells (Pettit *et al.*, 1987). McGaw *et al.* (2001) also demonstrated that *C. imberbe* and *C. molle* both had anti-inflammatory and and antischistosomal activity. Katerere *et al.* (2003) has shown that pentacyclic triterpenes from *C. imberbe* have antibacterial activity

against *Mycobacterium fortuitum*. Angeh *et al.* (2007) observed the antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from *C. imberbe*. The results obtained in the study done by Simon *et al.* (2012) justify the use of *C. molle* as a useful and effective agent for the control of gastrointestinal nematodes in livestock production, particularly in a rural setting. The aqueous and methanol extracts of *C. molle* were also screened by Ojewole (2008) for inhibitory effects against HIV-1 reverse transcriptase.

Conclusion

C. molle leaf extracts contained at least five different antimicrobial compounds. The acetone extracts were generally the most active and extracts were generally more active against fungi than bacteria. There was some evidence of selectivity because not all antimicrobial compounds based on the R_f determined by bioautography compounds were active against all pathogens.

Five antimicrobial compounds were isolated but only one compound was present in a sufficient quantity and purity to determine the structure as taraxerol. Taraxerol had a higher activity against some bacteria and fungi than the positive controls gentamicin and amphotericin B. Most of the activities on this compound has already been described in the literature.

It may be useful to use a larger quantity of material in order to isolate and characterize antimicrobial compounds present in low concentrations.

Some of the crude extracts had even higher activities indicating the presence of synergistic reactions. The excellent MIC of 0.04 mg/ml of the acetone extract against *Penicillium janthenillium* and the good total activity of 775 ml/g indicates that this extract may be useful in controlling this important plant fungal pathogen. It would also be useful to determine the animal and environmental safety of extracts to evaluate the use in combating plant fungal pathogens on food crops.

CHAPTER 7

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CHAPTER 8

APPENDIX

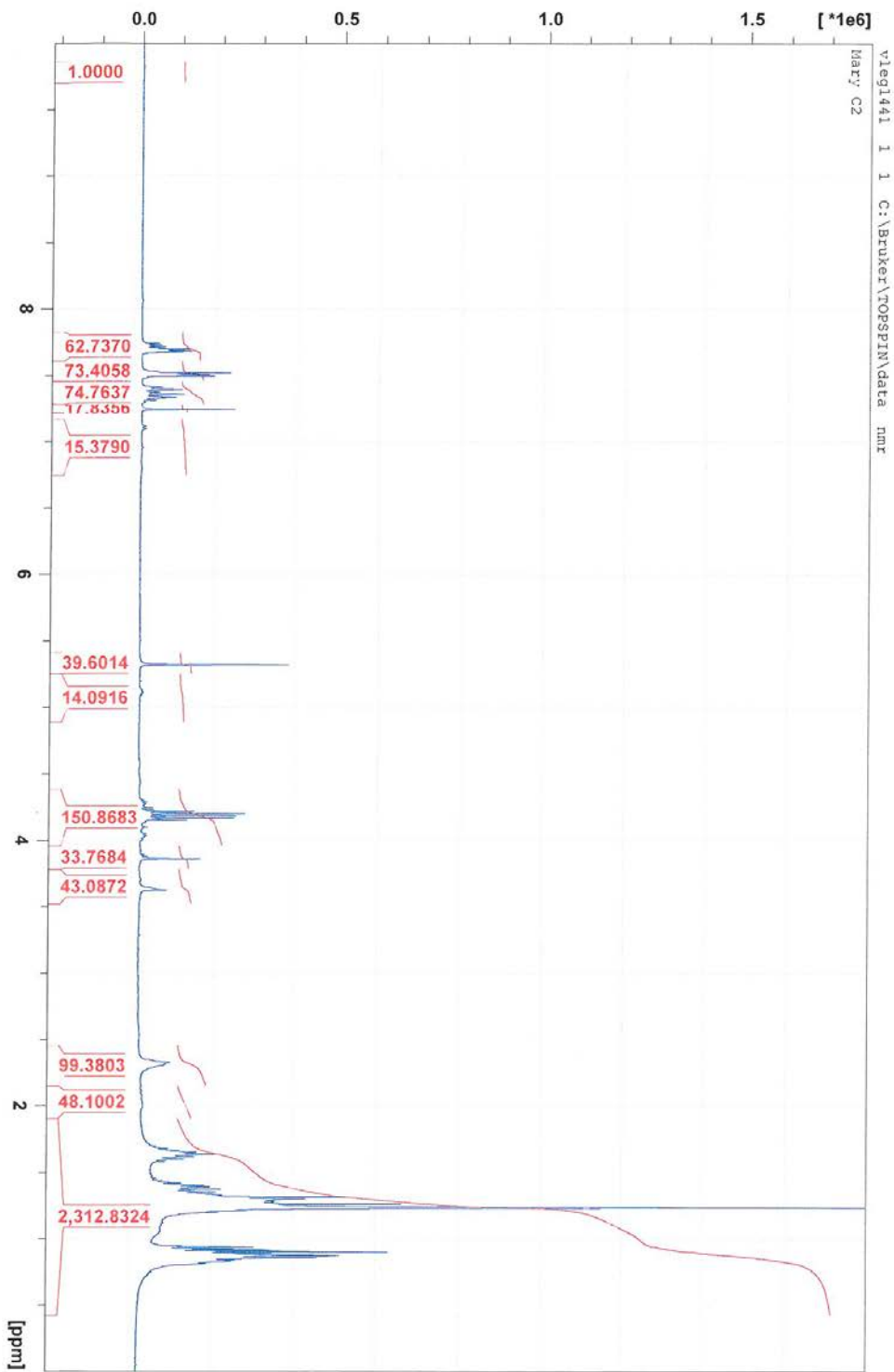
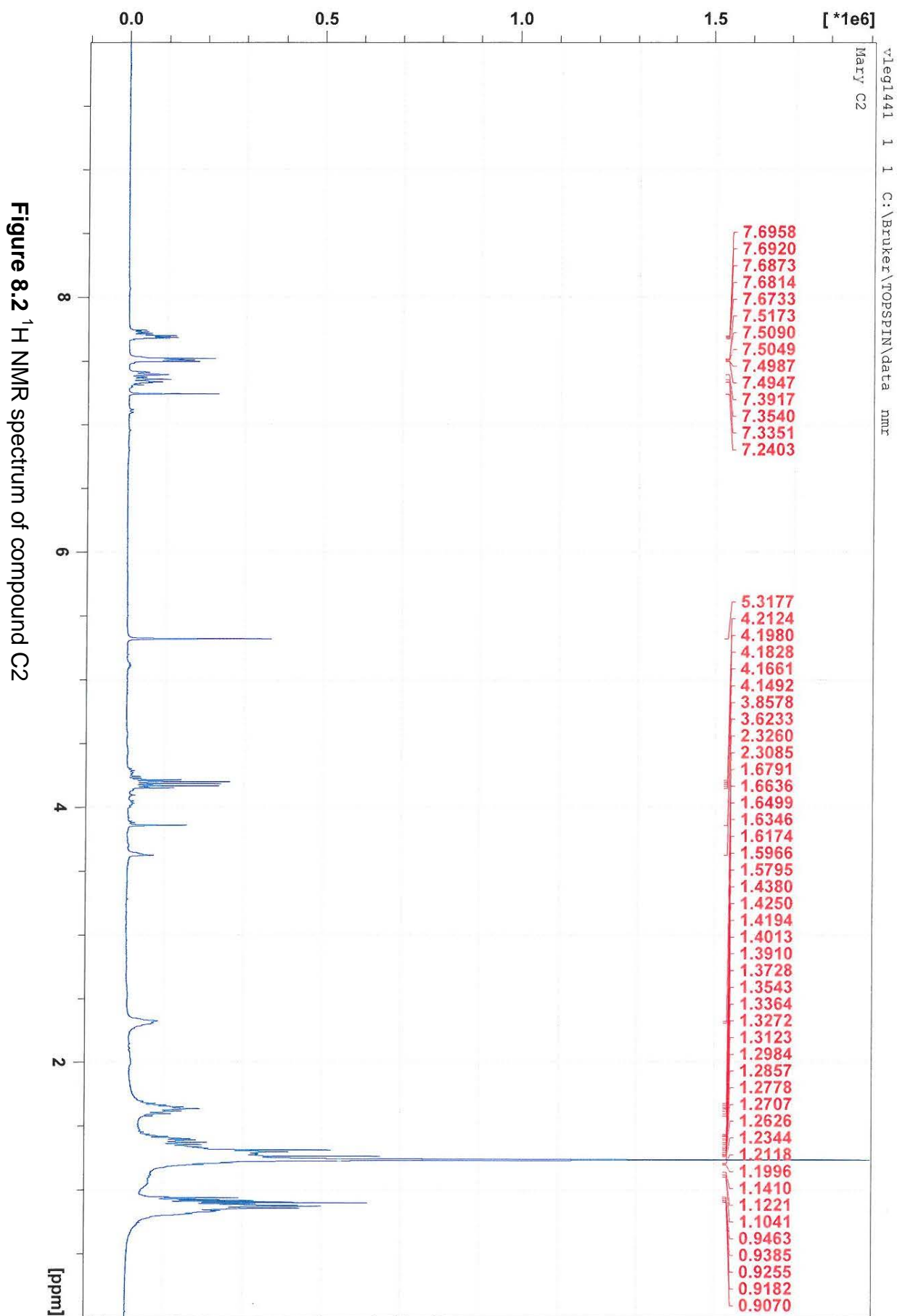
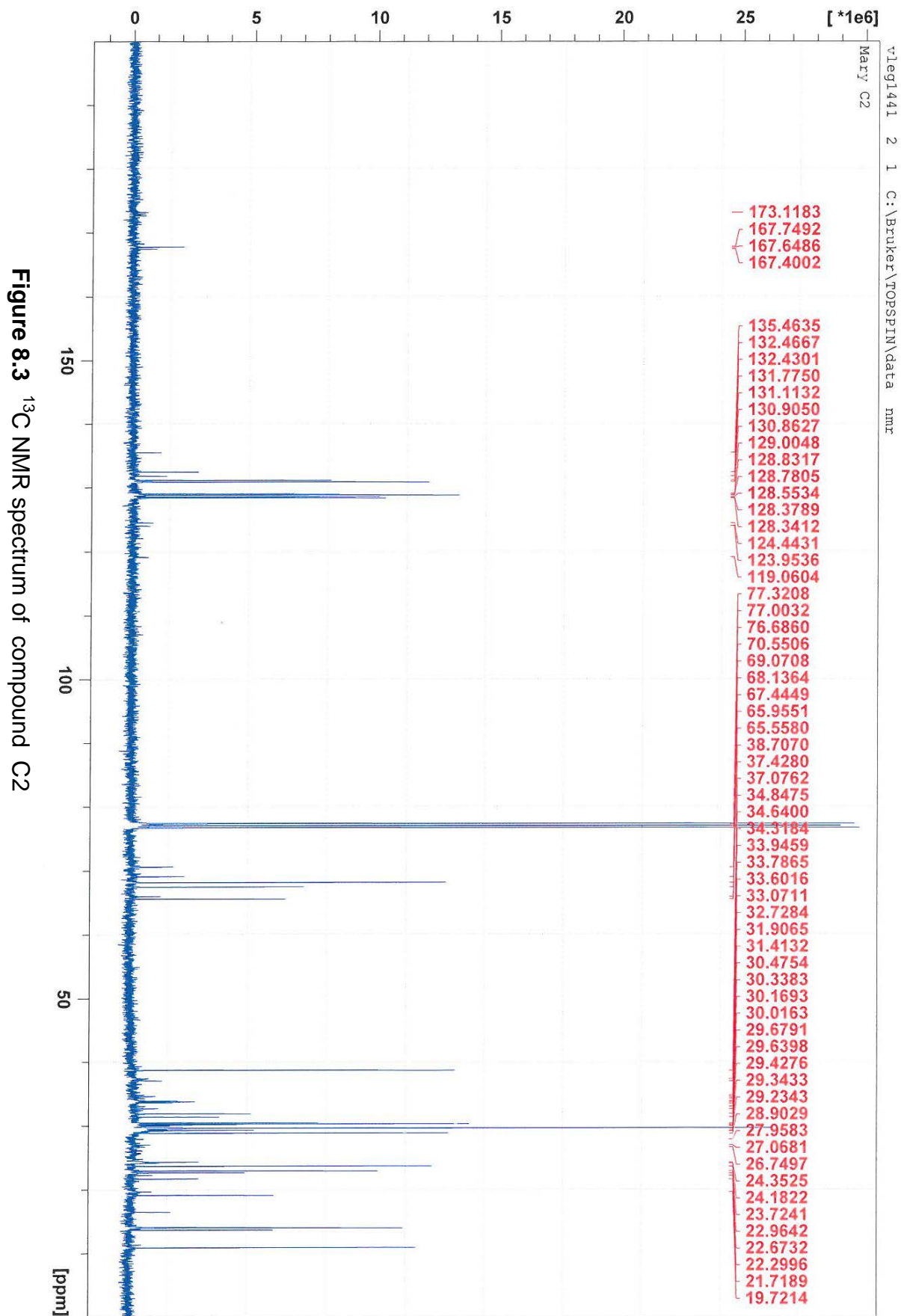
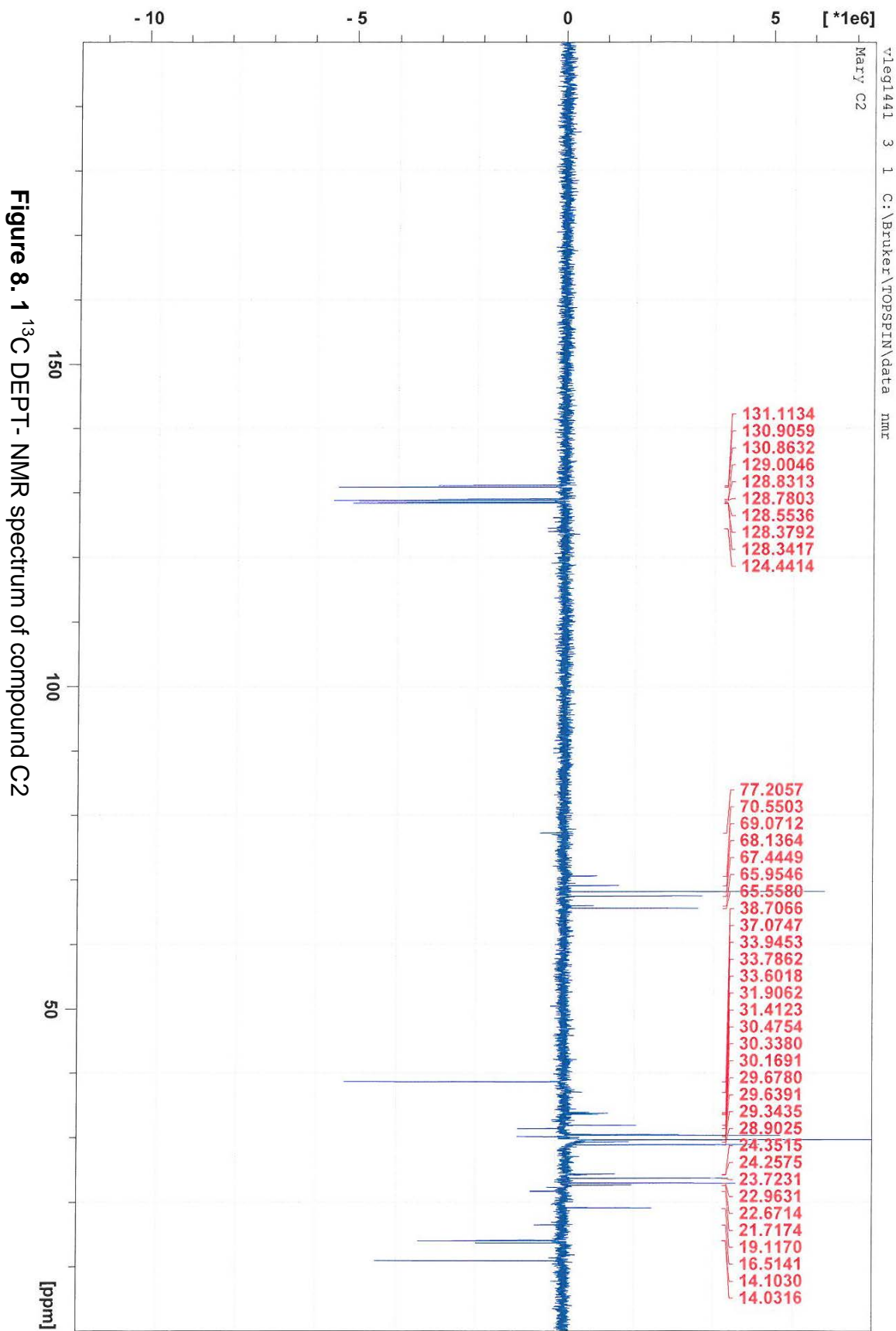


Figure 8.1 ^1H NMR spectrum of compound C2







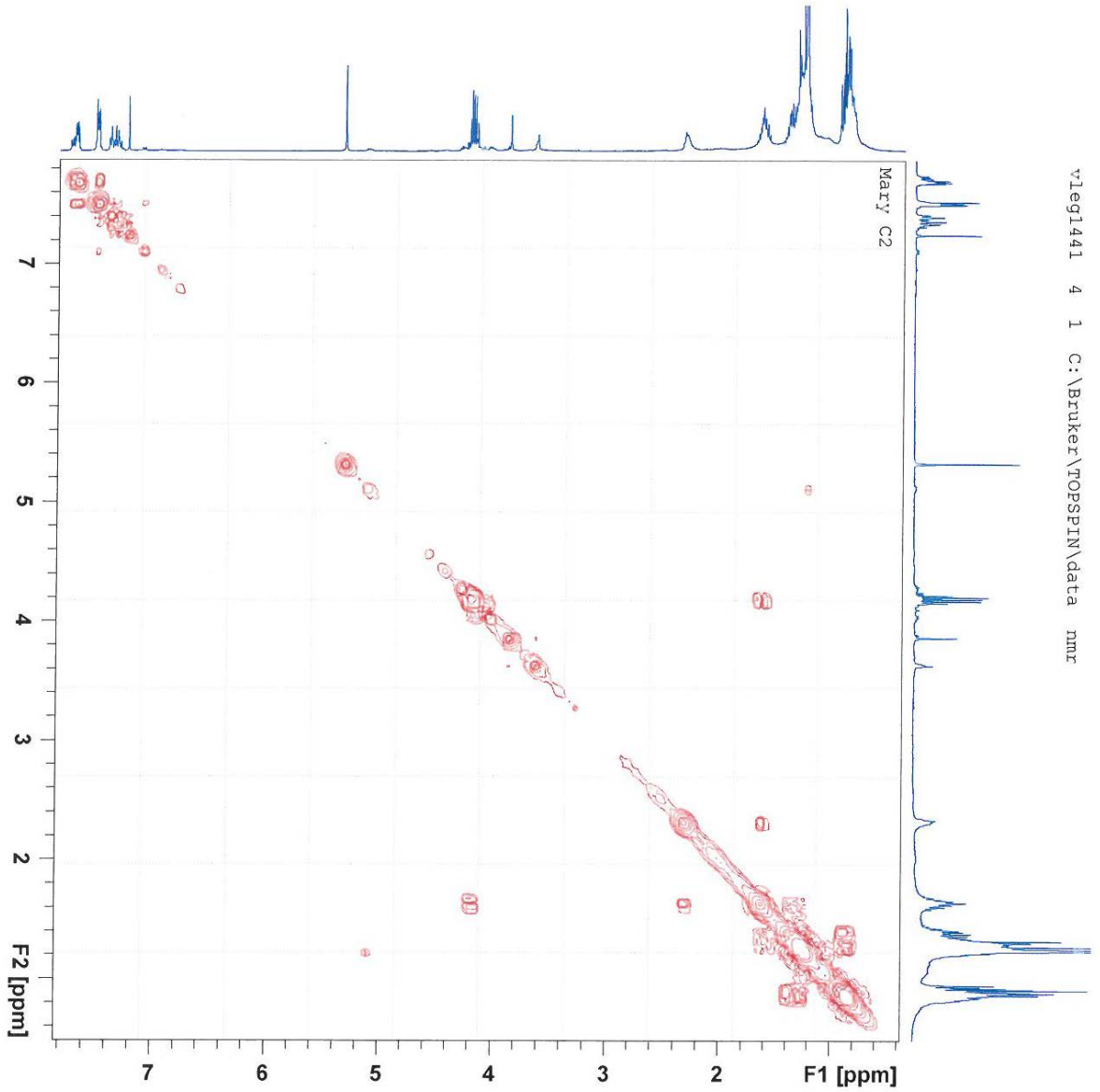


Figure 8. 2 gCOSY spectrum of compound C2

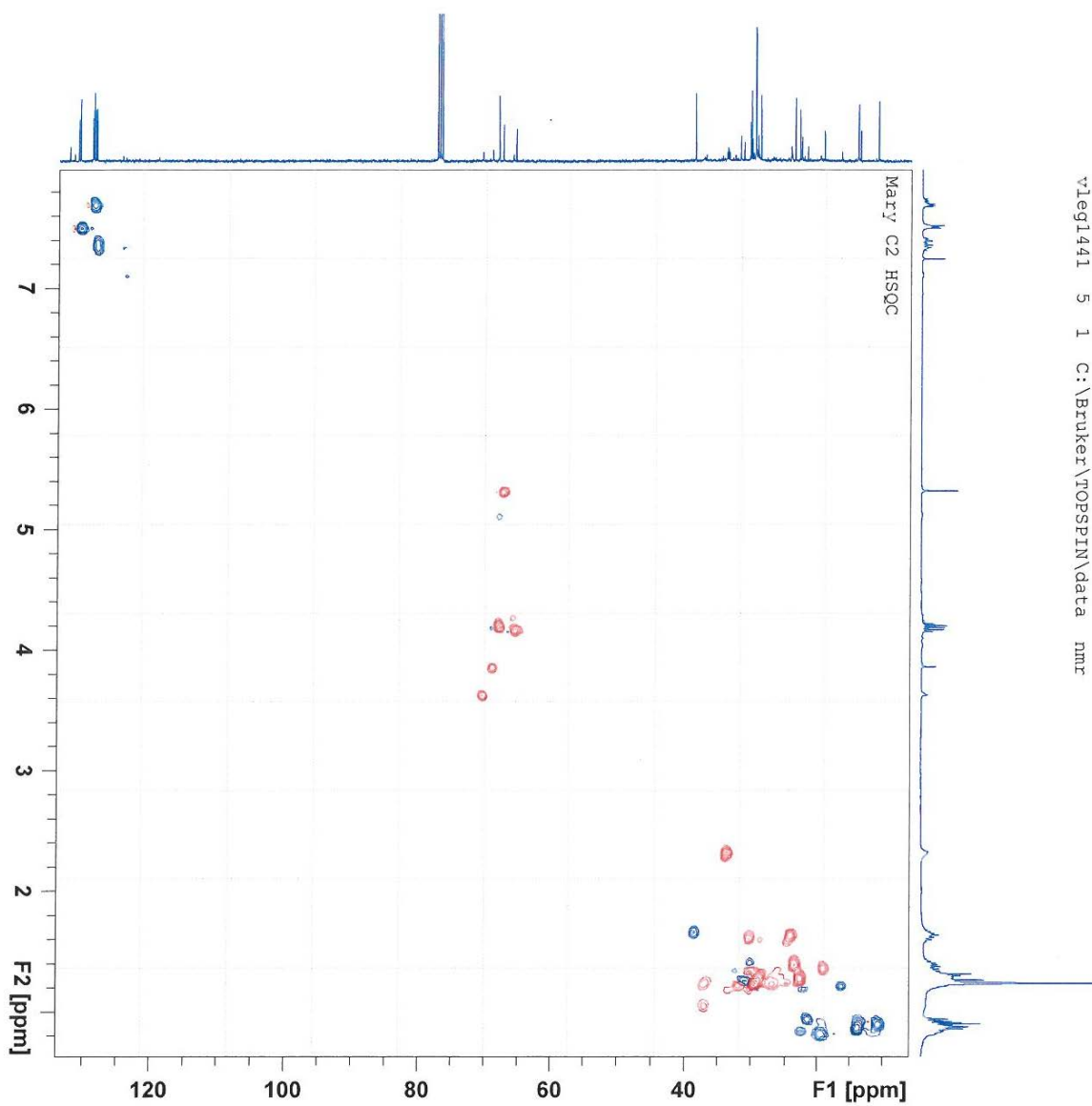


Figure 8. 3 HSQC spectrum of compound C2

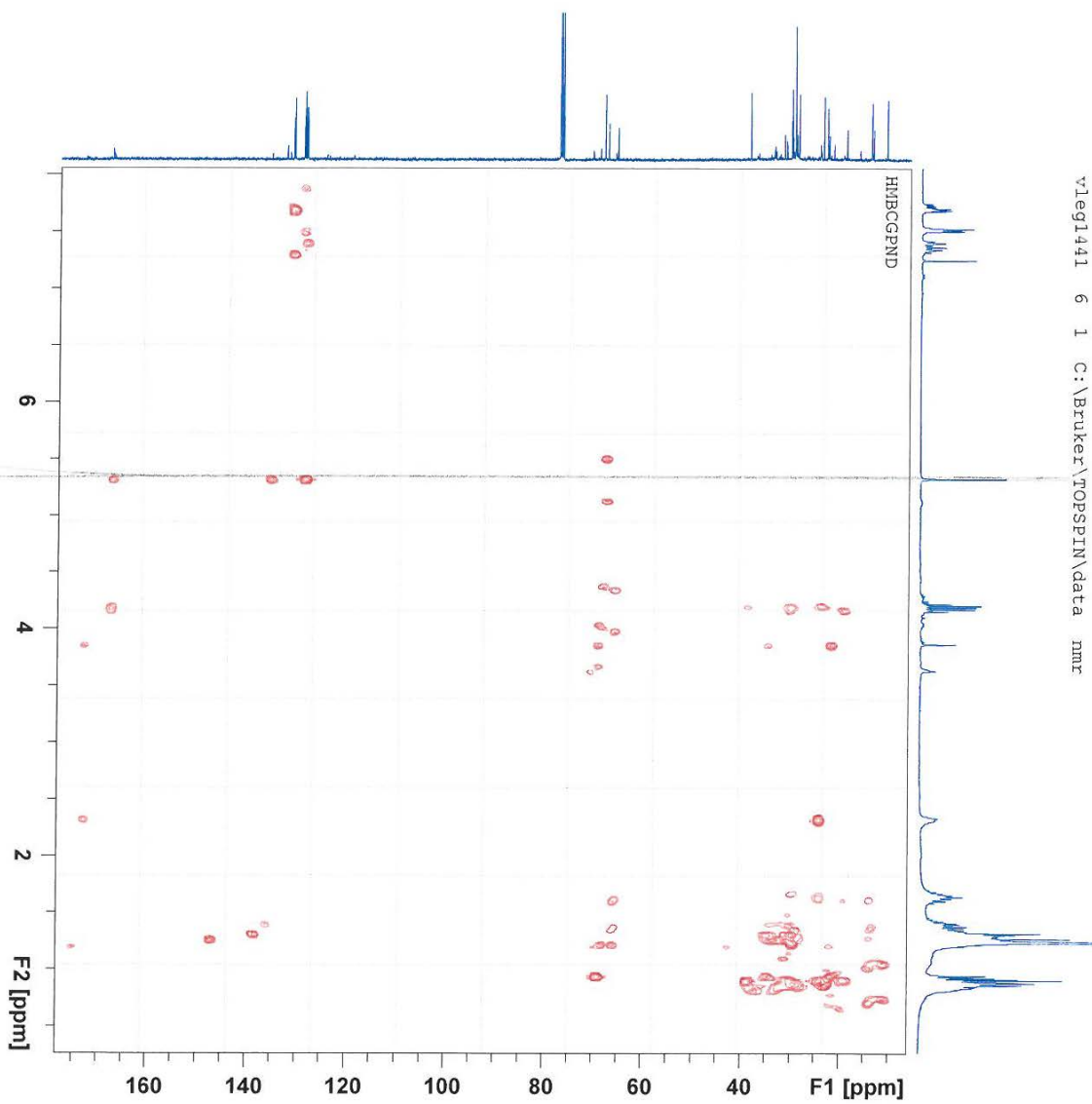


Figure 8. 4 HMBC spectrum of compound C2

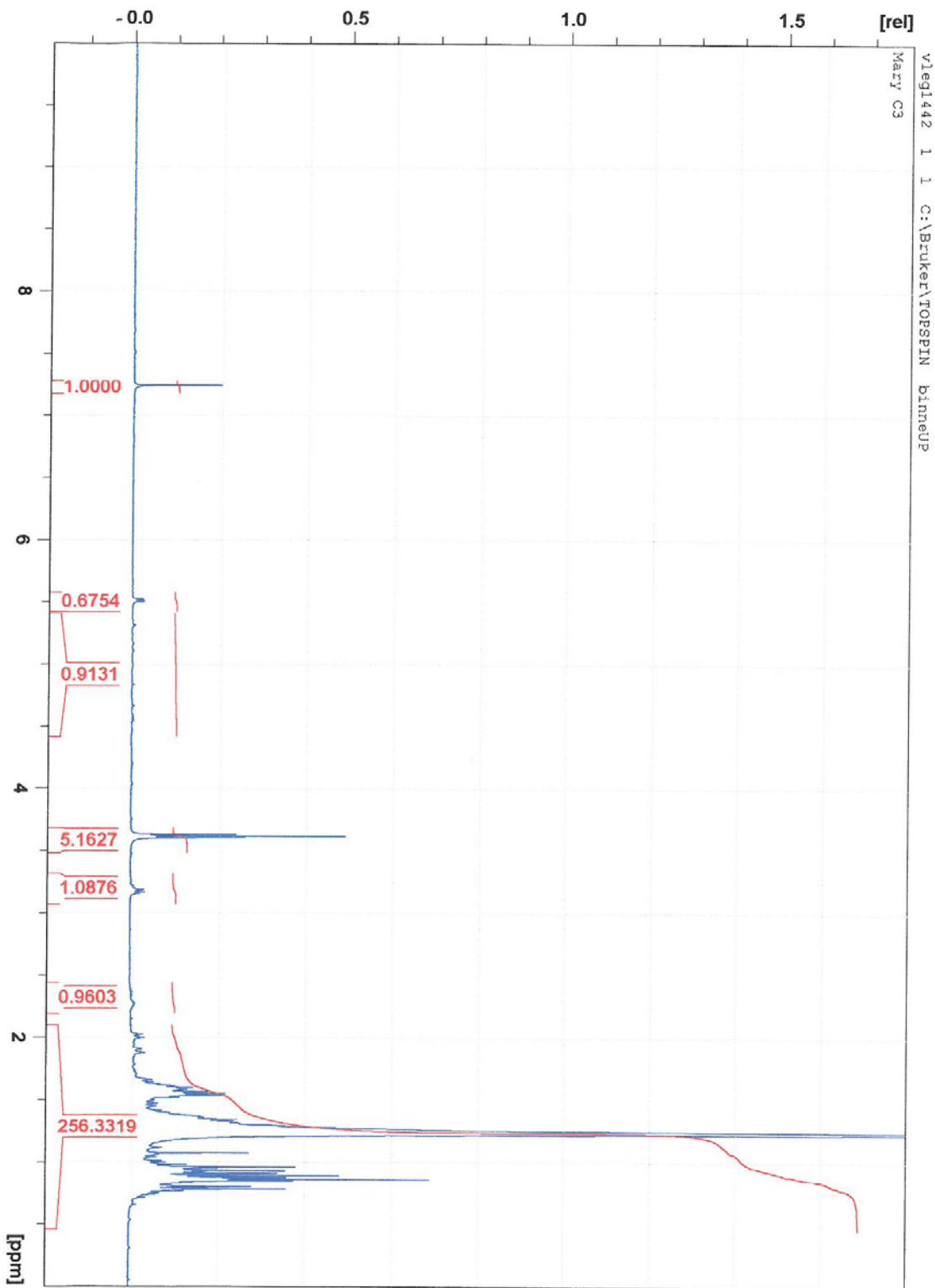
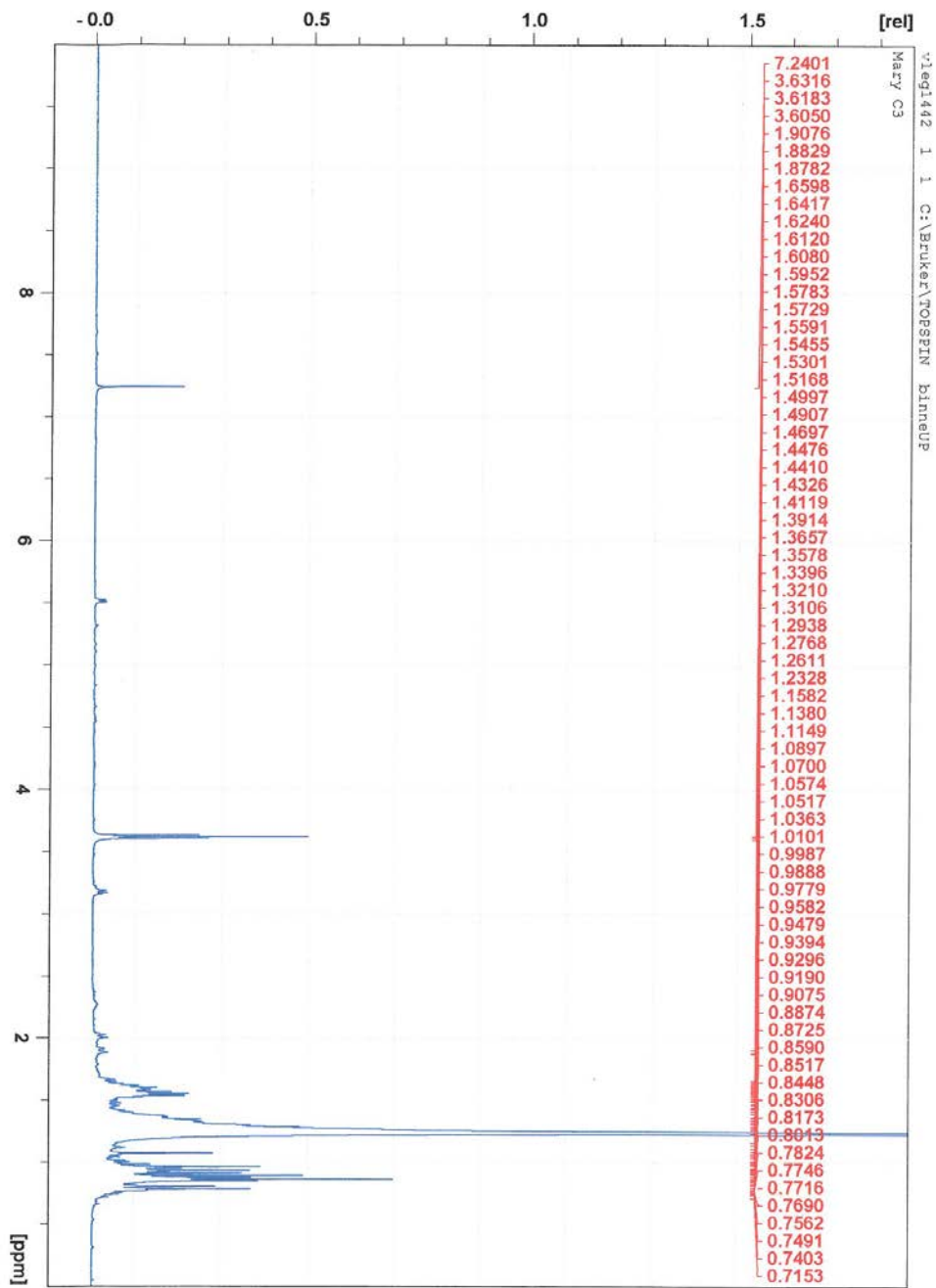


Figure 8.5 ^1H NMR spectrum of compound C3



Figure 8. 6 ^1H NMR spectrum of compound C3



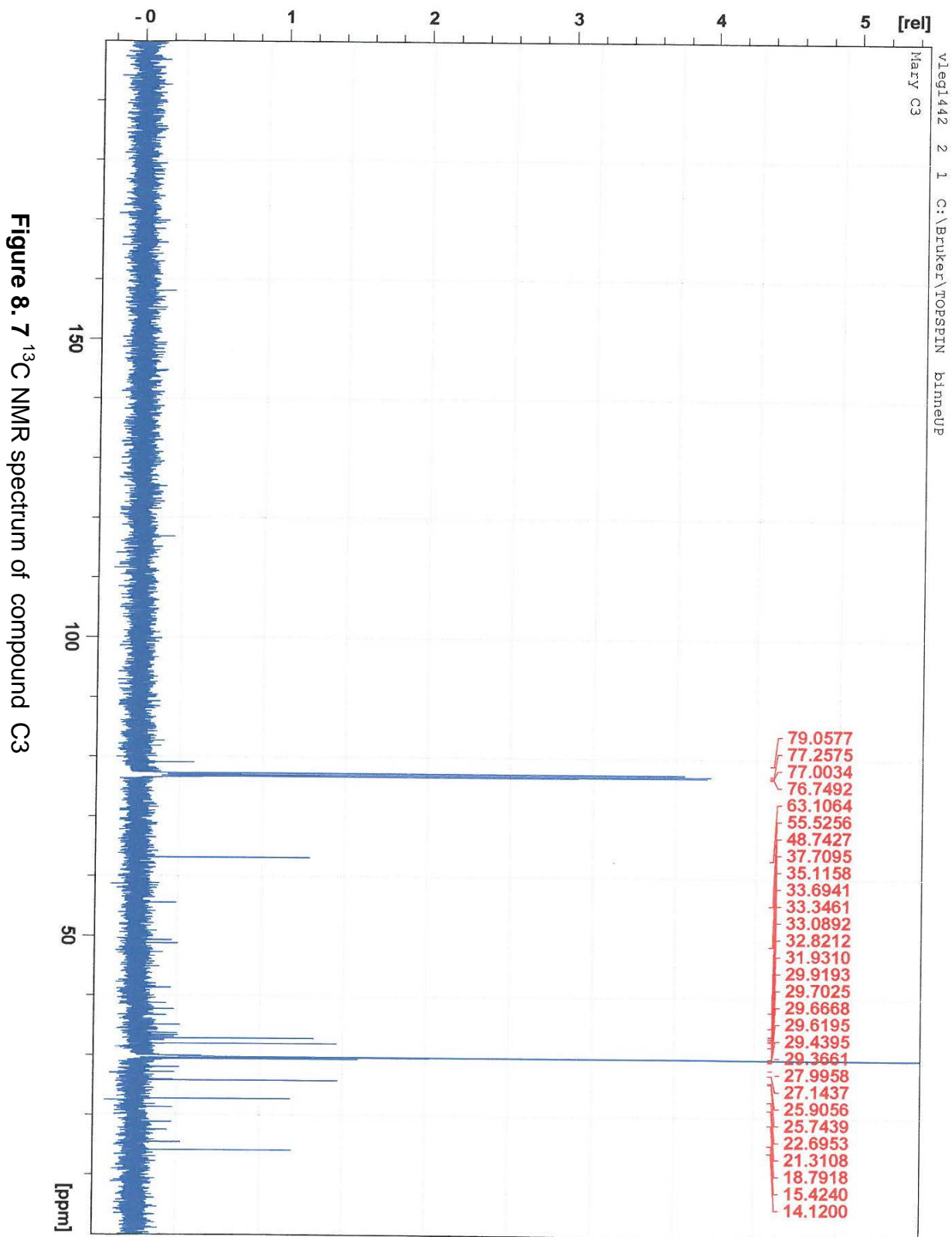
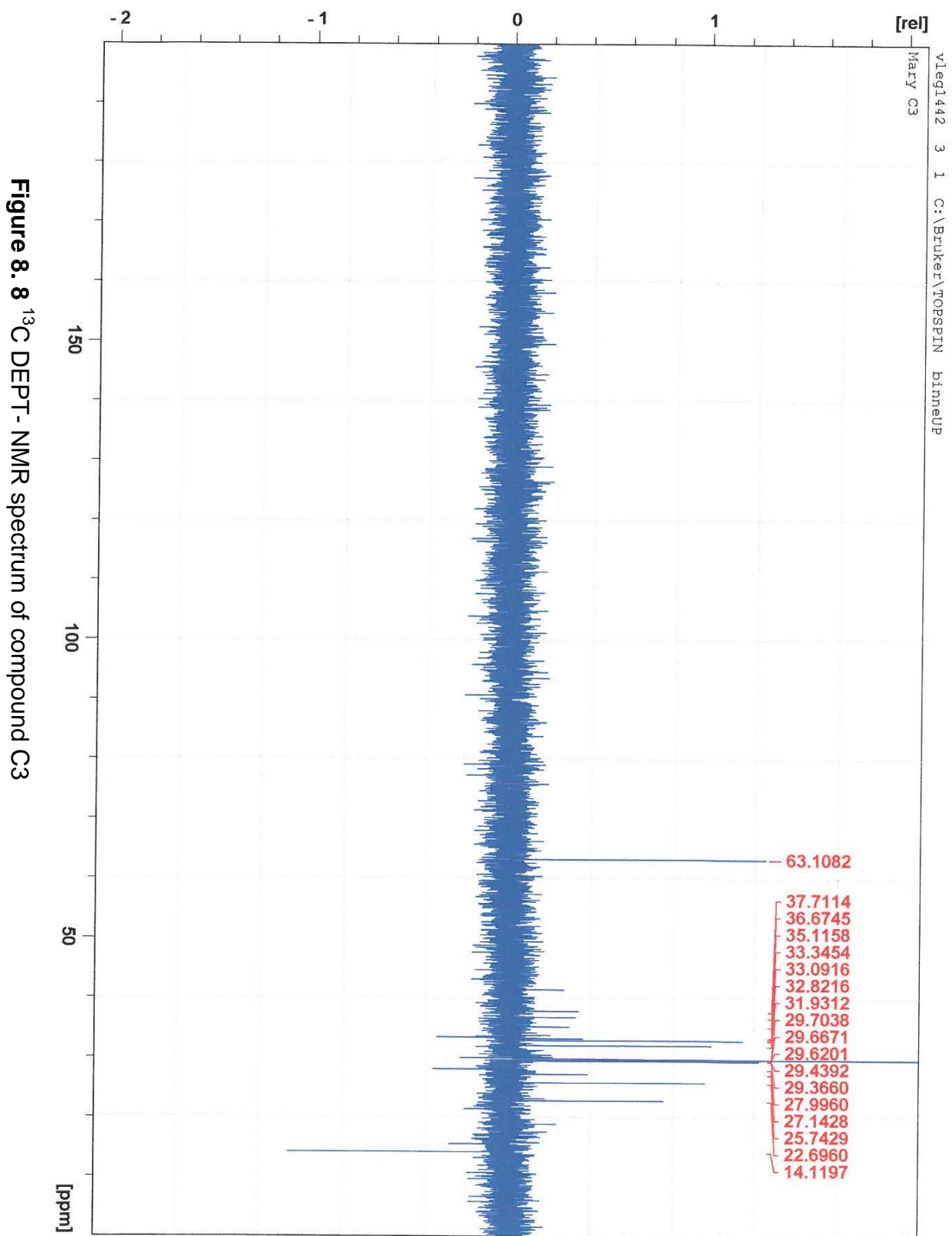


Figure 8.7 ^{13}C NMR spectrum of compound C3



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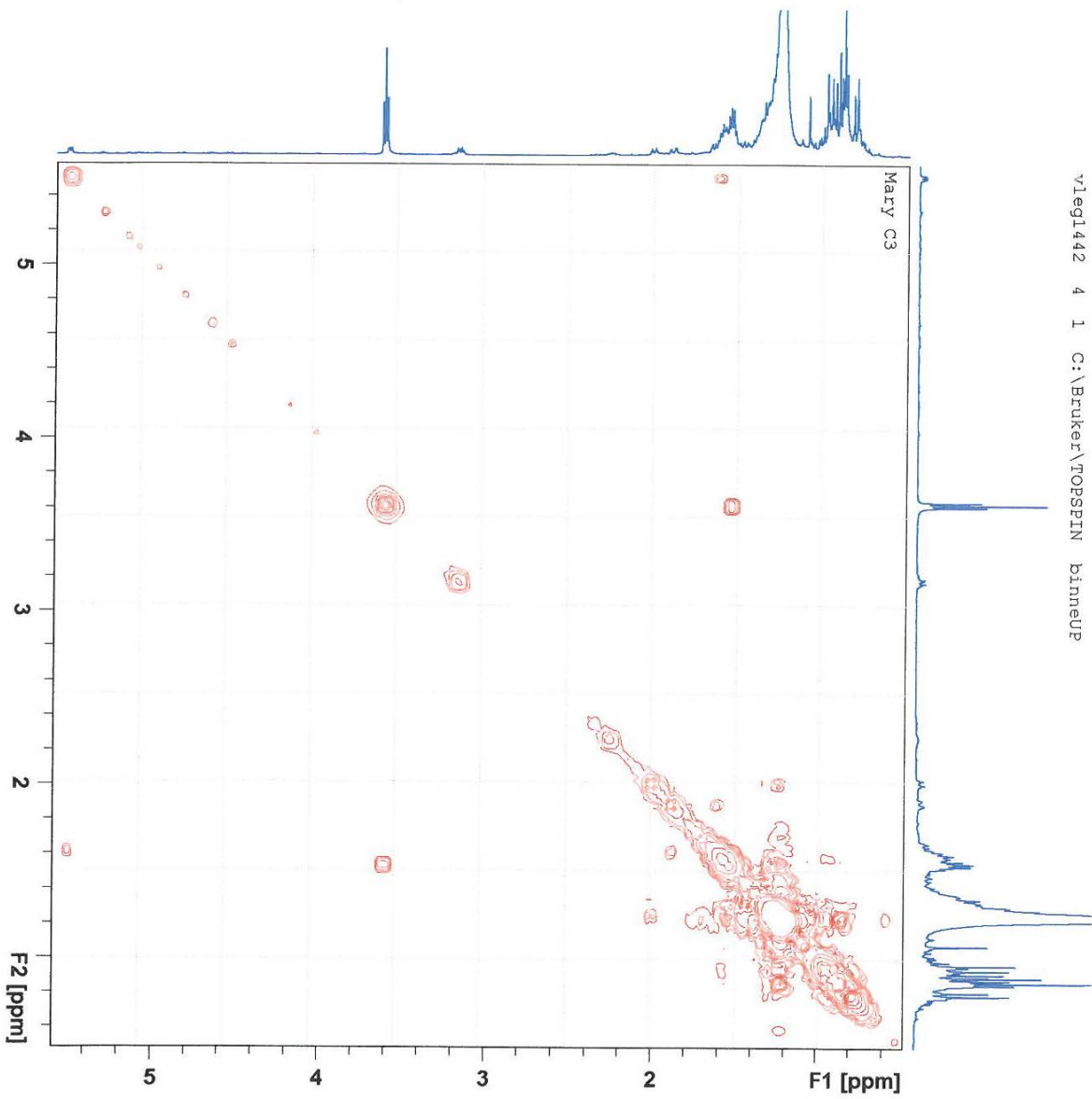


Figure 8.9 gCOSY spectrum of compound C3

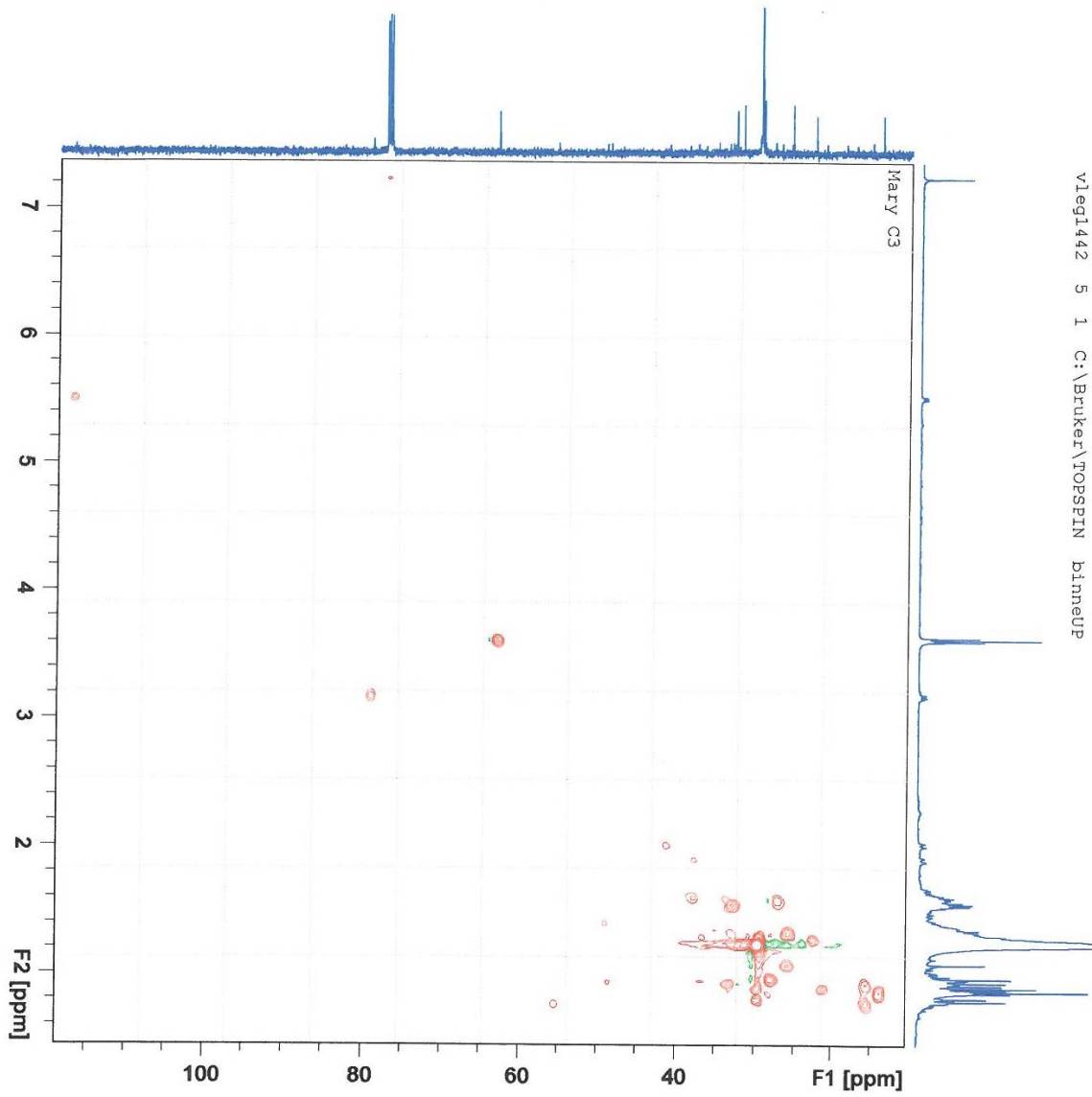


Figure 8. 10 HSQC spectrum of compound C3

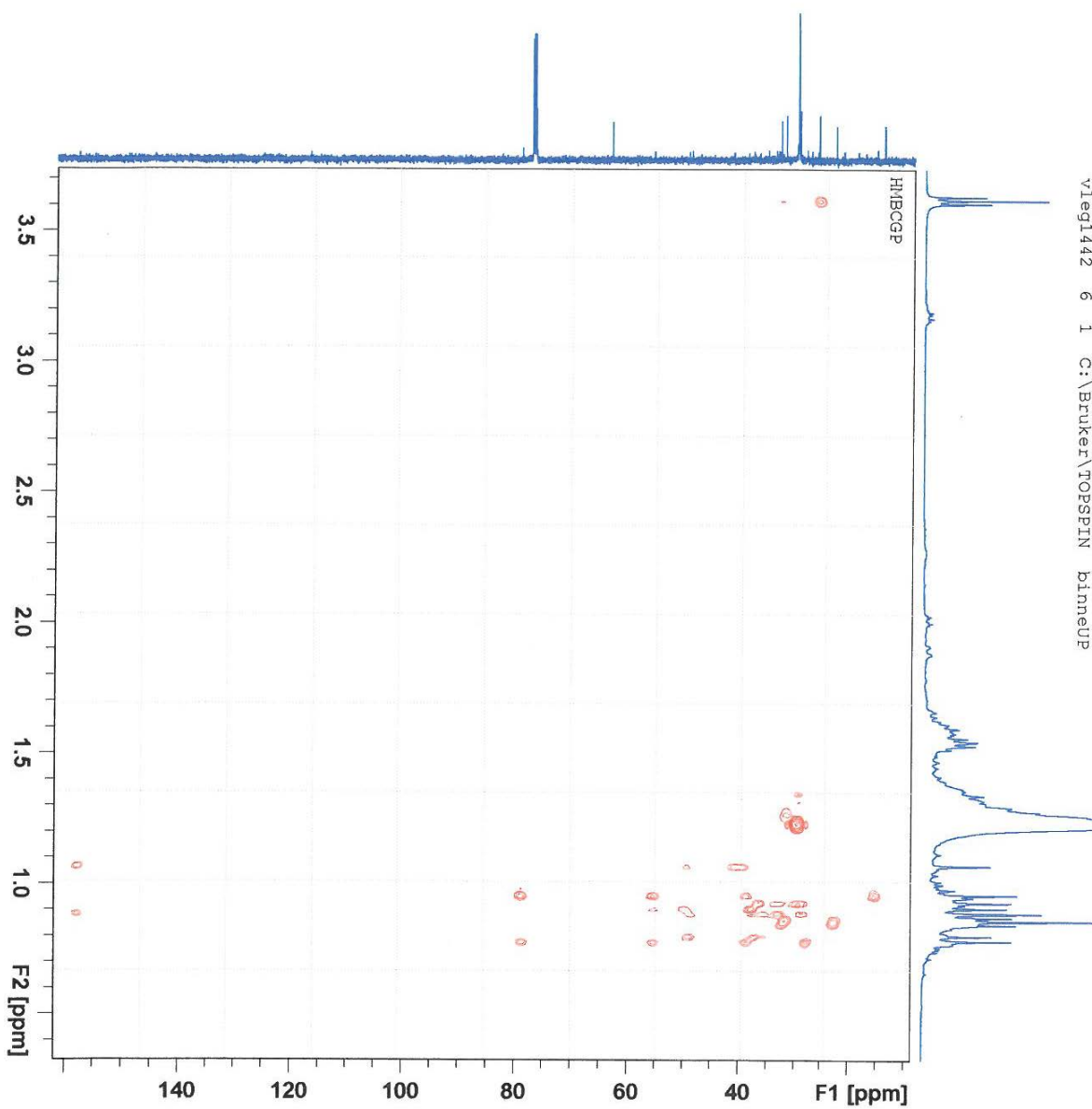
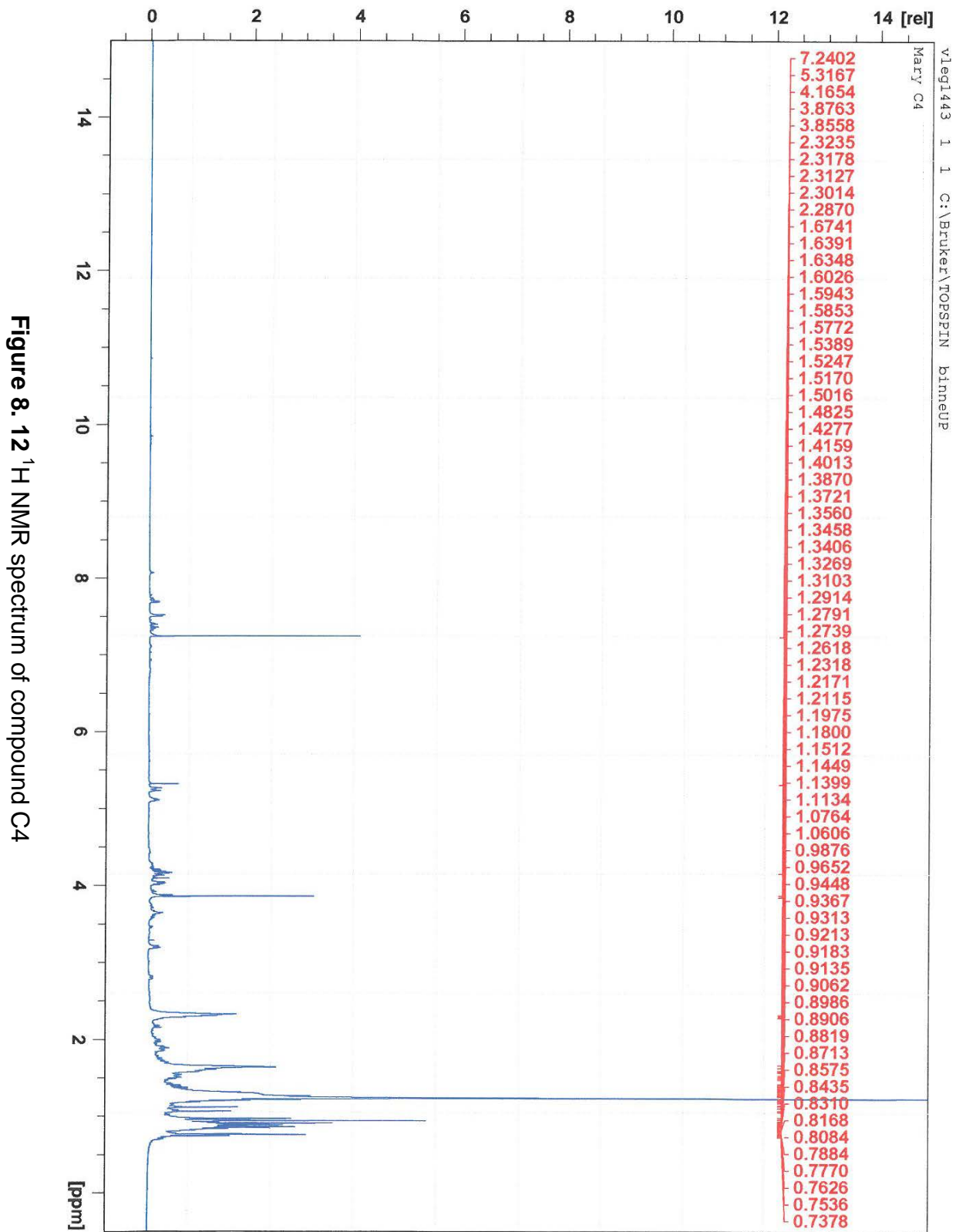


Figure 8. 11 HMBC spectrum of compound C3



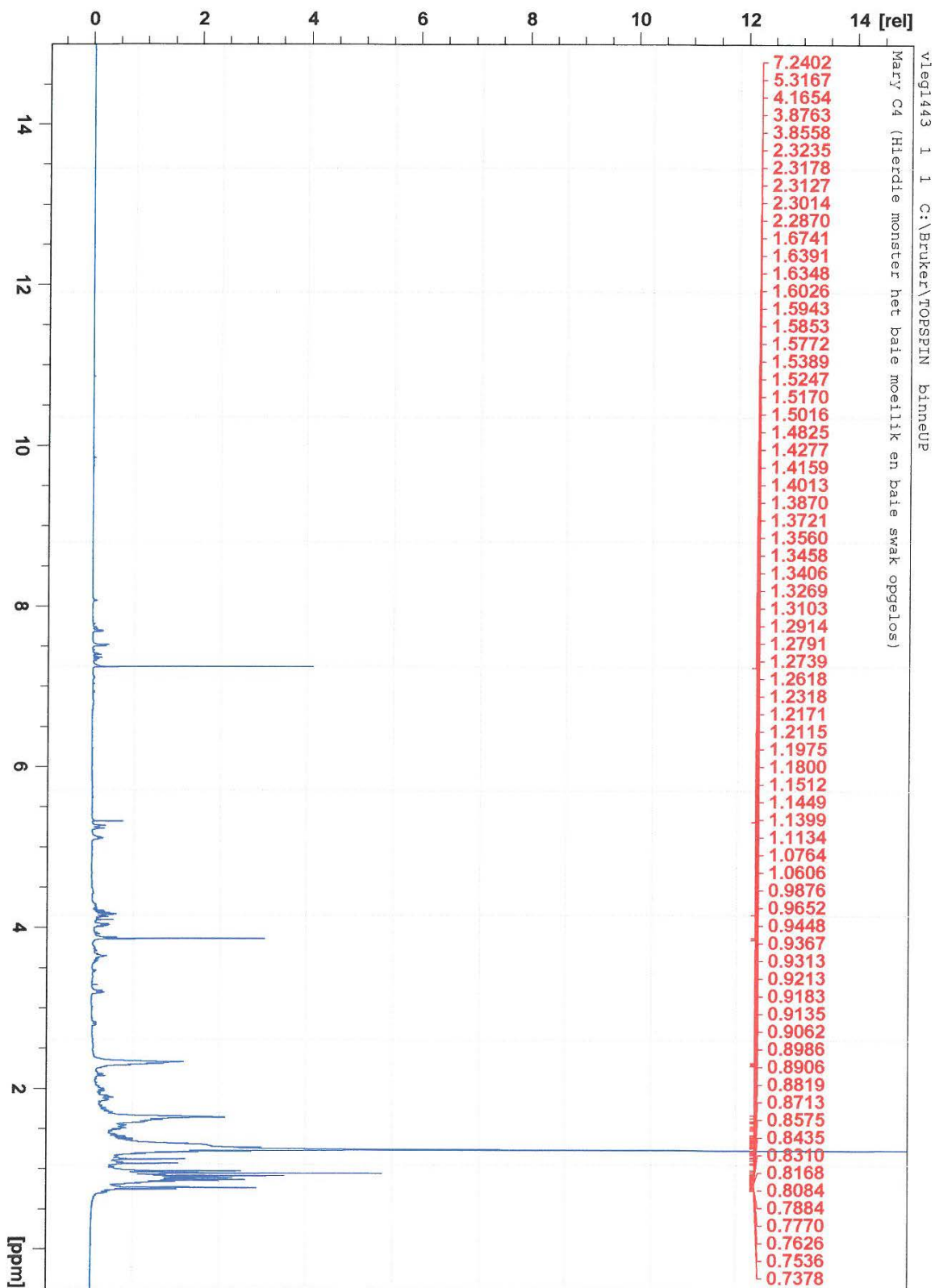


Figure 8. 13 ¹H NMR spectrum of compound C4